Differential Expression of Differentiation, Key Stemness and Pathways Genes and MicroRNAs and the role of Tgf-β in Embryonal Carcinoma Stem Cells

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By

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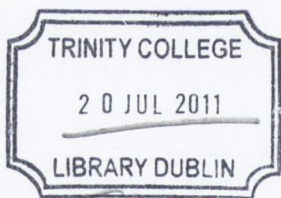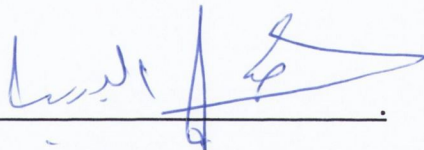
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Salah Elbaruni

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DEDICATION

I would like to dedicate this thesis to my wife, my parents and my three kids (Fatima, Mohammed and Taha) for unwavering support at all times. Whatever success I have had in life is mainly due to Allah and due to their supplications.

الإهداء

أهدى هذا البحث إلى زوجتى ووالدي وإلى أبنائي فاطمة ومحمد وطه الذين لم يترددوا فى تأيدى وتشجيعى. وكل ما حققته من نجاح فى هذه الحياة يعود الفضل إلى الله وإلى دعائهم.

Acknowledgements

General Aims

The study of self-renewal and differentiation of cancer stem cell lines (nullipotent and pluripotent) in this project started with several aims;

- First, to identify specific genes, pathways and miRNAs whose expression patterns are differentially regulated during early differentiation of CSCs.

- Second, by analysing pluripotent and nullipotent CSCs, we hypothesised that novel pluripotent-specific molecular markers and miRNAs could be identified.

- Third, we also hypothesised that specific miRNAs regulate early CSC differentiation specifically.

- Fourth to identify targetable CSC-specific events for use in anti-cancer therapeutics.

- Fifth, our overall aim was the removal of stemness from CSCs by Knocking down or overexpressing specific genes or pathways whose expression in CSCs facilitates survival or self-renewal.

In summary, this project aims to identify gene and miRNA targets that may ultimately exploited to remove stemness from these CSCs to remove the tumor regenerative ability.

Abstract

Cancer stem cells (CSCs) identified in multiple malignancies has fuelled the belief that they drive primary tumourigenesis. Their persistence post-intervention contributes to metastasis, recurrence, self-renewal, thus differentiating and regenerating tumours. CSCs occur in brain, breast, prostate and also in head and neck tumours. It is now widely believed that CSC stemness is key in malignancy, as self-renewal and differentiation of both normal stem cells (NSCs) and CSCs of similar potency involves almost identical events, we hypothesise that it is regulation of differentiation, rather than differentiation itself, that is aberrant in CSCs.

Additional strategies are required to reduce further the rates of mortality from cancer. For example, one particular strategy involves targeting stem-like cells required for normal tissue renewal that are a likely cell of origin of tumours. Addressing this hypothesis, we have characterised expression of key stemness events during early CSC differentiation. We believe that identification of CSC-specific events could be developed to achieve targeted removal of CSC stemness in a patient-orientated manner applicable to cancer therapeutics. Cancer stemness mirrors stemness of normal stem cells (NSCs) in terms of expression of key stemness genes such as Oct4, Nanog and Sox2 and pathways such as Wnt, Notch, Tgf-β, Snail and Pten, which show regulated expressed within 1 week of differentiation. Postulating that the difference between NSCs and CSCs relates to regulation during early differentiation, we have characterised novel expression of key stemness events during early CSC differentiation.

Our overall aim is identification of targetable CSC-specific events for use in anti-cancer therapeutics, which is hampered by commonalities between normal and cancer stem cells of similar potency. Therefore, in this study, we examined mRNA, pathways and microRNA (miRNA) regulation during early differentiation of human teratocarcinoma cells (Pluripotent NTera2 and Nullipotent 2102Ep). mRNA, pathways and miRNAs were differentially expressed in early differentiation and found to be CSC-specific. Through analysis of early differentiation gene events, we have identified several genes, pathways and miRNAs that are key to CSC biology. Furthermore, we have identified key stemness genes, pathways and miRNAs regulated in highly malignant nullipotent CSCs. We believe that the functional knockdown or overexpression of these genes may facilitate removal of stemness from CSCs in a manner applicable to cancer therapies.

Also through analysis of early differentiation gene events, we have identified that Tgf-β was up-regulated in differentiated pluripotent cells and not altered in differentiation of nullipotent cells. Differentiating nullipotent cancer stem cells may be a suitable therapy to cure malignant cancer stem cells. Therefore, Tgf-β was used to differentiate nullipotent cancer stem cells by up-regulating Tgf-β-R2 and to remove differentiation capacity from pluripotent cancer stem cells by knocking down the Tgf-β-R2. Our data demonstrates that the expression of differentiation marker genes and key stemness genes responded to knocking-down and overexpression of Tgf-β-R2, but was not sufficient to differentiate nullipotent CSCs and maintain self-renewal of pluripotent CSCs.

Publications

- Novel Cancer Stemness Regulation: Co-ordinated Upregulation of Sox3 During 'Differentiation' of Pluri- and Nullipotent Teratocarcinoma Cancer Stem Cells (IMM 10th Annual Meeting - Poster Competition Abstracts 9/11/2007).

- Early Regulation of 'Early Stemness' Gene Events in Pluripotent Cancer Stem Cells (Poster presentation, pathological Society Winter Meeting, Oxford: 8-9/1/2008).

- Characterisation of Novel 'Early Stemness' Gene Events Specific to Highly-Malignant Cancer Stem Cells (Oral presentation, pathological Society Winter Meeting, Oxford: 8-9/1/2008).

- Characterisation of Early Stemness Regulation in Teratocarcinoma Stem Cells (Poster presentation, USCAP 2008).

- Differential Regulation of Key Stemness Genes During Early Differentiation of Teratocarcinoma Stem Cells (Poster presentation, USCAP 2008).

- Regulation of Toll-like Receptors in Cancer Stemness: a Pro-Inflammatory Switch Model (Poster presentation, USCAP 2009).

- Downregulation of miRNAs in Pluripotent Cancer Stem Cells is mirrored in advanced ovarian serous carcinoma (Poster presentation, USCAP 2009) **Biobank Ireland Prize**.

- Analysis of Signaling Events at 3 Days Differentiation Reveals Novel Hierarchal Regulation During Early Differentiation of Embryonal Carcinoma Stem Cells (Postgraduate Research, 2nd School of Medicine Day 11/12 2008).

- Characterisation of Early Stemness Regulation in Embryonal Carcinoma Stem Cells (Poster presentation, Coombe Women's Hospital research symposium 2008).

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Abbreviations

ES	Embryonic stem cells
F12-K	F12-K Nutrient Mixture Kaighn's Modification
FBS	Foetal Bovine Serum
FCS	Fetal Calf Serum
g	Gram
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
Gata6	Gata binding protein 6
GLI1	Glioma-associated oncogene homolog 1
H₂O	Water
hES	Human embryonic stem cells
HMG	Mobility group box
ICM	Inner cell mass
IMS	Industrial Methylated Spirits
IPS	Induced pluripotent cells
KN	Knockdown
LIF	leukaemia inhibitory factor
MEF	Embryonic Fibroblast
MgCl₂	Magnesium chloride
mins	Minutes
miRNA	MicroRNA
mM	Milimolar
Mock	Mock-transfected cells (cells treated with Transfection Reagent)
mRNA	Messanger RNA
mV	Millivolts
NaCl	Sodium chloride
Nanog	Tir na nOg
Ncam1	Neural cell adhesion molecule1
ncRNA	Non-coding genes

ng	Nanogram
Ng	Silencer Negative control
NICD	Notch intracellular domain
NSCs	Normal stem cells
Nt	Nucleotides
NTC	Non-template control
NTCs	Non-transfected control cells
Nulli-	Nullipotent cells
°C	Degrees Celsius
Oct4	Octamer-binding transcription factor4
OD	Optical density
OV	Overexpression
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PIN	Prostatic intra-epithelial neoplasia
Plg	Plasminogen precursor
Pluri-	Pluripotent cells
PreAmp	Preamplification
Pten	Phosphatase and tensin homologue deleted on chromosome ten
PVDF	Polyvinylidene diflouride
RA	Retinoic Acid
RIPA buffer	Radioimmunoprecipitation assay buffer
RLE	Relative log expression
RNA	Ribonucleic Acid
Rpm	Revolutions per minute
RT	Reverse Transcription
SDS	Sodium dodecyl sulphate
Shh	Sonic Hedgehog

siRNAs	Small interfering RNAs
SM α-actin	Smooth muscle α-actin
Sox2	SOX gene family
SRY	Sex-determining Region Y
T0	Time zero
TBS	Tris Buffered Saline
TE	Tris-EDTA
TGF-β	Transforming growth factor beta
Tgf-β-R1	Transforming growth factor, beta receptor I
Tgf-β-R2	Transforming growth factor, beta receptor II
TLDAs	TaqMan Low Density Arrays
TRIS	Tris(hydroxymethyl)methylamine
Undiff or U	Undifferentiated
UV	Ultra Violet
WHO	The World Cancer Report
WT	Whole Transcript
β-ME	β-Mercaptoethanol
μg	Microgram
μl	Micorliter
100%	One hundred percent
&	And
–RA	Non Retinoic Acid -treated cells
+RA	Retinoic Acid –treated cells
±RA	Non or Retinoic Acid treated cells
1W	One week
2W	Two weeks
3D	Three days

Chapter One

General Introduction

1.1 Overview

Despite impressive advances in the treatment of malignancies, the number of deaths due to cancer rises yearly. Today, one in three people will develop a malignancy in their lifetime. Thus cancer will affect every individual in the world, either directly or indirectly. To address this, new cancer treatment strategies must be developed.

One potential avenue for novel cancer therapy development is targeting of cancer stem cells (CSCs). CSCs are cancer cells that display the defining stem cell properties of both self-renewal and differentiation. CSCs have been identified in numerous cancers to date. CSCs have highly increased cancer properties compared to non-stem like cancer cells. These include rates of tumor development and resistance to hypoxia and chemotherapy drugs. Today, CSCs are widely accepted as the likely driving cell for tumorigenesis. These traits are highly active in undifferentiated CSCs and lost upon differentiation. Therefore, removing stemness (the ability to self-renew and differentiate) by force-differentiating CSCs is a potential avenue of anti-cancer therapy development. Targeting CSCs therapeutically involves the characterization of the specific genes and molecular pathways active in CSCs.

In this chapter, a detailed background of the subject area is presented. The general area of stem cell biology is initially discussed. The concept of cancer stemness and the relationship between CSCs and several properties of malignancy is then described. Subsequently the teratocarcinoma model of cancer stemness is evaluated. Finally, the specific genes and pathways assessed in this study are explained.

In this study the characterization of a CSCs model is undertaken. This takes place at two levels:
1) analysis of key genes and pathways involved in stemness and 2) analysis of microRNA regulation of CSC differentiation. Once characterised, a specific molecular pathway, TGF-beta signaling, is selected for functional analysis. The protocol for this functional analysis is successfully developed and is now available for further analysis of any gene of interest by our group. Ultimately, this will identify novel regulators of key stemness regulators, validating our approach.

1.2 Stem Cells

1.2.1 History of Stem Cells

Most cells in the body divide symmetrically by mitosis to produce identical copies of themselves. The term 'stem cell' is used to describe any cell that can divide asymmetrically to produce an identical copy of itself (self-renewal) and a 'mature' or 'differentiated' cell (differentiation). Under different circumstances, stem cells can divide symmetrically or asymmetrically as required. This facilitates maintenance of the stem cell population and production of differentiated cells required for the body during general growth and repair.

Stem cells are defined by their origin and potency. In terms of potency there are three main classes of stem cell. 'Unipotent' stem cells can differentiate to form cells from one tissue type only: it is thought that epidermal layers such as skin are maintained by unipotent stem cells (Blanpain *et al.*, 2007). 'Multipotent' stem cells can differentiate to form several cell types representative of a particular system: for example, mesenchymal stem cells can develop into the different classes of blood and bone marrow cells (Hock, 2010). Most stem cells in the body are uni- or multipotent and are referred to as 'adult' stem cells. The final class is the 'pluripotent' or 'totipotent' stem cell. These stem cells can differentiate to form cells representative of all three germ layers. Specifically, totipotent stem cells can form any cell type while pluripotent cells can form almost every cell type. (For example, in the embryo, totipotent cells form the embryo and placenta while pluripotent cells form the embryo alone).

To date, three sources of pluripotent cell exist. Embryonic stem (ES) cells are derived from the inner cell mass of the developing embryo and form benign teratomas in immuno-compromised mice (Ramahlo-Santos *et al.*, 2002). Embryonic cancer (EC) cells are derived from teratocarcinomas and embryonal carcinomas and are highly malignant (Andrews *et al.*, 2002 and Andrews 2005). Induced pluripotent (IPS) cells are adult stem cells overexpressing pluripotency transcription factors and are benign (Rashid *et al.*, 2010).

There are different types of stem cell:

1- Totipotent stem cells: the zygote that is able to produce the embryo and placenta.

2- Pluripotent stem cells: embryonic stem cells that give rise only to the embryo.

3- Multipotent stem cells: stem cells that give rise to one of the three germ layers.

4-Unipotent stem cells: tissue committed stem cells, which give rise to cells building particular tissues (Lemoli *et al.,* 2005).

The first stem cells were discovered in the early 1900's, but it was some time before stem cells were isolated from blood, as it was found that some cells could generate blood cells (Till *et al.,* 1961). The first pluripotent ES cells were isolated from mice in 1981 (Evans *et al.,* 1981) and the first successful derivation of pluripotent human ES (hES) cells was in 1998 (Thomson *et al.,* 1998). In this case the authors isolated inner cell mass (ICM) cells by plating onto mitotically inactivated Mouse Embryonic Fibroblast (MEF) 'feeder' cells. Two years later another group confirmed that hES cells could be efficiently derived from surplus embryos demonstrating at the same time the differentiation potential of hES cells under *in-vitro* conditions (Reubinoff *et al.,* 2000). The derivation of hES involves the destruction of a human embryo, which has major ethical considerations.

In the body, stem cells are extremely rare: for example, the frequency of 'haematopoietic' stem cells in the bone marrow is 1 per $10^4–10^5$ bone marrow cells. The estimated number of heart stem cells varies in recent papers from 0.5% to 500–600 cells among all heart cells (Beltrami *et al.,* 2003 and Laugwitz *et al.,* 2005). Only tissues such as skin or gut contain higher number of stem cells due to their regenerative needs but these cells are as yet not very well defined.

1.2.2 Normal stem cells (NSCs) and Cancer stem cells (CSCs)

The term CSC is used to refer to any cancer cell with stem cell properties. By definition, stem cells can self-renew and differentiate. This is generally demonstrated in cell culture through passaging of cells (self-renewal) and stimulation of differentiation using mutagens such as retinoic acid (RA) or withdrawal of growth factors such as 'leukaemia inhibitory factor' (LIF) or bone morphogenic proteins (BMPs) from the cell culture media. As with any cancer analysis, a non-cancer comparator must be studied. Cancer stemness research, then, must consider both CSCs and non-malignant or 'normal' stem cells (NSCs). At the time of writing, our department and university does not conduct analysis on hES cells, although published hES

data is reviewed for comparison to cancer data. Cancer stem cells are discussed in detail in Section 1.3 while their relationship with NSCs is now described.

In the study of cancer stemness, non-malignant NSCs share many properties with comparable CSCs such as cell division and gene expression profiles (Andrews *et al*, 2005). Stem cells respond to signals from their environment, or niche, that tell them when to remain in a self-renewal state and when to divide and differentiate into needed cell types. The majority of stem cell analysis, whether of NSCs or CSCs, is focused on the mechanisms of self-renewal, differentiation and the switch from one to the other. Both CSCs and NSCs share this mechanism of self-renewal and differentiation (Clarke *et al.*, 2006). For example, EC cells are considered to be so similar to ES cells that they are used as an easily cultured model of ES biology (Josephson *et al.*, 2007).

Several types of NSC have been characterised to date. Cells from neuronal tissues can be transformed into hematopoietic cells under stress conditions due to myeloablation (Bjornson *et al.*, 1999). At the same time, skeletal muscle stem cells gave rise to hematopoietic cells after transplantation *in-vivo* (Jackson *et al.*, 1999). This phenomenon is called trans-differentiation. Cell fusion is cited as an explanation for the observed stem cell plasticity (Terada *et al.*, 2002). It has been shown that under some circumstances, cells of different lineages can fuse with each other and that the new cell can acquire characteristics of one of them. Particularly, it has been noted that myeloid cells, monocytes and macrophages are likely to fuse with other cell types (Camargo *et al.*, 2004). This would partially explain why NSCs could so easily trans-differentiate into other cell types.

NSCs offer a lot of promise and expectations for developing new cell-based therapeutics. Despite the difficulties in their isolation and *in-vitro* culture, tremendous progress has been made during the last several years. These new discoveries will bring stem cells closer to the patients' bed and will give hope to patients suffering from untreatable diseases.

Cancer stem cell populations from multiple different malignancies can self-renew, differentiate and regenerate malignant tumours (Al-Hajj *et al.*, 2003, Hemmati *et al.*, 2003, Galli *et al.*, 2004, Richardson *et al.*, 2004, Singh *et al.*, 2004, Collins *et al.*, 2005, Szotek *et al.*,

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2006, Li *et al.*, 2007 and Prince *et al.*, 2007). A single such CSC can form a *de-novo* tumour *in-vivo* suggesting that CSCs can drive primary tumourigenesis and contribute to metastasis and recurrence (Kleinsmith *et al.*, 1964 and Al-Hajj *et al.*, 2003). Tumours containing high concentrations of undifferentiated stem cells are considered to be highly malignant and differentiated tumours less malignant (Andrews, 2002 and Andrews *et al.*, 2005). Compromising the CSC undifferentiated state must be addressed as a potential anti-cancer therapy. However, similarities between CSCs and their NSC counterparts complicate targeting of CSCs in a manner that does not affect the normal stem cell population. Due to these similarities and the reported resistance of CSCs to certain anti-cancer therapies, clinical inhibition of CSCs has not been achieved to date (Andrews, 2002 and Andrews *et al.*, 2005).

One of the defining properties of CSCs is their ability to regenerate a new tumor when as little as one cell is introduced into an immuno-compromised mice (Andrews *et al.*, 1982). As such, CSCs have the capability of growing a new tumor from a single cell missed by chemotherapy or surgery. They also promote the metastasis of a cancer to new sites around the body. Thus CSCs are thought to be key components of primary, metastatic and chemo-resistant, recurrent disease. CSCs are characterized by multi-drug chemoresistance (An *et al.*, 2009 and McDermott *et al.*, 2010). *In-vitro* chemotherapy with differentiating agents reduces the number of the CSCs in a tumor (Roy *et al.*, 2010).

While the properties of differentiation of different classes of CSC can differ substantially, NSCs and CSCs of similar differentiation capability or 'potency' are strikingly similar. This is particularly true of pluripotent EC and ES cells (Andrews *et al.*, 2005). Thus characterisation of the differences between NSCs and CSCs is required to define specific targets for therapy. Without specific targets, the NSC population required for normal growth could be eliminated during intervention, with potentially lethal implications for the patient. In defining traits of stem cells, much research focuses on the differentiation and self-renewal of NSCs and CSCs. The differences between NSC and CSC differentiation may reveal new targets for developing therapeutics to treat cancer more effectively (Clarke *et al.*, 2006).

Since CSCs share common properties with NSCs, it is not surprising that they have overlapping regulatory mechanisms. Many studies have demonstrated that a 'plethora' of genes and signaling pathways are involved in the regulation of the processes (reviewed by

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Andrews *et al.,* 2005). The Sonic Hedgehog (Shh), Notch and Wnt signal transduction pathways play a major role in stem cell regulation. Studies on the molecular pathways that, when altered, could give rise to CSCs are of great interest for stem cell therapy. The so-called brain cancer stem cell, with high-proliferative capacity, self-renewal properties and multilineage potential, could be responsible for tumor development (Flores *et al.,* 2009). The signal transduction pathways assessed in this study will explained in detail latter.

1.3 Cancer and Cancer Stem Cells: An Overview

The growth and development of each cell in the body is tightly regulated. In most cases, abnormal cells are recognized and removed by the immune system. Rarely, a defective cell will acquire the ability to grow in an uncontrolled manner. In these cases a body of cells or tissue is formed. In many cases this body of cells is termed 'benign' and can be removed with little danger to the individual. However, in some cases these defective cells develop into a malignancy or cancer.

Cancer is one of the most devastating diseases worldwide, where more than 10 million new cases of cancer are reported each year. The World Cancer Report (WHO, 2000) indicated that 5.3 million men and 4.7 million women are develop malignancy annually, which is expected to grow by 50% by 2020. The World Health Organization estimates that 6.2 million people died of cancer in 2000 and 7.6 million in 2005 and 84 million people will die in the next 10 years if action is not taken (WHO).

Tumours consist of a heterogenous collection of cells that contribute differently to the generation of the tumour. This spawned the 'Cancer Stem Cell theory'. This theory suggests that some, and possibly all, tumours are derived from a population of cells with properties similar to stem cells (Reya *et al.,* 2001). Such cells can self-renew in a similar manner to somatic cells. Non-malignant or 'normal' stem cells contribute to the constant growth and development of the body. Cancer stem cells, similarly contribute to the growth and development of tumours. When injected into immunocompromised mice, a single CSC is sufficient to generate a tumour. This has led to the belief that CSCs are the driving force behind tumourigenesis. Undifferentiated CSCs represent a small proportion of the tumour, the

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size of which appears to be related to the aggressiveness of the specific malignancy (Quintana *et al.*, 2008). CSCs have been isolated from multiple types of cancer, suggesting that they are indeed responsible for most, if not all, malignancies. Worryingly, CSCs appear to be ideally suited to generating tumours, displaying rapid growth through extensive rounds of self-renewal and differentiation, chemoresistance and an ability to grow in the low oxygen 'hypoxic' environment found in tumours (Andrews *et al.*, 2005 and Berry, 2008). Clearly, CSCs are an aspect of cancer biology that must be fully characterized to allow specific CSC targeting as part of cancer treatment.

1.3.1 Cancer Stem Cells and Metastasis

During their development, many cancers have the ability to spread to another site in the body or 'metastasis'. Only malignant tumor cells have the capacity to metastasize (Thomas *et al.*, 1999, Anjomshoaa *et al.*, 2009 and Tsuyuki *et al.*, 2010). Metastases may occur via the blood or the lymphatic system or through both routes. CSCs are dramatically more efficient at tumourigenesis than differentiated tumour cells (Reya *et al.*, 2001). In general, CSCs can regenerate tumours in immuno-compromised mice as quickly as three to four weeks while differentiated cells take many months (Kleinsmith *et al.*, 1964 and Al-Hajj *et al.*, 2003). This leads to a widely respected hypothesis that CSCs are responsible for metastasis. Experimental evidence for CSC involvement in metastasis has been obtained in breast cancer (McDermott *et al.*, 2010). Two theories of CSC metastatic mechanisms are currently proposed, both of which address the circulating tumour cell (CTC) model (Allen *et al.*, 2010). This model proposes that cancer cells must have the ability to circulate the body, most likely in the bloodstream, in order to develop tumours at new sites. The first theory proposed that undifferentiated CTCs are undifferentiated CSCs that can remain dormant while circulating, undergoing extensive self-renewal and differentiation to generate a new tumour upon arrival at the metastatic site. The second suggests that differentiated cancer cells circulate and can de-differentiate upon arrival at the metastatic site (Martin *et al.*, 2010). From these de-differentiated (undifferentiated) CSCs, a tumour can form as before. This model is receiving increased attention due to the recently established relationship between CSCs and epithelial mesenchymal transition (EMT), a process that is essential for metastasis (reviewed in Kalluri and Weinberg, 2009). In terms of CSC biology, EMT is one potential mechanism of de-differentiation, which is highly controversial among stem cell biologists (Bapat *et al.*, 2010).

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Whether metastasis occurs through differentiation, de-differentiation or both, CSCs are a very strong candidate driving force behind the metastatic disease process.

1.3.2 Cancer Stem Cells and Recurrence

Some malignancies, such as ovarian cancer, have highly successful treatment regimes for primary disease, only to be thwarted by high mortality levels due to chemoresistant metastatic disease. Up to 80% of ovarian cancer patients will recover from primary disease. In contrast, up to 80% of these women will develop recurrent chemoresistant disease from which they will not recover (Jemal *et al.*, 2008, Bray *et al,* 2002 and Parkin *et al.*, 2005). Due to their chemoresistant and tumour regenerative properties, CSCs are a candidate cell from which recurrent disease may arise. Ovarian cancer is treated with surgery in combination with cisplatin and paclitaxel chemotherapy drugs. Our lab and others have shown that CSCs can survive, and in some cases thrive, in high concentration of cisplatin and paclitaxel (Mayer *et al.*, 2005). As such, it is entirely possible that a small population of CSCs could survive surgical and chemotherapy intervention. We have already mentioned that single CSCs are sufficient to regenerate malignant disease in marine model. Additionally, each cell that develops from the chemoresistant CSC (that survives primary treatment) may itself be chemoresistant. As such, we can see how CSCs are ideally suited to drive chemoresistant recurrent disease, with devastating consequences for the patient. Clearly, CSCs must be specifically targeted to increase survival rates for cancer patients.

1.3.3 Cancer Stem Cells and Cancer diagnosis

Cancer stem cell research is geared towards cancer diagnosis and therapy. Generally, the earlier a cancer is detected the better the prognosis for the patient. Many malignancies are asymptomatic, where patients only present with advanced disease. As the driving force of tumourigenesis, CSCs are an obvious target for detection strategies. Ultimately, detection of the CSC markers may allow the early detection of malignancy. However, before this can be facilitated we must characterize the molecular biology of CSCs (Bapat, 2010).

A good example of molecular diagnostics is breast cancer. When women are diagnosed with breast cancer they are immediately triaged into one of several treatment groups (Stone *et al.,*

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2007). This is based on the observation that certain breast cancer patients responded to certain treatments and not to others. Starting with the breast cancers that expressed or lacked expression of the Her2neu gene, this has developed into advanced molecular diagnostics with improved survival rates (Ross *et al.*, 2002). Ovarian cancer may similarly be open to triage based on molecular diagnostics. In recent years epithelial ovarian cancer (EOC) has been divided into two classes. Type I EOC is believed to the laden with CSCs, which are absent in type II (Pan *et al.*, 2008). Type 1 cells were recently found to have increased chemoresistance (Alvero *et al.*, 2009a). When introduced into immuno-compromised mice, type 1 cells efficiently generated tumours with high levels of vasculature (Alvero *et al.*, 2009b). The treatment of EOC looks to be heading towards triaging into types I and II, from which patients will receive different treatment regimes. In overview then, a comprehensive understanding of CSCs biology could realistically lead to sensitive detection of malignancies containing CSCs, with obvious benefits for the patient.

1.3.4 Cancer Stem Cells and Gene Therapy

At the moment it is hard to protect healthy cells from the harmful effects of cancer treatment. The aim of such treatment is the prevention of side effects of cancer treatment, while identifying anticancer drugs that can be directed to kill or eradicate only cancer cells. The major question we have to answer is how to target cancer cells or cancer stem cells without affecting normal cells? One such approach is targeted gene therapy.

Gene therapy uses genetic engineering for the introduction or elimination of specific genes by using molecular biology techniques to physically manipulate genetic material to alter or supplement the function of an abnormal gene by providing a copy of a normal gene, to directly repair such a gene, or to provide a gene that adds new functions or regulates the activity of other genes. In theory, targeting of a specific gene required by CSCs could eliminate CSCs without harming other cells in the body.

The potential success of gene therapy technology depends not only on the delivery of the therapeutic transgene into the appropriate human target cell, but also on the ability of the gene to function properly in the cell (Morgan *et al.*, 2006 and Kallai *et al.*, 2010). Before gene therapy technology can be used, genes must be analysed to identify specific gene targets.

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Therefore, in this study those genes involved in CSC differentiation were analysed to identify of targetable CSC-specific events for use in anti-cancer therapeutics.

1.4 Embryonal carcinoma stem cells: the teratoma model of stemness.

The embryonal carcinoma (EC) model is the most studied cancer stemness system available today (Josephson *et al.*, 2007). Originally derived from human teratocarcinoma by Peter Andrews and colleagues, it is so well characterised that EC cells have been proposed as an internal comparator system for all pluripotent stem cell analysis (Josephson *et al.*, 2007).

1.4.1 Teratoma and Malignant teratoma (Teratocarcinoma)

The Greek term 'teratoma' refers to a 'monstrous' tumor, which perfectly characterizes teratomas. These germ cell tumours are composed of different types of tissue, representing all three germ layers. At the macroscopic level, malignant teratomas or 'teratocarcinomas' can contain hair, teeth and even primitive body parts, demonstrating the degree of development involved (Liberis *et al.*, 2008 and Ohta *et al.*, 2009). Teratocarcinomas are most frequently observed in the testis and more rarely in the ovary and may be mature (well-differentiated) or primitive (immature). These can range from benign (mature, dermoid and cystic) to malignant (immature and solid).

In rare cases a pure 'embryonal carcinoma' can occur, which is described as being almost totally composed of undifferentiated EC cells. In terms of CSC study, embryonal carcinomas are another important cancer type. Malignant germ cell tumours are generally composed of teratomas and embryonal carcinomas in varying proportions (O'Hare, 1978). Embryonal carcinomas are considered to be reproductive cells that have lost regulation. This can lead to the development of a mostly undifferentiated EC (Andrews *et al.*, 2005).

Teratocarcinomas and embryonal carcinomas have become very important in the analysis of CSCs. The best characterised CSC model is that of EC cells, which are derived from

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teratocarcinomas (Josephson *et al.*, 2007). As early as 1964, a single teratocarcinoma cell was isolated and transplanted to a new host mouse and found to be sufficient to regenerate a new tumour (Kleinsmith *et al.*, 1964). Extensive studies during the 1970s also showed a close relationship between EC cells from murine teratocarcinomas and the non-malignant stem cells of the ICM (inner cell mass) cells of the blastocyst stage of early mouse embryos (Dunia *et al.*, 1979).

1.4.2 Embryonal Carcinoma Stem Cells

EC cells are the pluripotent or nullipotent stem cells of EC cancers, which have the ability to self-renewal and to differentiate (pluripotent) or to avoid differentiation (nullipotent) during tumor development, as will now be described. Once differentiated, these cells lose their tumorigenicity, indicating that undifferentiated EC cells represent a key component of this malignancy (Andrews *et al.*, 2005). To date, EC cells are the best characterised CSC model and have be used to elucidate much of our current understanding of CSC biology. There are two types of EC cell, pluripotent and nullipotent, as now described.

1.4.3 Pluripotent and Nullipotent Embryonal Cancer Stem Cells

Pluripotent stem cells have the capacity to self renew and to differentiate to cells representative of all three somatic germ layers. Embryonic stem cells are the most studied pluripotent stem cell. Human EC cells are also pluripotent and closely resemble ES cells (Draper *et al.*, 2002, Henderson *et al.*, 2002 and Andrews *et al.*, 2005). Nullipotent CSCs have been isolated from embryonal carcinomas. These cells exhibit all the highly-malignant properties of CSCs (efficient tumor generation, hypoxia-resistance and chemo-resistance etc) but can avoid differentiation. This, alarmingly, results in the generation of an almost entirely undifferentiated tumor. As such, embryonal carcinomas, and nullipotent EC cells, are considered to be highly-malignant. In this study, we explore the nature of the genetic changes that promote nullipotency by comparing two human EC cell lines: a 'pluripotent' line, NTera2 and a 'nullipotent' line, 2102Ep.

1.4.4 Pluripotent Cell line (NTera2)

Pluripotent stem cells are derived from pre-implantation embryos, primordial germ cells or teratocarcinomas. The Human NTera2 cell line is pluripotent and malignant and was originally

derived from a human testicular teratocarcinoma. The parental NTera2 line was established in 1980 from a nude mouse xenograft of the Tera-2 cell line (Andrews *et al.*, 1984, Thomson *et al.*, 1996 and Thomson *et al.*, 1998). This clone differentiates along neuroectodermal lineages after exposure to RA (Andrews *et al.*, 1982 and Andrews *et al.*, 1984).

Pluripotent stem cells are, by definition, functionally pluripotent: they can produce structures containing tissues representative of all three germ layers. Several studies have definitively demonstrated upregulation of maker genes indicative of endodermal, mesodermal and ectodermal differentiation post-RA induced differentiation (Andrews 2002 and Andrews *et al.*, 2005). The studies have generally assessed gene expression changes at approximately 1 week differentiation or later. However, the characterisation of earlier events in differentiation has received less attention. We initially asked whether markers of differentiation were detectably upregulated earlier than 1 week differentiation in pluripotent NTera2 CSCs.

1.4.5 Nullipotent Cell line (2102Ep)

The human EC cell line 2102Ep is "relatively" nullipotent and expresses most of the same genes as undifferentiated hES and NTera2 cells (Matin *et al.*, 2004 and Josephson *et al.*, 2007). The 2102Ep cell line was originally derived from a testicular teratocarcinoma and reproducibly forms EC tumors when injected into immuno-compromised mice. NTera2, but not 2102Ep, EC cells differentiate in response to RA (Andrews *et al.*, 1982 and Andrews *et al.*, 1984). A hybrid derived by fusion of these cells differentiates in response to retinoic acid but, unlike the parental NTera2 line, does not form terminally differentiated neurons (Andrews *et al.*, 2005). This implies that the nullipotent EC cell line, 2102Ep, differs in expression of at least two functions in comparison with the NTera2 pluripotent line, one affecting commitment to differentiation, and one affecting terminal neural differentiation (Bahrami *et al.*, 2005).

To balance self-renewal and differentiation, EC and ES cells must carefully control the levels of three transcription factors, Nanog, Sox2, and Oct4 (Pereira *et al.*, 2006). Together, these are referred to as the "master pluripotency regulators". Their importance is such, that they are described in detail in specific sections later (section 1.5). In this project, the two cell lines (NTera2 and 2102Ep) were used to explore the genetic changes when the cells are stimulated to differentiate.

1.5 Molecules Assessed

1.5.1 Key Stemness Genes (Oct4, Sox2 and Nanog)

The most important regulators of pluripotency are Oct4, Sox2 and Nanog, which act in concert to maintain the self-renewal state. As we will now detail, loss of any of these genes is sufficient to remove pluripotency from ES and EC cells. Oct4 and the other two key stemness genes (Sox2 and Nanog) play a crucial role in pluripotent cells. Knockdown of any of this trio of key stemness genes drives pluripotent cells towards differentiation, while their over-expression in pluripotent cells leads to maintenance of the self-renewal state following stimulation to differentiation (Hyslop *et al.,* 2005 and Fong *et al.,* 2008).

1.5.1.1 Octamer-binding transcription factor 4 (Oct4)

Oct4 is an abbreviation of Octamer-4. Octamer-binding transcription factor 4 plays a critical role for maintaining the pluripotent and the self-renewing state of stem cells. The POU domain transcription factor OCT4 is a key regulator of pluripotency in the early mammalian embryo and is highly expressed in the inner cell mass of the blastocyst (Babaie *et al.,* 2007). The Oct4 gene is expressed in human cancers, embryonic stem cells, germ cells and tumor cells but not in cells of differentiated tissues (Nichols *et al.,* 1998, Tai *et al.,* 2005 and Suo *et al.,* 2005) and is frequently used as a marker for undifferentiated pluripotent cells. Oct4 expression is rapidly downregulated during formation of the trophoblast lineage (Babaie *et al.,* 2007). Oct4 plays a critical role in maintaining pluripotency and the self-renewing state of stem cells and is expressed in human cancers. Pseudogenes Oct4-pg5 and Oct4-pg1 are involved in the regulation of Oct4 gene activity, which may pertain to carcinogenesis (Suo *et al.,* 2005). Oct4 and SF-1 are co-expressed in undifferentiated human embryonal carcinoma NCCIT cells and the downregulated during retinoic acid-mediated differentiation (Yang *et al.,* 2007).

1.5.1.2 Sox2

Sox2 is a member of the SOX gene family of developmental regulators. Sox genes are specifically those that match the high mobility group box (HMG) of a gene involved in sex determination called SRY. SRY (Sex-determining Region Y) is a sex-determining gene on the Y chromosome in humans and other primates (reviewed in Wang *et al.,* 2010). The SRY gene encodes testis determining factor, which is also referred to as the SRY protein (reviewed in Wang *et al.,* 2010). *In vitro* Sox is capable of inducing oncogenic transformation of fibroblast cells and *in vivo* Sox genes are associated with a large number of tumour types (Dong *et al.,* 2004). Sox2 is a transcription factor that is essential to maintain self-renewal of undifferentiated embryonic stem (ES) cells and also Sox-2 plays an important role in supporting gene expression in ES cells, especially by forming a complex with embryonic Oct4 (Tomioka *et al.,* 2002).

Sox2 appears to be essential for multipotent stem cell types in the early embryo (blastocyst). Sox2 is one of the transcription factors involved in the specification of the three embryonic cell lineages. Sox2 is required to maintain cellular pluripotency both in the developing embryo and in embryonic stem cells. Expression of both genes (Sox2 and Oct4) are required in the inner cell mass (ICM) and epiblast. An adjacent pair of highly conserved Octamer- and Sox-binding sites was found to be essential for activating pluripotential state-specific gene expression (Kuroda *et al.,* 2005). High levels of Sox2 expression are detected in undifferentiated EC stem cells (NTera2) and is down-regulated when differentiated with retinoic acid for 24h (Stevanovic, 2003).

1.5.1.3 Nanog

Nanog (named after the Tir na nOg legend) is a gene expressed in ES cells and is a key factor encoding a homeodomain-bearing transcription factor required for maintaining the pluripotency and undifferentiated state of stem cells (Kuroda *et al.,* 2005). Overexpressing Nanog allows ES cells to self-renew in the absence of the otherwise obligatory LIF and BMP signals (Chambers *et al.,* 2004). Nanog functions in concert with Oct4 and Sox2 to establish ES cells identity. Embryonic stem cells are controlled by co-operation of three transcription factors, known as Oct4, Sox2 and Nanog. The transcription factors Oct4, Sox2, and Nanog

have essential roles in early development and are required for the propagation of undifferentiated ES cells in culture. Oct4 and Nanog are highly expressed in the testicular germ cell tumor (seminoma) and breast carcinoma and expressed at very low levels in the normal testis and breast tissue (Ezeh *et al.*, 2005).

The expression of these three key stemness genes was assessed in this study during differentiation of both cell lines (pluripotent and nullipotent) over a differentiation time period and used to establish normal gene expression patterns for use in the knockdown and overexpression analyses in chapter three.

1.5.2 Differentiation Markers (Ncam1, Eno3, Afp and Gata6)

The differentiation of stem cells must be confirmed during all analyses. In ES and EC cells, this is shown through downregulation of pluripotency genes Oct4 and Nanog and upregulation of genes indicative of endodermal, mesodermal and ectodermal differentiation.

1.5.2.1 Ectoderm Marker (Ncam1)

Ncam1, called Neural Cell Adhesion Molecule 1 or cluster of differentiation 56 (CD56), is a homophilic binding glycoprotein expressed on the surface of nerve cells (neurons), glia (neuroglia) and skeletal muscle. Ncam1 has been implicated as having a role in cell-cell adhesion, neurite outgrowth, synaptic plasticity, learning and memory. Ncam1 is known to play important roles in cell migration, neurite growth, axonal guidance, and synaptic plasticity. Disturbance of these neuro-developmental processes is proposed as one etiology for mood disorder (Arai *et al.,* 2004).

Ncam1 protein is not expressed within the neural tube early in human embryos, but it is expressed in the surrounding and later in differentiated neurons of the CNS (Deak *et al.,* 2005). Ncam1 is expressed during primitive neuroectoderm formation. The neural cell adhesion molecule appears on early embryonic cells and is important in the formation of cell collectives and their boundaries at sites of morphogenesis. Later in development, it is found on various differentiated tissues and is a major cell adhesion molecule mediating adhesion among

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neurons and between neurons and muscle. The differentiation marker gene Ncam1 (ectoderm) is expressed after 3 days of differentiation in hES cells (Abeyta *et al.*, 2004).

1.5.2.2 Mesoderm Marker (Eno3)

Enolase 3 (Eno3) is a marker of mesodermal differentiation that is expressed in adult human muscle. Over 90% of enolase activity is accounted for by the beta-enolase subunit, the protein product of the Eno3 gene (Comi *et al.*, 2001). Four major enolase isozymes have been identified in human tissues. The M isozyme is the major form found in skeletal muscle and heart extracts. Eno3 is one of the three genes involved in the determination of human enolase (Pearce *et al.*, 1976). The major portion of the elevated plasma beta-enolase was derived from heart muscle (Usui *et al.*, 1989). This gene was identified as being rapidly upregulated in RA-treated NTera2 cells in a microarray study and was exploited as a useful mesoderm marker (Gallagher *et al.*, In prep).

1.5.2.3 Endoderm Marker (Afp)

Alpha-fetoprotein (Afp) was first identified in human fetal sera in 1956 (revieded by Crandall, 1981). Alpha-fetoprotein is the most abundant serum protein in the developing embryo. It is secreted by the visceral endoderm. Afp gene regulatory elements might serve to effectively drive reporter gene expression in developing endodermal tissues (Kwon *et al.*, 2006). Afp is synthesized by the yolk sac endoderm of the embryo, fetal liver hepatocytes and in liver tumors (Abelev, 2001). Afp is expressed in the genome of mesenchymal stem cells at an early stage (Sato *et al.*, 2005). Increased Afp gene expression occurs in humans suffering from chronic liver disease and is considered to be a marker for hepatocellular carcinoma (Hellerbrand *et al.*, 2001 and Hu KQ *et al.*, 2004). Afp is expressed in both immature teratomas and mature teratomas (Hiroshima *et al.*, 2001). Afp is upregulated through a cascade involving the Gata6 gene, which was also used as an endodermal differentiation marker.

1.5.2.4 Endoderm marker (Gata6)

Gata6 belongs to a family of zinc finger transcription factors that play important roles in transducing nuclear events that regulate cellular differentiation and embryonic morphogenesis in vertebrate species. Gata6 is required for establishment of the endodermally derived bronchial epithelium (Morrisey et al., 1998).

Several studies have suggested that Gata6 has an integral role in controlling development of the mammalian liver (reviewed in Morrisey et al., 1998). Gata6 is expressed in multiple tissues that impact development of the liver, including the heart, septum transversum mesenchyme, and vasculature; all are relatively unaffected by loss of Gata6, which is consistent with a cell-autonomous requirement for Gata6 during hepatogenesis (Zhao et al., 2005 and Sumi et al., 2007). Gata6 and Gata4 are functionally redundant during hepatic specification, but Gata6 alone is available for liver bud growth and commitment of the endoderm to a hepatic cell fate (Zhao et al., 2005). Gata6 can act as a positive or negative regulator of smooth muscle cell SMC-specific gene expression.

The Gata-4/5/6 family of transcription factors are important for the development of the cardiovascular system and the visceral endoderm (reviewed in Morrisey et al., 1998). Gata6 is the only family member expressed at high levels in vascular smooth muscle cells and is important for controlling the phenotype of these cells following vascular injury (Yin et al., 2004). Gata6 plays a critical role in the maintenance of the differentiated phenotype in vascular smooth muscle cells (Du et al., 2003). The high level of Gata6 expression in vascular smooth muscle explains the relatively low levels of telokin expression in the vascular system (Yin and Herring 2005). Overexpression of Gata6 in smooth muscle cells selectively inhibited expression of endogenous telokin, while simultaneously increasing expression of other smooth muscle proteins (Yin et al., 2004). Gata6 can interact directly with Serum-response factor (SRF)-associated myocardin to further enhance or modulate promoter activity. SRF is a member of the MADS (MCM1, Agamous, and Deficiens, SRF) box family of transcription factors that are important regulators of many genes associated with cell growth and differentiation. SRF is required for smooth muscle differentiation (Wang et al., 2001 and Wang et al., 2003). SRF is enriched in cardiac, skeletal, and smooth muscle progenitor cells during embryogenesis, as well as in terminally differentiated adult muscle cells (Croissant et

al., 1996 and Belaguli *et al.*, 1997). Targeted disruption of the mammalian SRF gene leads to malformation of the mesoderm, indicating a critical role for SRF in mesoderm development (Weinhold *et al.*, 2000).

Gata6 expression in vascular smooth muscle cells is rapidly down-regulated upon mitogen stimulation or vascular injury (Du *et al.*, 2003). Adenovirus-mediated Gata6 gene transfer to the vessel wall after balloon injury partially inhibited lesion formation and reversed the down-regulation of Sm-MHC, smooth muscle α-actin (SM α-actin), calponin, vinculin, and metavinculin expression that is normally associated with injury-induced vascular smooth muscle cells phenotypic modulation (Du *et al.*, 2003).

In this study, we hypothesized that the expression of the three germ layer marker genes would be altered in pluripotent but not in nullipotent cells. All the markers of differentiation were expected to increase following differentiation treatment. Expression of differentiation marker genes Ncam1 (ectoderm), Eno3 (mesoderm) and Afp and Gata6 (endoderm) were studied during RA-induced differentiation of pluripotent and nullipotent human EC cells over a period of time and to establish normal gene expression patterns for comparison with knockdown and overexpression analyses.

1.5.3 Key Stemness pathways

Stem cells commonly employ a group of signal transduction pathways during their self-renewal and differentiation. In most cases, genes of importance to stem cells affect their potency and/or differentiation via regulation of these signal transduction pathways. In this section we detail these pathways, which will be used as read-outs for functional analyses later.

In general, signal transduction pathways intercept a signal or 'ligand' via a receptor on the cell surface and transmit this signal to the nucleus via a mediator molecule whereupon gene expression in the nucleus is altered. Our group's EC differentiation microarray data reported that four pathways (TGF-β, Shh, Notch and Wnt) as well as key stemness modulator Snail and the cancer modulator Pten were all highly up-regulated in early (3 Days) differentiation of pluripotent EC cells (Gallagher *et al.*, 2011). Therefore, these pathways and modulators were chosen to be analysed in pluripotent and nullipotent CSCs.

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1.5.3.1 Transforming growth factor beta pathway (TGF-β)

The transforming growth factor beta (TGF-β) signaling pathway is involved in many cellular processes in both the adult organism and the developing embryo including cell growth, cell differentiation, apoptosis, cellular homeostasis and other cellular functions (reviewed in Clark *et al.*, 2009). TGF-β can be found in many different tissue types, including brain, heart, kidney, liver and testes (reviewed in Clark *et al.*, 2009). Overexpression of TGF-β can induce renal fibrosis, causing kidney disease and diabetes.

The TGF-beta pathway incorporates several signaling pathways that share most components of a central signal transduction engine. The general signalling scheme is rather simple: upon binding of a ligand, an activated plasma membrane receptor complex is formed, which passes on the signal towards the nucleus through a phosphorylated receptor SMAD (R-SMAD) (**Figure 1.1**). In the nucleus, the activated R-SMAD promotes transcription in a complex with a closely-related helper molecule termed the CO-SMAD. TGF-βR signaling is regulated by both positive and negative acting sites in the type I and type II receptors (TGF-beta-R1 and TGF-beta-R2) (Heldin *et al.*, 1997). TGF-β superfamily ligands bind to two different serine/threonine kinase receptors type I and type II receptors (TGF-beta-R1 and TGF-beta-R2). Upon ligand binding, type I receptors specifically activate intracellular Smad proteins. R-Smads are direct substrates of type I receptors; Smads 2 and 3 are specifically activated by activin/nodal and TGF-β type I receptors, whereas Smads 1, 5 and 8 are activated by BMP type I receptors (Ten Dijke *et al.*, 1994, Miyazawa *et al.*, 2002 and Clark *et al.*, 2009). About 30 proteins have been identified as members of the TGF-β superfamily in mammals (Kingsley, 1994 and Ten Dijke *et al.*, 1994). R-Smads form complexes with Co-Smads and translocate into the nucleus, where they regulate the transcription of target genes. AR-Smads bind to various proteins, including transcription factors and transcriptional co-activators or co-repressors, whereas BR-Smads interact with other proteins less efficiently than AR-Smads (Heldin *et al.*, 1997 and Miyazawa *et al.*, 2002). The mechanisms of TGF-β superfamily signaling is important for the development of new ways to treat various clinical diseases in which TGF-β superfamily signaling is involved.

Microarray data in our lab demonstrated that TGF-β pathway was up-regulated during the differentiation of pluripotent EC cells and my analysis demonstrates that regulation of TGF-β pathway is achieved through up-regulation in differentiation in pluripotent cells and is not altered in differentiation of nullipotent CSCs. Up-regulation of TGF-β in differentiated pluripotent (NTera2) cells and its unaltered expression in differentiated nullipotent (2102Ep) cells demonstrates that TGF-β plays an important pathway in this study.
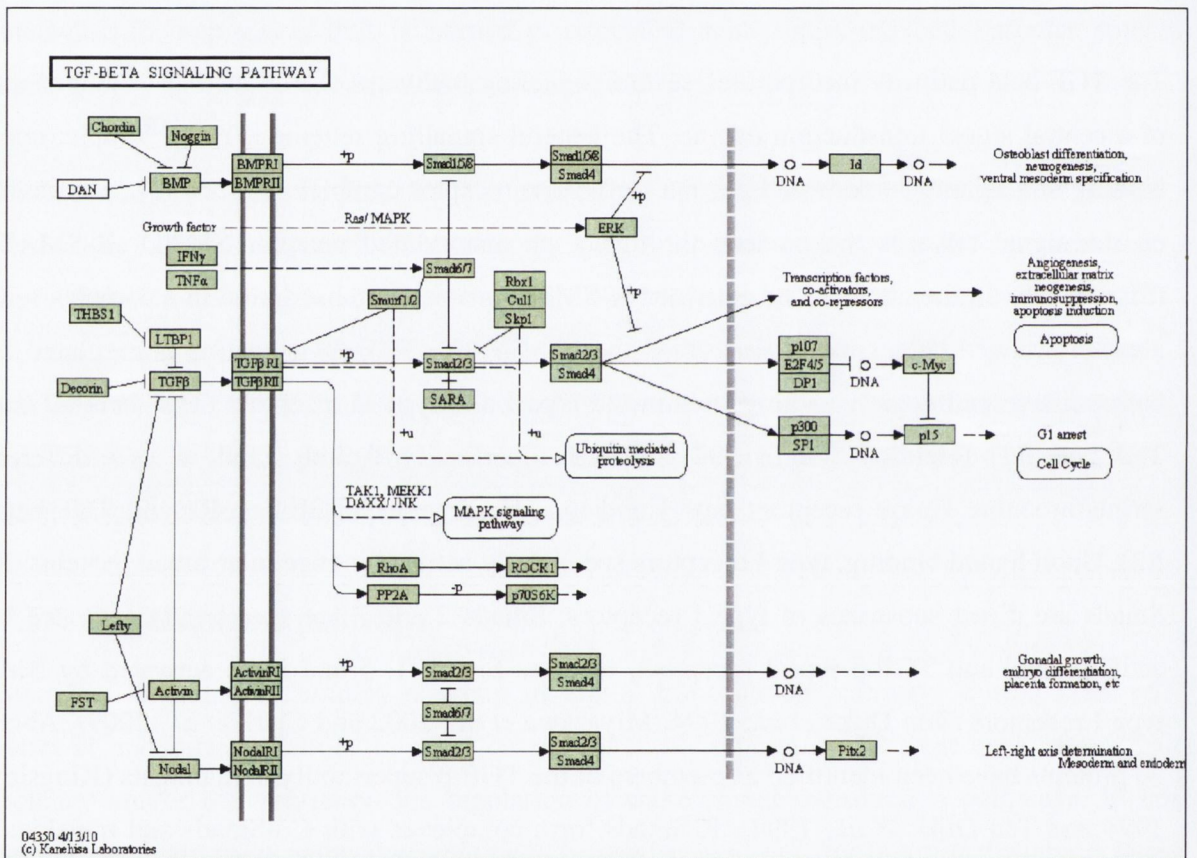
Figure 1.1 Signaling of the TGF-β pathway from receptors to nucleus. The TGF-β pathway has four receptors (BMP, TGF-β, Activin and Nodal) and each receptor includes two types, I and II. Ligand binding to receptors induces phosphorylation and activation of receptor I by receptor II. Then receptor I phosphorylates two SMADs and these two SMADs bind to another SMAD mediator and are translocated to the nucleus. (www.genome.jp).

1.5.3.2 Sonic Hedgehog Pathway (Shh)

Sonic hedgehog (Shh) belongs to the Hedgehog family of signaling molecules that were identified by their homology to the *Drosophila melanogaster* (the fruit fly) segment polarity gene hedgehog in 1978. Shh is one of three proteins in the mammalian signaling pathway family called hedgehog (Dorus *et al.*, 2006). Shh is a highly conserved gene. In mammals, Shh encodes a signaling molecule that plays a central role in developmental patterning, especially of the nervous system and the skeletal system (Dorus *et al.*, 2006). Shh controls cellular differentiation and proliferation in a variety of tissues (Ingham *et al.*, 2001 and McMahon *et al.*, 2003). Hedgehog signaling in thymocytes maintains expression of the transcription factor FoxA2 on pre-TCR signal transduction (Rowbotham *et al.*, 2009). Autoproteolytic cleavage of Shh generates the active NH_2-terminal peptide (N-Shh) that regulates proliferation and differentiation of different cell types in the gut mesenchyme and in neural crest–derived cells (Bitgood *et al.*, 1995). Shh controls the proliferation and differentiation of neural crest cells, human pancreatic stellate cells and mesenchymal cells in the mouse (Yu *et al.*, 2002, Fu *et al.*, 2004 and Bailey *et al.*, 2008). It promotes proliferation and inhibits the differentiation of neural crest cells (Fu *et al.*, 2004). The Hedgehog family of signaling molecules functions in the development of numerous tissues by regulating cellular differentiation and proliferation. Studies have demonstrated that the different components of the Hedgehog signaling pathway are expressed in the human thymus suggesting a role for Sonic hedgehog in human intrathymic T cell maturation (Gutiérrez-Frías *et al.*, 2004). Sonic hedgehog signaling controls many aspects of ontogeny, orchestrating congruent growth and patterning. It controls the behaviour of cells with stem cell properties in the mouse embryonic neocortex, and is implicated in the control of cell proliferation in the adult ventral forebrain and in the hippocampus (Palma *et al.*, 2005). The sonic hedgehog transcription pathway controls cell division of adult stem cells and has been linked to the formation of specific kinds of cancerous tumours such as pancreatic cancer and prostate cancer (Datta *et al.*, 2006 and Bailey *et al.*, 2008).

Mechanistically, the extracellular protein Shh binds to and blocks Patched (PTCH1), a transmembrane receptor, which relieves the inhibition of another transmembrane protein, Smoothened (SMO). Like PTC1, SMO is an obligate component of the pathway, being required for all aspects of HH signal transduction. SMO goes on to activate glioma-associated oncogene homolog 1 (GLI1) and GLI2. GLI-family protein, named Cubitus interruptus (CI).

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CI is present in a complex with the COS2 scaffold protein and then CI transcription factors that travel into the nucleus to activate the expression of genes (**Figure 1.2**).
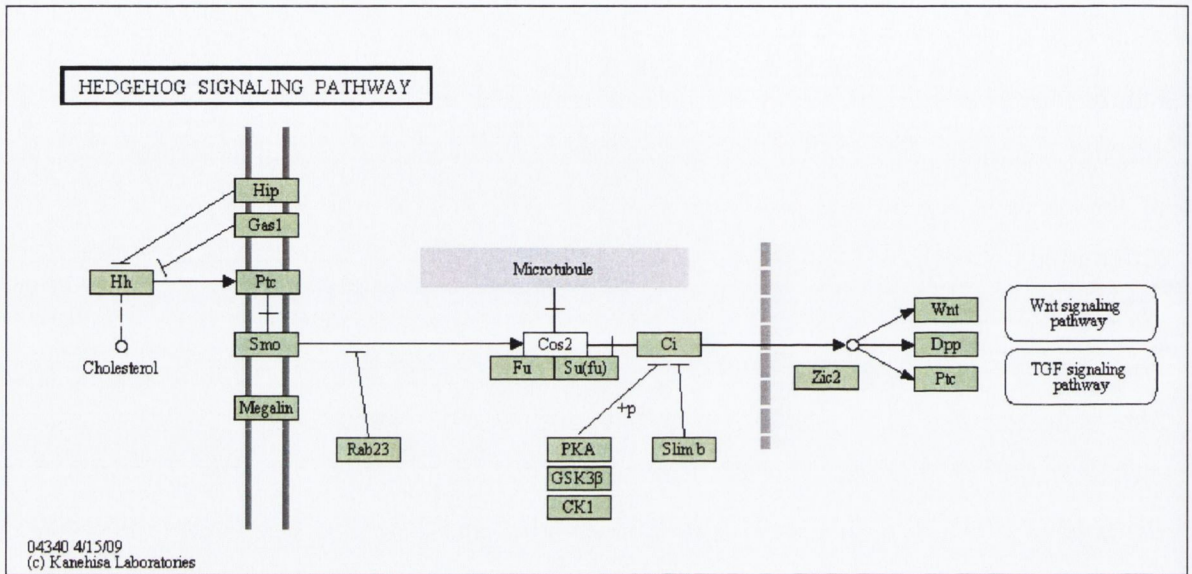
Figure 1.2 Signaling of the Shh pathway. A member of the Hh family binds to patched (Ptc), thus releasing smoothened (Smo) to transduce a signal. Transcriptional activation occurs through the GLI family of proteins resulting in activation of target genes (www.genome.jp).

The hedgehog signaling pathway regulates many processes of development and tissue homeostasis (Nüsslein-Volhard *et al.*, 1980 and Jiang *et al.*, 2008). Activation of hedgehog signaling has been reported in about 30% of human cancers including ovarian cancer assessed by Xie *et al.* (2008). The importance of the Hedgehog signaling pathway in tumorigenesis was established through the discovery of inactivating mutations in the Ptch gene in patients with familial (Gorlin's syndrome) basal cell carcinomas and sporadic BCC (Hahn *et al.*, 1996 and Dahmane *et al.*, 1997). The absence of the ligand Hh, hedgehog receptor (PTCH1 or PTCH2) inhibits smoothened (SMO) signaling. When Hh binds to PTCH1, SMO is able to signal, eventually resulting in formation of activated transcriptional factor Gli (Gli1 and Gli2) molecules and elevated expression of the target genes. Overexpression of the main Hh member Sonic hedgehog (Shh), leading to activation of Smo, has been identified in some gastro-intestinal cancers and pancreatic adenocarcinomas (Berman *et al.*, 2003 and Thayer *et al.*, 2003). Shh and TGF-β are capable of inducing Gli expression (Dennler *et al.*, 2007). Inhibition of hedgehog signaling has been pursued as an effective strategy for cancer treatment

including an ongoing clinical trial in solid tumors, such as ovarian cancer (Von Hoff *et al.*, 2009).

1.5.3.3 Notch Signaling Pathway

The Notch gene was discovered in 1917 in *Drosophila melanogaster*. The Notch signaling pathway is a highly conserved cell signaling system present in most multicellular organisms and plays an important role in neural, vascular, muscular, and endocrine differentiation during embryogenesis (Artavanis-Tsakonas *et al.*, 1999 and Shawber *et al.*, 2004). The Notch signalling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life (Shawber *et al.*, 2004). Notch pathways have been shown to be important in the process of neurogenesis and also in the regulation of self-renewal in ES cells (Shawber *et al.*, 2004). Notch affects the implementation of differentiation, proliferation, and apoptotic programs, providing a general developmental tool to influence organ formation and morphogenesis (Artavanis-Tsakonas *et al.*, 1999). Recent studies have identified that Notch signals control differentiation and self-renewal and control germline stem in *Drosophila* ovary (Song *et al.*, 2007, Lin *et al.*, 2008, and Xie *et al.*, 2008). Notch signaling is also linked to cancer (Miele *et al.*, 2006). The Notch signaling pathway is a conserved intercellular signaling mechanism that is essential for proper embryonic development in numerous metazoan organisms (Miele *et al.*, 2006). Notch signaling pathways have been implicated in the self-renewal and proliferation of hematopoietic stem cells and involved in the maintenance of undifferentiated mouse ESCs (Nemir *et al.*, 2006 and Cerdan *et al.*, 2010).

Mechanistically, Notch is a cell-surface receptor whose ligand 'Delta' is also expressed on the cell surface. Binding of Delta to Notch activates cleavage of Notch at the membrane, thereby releasing the Notch intracellular domain (NICD), which migrates to the nucleus where it functions in transcriptional regulation. The NICD translocates to the nucleus, where it forms a complex with the DNA binding protein CSL, displacing a histone deacetylase (HDAc)-co-repressor (CoR) complex from CSL. Components of an activation complex, such as MAML1 and histone acetyltransferases (HATs), are recruited to the NICD-CSL complex, leading to the transcriptional activation of Notch target genes (**Figure 1.3**).
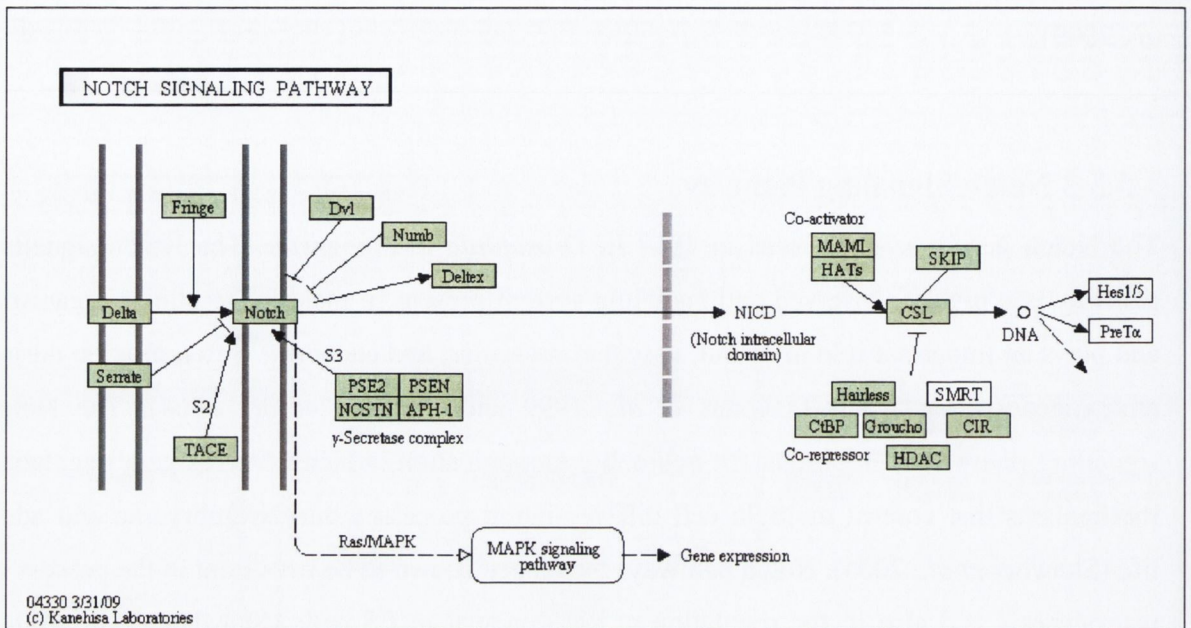
Figure 1.3 The Notch proteins are single-pass receptors that are activated by Delta at the membrane to liberate the Notch intracellular domain (NICD). The NICD translocates to the nucleus, where it functions in transcriptional regulation. NICD forms a complex with the DNA binding protein CSL. The NICD-CSL complex stimulates to the transcriptional activation of Notch target genes (www.genome.jp).

1.5.3.4 Wnt Pathway

Wnt signaling is a critical regulatory pathway in development and disease (**Figure 1.4**). Wnt signaling pathways have been implicated in self-renewal and proliferation of hematopoietic stem cells (Cerdan *et al.*, 2010). Wnt proteins have widespread roles in tissue differentiation and organogenesis. Loosing a signal from Wnt receptors at the cell surface results in perpetuation of the undifferentiated state (Bioani and Schloer 2005). The signaling of this pathway also plays a role in normal adult tissues and in carcinogenesis such as prostate cancer (Wang *et al.*, 2010).

Traditionally, it is assumed that Wnt proteins can act as stem cell growth factors, promoting the maintenance and proliferation of stem cells (Willert *et al.*, 2003). Wnt signalling inhibits neural differentiation of embryonic stem cells and self-renewal of haematopoietic stem cells (Haegele *et al.*, 2003 and Reya *et al.*, 2003). Wnt signaling is important in stem cell differentiation. Studies suggested that Wnt signaling induces differentiation of pluripotent stem cells into mesoderm and endoderm progenitor cells and Wnt proteins act to maintain the

undifferentiated state of stem cells (Nusse, 2008). Activation of the Wnt pathway in mouse embryonic stem cells induces differentiation into multipotent mesoderm and endoderm cells (Bakre *et al.*, 2007).
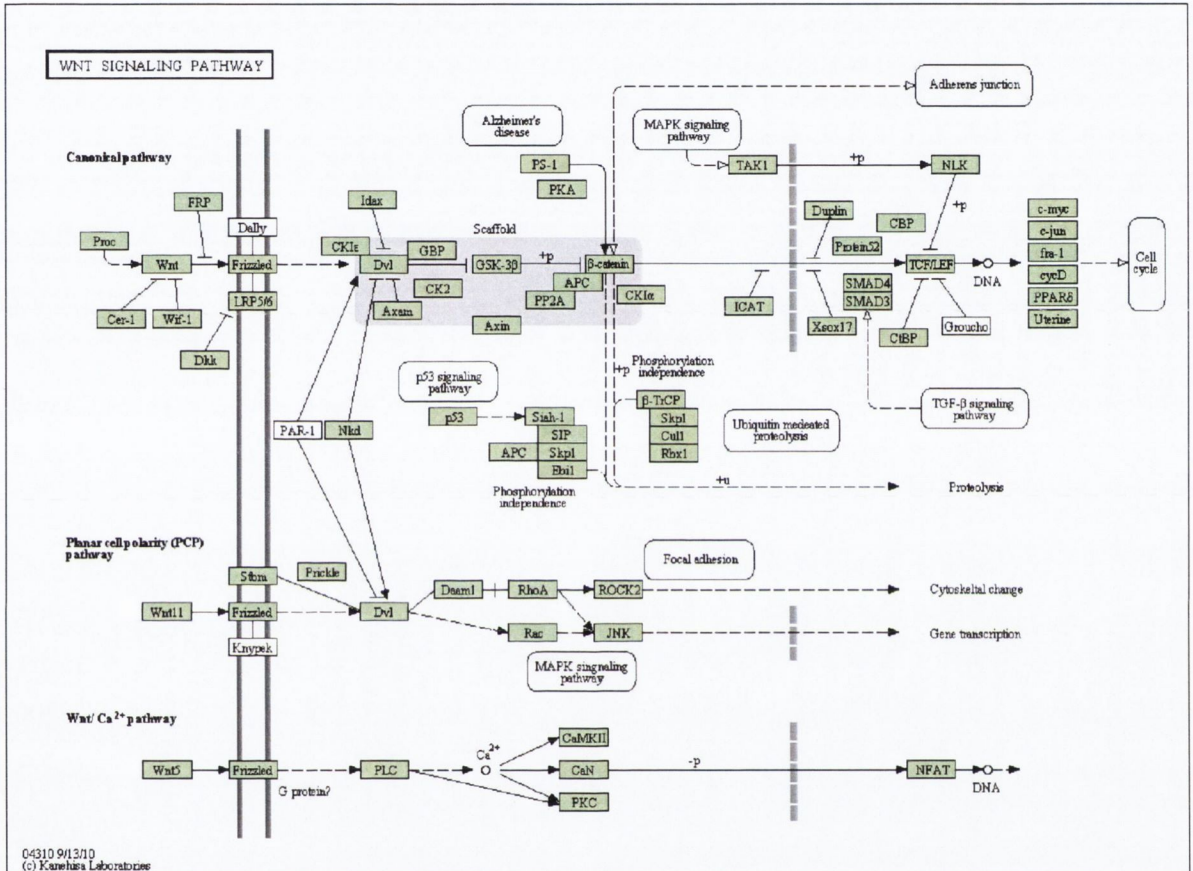
Figure 1.4 The Wnt signaling pathway is one of the most important pathways in stem cell development. In three branches, Wnts act as signaling ligands that are received by membrane-bound receptors such as 'frizzled' proteins. This interaction stimulates the downstream activation of a complex network of modulatory genes that is not fully characterised to date. Wnt is so important that it can stimulate other stemness-related pathways such as TGF-β signalling.

1.5.3.5 Snail

The transcription factor Snail was first described in *Drosophila*, where defects in the invagination of the presumptive mesoderm and of germ band retraction were seen in mutant embryos (Grau *et al.*, 1984). While not a pathway, Snail is such an important modulator in stem cell biology that it is treated with the same importance as the other pathways detailed here. The Snail gene family has been described as playing an important role in development and cancer. Expression of Snail transcription factors can be induced by a variety of different pathways that act on the transcription of these genes (De Craene *et al.*, 2005). Snail is required for mesoderm and neural crest formation during embryonic development and has been implicated in the epithelial-mesenchymal transition (EMT) associated with tumour progression (Blanco *et al.*, 2002). Silencing of Snail blocks TGFbeta(1)+AngII induced epithelial to myofibroblast transformation (Saad *et al.*, 2010). Notch signalling affects Snail in cultured proximal tubular epithelial cells (Saad *et al.*, 2010). Additionally, Snail expression is altered during epithelial-mesenchymal transition in gastric cancer (Rosivatz *et al.*, 2002 and Saad *et al.*, 2010).

Snail is highly expressed in breast carcinoma. Snail may predict a poor outcome in patients who have breast carcinoma metastasis (Elloul *et al.*, 2005). Snail has been found to evoke tumorigenic and invasive properties in epithelial cells on overexpression (Cano *et al.*, 2000). Snail is expressed at the invasive front of skin tumours induced by chemical carcinogenesis in the mouse (Cano *et al.*, 2000). Snail is expressed in infiltrating ductal carcinomas with lymph node metastases, where expression inversely correlates with tumour grade (Blanco *et al.*, 2002). Several studies showed a relationship between Snail expression and cell differentiation *in-vitro* and during early embryonic development (Sefton *et al.*, 1998 and Cano *et al.*, 2000). Well differentiated cell lines derived from human breast and colon carcinomas do not express Snail mRNA, whereas it is expressed in de-differentiated cell lines from breast and melanoma (Cano *et al.*, 2000 and Batlle *et al.*, 2000).

1.5.3.6 Pten

Phosphatase and tensin homologue deleted on chromosome ten (Pten) has a wide function as a tumor suppresser gene and is mutated in many human sporadic cancers and in hereditary cancer syndromes (Premkumar *et al.,* 2006). Having identified Pten on our EC microarrays, we felt that it should be included in our analyses, such is its broad role in development and cancer. The protein encoded by this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (Steck *et al.,* 1997). Pten is a major negative regulator of the PI3K and Akt signaling pathway. Upregulating Pten in glioma cells and in nude mice tumors downregulates PI3K and Akt signaling pathways (Korkaya *et al.,* 2009 and Dasari *et al.,* 2010). Pten is inactivated in many human cancers. For example, Pten suppresses leukaemia stem cells and induces cell-cycle arrest of leukaemia cells (Chen *et al.,* 2010). Pten deletion causes acceleration of chronic myeloid leukaemia development. Overexpression of Pten delays the development of chronic myeloid leukaemia and B-cell acute lymphoblastic leukaemia and prolongs survival of leukaemia mice (Peng *et al.,* 2010). Mutations in Pten are associated with breast cancer (Lynch *et al.,* 1997). During tumor development, mutations and deletions of Pten occur that inactivate its enzymatic activity leading to increased cell proliferation and reduced cell death. Frequent genetic inactivation of Pten occurs in glioblastoma, endometrial cancer, prostate cancer, and reduced expression is found in many other tumor types such as lung and breast cancer (Rhei *et al.,* 1997 and Lynch *et al.,* 1997). In prostate tumors, loss of Pten expression predicts progression to invasive and metastatic disease (Schmitz *et al.,* 2007). Several groups have shown that the cancer pathway Pten is regulated in EC cells (Di Cristofano *et al.,* 1998 and Korkaya *et al.,* 2009).

1.6 MicroRNAs

As well as analysis of gene expression, this thesis explores another aspect of molecular regulation, that of microRNA regulation. MicroRNAs (miRNAs) are single-stranded, non-coding RNA molecules, about 21–23 nucleotides in length and are remarkably similar to small interfering RNAs (siRNAs) (Bernstein *et al.*, 2001). MiRNA regulate gene expression post-transcriptionally. This is usually achieved by miRNAs biding to specific sites within target mRNAs. This results in suspension of the mRNA within the cell or, in rarer cases, mRNA degradation.

MiRNAs are encoded by genes that are transcribed from DNA but not translated into protein (non-coding RNA). The first miRNA (Lin-4) was discovered in 1993 and the second miRNA (let-7) was discovered in 2000 in *C. elegans* (Lee *et al.*, 1993, Reinhart *et al.*, 2000 and Pasquinelli *et al.*, 2000). Since then, different sets of miRNAs have been found to be expressed in different cell types and tissues (Lagos-Quintana *et al.*, 2002). Recently, complementary theories have been introduced, such as the ability of miRNAs to target the mRNA coding region and genomic promoter region to inhibit or promote gene expression respectively (Tay *et al.*, 2008 and Place *et al.*, 2008). The specific mechanisms of miRNA-induced gene silencing and activation have not yet been fully elucidated and are subject to much scrutiny and revision.

1.6.1 The Transcriptional Mechanism of microRNA

The bio-synthesis of miRNAs is shown in Figure 1.5. MiRNAs are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail. Then the Pri-microRNAs are cleaved by the RNaseIII 'Drosha' and co-factor, DGCR8 (DiGeorge syndrome critical region 8 gene) to pre-microRNA (Lee *et al.*, 2004, Gregory *et al.*, 2005 and Zhou *et al.*, 2007). Pre-microRNAs are exported from the nucleus to the cytoplasm by Exportin5 in a Ran-GTP dependent manner. Exportin5 is a protein encoded by the XPO5 gene while Ran is a small protein involved in transport into and out of the cell nucleus (Cai *et al.*, 2004, Lee *et al.*, 2004 and Murchison *et al.*, 2004). In the cytoplasm, pre-microRNAs are further cleaved by another RNaseIII, 'Dicer',

with cofactors (TRBP and PACT in humans), to remove the loop sequence, forming a short-lived asymmetric duplex intermediate (microRNA: microRNA*) (Gregory *et al.,* 2006). The microRNA and microRNA* duplex is in turn loaded into the RNA Induced Silencing Complex (RISC) (Schwarz *et al.,* 2002). The two complementary miRNAs are formed but only one is integrated into RISC and the other degraded (Eulalio *et al.,* 2009) (**Figure 1.5**). MiRNA are partially complementary to mRNA and bind to down-regulate gene expression (Bartel, 2004 and Bartel, 2009).
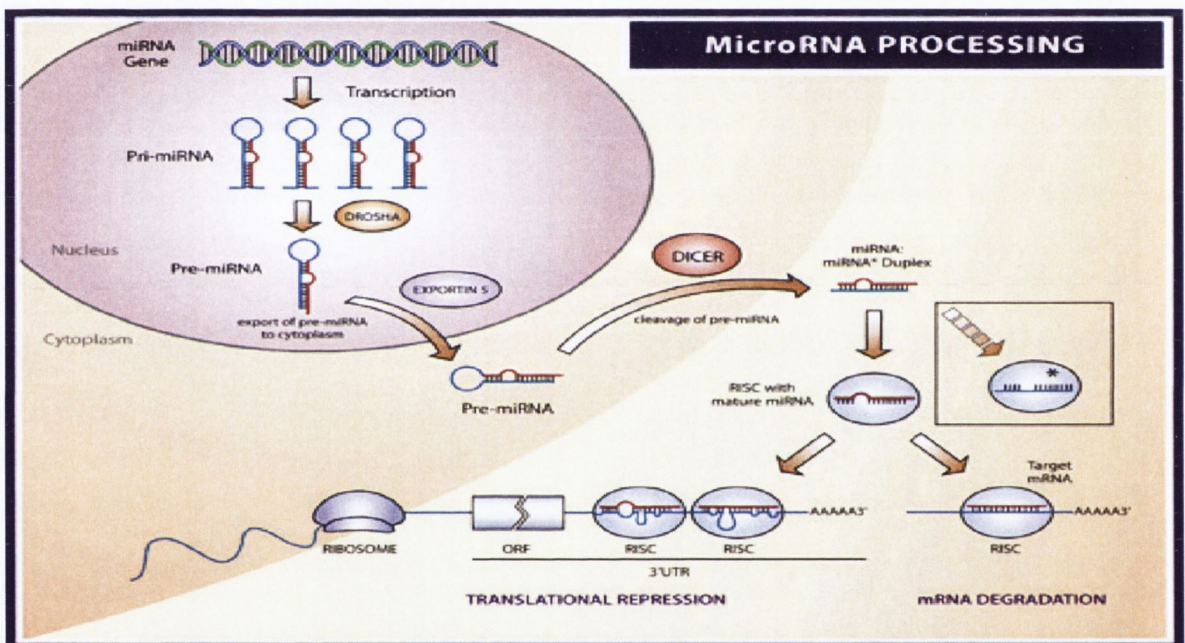
Figure 1.5 miRNA is initially transcribed as pri-miRNA in the nucleus. Pri-miRNA is then cleaved by Drosha and DGCR8 to for pre-microRNA. Pre-microRNA is exported to the cytoplasm by Exportin5 to be cleaved by Dicer with cofactors. The miRNA duplex is in turn loaded into the RISC. Thereafter, the miRNA can bind to mRNA targets perfectly (leading to degradation) or imperfectly (leading to suspension) (Marligen Biosciences www.marligen.com).

1.6.2 Small RNAs control gene expression and affect mRNA transcription

MiRNAs bind to the 3' untranslated region of target genes with imperfect complementarity to prevent their translation. The key component of the RISC is an Argonaute protein (Ago). In the RISC, miRNAs are loaded onto Ago. Ago proteins bind miRNA fragments and the Ago proteins directly interact with the miRNA (Pillai *et al.*, 2004 and Behm-Ansmant *et al.*, 2006). One strand of miRNA is removed via a bypass mechanism allowing Ago to bind the target mRNA. The binding of Ago to target mRNA inhibits translation (Bartel, 2004 and Bartel, 2009). Up to 30% of human genes are regulated by miRNAs (Lewis *et al.*, 2005). Recent data shows that the miRNA loaded RISC can silence translation by targeting sites along the coding region of the mRNA strand complementary to the loaded miRNA (Tay *et al.*, 2008). Controversy also surrounds the methods of mRNA translation silencing by RISC after miRNA-mRNA alignment. One proposed method is by inhibiting translation before initiation by aggregating mRNAs in P bodies or elsewhere (Eulalio *et al.*, 2007). A second theory, gaining much support, is the inhibition of translation by the RISC at a polysomal level after initiation. This has been supported by evidence of the RISC co-precipitating with polysomes in laboratory experiments. These two methods possibly work cooperatively or in parallel. A common feature of miRNA-mRNA hybridisation is the common imperfection of complementarity of the two sequences. The resulting bulge formations are thought to prevent RISC from cleaving the target mRNA. Instead the target is preserved and therefore 'delayed' for expression (Rana, 2007).

1.6.3 Role of miRNA in Stem cells and CSCs

MiRNAs are essential regulators in stem cells, CSCs and malignancy in general (Pasquinelli *et al.*, 2005). Indeed, miRNAs have roles in every biological process in which they have been studied to date. Different populations are expressed in self-renewing and differentiating hES cells and CSCs and in normal versus malignant tissues (Esquela-Kerscher *et al.*, 2006). Several miRNAs has been found to have links with cancer (McManus, 2003). Several groups have demonstrated that overexpression and 'underexpression' of miRNAs are linked to certain cancers (reviewed in Esquela-Kerscher *et al.*, 2006). Several groups have demonstrated that different populations of miRNA are found in many cancers when compared to appropriate normal tissues (reviewed in Esquela-Kerscher *et al.*, 2006). MiRNAs are clearly involved in malignancy and stemness and may thus be key components of cancer stemness. Studies report

that a population of mammalian miRNAs is downregulated in differentiating ES cells, suggesting that they are involved in the maintenance of a pluripotent state (Suh *et al.,* 2004 and Pasquinelli *et al.,* 2005). miRNAs -134, -296 and -470 are upregulated upon RA-induced differentiation of mouse ES cells (Tay *et al.,* 2008). MiRNAs are likely to represent a key target group for specific inhibition of CSCs. However, prior to this study the involvement of miRNAs in early differentiation of CSCs had not been established. Therefore, we analysed the expression of more than 300 miRNAs in both cell lines (pluripotent and nullipotent) in both states (undifferentiated and differentiated) in this study.

MicroRNA expression was assessed using the microRNA TaqMan kit, version 1, from Applied Biosystems, to which our lab was given pre-launch access by the company. This kit contained assays for 330 miRNAs (listed in Appendix Table **7**) known to exist at that time (2007). This was the only miRNA qPCR product available at that time.

1.7 Knockdown and overexpression of molecules of interest

The final aspect of this project involves the knockdown and overexpression of key stemness molecules. Gene knockdown and gene overexpression are genetic techniques in which an organism or cell is engineered to carry genes that have been made inoperative or over-operative respectively. Transfection is the process of introducing nucleic acids (siRNA or plasmid DNA) into cells. There are several different transfection methods used to introduce siRNA or plasmid DNA into cells that depend, in part, on the cell lines and the types of experiments; such as chemical, physical and viral-based delivery system;

1- Chemical (liposome-mediated transfection)

Liposomes are synthetic analogues of the phospholipid bilayer of the cellular membrane. These compounds contain a number of the physical characteristics of phospholipids including the presence of hydrophobic and hydrophilic regions of each molecule which allows for the formation of spheroid liposomes under aqueous conditions. In the presence of DNA or RNA, liposomes are capable of interacting with and encapsulating the nucleic acids thereby creating an efficient delivery system. The liposomal charge, composition and structure, defines the affinity of the complex for the cellular membrane. Under specific conditions, the liposome

complex is able to interact with the cell membrane, which enables its uptake by endocytosis and subsequent release into the cellular cytoplasm.

2- Physical

Electroporation is a technique that employs the use of an electrical field to create transient pores, known as electropores, in the cellular membrane which enables the delivery of charged molecules like RNA or DNA to the cytoplasm and nuclei of the targeted cells.

3- Virus-mediated gene delivery

DNA can be introduced into cells by viral transduction technique using viruses as carriers. This technique is beneficial for transfection but also carry significant bio-hazardous risks.

In this study, a chemical transfection methodology was employed by means of commercially available lipid based transfection agents. Protocols were optimised by other members of the lab and were available for the transfections carried out in this project. Two different transfection agents were used in this study to knockdown and overexpress a specific target protein TGF-beta-Receptor2; Lipofectamine RNAiMAX was used optimally for pluripotent cells (NTera2) and Lipofectamine 2000 for nullipotent cells (2102Ep). The gene knockdown and overexpression techniques developed in this study allow essential investigation of the functional effects of target genes on the stemness of each CSC cell line (NTera2 and 2102Ep).

In this study, we analysed two EC CSC lines (pluripotent NTera2 and nullipotent 2102Ep) to identify genes and miRNAs regulating early differentiation with an ultimate aim of targeting these to remove or reduce stemness from CSCs. We have determined that a subset of stemness-associated genes is involved in regulation of early differentiation in CSCs. The involvement of these genes in early differentiation links them to regulation of CSCs, a key area of interest to our group.

Chapter Two

Materials and Methods

2.0 Materials and methods

This chapter of the thesis gives a detailed account of the procedures that were followed in completing the experiments discussed in the thesis. For some newer techniques some, background information is also provided. Several of the techniques are used in a number of chapters. Where this occurs, the full description of the technique is restricted to this chapter. ID numbers of the assays, antibodies and cloned Plasmid DNA used in this study.

2.1 Media and chemicals

2.1.1 Dulbecco's Modified Eagle Media (DMEM)

Human Embryonal Carcinoma (EC) ('classical stem cell' gonadal tumours) CSCs, originally derived from well (pluripotent 'NTera2') and poorly-differentiated (nullipotent '2102Ep') tumours, were routinely grown at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Media (DMEM) (GIBCO invitrogen Ireland), supplemented with 10% Fetal Calf Serum (FCS), 1% L-Glutamine and 1% Penicillin-Streptomycin (GIBCO invitrogen Ireland). NTera2 cells were removed from the tissue culture flask using a cell–scraper (because NTera2 cells grow in clumps that must be separated). 2102Ep cells were harvested using Trypsin-EDTA (GIBCO invitrogen Ireland). Differentiation was facilitated by the addition of 0.01mM Retinoic Acid (RA). Undifferentiated cells were split every 3-4 days and differentiated cells were re-fed every 3 days with fresh media containing RA.

Both cell types were grown in parallel in differentiated and undifferentiated states. Cells were thawed from storage in liquid nitrogen, initially grown in T-25cm^2 flasks. Cells were grown to 80% confluence where upon they were transferred to a T-75cm^2 flask: cells were generally divided 1:3 or 1:4. Almost confluent flasks were harvested at time zero (T0) and at three days (3D), one week (1W) and two weeks (2W) in the undifferentiated and differentiated states, pelleted and frozen at –80°C. Pellets generally contained $5 \times 10^6 - 1 \times 10^7$ cells.

2.1.2 Opti-MEM® I Reduced Serum Media

Opti-MEM® I Reduced Serum Media was used for transfection agents to knockdown and overexpress genes in both cell lines (NTera2 and 2102Ep). Most cells were grown in serum-supplemented media whereas; Opti-MEM I is a minimum of 50% reduction in serum. Opti-MEM is a modification of Eagle's Minimum Essential Media, buffered with HEPES and sodium bicarbonate and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements and growth factors (Bio-Sciences). This media was used only in transfection studies.

2.1.3 Cell culture protocols and counting

Cells harvested from a T-75 flask were washed with preheated PBS and pelleted in DMEM media. Cells were resuspended in 4mls preheated DMEM medium and kept incubated at 37°C constantly agitating on an agitator to prevent cell adhesion. Two 50µl suspensions were transferred to two fresh sterile 1.5mls microtubes. The two suspensions were diluted with 50µl DMEM media. An equal volume (100µl) of Trypan Blue 0.4% Stain was then added. 10µl of the stained cells were transferred to a haemocytometer (chamber) and covered with a cover glass. Cells were counted in the four outer corner 1mm^2 squares of the haemocytometer under the bright light microscope (**Figure 2.1**). The two numbers were averaged and the cell concentration of the original suspension determined using the following conversion {Cells/ml = (dilution factor). 2500}. A quantity of $6X10^3$ cells (6000 cells/100µl) was plated in a 96-well plate and 168,000 cells/3.2mls were plated in 6-well plate. Cells were seeded in triplicate into 6- or 96-well plates and incubated at 37°C and 5% of CO_2 for 24hrs.
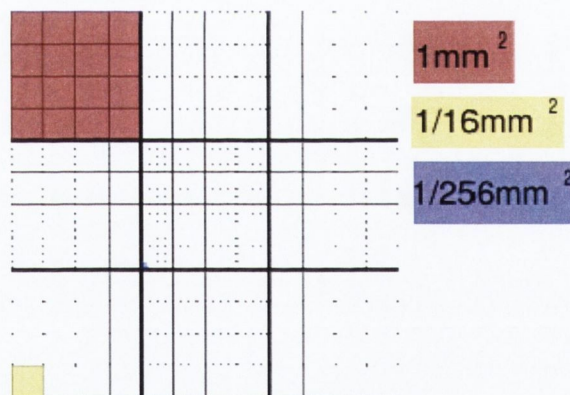
Figure 2.1 Haemocytometer illustrating the different squares within it that are used to count cells (http://commons.wikimedia.org/wiki/File:Haemocytometer_grid.svg).

2.2 Cell Transfection: Principles and Approaches

Transfection is the process of introducing nucleic acids (plasmid DNA or siRNA) into cells. There are several different techniques which can introduce plasmid DNA or siRNA into cells, including biological particules (viruses), electroporation and chemical transfection. In this study, a chemical transfection methodology was employed by means of commercially available lipid based transfection agents. For eukaryotic cells, transfection is better achieved using cationic lipids, because the cells are more sensitive. The popular agent used is Lipofectamine. The efficient method is the inclusion of the plasmid DNA or siRNA to be transfected in liposomes. A liposome is a tiny intracellular membrane-enclosed vesicle that transports substances within a cell. These have a positive surface charge and are made out of the same material as a cell membrane. Generally liposomes are filled with nucleic acids and fuse with the cell membrane, releasing the nucleic acids into the cell. All transfection agents used were cationic lipid formulations and employed the mechanism of cationic lipid-mediated transfection. This mechanism of transfection relies on the structure of cationic lipids, a positively charged head group and one or two hydrocarbon chains. Cationic lipids are often formulated with a neutral co-lipid, which results in a unilamellar liposomal structure with a positive surface charge when formulated in water (**Figure 2.2**). The positively charged surface allows for the interaction between the lipid and the phosphate backbone of the nucleic acid, known as the transfection complex. Additionally, the positive surface charge of the liposomes also mediates the interaction of the nucleic acid and the cell membrane, allowing for fusion of the transfection complex with the negatively charged cell membrane. The transfection complex is then thought to enter the cell through endocytosis. Endocytosis is the process where a localised region of the cellular membrane uptakes the transfection complex by forming a membrane bound/intracellular vesicle. Transfections of plasmid DNA and siRNA in 6-well plates was performed using two different transfection agents: Lipofectamine™ 2000 (Invitrogen) for 2102Ep cells and Lipofectamine™ RNAiMAX (Invitrogen) for NTera2 cells respectively.
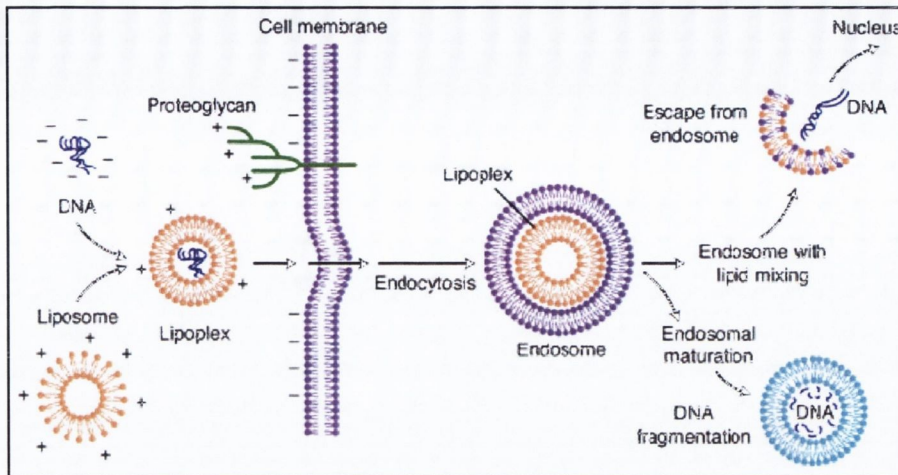
Figure 2.2 Cationic lipids forming micellar structures called liposomes are complexed with nucleic acids to create lipoplexes. The structures fuse with the cell membrane. The complexes are internalised by endocytosis, resulting in the formation of a double-layer inverted micellar vesicle. During the maturation of the endosome into a lysosome, the endosomal wall might rupture, releasing the contained nucleic acids into the cytoplasm and potentially towards the nucleus. (www.journals.cambridge.org).

2.2.1 Efficient transfection of 2102Ep cells in a 6-well plates

Lipofectamine ^{TM} 2000 Transfection Reagent (invitrogen) and a full ORF expression cloned plasmid DNA (victor pDEST26) (Imagenes, Germany) were used to overexpress TGF-beta-R2 and Gapdh in 2102Ep cells. OptiMEM I media, pre-heated to room temperature, was used via the following protocol. First, 7.6µl of Lipofectamine 2000 Transfection Reagent was added to 307µl OptiMEM I media and incubated at room temperature for 10min. Second, 3.9µl of cloned plasmid DNA was added to 316µl OptiMEM I media, which was incubated at room temperature for 10min. The first and the second mixtures (Lipofectamine 2000 and cloned plasmid DNA) were mixed and incubated at room temperature for 10min to facilitate formation of transfection complexes. Old media (DMEM media) was removed from pre-seeded 6-wells plates (plated with 168,000 cells) and the cells washed with OptiMEM I media. 633µl of Transfection Media was transferred to each plate. 2.533ml of OptiMEM I media was used to top up the Transfection Media. The plate was gently rocked and the cells incubated at 37°C and 5% CO_2 for 4hrs. After 4hrs incubation, Transfection Media was removed and replaced with 3mls pre-warmed DMEM media (±RA) and incubated at 37°C for 3days. Non-Transfected Control (N-TC cells) cells and Mock (OptiMEM I media with only Lipofectamine 2000 Transfection Reagent) cells were used as specificity controls.

2.2.2 Efficient transfection of NTera2 cells in a 6-well plate

Lipofectamine™ RNAiMAX Transfection Reagent (invitrogen) and Silencer Select siRNAs were used to knockdown TGF-beta-R2 and Gapdh in NTera2 cells. Silencer Negative control siRNAs (Ambion), which do not target any gene products, were used to as controls to assess the specificity of knockdowns.

OptiMEM I media was pre-heated to room temperature and the following protocol was used. First, 5.15μl of Lipofectamine RNAiMAX Transfection Reagent was added to 308μl OptiMEM I media and incubated at room temperature for 10min. Second, 2.5μl (19nmol) of Silencer Select siRNAs or Silencer Negative control siRNAs were added to 317μl OptiMEM I media and incubated at room temperature for 10min. The first and the second mixtures (Lipofectamine RNAiMAX and siRNAs) were mixed and incubated at room temperature for 10min to facilitate formation of transfection complexes. Old media (DMEM media) was removed from the pre-seeded 6-well plates (plated with 168,000 cells), and the cells washed with OptiMEM I media. 633μl of Transfection Media was added to each plate, which was toped up with 2.533ml of OptiMEM I media. The plate was gently rocked and the cells incubated at 37°C for 4hrs. After 4hrs incubation, Transfection Media was removed and replaced with 3mls pre-warmed DMEM media (±RA) and incubated at 37°C for 3days. Negative control cells (Silencer Negative control siRNA and OptiMEM I media with Lipofectamine RNAiMAX Transfection Reagent) were used as controls to assess specificity of knockdowns.

The overexpressed and knocked-down cells were collected and total RNA and Protein isolated using mirVana ™ PARIS ™ Kit (Ambion).

2.3 Genetic Techniques

Total RNA, miRNA and protein was isolated in a laminar flow hood, using appropriate sterile technique (gloves changed regularly and RNase-free disposable sterile tubes and pipettes) to reduce the possibility of crossover contamination and degradation of nucleic acid (RNA, miRNA and protein). Nucleic acids were purified using the RN*easy* mini Kit (Qiagen, UK and Ireland), *mir*Vana miRNA Isolation Kit (Ambion) and *mir*Vana ^{TM} PARIS ^{TM} Kit (Ambion) according to the following protocols.

2.3.1 RNA Purification using RN*easy* mini Kit

Cells were harvested at time zero (T0), three days (3D), one week (1W) and two weeks (2W), were pre-treated by adding of 600µl of buffer RLT containing ß-Mercaptoethanol, to facilitate subsequent lysis, and cell lysates added to a filter column (QIAshredder), which removed excessive cell debris. The flow-through from this column, which was centrifuged for 2min at maximum speed, was mixed with an equal volume of 70% ethanol and added to a new nucleic acid binding column (RNeasy). Following centrifugation for 15sec at 8000 xg, wash solutions buffer RW1 and buffer RPE were added sequentially. RNA was eluted from the binding column with RNase-free water. The quality of RNA assessed using the NanoDrop spectrophotometer. Samples were stored at –80ºC.

2.3.2 miRNA Purification using *mir*Vana miRNA Isolation Kit

Total RNA (including small species RNAs) was isolated using the *mir*Vana™ miRNA Isolation Kit (Ambion). Cells were harvested at time zero (T0) and three days (3D) ±RA by adding 600μl of Lysis/Binding Buffer. Once harvested, cells were vortexed to completely lyse the cells. 100μl of elution solution was pre-heated to 95°C on a heat block to use at the end of the procedure. 1/10 volume of miRNA Homogenate Additive was added to the mixture and incubated on ice for 10min. The rest of the protocol was performed at room temperature. Equal volume of Acid-Phenol:Chloroform were added to the mixture, which was vortexed for 1min and centrifuged for 5min at 10,000xg speed. The upper aqueous phase was carefully removed and transferred to a fresh sterile 1.5mls tube. 1.25 recovered volumes of 100% ethanol were added to the aqueous phase and mixed well. Contents were applied to Filter Cartridges and centrifuged for 15sec at 10,000g. Following centrifugation for 15sec at 10.000xg, Wash Solution 1 (700μl) and Wash Solution 2 (500μl) were added sequentially. Filter Cartridge were centrifuged for 1min at 10.000xg to dry. RNA was eluted from the Filter Cartridge with 100μl of 95°C pre-heated Elution Solution. The quality of RNA was assessed using the NanoDrop spectrophotometer. Samples were stored at –80°C.

2.3.3 RNA and Protein Purification using *mir*Vana ™ PARIS ™ Kit

The knocked-down or overexpressed (TGF-beta-R2 and Gapdh) EC cells were harvested after three days (3D) ±RA treatment. Total RNA and Protein isolated from the same lysate sample using the *mir*Vana ™ PARIS ™ Kit (Ambion).

The *mir*Vana PARIS Kit is a versatile procedure that permits quantitative recovery of native protein and all RNA species (including small RNAs such as miRNA, siRNA, snRNA, and snoRNA), from the same sample (www.ambion.com). This Kit procedure begins with homogenization of samples with a special Cell Disruption Buffer that includes nonionic detergent. Protein remains intact, so a portion of the lysate can be used directly for common applications such as Western Blotting and total RNA is extracted using a highly efficient acid-phenol:chloroform. The following is the protocol:

The seeded 6-well plates were placed on ice and media discarded. Cells were washed with 500µl ice cold PBS and 400µl of ice cold Cell Disruption Buffer added to each well. Cells were then harvested by cell scraping. The lysates were transferred to fresh sterile 1.5mls microtubes and vortexed to completely lyse the cells. 100µl of the lysate was transferred to fresh sterile 1.5mls microtubes for RNA purification. The leftover lysate was kept on ice for 10min, centrifuged for 2min at 4°C at Max Speed and stored at -80°C for protein analysis.

100µl of 2X Denaturing solution was added to the 100µl lysate for RNA purification, mixed well and incubated on ice for 5min. The rest of the protocol was performed at room temperature. 200µl of Acid-Phenol:Chloroform was added to the mixture, which was vortexed for 1min and centrifuged for 5min at max speed. The upper aqueous phase was transferred to a fresh sterile 1.5ml tube. 1.25 recovered volumes of 100% ethanol were added to the aqueous phase and mixed well. This was applied to a Filter Cartridge and centrifuged for 30sec at 10,000g. Following centrifugation, Wash Solutions 1 (700µl) and Wash Solution 2 (500µl) were added sequentially. Filter Cartridge were centrifuged for 1min at 10000g to dry. RNA was eluted from the Filter Cartridge with 100µl of Elution Solution (95°C). The quality of RNA was assessed using the NanoDrop spectrophotometer. Samples were stored at –80°C.

All RNAs products were electrophoresed on 1% agarose gels (VWR Scientific) stained with ethidium bromide solution (Sigma) and visualized under ultraviolet light (UV) using a Digital Imaging System.

2.3.4 The TaqMan® Gene Expression Cells-to-CT™ Kit

The TaqMan® Gene Expression Cells-to-CT™ Kit was used to perform expression analysis directly from cultured cells without RNA purification. This kit saves time and offers a simple workflow that is suitable for a few samples or can be easily incorporated into automated, high throughput applications. Therefore, this kit was used to design the knockdown and overexpression experiment in a 96-well plate format. Featuring a unique method for lysing cultured cells while removing genomic DNA and preserving RNA integrity, the TaqMan Gene Expression Cells-to-CT Kit contains reverse transcription (RT) reagents for cDNA synthesis and TaqMan® Gene Expression Master Mix for real-time PCR analysis.

Cells were seeded ($6X10^3$ cells/100µl) in a 96-well plate and following a defined time period, depending on experimental conditions, medium was removed from the cells. Cells were washed with PBS and 50µl of Lysis Buffer were added to each well, which was mixed well by pipetting up and down 5 times. Plates were incubated at room temperature for 8min. 5µl of Stop Solution was then added to each well, which was again mixed by pipetting up and down 5 times. The plates were incubated at room temperature for 2min, sealed with a film and stored at -80°C.

cDNA synthesis was carried out in a 96-well plate (Applied Biosystems) containing 25µl 2xRT buffer, 2.5µl 20xRT Enzyme Mix, 10µl Lysated and dH_2O in a final volume of 50µl. The RT-PCR experiments were performed using the Gene Amp PCR System (Applied Biosystems USA). Thermocycling conditions were as follows: 37°C for 60mins and 95°C for 5mins and the cDNA plate sealed and stored at -80°C.

The TaqMan Universal PCR Master Mix was used to analyse gene expression using the 7500 Real Time PCR System or 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) as mentioned in section 2.3.8.

2.3.5 Plasmid DNA Purification

Full ORF plasmids for TGF-beta-R2 and Gapdh were purchased from Imagenes (Germany). These plasmids are pre-cloned into constitutively expressing mammalian expression systems, namely a pDEST26 vector, which contains both T7 and Sv40 promoters. Bacterial samples from imaGenes were grown overnight at $37^{\circ}C$ (shaking) in Luria-Bertani broth (LB broth) (Invitrogen) containing 100µg/ml of ampicillin. QIAprep Miniprep Kit (QIAGEN) was used for plasmid DNA purification. 5 ml of the cultured broth was removed into a sterile 15ml falcon tube and centrifuged at 3000g for 10min. The supernatant was removed and the pellet resuspended in 250µl Buffer P1. The contents were transferred into a sterile 1.5ml tube. 250µl of Buffer P2 was added and mixed by inverting 4-6 times. 350µl of Buffer N3 was added and mixed immediately by inverting 4-6 times and centrifuged for 10min at 13,000 rpm. Supernatants were applied to a QIAprep spin column and centrifuged for 60sec at 13,000 rpm. The flow through was discarded and 500µl of Buffer PB added to the spin column and centrifuged for 60sec at 13,000 rpm. The flow through was again discarded and 750µl of Buffer PE was added to the spin column and centrifuged for 60sec at 13,000 rpm. The flow through was discarded and the spin column centrifuged for 60sec at 13,000 rpm to dry. The spin column was transferred into sterile 1.5mls tubes and 50µl of RNase-free water added. Spin columns were left for 1min at room temp in 1.5mls tubes and centrifuged for 1min at 13,000 rpm. The plasmid DNA, which was eluted from the Spin column with 50µl RNase-free water was electrophoresed on 1% agarose gel (VWR Scientific) stained with ethidium bromide solution (Sigma) and visualized under ultraviolet light (UV) using a Digital Imaging System (UVP, USA). Plasmid DNA products were stored at $-20^{\circ}C$.

Plasmid DNA products were electrophoresed on 1% agarose gel (VWR Scientific) stained with ethidium bromide solution (Sigma) and visualized under ultraviolet light (UV) using a Digital Imaging System (UVP, USA).

2.3.6 NanoDrop® ND-1000

Nucleic acid concentration and quality was determined using the NanoDrop® ND-1000 spectrophotometer (Thermofisher Scientific In., Waltham, MA, USA). The NanoDrop® ND-1000 is a full-spectrum (220-750nm) spectrophotometer that measures 1-2μl samples with high accuracy and reproducibility (**Figure 2.3**). It utilises a patented sample retention technology that employs surface tension alone to hold the sample in place. In addition, the ND-1000 has the capability to measure highly concentrated samples without dilution (50X higher concentration than the samples measured by a standard cuvette spectrophotometer).
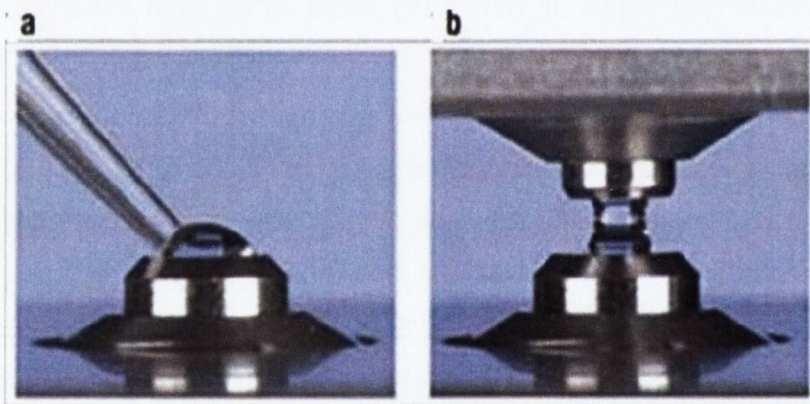
Figure 2.3 NanoDrop® spectrophotometer. To measure Nucleic acid, 1-2ul of sample is pipetted directly onto the lower (measurement) surface (a). An upper pedestal automatically engages the sample, forming a liquid column of mechanically-controlled path length (b) (www.biotechniques.com).

2.3.7 RT- PCR (cDNA Synthesis)

RT-PCR is a rapid, sensitive method for the amplification and reverse transcription (RT) of total RNA to single-stranded cDNA. The High capacity cDNA Archive kit (Applied Biosystems, UK) was used to perform single-stranded cDNA synthesis.

Amplification was carried out in 0.2 ml MicroAmp reaction tubes (Applied Biosystems) containing 10µl 10xRT buffer, 4µl dNTP Mix, 10µl 10xRT Random primers, 5µl RT enzyme, 0.2-0.5µg purified RNA and dH_2O in a final volume of 100µl. The RT-PCR experiments were performed using Gene Amp PCR System (Applied Biosystems, USA). Thermocycling conditions were as follows: 25°C for 10 minutes and 37°C for 120 minutes.

2.3.8 Real Time - PCR

TaqMan® Universal PCR Master Mix (Applied Biosystems, USA) was used to analyse gene expression (mRNA) using the 7500 Real Time PCR System or 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). Triplicate reactions were carried out in 96-well Optical Reaction plates with Barcode or in MicroAmp Optical 384-well Reaction plates with Barcode (Applied Biosystems) containing 10µl TaqMan Universal PCR Master Mix, 1µl Primer-Probe (Applied Biosystems), 5µl cDNA and 4µl dH_2O in a final volume of 20µl in each well reaction. All primer-probes used for Real Time-PCR were supplied by Applied Biosystems (Appendix Table **8**). In chapter 3, TGF-β, Wnt, Snail, Notch and Shh pathways were assayed using marker genes selected from our group's microarray data (Gallagher *et al.*, 2011). Specifically, assays for TGF-β-R2, Wnt5a, Snail2, Notch2 and Shh were used to model expression of TGF-β, Wnt, Snail, Notch and Shh pathways respectively (Appendix Table **8**).

Quantitative miRNA realtime PCR analysis was carried out using the TaqMan® microRNA assay early-access panel (Applied Biosystems) as per manufacturer's instructions. This panel included assays for each of the 330 human miRNAs known at the start of the study (Appendix Table **7**). The protocol detects mature miRNAs using looped-primer real time PCR involving three steps: reverse-transcription (RT), pre-PCR amplification and real-time PCR (Livak *et al.*, 2001). Each RT contained 10ng total RNA. Triplicate reactions were carried out in 384-well

Optical Reaction plates with Barcode (Applied Biosystems) containing 5µl TaqMan Universal PCR Master Mix, 0.5µl Primer-Probe (Applied Biosystems), 2.5µl cDNA and 2µl dH$_2$O in a final volume of 10µl in each well reaction. All primer-probes used for miRNA Real Time-PCR were supplied by Applied Biosystems (Appendix Table 7).

All reactions were carried out in triplicate in parallel with a non-template (NTC) control and using Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and beta-2-microglobulin (B2M) (for mRNA), miR-16 and let-7a (for miRNAs) as internal controls on the 7500 real time PCR system (AB) or 7900HT Fast Real-Time PCR System. Data was normalised using Gapdh and B2M for gene expression and miR-16 and Let-7a for miRNA expression. Gene expression fold change values were calculated using the 2^{-ddCt} method (Livak *et al.*, 2001).

2.3.8.1 mRNA and miRNA Calculation

Gapdh and B2M were used as internal controls to normalise gene expression data and miR-16 and Let-7a were used to normalise miRNA expression data. The 2^{-ddCt} method was used to calculate fold change values for gene and miRNA expression. The values at T0 were equated to 100% (T0=100) and values at all other time points expressed (3 day, 1 week and 2 weeks) relative to this. Data presented here represents changes in expression upon differentiation compared to levels in the undifferentiated state. Where expression was undetected in either state, data is presented as dCt, the basic expression level normalised using an internal control. For example, using this method, a dCt undifferentiated value indicates that the gene was expressed in the undifferentiated state but dropped to undetectable levels upon differentiation. Data shown are representative of at least three biological replicates.

2.3.8.2 Calculation of Percentage Knockdown

In knockdown work, Gapdh expression was used to design the knockdown experiment. Thus B2M was used as the internal control to normalise genes expression data. The 2^{-ddCt} method was used to calculate values fold change of gene expression. Knockdown values of Negative Control (Silencer Negative control siRNA) or non-transfected controls (NTCs) were equated to 100% and expression in knockdown samples presented relative to this. This was achieved via the calculation % Expression $=100(2^{-ddCt})$.

2.3.8.3 Calculation of Percentage Overexpression

As Gapdh was used to optimise overexpression analyses, B2M was used as an internal control to normalise genes expression data. 2^{-ddCt} method was used to calculate fold change values for gene expression. Overexpression values of Non-Transfected Control (NTC cells) cells and Mock (Media with only Transfection Reagent) cells were used to calculate fold change values for overexpression analysis. NTC were equated to 100% and expression in overexpression and mock samples presented relative to this. This was achieved via the calculation % Expression $=100(100-2^{-ddCt})$.

2.4 Western blot analysis

Western blotting is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are detected using antibodies specific to the target protein (Towbin *et al.*, 1979 and Renart *et al.*, 1979).

2.4.1 Protein quantification

Protein was isolated using the *mir*VANA™ PARIS™ kit, as previously described. Just prior to electrophoresis, samples were mixed with 2X Lammeli buffer (Sigma) to a final concentration of 1X and boiled for 5mins. Protein content of BSA standards and cell extracts was measured using the BCA™ Protein Assay Kit (Thermo Scientific). The Thermo Scientific Pierce BCA™ Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. The assay involves a two step process: one, the chelation of copper with protein in an alkaline environment, which results in the reduction of copper (Cu^{2+}) to cuprous cation (Cu^{1+}), and the formation of a light blue complex and two, the chelation of the cuprous cation (Cu^{1+}) from step one with BCA producing an intense purple colour. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations.

2.4.2 BCA™ assay protocol

Protein concentrations were assessed by BCA assay. The initial set up involved diluting protein extract samples 1:5 with H_2O and preparing Bovine Serum Albumin (BSA) standards with H_2O as per manufacturer's instructions. An additional sample included was Radio-Immunoprecipitation Assay buffer (RIPA buffer) diluted 1:5 with H_2O, which was used as a blank for the protein extracts. RIPA buffer is one of several commercially available lysis buffers, which enables efficient cell lysis and protein solubilization of our cells while avoiding protein degradation. The BCA™ working reagent was prepared by mixing 50 parts of BCA™ Reagent A with 1 part BCA™ reagent B. Each of the standards and extracts (10μl) was pipetted into the wells of a 96-well plate in triplicate and mixed with BCA™ working reagent (200μl). The plate was incubated at 37°C for 30mins. The plate was cooled to room temperature prior to absorbance being measured using the Sunrise TECAN microplate reader at 562nm. Protein standards were used to construct a standard curve, which was subsequently used to determine protein concentration of the cell extracts.

2.4.3 SDS polyacrylamide Gel Electrophoresis

SDS-PAGE was conducted according to the method of Laemmli (Laemmli, 1970), as modified by Studier (Studier, 1973). Protein extract samples (30μg) and appropriate prestained (Lonza Group) and biotinylated (Bio-Rad, Hercules) protein markers were loaded into separate wells. Gel electrophoresis was performed at a constant current of 120mV. Samples were first run through an upper gel, known as the stacking gel (1.3ml 30% bisacrylamide mix, 1ml 1M Tris pH 6.8, 80μl of 10% sodium dodecyl sulphate (SDS), 80μl 10% ammonium persulphate (APS) and 8μl TEMED made up to 8ml with H_2O), which condenses the proteins to form thin, sharply defined bands. Then the samples were resolved by size using 8-12% polyacrylamide gels (required volume of 30% bisacrylamide mix, 2.5ml 1.5M Tris pH 8.8, 100μl of 10% SDS, 100μl 10% APS, 6μl TEMED made up to 10ml with H_2O).

2.4.4 Transfer of Proteins to membrane

The resolved proteins were transferred to Immobilon™ polyvinylidene diflouride (PVDF) membrane (Millipore, Billerica) using a wet transfer system, with all components soaked beforehand in cold transfer buffer (25mM Tris-HCl pH8.0, 0.2M glycine, 20% methanol). The gel was placed on a layer of filter paper and sponge overlaid with the membrane. A second piece of filter paper was placed on top followed by a second sponge. The entire assembly was placed in a cassette, the chamber filled with transfer buffer and a constant current of 100mV was applied for 1hr.

2.4.5 Antibody blotting

Prior to antibody blotting, the membranes were blocked in blocking buffer (5% (w/v) non-fat dried milk in 1% (v/v) Tris Buffered Saline (TBS)-Tween) for 1hr at room temperature to remove non-specific binding. Primary antibodies were prepared with blocking buffer using a 1:250,000 – 1:100 dilution as appropriate. The membranes were either left shaking in the primary antibody for 1hr at room temperature or overnight at 4°C. After the appropriate incubation period the membranes were washed in 1% TBS-Tween for 5 minutes, three times, on a rocking platform at room temperature. The secondary antibodies were prepared in blocking buffer using 1:1000 – 1:200 dilutions. To this solution, a biotinylated secondary antibody for marker detection was also added. The membranes were left shaking in the secondary antibody solution for 1hr at room temperature. The membranes were then washed in 1% TBS-Tween for 5 minutes on a rocking platform at room temperature three times. Blots were developed by enhanced chemiluminesence (ECL). The membranes were left in working solution for 2mins and then placed in a project clear membrane to read in the Luminescent Image Analyzer (General Electric). ID numbers and supplier's name for each antibody used in this study see Appendix Table **8**.

Chapter Three

Expression of Markers of Differentiation and Key Stemness Genes and Pathways in Embryonal Carcinoma Stem Cells

3.1 Chapter introduction

3.1.1 General overview

Stem cells can divide asymmetrically, producing two daughter cells with different properties: one identical cell and one cell programmed to differentiate into mature cell types. Only stem cells can divide asymmetrically and both normal stem cells (NSCs) and cancer stem cells (CSCs) use this mechanism to self-renew and differentiate (Clarke *et al.*, 2006). Similarities between CSCs and NSCs during self-renewal and at 1 week differentiation are striking and have facilitated identification of key stemness processes (Schoenhals *et al.*, 2009). **This similarity makes identification of cancer-specific target molecules for use in cancer therapeutics very difficult**. Several studies have shown that CSCs occur in brain, breast, prostate, colon, pancreatic and head and neck malignancies and through extensive self-renewal and differentiation (defined as 'stemness'), may drive tumour growth (Hemmati *et al.*, 2003, Li *et al.*, 2007 and Prince *et al.*, 2007). A single CSC is sufficient to form a *de novo* tumour (Al-Hajj *et al.*, 2003). Theoretically CSCs have the capability of generating a new tumor from a single cell missed by anti-cancer therapies. This may play a key role in the promotion of metastasis of cancer to new sites around the body. Thus CSCs remaining post-cancer therapy may lead to metastasis and recurrence of the tumor. Almost all cancer treatments have side effects. Cancer stem cells may be capable of quietly living within the body for years and are able to start tumor growth and to metastasis, even after a patient has been successfully treated with chemotherapy (Collins *et al.*, 2005). Therefore, it is now widely believed that CSC stemness is a key component of malignancy.

Pluripotent stem cells are undifferentiated cells with a unique capacity to renew themselves and to differentiate into cells of all three somatic germ layers (Ulloa-Montoya *et al.*, 2005). Embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, are the malignant counterparts of pluripotent embryonic stem (ES) cells. Nullipotent stem cells are poorly-differentiating cells (Ulloa-Montoya *et al.*, 2005). In terms of malignancy, differentiation status is linked to the grade of the tumour. Tumours containing high numbers of differentiated tissues, such as those that develop from pluripotent CSCs, are considered to be malignant but of low grade. In contrast, tumours containing high numbers of undifferentiated cells, such as those that develop from nullipotent EC cells, are considered to be highly malignant and high grade. The EC model is the best characterised CSC model

available and the only one to allow comparative analysis of poorly-differentiating and well-differentiating CSCs and NSCs (Andrews, 2001 and Andrews *et al.*, 2005).

3.1.2 Markers of Differentiation, Key stemness genes and Key Stemness Pathways

In this work, we describe characterisation of early cancer stemness events in pluripotent (NTera2) and nullipotent (2102Ep) EC cells to define key events that can be used to indicate changes in stemness during subsequent experiments. Expression of key stemness, differentiation and cancer genes was assessed over time to establish normal gene expression patterns in EC cells for use in subsequent knockdown analyses.

In stem cells, the self-renewal state is characterised by low expression of markers of differentiation (Ncam1, Eno3, Gata6 and Afp) and high expression of key pluripotency genes (Oct4, Nanog and Sox2) and stemness pathways (Wnt, Notch, Snail, Shh etc). These genes are expressed in a converse fashion during differentiation, which is also characterized by increased expression of markers of differentiation (Josephson *et al.*, 2007). Differentiation in stem cells can be detected both morphologically and by demonstration of increased expression of differentiation markers and decreased expression of stemness genes and pathway marker genes (Andrews *et al.*, 2005 and Josephson *et al.*, 2007).

Cancer stemness mirrors stemness of NSCs. This is particularly true of pluripotency. Pluripotent CSCs and NSCs are similar in terms of expression of key stemness genes such as Octame-4 (Oct4), Tir na nOg (Nanog) and a member of the SOX gene family (Sox2) and differentiation marker genes such as ectoderm marker gene neural cell adhesion molecule1 (Ncam1), mesoderm marker gene enolase 3 (Eno3) and endoderm marker genes Alpha-fetal protein (Afp) and Gata binding protein 6 (Gata6) (Josephson *et al.*, 2007). These genes all show regulated expression within 1 week of differentiation (Morrisey *et al.*, 1998). Our hypothesis was that early regulation events, such as signal transduction and transcriptional activation, may be the key difference between CSCs and NSCs. We initially asked whether markers of differentiation were detectably upregulated earlier than one week post differentiation in CSCs and NSCs.

Recent studies have demonstrated that a collection of key signaling pathways is involved in the regulation of stemness processes. Sonic Hedgehog (Shh) is a secreted protein that has been described as a regulator of cell-fate determination and body-segment polarity

(Ruiz Altaba *et al.,* 2002). Sonic Hedgehog regulates expression of the cascading Shh pathway. Notch pathways have been shown to be important in the process of neurogenesis and also in the regulation of self-renewal in embryonic stem (ES) cells, which are pluripotent NSCs (Lennington *et al.,* 2003). Expression of Snail transcription factors can be induced by a variety of different pathways that act on the transcription of these genes (De Craene *et al.,* 2005). Several groups have shown that cancer pathways Pten and Tgf-β are regulated in EC cells (Kishimoto *et al.,* 2003). Pten is a phosphatase that has a wide function as a tumor suppresser gene; it is mutated in many human sporadic cancers and in hereditary cancer syndromes (Premkumar *et al.,* 2006). The transforming growth factor signaling pathway is involved in many cellular processes including cell growth, cell differentiation, apoptosis, cellular homeostasis and other cellular functions (Hoshino *et al.,* 2010 and Mantel *et al.,* 2011). An analysis of the involvement of these pathways in CSC differentiation is presented in results section of this chapter.

3.2 Chapter Hypotheses

Cancer and normal stem cells of similar potency employ similar mechanisms to facilitate differentiation. This is particularly true of undifferentiated stem cells and those that have been differentiated for one to two weeks. However, while these cells employ similar mechanisms, the resulting phenotypes are radically different in terms of tissue organization. **Thus we hypothesise that CSCs and NSCs are likely to regulate common differentiation mechanisms differently**.

As regulatory events usually occur earlier than non-regulatory events in biological systems, we hypothesised that our data could be enriched for regulatory events through analysis of early differentiation. Addressing this, we assessed normal expression of stemness-related genes and pathways during EC cell differentiation.

Nullipotent 2102Ep cells are believed to have a similar differentiation mechanism to pluripotent NTera2 cells (Andrews *et al.,* 2005). However, in 2102Ep cells there is a 'lesion' or break in this differentiation mechanism (Gallagher *et al.,* 2009). As a result of this lesion, 2102Ep cells cannot fully differentiate whereas NTera2 cells can. In essence, therefore, 2102Ep cells are 'broken' NTera2 cells, a break that increases the grade of the resultant tumours. We hypothesise that analysis of early differentiation will allow us to characterise the '2102Ep differentiation lesion'. Once molecularly characterised, we propose that genes involved in the lesion may be manipulated to force differentiation upon these nullipotent cells, thus decreasing the grade of the tumours they generate.

3.3 Chapter Aims

The aim of this study was to identify specific genes and pathways whose expression patterns are differentially regulated during early differentiation of CSCs. Furthermore, by analysing pluripotent and nullipotent EC cells, we hypothesised that novel pluripotent-specific molecular markers could be identified. Our overall aim is identification of targetable CSC-specific events for use in anti-cancer therapeutics, which is hampered by commonalities between normal and cancer stem cells of similar potency.

The nullipotent phenotype of 2102Ep cells is likely to result from a '2102Ep differentiation lesion'. We aim to assess if key stemness genes and pathways are involved in this lesion. This will be achieved by comparative analysis of the early differentiation response of NTera2 and 2102Ep cells.

3.4 Chapter Materials and Methods

Full details of methods used are described in chapter 2. Briefly, two embryonal carcinoma (EC) cancer stem cell (CSC) lines (pluripotent NTera2 and nullipotent 2102Ep) were grown in DMEM media at 37°C and 5% CO_2. Cells were stimulated to differentiate by adding 0.01mM Retinoic Acid (RA). Cells were harvested at time zero (T0), three days (3D), one week (1W) and two weeks (2W) ±RA. The harvested cells were lysed and total RNA isolated using the RN*easy* mini Kit. The quality of RNA was assessed using a NanoDrop spectrophotometer. Single-stranded cDNA synthesis was performed using the high capacity cDNA Archive kit and performed using the Gene Amp PCR System. Thermocycling conditions were as follows: 25°C for 10 minutes and 37°C for 120 minutes. Gene expression was analysed using TaqMan® Universal PCR Master Mix and pre-designed assays via the 7500 Real Time PCR System or 7900HT Fast Real-Time PCR System (All technologies: Applied Biosystems, USA).

All reactions were carried out in triplicate in parallel with non-cDNA and non-template (NTC) controls using Gapdh as an internal control. Data was normalised using Gapdh gene expression. Gene expression fold change values were calculated using the 2^{-ddCt} method (Livak *et al.*, 2001).

TaqMan qPCR data was remarkably consistent in terms of the temporal patterns of expression: genes alterations occurred at the same time point and qualitative direction (upregulation or downregulation) across multiple biological replicates. Data is shown proportional to time zero (T0 were equated to 100%) and values at all other time points expression relative to T0. This was achieved via the calculation % Expression $=100(100-2^{-ddCt})$.

3.5 Chapter Results

Both cell types (Pluripotent NTera2 and Nullipotent 2102Ep) were grown in the undifferentiated (non-RA-treated) and retinoic acid (RA)-induced differentiated (RA-treated) states and harvested after 3, 7 and 14 days. Total RNA was isolated and single-stranded cDNA synthesis performed as described in Material and Methods. We first tested that our normalization gene for quantitative PCR (qPCR, TaqMan) was not affected by differentiation status. Internal control gene (Gapdh) expression was successfully amplified and analysed using Real Time PCR and was not affected by differentiation status (**Figure 3.1**). Non-template (NTC) controls using Gapdh were included in all analyses. Thus Gapdh was employed as a suitable internal control gene for qPCR normalization.
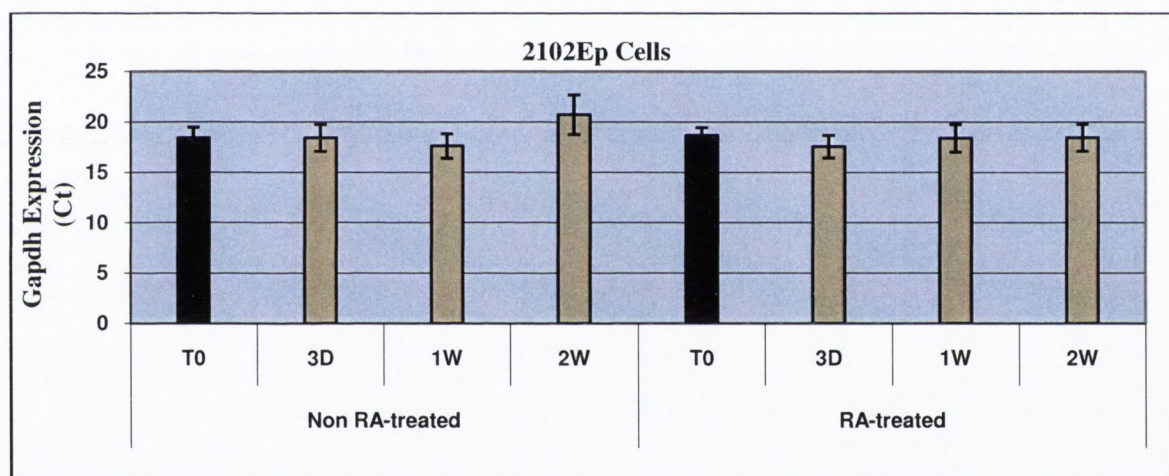
Figure 3.1 Expression of internal control Gapdh used in nullipotent CSCs in non-retinoic acid treated cells (non-RA-treated) and retinoic acid treated cells (RA-treated) over a period from time zero (T0), 3 days (3D), one week (1W) and two weeks (2W). Internal gene (Gapdh) was detected and were unaffected by differentiation status, confirming their suitability as endogenous controls.

3.5.1 Expression of differentiation marker genes (Ncam1, Eno3 and Afp and Gata6) in NTera2 and 2102Ep CSCs

Nullipotent cells are self-renewing cells, which have almost no potential for differentiating layers (Chambers *et al.*, 2004). In nullipotent (2102Ep) cells our hypothesis expected that all three germ layer marker genes would remain inactivated despite differentiation treatment. However, all the markers of differentiation were expected to increase following differentiation treatment in pluripotent cells (NTera2). Expression of differentiation marker genes Ncam1 (ectoderm), Eno3 (mesoderm) and Afp, Gata6, Gata4 and Hnf4 (endoderm) were studied during RA-induced differentiation of pluripotent and nullipotent human EC cells over a period from 3-14 days. Thus we hypothesised that the expression of the three germ layer marker genes would be altered in pluripotent but not in nullipotent cells.

In pluripotent cells, the ectoderm marker gene (Ncam1) increased coordinatedly from 700% fold after 3 days to 40^3% fold at 1 week peaking at 90^3% fold after 2 weeks (**Figure 3.2A**). Mesoderm marker gene (Eno3) increased 400% fold after 3 days, peaking at 20^3% fold after 1 week (**Figure 3.2A**). The endoderm marker gene Afp was observed to increase linearly while Gata6 rapidly increased to 60^3% fold after 3 days differentiation. The increase of Afp continued to 300% fold after 1 week and 2 weeks. Gata6 increased to 20^4% fold by 1 week and 2 weeks differentiation (**Figure 3.2A**). Thus all three markers genes were upregulated upon differentiation as expected.

In contrast, in nullipotent cells neither ectoderm nor mesoderm marker genes (Ncam1 and Eno3) were regulated over time, where expression fluctuated uncoordinatedly (**Figure 3.2B**). Neither ectoderm nor mesoderm marker genes were altered in nullipotent cells. However, unexpected results were achieved. Endoderm marker genes (Afp and Gata6) increased when we tested the ability of nullipotent cells to differentiate. Afp expression was tightly regulated, demonstrating co-ordinated increased expression over 2 weeks 'differentiation' (**Figure 3.2B**). Gata6 was observed to increase after 3 days differentiation and increased further to 10^5% fold and 70^5% fold by 1 week and 10^6% by 2 weeks respectively (**Figure 3.2B**).

Expression of Gata6 was substantially higher than Ncam1 and Ncam1 higher than Eno3 and Afp in pluripotent cells. Other endoderm marker genes Hnf4 and Gata4 were not altered in both cell lines (data not shown).
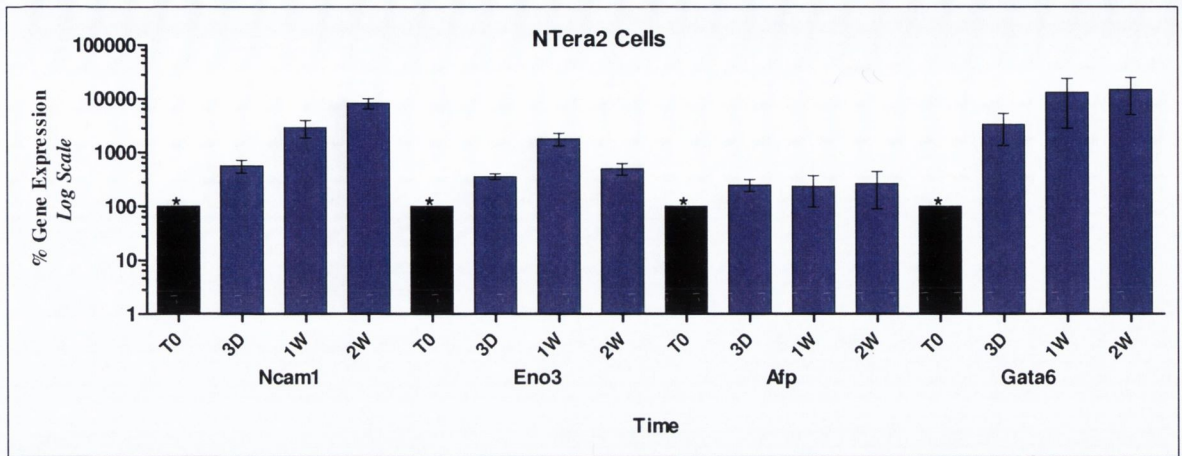
Figure 3.2A Trend Expression Analysis of Differentiation Markers (Ncam1, Eno3, Afp and Gata6) during Differentiation in NTera2 (Pluripotent) CSCs over a period from time zero (T0), 3 days (3D), one week (1W) and two weeks (2W).
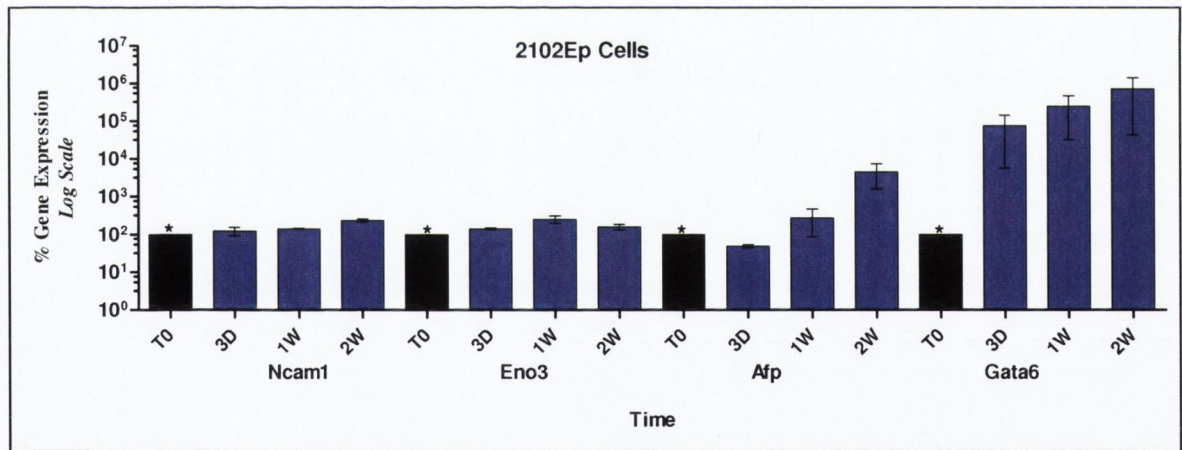
Figure 3.2B Trend Expression Analysis of Differentiation Markers (Ncam1, Eno3, Afp and Gata6) during Differentiation in 2102Ep (Nullipotent) CSCs over a period from time zero (T0), 3 days (3D), one week (1W) and two weeks (2W).

3.5.2 Expression of Pluripotency Genes (Oct4, Nanog and Sox2) in NTera2 and 2102Ep CSCs

Oct4, Nanog and Sox2 are reportedly downregulated in the early differentiation of pluripotent EC cells and are highly expressed in the inner cell mass of the blastocyst but not in cells of differentiated tissues and 'differentiated' nullipotent cells (Hyslop *et al.*, 2005 and Fong *et al.*, 2008). The expression of these key stemness genes was studied during differentiation of pluripotent and nullipotent cells over time from 3 to 14 days. Both Oct4 and Nanog acted in parallel and were co-ordinately downregulated in pluripotent cells over time after being stimulated to differentiate. Both were decreased early from - 70% to -80% fold after only 3 days and decreased further to -80% to -90% fold by 1 week and -80% to -85% fold after 2 weeks (**Figure 3.3A**). An unexpected result, Sox2 did not change in pluripotent cells over time after being stimulated to differentiate (**Figure 3.3A**). All genes demonstrated fluctuating uncoordinated expression in nullipotent cells over time after being stimulated to differentiate (**Figure 3.3B**). Thus stemness marker genes were downregulated in pluripotent CSCs but not nullipotent CSCs, as expected.
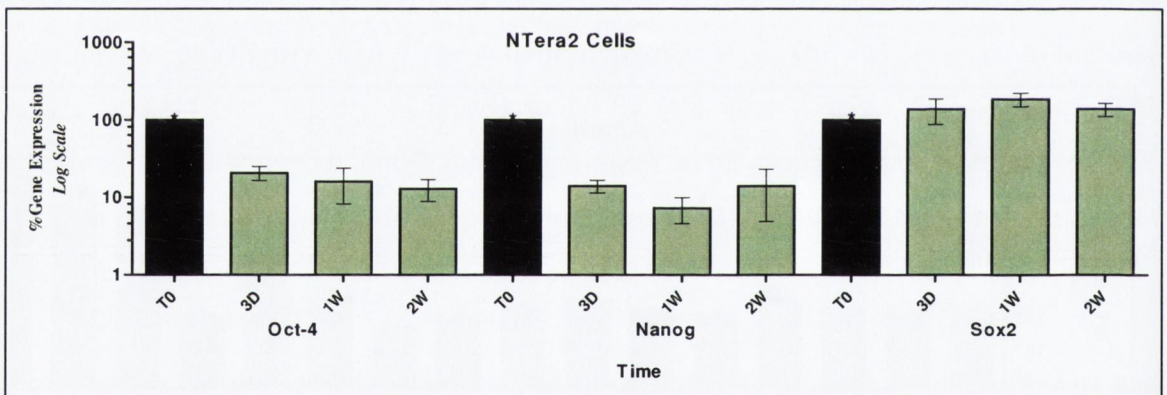
Figure 3.3A Trend Expression Analysis of Key Stemness Genes (Oct4, Nanog and Sox2) during Differentiation in NTera2 (Pluripotent) CSCs over a period from time zero (T0), 3 days (3D), one week (1W) and two weeks (2W).
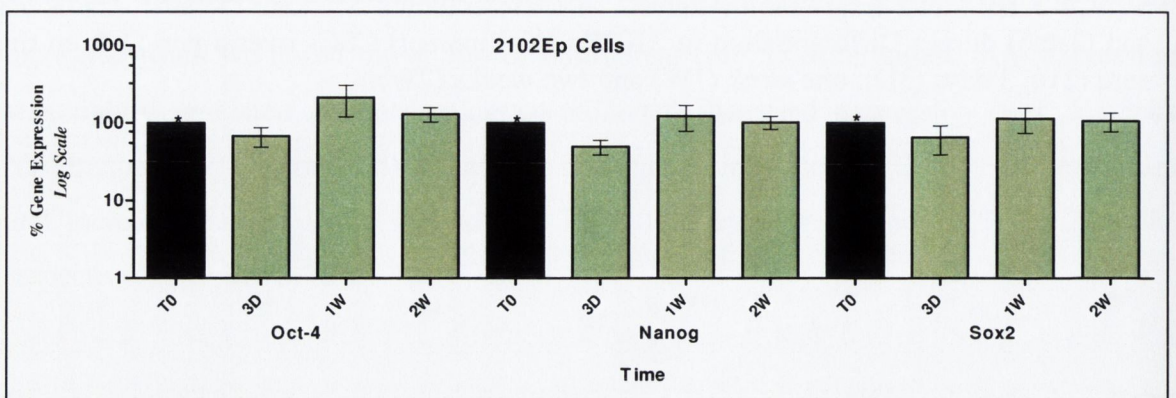
Figure 3.3B Trend Expression Analysis of Key Stemness Genes (Oct4, Nanog and Sox2) during Differentiation in 2102Ep (Nullipotent) CSCs over a period from time zero (T0), 3 days (3D), one week (1W) and two weeks (2W).

3.5.3 Expression of Key Stemness Pathways and Cancer Pathways (Wnt5a, Notch, Snail, Pten, Shh and Tgf-β) in NTera2 and 2102Ep CSCs

Expression of key stemness and cancer pathways (Wnt5a, Notch, Snail, Pten, Shh and Tgf-β) were studied during RA-induced differentiation of pluripotent and nullipotent cells over a period from 3 days to 2-4 weeks. These pathways have been demonstrated to play an important role in stemness and to be co-ordinatedly regulated to a high level of expression after 1 week differentiation (Cano *et al.*, 2000, Nemir *et al.*, 2006, Bailey *et al.*, 2008 and Korkaya *et al.*, 2009). In contrast to key stemness genes, key stemness pathways (Wnt5a, Notch, Snail and Shh) and cancer pathways (Pten and Tgf-β) were up-regulated in differentiated pluripotent cells. Few pathways showed altered expression in nullipotent EC cells. Wnt5a, Notch, Pten and Shh were regulated in both differentiated cell lines whereas, Snail and Tgf-β were regulated in differentiation of pluripotent cells but fluctuated uncoordinatedly or downregulated in differentiation of nullipotent cells.

Almost all expression levels of pathways such as Wnt5a, Notch, Snail and Pten were regulated to similar levels (observed after 3 days, continued to increase 200% fold by 1 week and 500% fold by 2 weeks) in differentiation of pluripotent cells (**Figure 3.4A**). The highest level of expression in pluripotent cells was Shh, which increased rapidly, reaching 20^3%fold after 3 days, 10^4% fold after 1 week and 80^3 fold by 2 weeks (**Figure 3.4A**). Tgf-β had the second highest level of expression in pluripotent cells after 3 days, continuing to increase 500% fold by 3 days, 10^3% fold by 1 week and 20^3 fold by 2 weeks (**Figure 3.4A**). In nullipotent cells, Shh was again the highest expressed pathway, increasing 200% fold after 3 days and 10^3% fold by 1 and 2 weeks (**Figure 3.4B**). Wnt5a, Notch and Pten were the second highest pathways expressed in nullipotent cells after 3 days and increased about 500% to 900% fold by 1 and 2 weeks (**Figure 3.4B**). An unexpected result, Tgf-β decreased 50% fold after only 3 days and had no changed after 1 and 2 weeks. (**Figure 3.4B**) Snail was not consistent over time after nullipotent cells being stimulated to differentiate (**Figure 3.4B**).
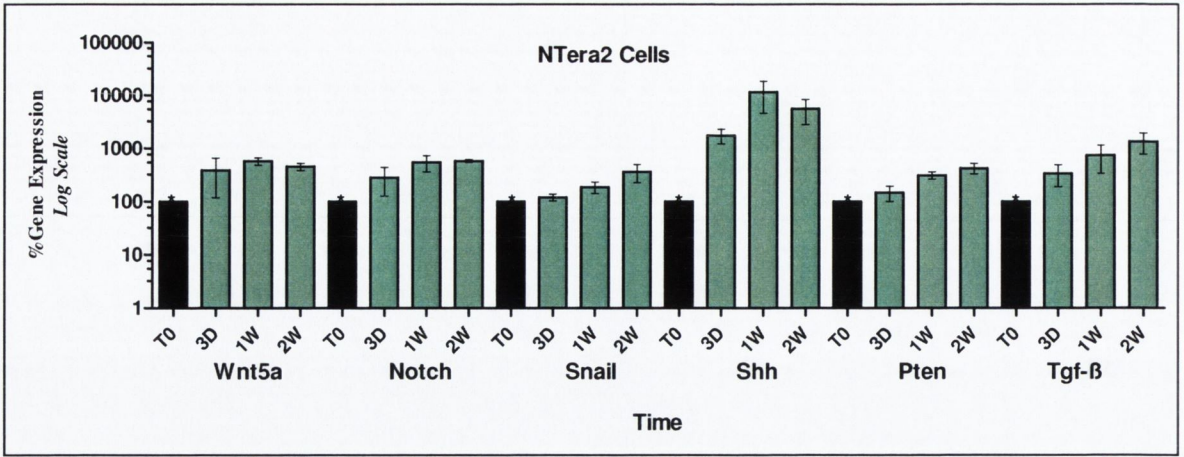
Figure 3.4A Trend Expression Analysis of Key Stemness and Cancer Pathways during Differentiation in NTera2 (Pluripotent) CSCs over a period from time zero (T0), 3 days (3D), one week (1W) and two weeks (2W).
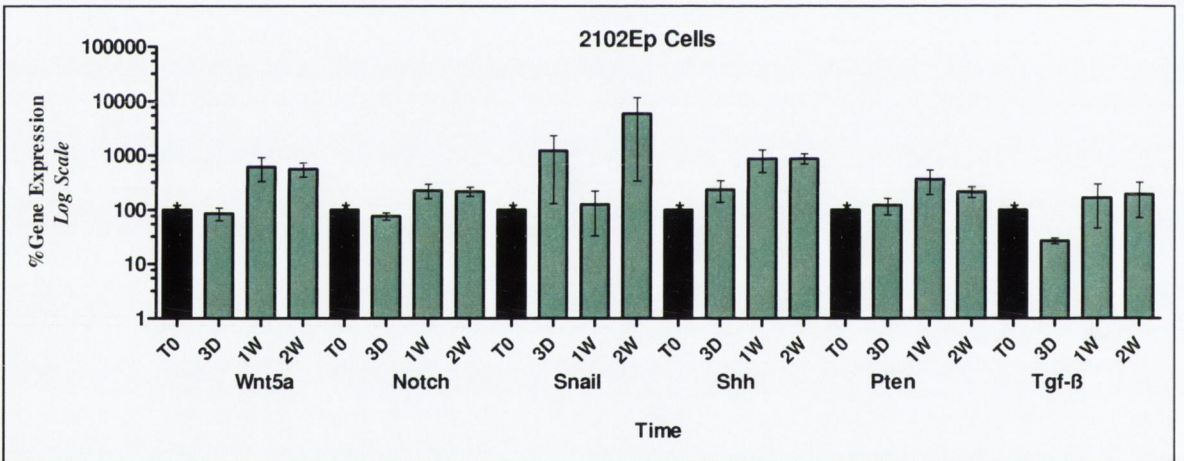
Figure 3.4B Trend Expression Analysis of Key Stemness and Cancer Pathways during Differentiation in 2102Ep (Nullipotent) CSCs over a period from time zero (T0), 3 days (3D), one week (1W) and two weeks (2W).

3.6 Chapter Discussion

Assessing early differentiation of EC cells is a novel approach by our group and as such has not been previously analysed. Addressing this, in this chapter we characterised the early differentiation of two types of EC cell: pluripotent NTera2 and nullipotent 2102Ep cells. Analysis was carried out in three broad categories:

- Effects on differentiation marker genes: Ncam1, Eno3 and Gata6

- Effects on pluripotency master genes: Oct4, Sox2 and Nanog

- Effects on key stemness pathways and modulators: TGF-β, Shh, Wnt, Notch, Snail and Pten

As expected, key processes were detectably regulated at 3 days differentiation in pluripotent CSCs. However, despite their lack of functional pluripotency, selected pluripotency, stemness and cancer genes were regulated during 'differentiation' of nullipotent cells. This suggests that nullipotent cells attempt to respond to differentiation through a mechanism that is ultimately non-functional: this supports the '2102Ep differentiation lesion' hypothesis. Additionally, activation of differentiation is not an 'all or nothing' process but rather a hierarchal mechanism.

Broadly, we posed two questions:

- Is each gene/pathway regulated during early differentiation?

- Is this regulation identical in NTera2 and 2102Ep cells?

Our results will now be discussed addressing each of these categories in turn, concluding with an overall commentary.

We first assessed the early differentiation responses of differentiation markers Ncam1, Eno3 and Gata6 in NTera2 and 2102Ep cells. Expression of all three markers of differentiation, Ncam1, Eno3 and (Afp and Gata6), was detectable in pluripotent cells. As expected, no change in expression was observed for Ncam1 or Eno3 in 2102Ep cells. Surprisingly, Afp and Gata6 were observed to be regulated over time in nullipotent cells, despite the lack of endodermal differentiation in the phenotype. Levels of expression of

each differentiation marker differed, suggesting that different concentrations of each gene were required during early differentiation. The level of expression required for Gata6, Ncam1 and Eno3 was achieved at an early time point in pluripotent cells. This data suggests that ectoderm (Ncam1) and mesoderm (Eno3) differentiation is functional in pluripotent cells but not in 2102Ep nullipotent cells. Endoderm (Afp and Gata6) differentiation was functional early in pluripotent cells and, surprisingly, also function in nullipotent cells. The latter result suggests that nullipotent CSCs (2102Ep) are attempting to differentiate along the endodermal lineage. This lineage may be an avenue for future attempts to remove stemness from this nullipotent CSC. As self-renewal (Nullipotent) cells had the capacity to alter endoderm genes Afp and Gata6 via addition of retinoic acid, regulation of ectoderm, mesoderm and endoderm development may be key to poorly differentiating teratocarcinoma. Hnf4 and Gata4 were not functional either in pluri- nor nullipotent cell lines. This is a fundamental change in our thinking on how stem cells are regulated.

We next assessed the regulation of Oct4, Sox2 and Nanog during early differentiation of EC cells.

Regulation of Oct4 and Nanog was down-regulated early in differentiation (after only 3 days) in pluripotent cells. As expected, Oct4 and Nanog expression was unaltered in nullipotent cells. In contrast, Sox2 was unaltered in either cell type. This was a surprising result as Sox2 is an absolute requirement of non-malignant pluripotent stem cells (Tomioka et al., 2002). We have since confirmed similar data with Peter Andrews group at the University of Sheffield (P Andrews, personal communication). Thus we have identified maintained Sox2 expression as a key difference between EC cells and their non-malignant comparators. As expected, our results show that Oct4 and Nanog regulation functions early in differentiation in pluripotent cells but not in nullipotent cells. This suggests that in teratocarcinoma CSC differentiation, Oct4 and Nanog are more important to stemness than Sox2. As expected, nullipotent cells did not alter regulation of any key stemness genes. These data have only been previously shown at later differentiation time points (Andrews et al, 2005). As such, comparison with similar data is not possible. This characterization is an important contribution to our understanding of EC biology.

Nullipotent malignant stem cells are capable of making identical copies of themselves in the organism or to metastasise to other organs. Knocking out key stemness genes from nullipotent cancer stem cells enables cells to differentiate and that could be give a great interest in the therapy of malignant cancer stem cells (Hyslop *et al.,* 2005 and Fong *et al.,* 2008). Knocking out key stemness genes to target malignant cancer stem cells will affect all stem cells (normal and malignant cells: Andrews, 2002 and Andrews *et al.,* 2005). Therefore, Oct4, Nanog, Sox2, which are common to NSCs and CSCs, are not suitable for therapy. However, targeting stemness of CSCs specifically is a potential avenue for cancer therapy.

Key stemness pathways and cancer pathways are up-regulated after 1 week differentiation of pluripotent EC cells. Our analysis demonstrates that regulation of key stemness pathways and cancer pathways is achieved through up-regulation early in differentiation (only after 3 days) in pluripotent cells. Wnt5a, Notch, Pten and Shh function early in differentiation in both cell types while Snail functions in pluripotent but not nullipotent cells. Once again, these processes have only been previously assessed at later time points, making comparison of our results impossible (Yu *et al.,* 2002, Fu *et al.,* 2004, De Craene *et al.,* 2005, Nemir *et al.,* 2006, Bailey *et al.,* 2008 and Cerdan *et al.,* 2010). Tgf-β is up-regulated in pluripotent and down-regulated in nullipotent cells. This highlighted TGF-beta (Tgf-β) signaling has been taken for further analysis (**Chapter 5**). Therefore Wnt5a, Notch, Snail, Pten, Shh and Tgf-β play key early and late roles in stemness of pluripotent malignant cells. In nullipotent malignant cells, Wnt5a, Notch, Shh and Pten play early and late key stemness roles. Therefore, Wnt5a, Notch and Pten, key pathways at 1 week differentiation, are also involved in early differentiation in pluri- and nullipotency, playing a rapid role in regulation of key stemness pathways in malignant cells. Shh appears to be necessary for pluri- and nullipotency, playing a very plastic regulatory role in malignant cells. Snail appears to only function in pluripotency, playing an early regulatory role in key stemness pathways in pluripotent but not nullipotent malignant cells. In contrast, Tgf-β appears to be required for pluripotency, playing an early regulatory role in cancer pathways in malignant cells, and the down-regulation in nullipotent malignant cells may be necessary to maintain the self-renewal state. In overview, this indicates a hierarchal activation of stemness pathways during differentiation of EC cells. Notably, this has not be described in comparable NSCs (**Figure 3.5**).
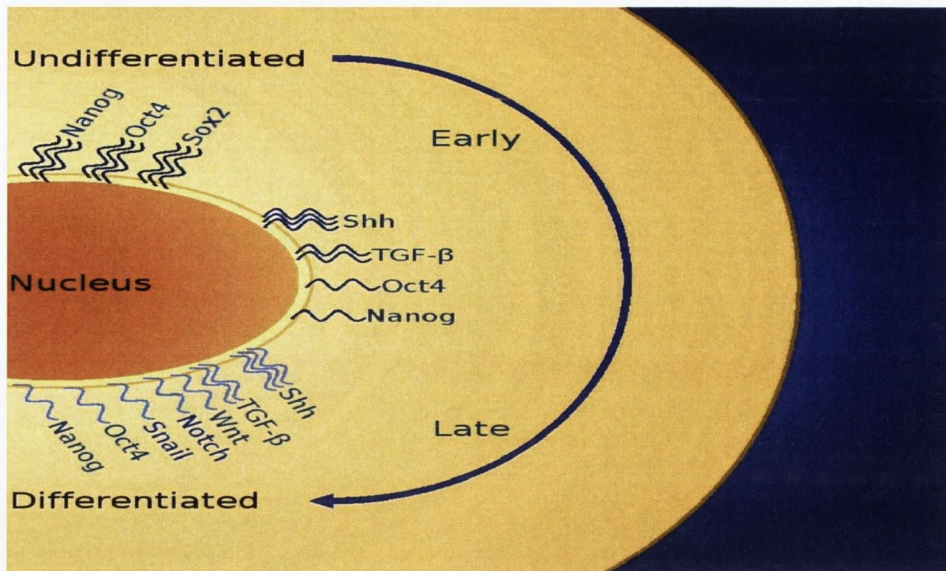
Figure 3.5 Hierarchal Regulation During Early Differentiation of Pluripotent ECs. The schematic represents the relative increases and decreases of stemness genes and pathways during early and later differentiation. In the undifferentiated state, EC cells express Oct4, Nanog and Sox2, which are downregulated quickly and substantially during early and through later differentiation. During early differentiation expression of Shh and TGF-beta signaling is activated and is later joined by expression of Wnt, Notch and Snail signaling. Activation/deactivation of genes is hierarchally regulated in terms of timing and levels of gene expression alterations. Higher numbers of waved lines indicate higher gene expression, reflecting the data generated in chapter 3.

Our data demonstrates that cancer stemness is regulated at 1 week and also at 3 days differentiation and probably even earlier. Interestingly, almost identical levels of change of expression were observed for Wnt, Notch, Snail and Pten in pluripotent cells, suggesting that these pathways may be controlled by one or more of the same signal transduction or transcriptional regulators. Most pathways were regulated in nullipotent cells, suggesting that pluri- and nullipotent cells may act similarly during self-renewal and/or early differentiation. The highest level of expression of Shh in both cell lines may indicate its role as leading regulator targeting early stemness or may in fact regulate the downstream activity of one or more the other pathways.

We and others have hypothesized that nullipotency may be due to a "differentiation lesion" (Andrews *et al.,* 2005 and Gallagher *et al.,* 2009). Our data indicate that the lesion in nullipotent differentiation may be related to the Snail and Tgf-β pathways or their upstream regulators: these two pathways are altered in NTera2 cells but unaltered in 2102Ep cells according to the data presented in this chapter. This has not been described in the most recent comprehensive study of 2102Ep cells (Josephson *et a.l,* 2007). Up-regulating Tgf-β signaling may enable the nullipotent cells to differentiate. Protocols that

differentiate nullipotent cells could be of therapeutic interest. Up-regulating the Tgf-β pathway may be a suitable therapy to treat malignant cancer stem cells. Knocking out Tgf-β pathway from pluripotent cancer stem cells may maintain self-renewal of the pluripotent cells. This may suggest that down-regulating Tgf-β in self-renewing nullipotent cells maintain the self-renewal state and up-regulating Tgf-β pathway in pluripotent cancer stem cells is important to differentiate the cells. These hypotheses are addressed functionally in chapter 5.

In overview, our analysis indicates that early differentiation can be characterised in EC cells. This characterization revealed a key difference (Sox2 expression) between cancer and normal cells, supporting our hypothesis. As we will see in chapter 5, our approach uncovers additional differences between cancer and normal cells. Additionally, we have confirmed that differentiation, pluripotency master genes and key pathways and modulators of stemness are all regulated from early in differentiation, validating our approach. Finally, we have identified novel differences between NTera2 and 2102Ep cells, which may be important to the 2102Ep "differentiation lesion".

Chapter Four

Differential Expression of miRNAs in Embryonal Carcinoma Stem Cells

4.1 Chapter introduction

4.1.1 General overview

Two embryonal carcinoma (EC) cancer stem cell (CSC) lines (pluripotent and nullipotent) were analysed to identify microRNAs (miRNAs) regulating early differentiation with an ultimate aim of identifying putative targets to remove or reduce stemness from CSCs. We have determined that a subset of stemness-associated miRNAs is involved in regulation of early differentiation (3 days) in CSCs. The involvement of these miRNAs in early differentiation links them to regulation of CSCs, a key area of interest to our group.

4.1.2 MicroRNAs

MicroRNAs are single-stranded RNA molecules of about 21–23 nucleotides in length, which regulate gene expression. MiRNAs are encoded by genes that are transcribed from DNA but are not translated into proteins (non-coding RNA): instead they are processed from primary transcripts known as *pri-miRNA* to short stem-loop structures called *pre-miRNA* and finally to functional miRNA (Gregory *et al.,* 2005).

Small RNAs, miRNAs were originally thought to have no function and perhaps be degraded bi-products of RNA isolation. More recently, miRNAs have been shown to be essential for direction of cell fates, in regulation of development, to control subtle or non-essential regulatory pathways, while others might help fine-tune the complex genetic network that builds a multicellular organism (Pasquinelli *et al.,* 2005). Subsequent studies have shown that miRNAs and cellular factors necessary for miRNA biogenesis are conserved in many organisms, suggesting the importance of miRNAs during developmental processes (Pasquinelli *et al.,* 2005 and Davis-Dusenbery *et al.,* 2010). The functions of miRNAs are not limited to the regulation of developmentally timed events. miRNA now appears to be important in the regulation of many fundamental processes.

MicroRNAs are clearly involved in malignancy and several groups have demonstrated that overexpression and underexpression of miRNAs are linked to cancers (reviewed in Esquela-Kerscher *et al.,* 2006 and Hui *et al.,* 2010).

Indeed, miRNAs have roles in every biological process in which they have been studied to date. Different populations are expressed in self-renewing and differentiating human hES cells and CSCs and in normal versus malignant tissues (Esquela-Kerscher *et al.,* 2006). Recent studies reported that a population of mammalian miRNAs is downregulated in differentiating embryonic stem cells, suggesting that they are involved in the maintenance of a pluripotent state (Suh *et al.,* 2004 and Pasquinelli *et al.,* 2005). The newly identified human ES-specific miRNAs may also serve as molecular markers for the early embryonic stage and for self-renewing human ES cells (Suh *et al.,* 2004). Evidence has recently emerged that deregulated miRNA activity is associated with human cancers. Several miRNAs has been found to have links with cancer. Non-coding (ncRNA) genes, which produce functional RNA molecules rather than encoding proteins, are associated with malignancy (McManus, 2003). Several groups have demonstrated that different populations of miRNA are found in many cancers when compared to appropriate normal tissues (Esquela-Kerscher *et al.,* 2006 and Li JH *et al.,* 2010). MiRNAs are clearly involved in malignancy and stemness and may thus be key components of cancer stemness. However, prior to this study the involvement of miRNAs in early differentiation of CSCs had not been established. MiRNAs are likely to represent a key target group for specific inhibition of CSCs.

4.2 Chapter Hypotheses

Cancer and normal stem cells of similar potency employ similar mechanisms to facilitate differentiation. This is particularly true of undifferentiated stem cells and those that have been differentiated for one to two weeks. However, while these cells employ similar mechanisms, the resulting phenotypes are radically different in terms of tissue organization. Thus we hypothesise that CSCs and NSCs are likely to employ different regulatory mechanisms. As regulatory events usually occur earlier than non-regulatory events in biological systems, we hypothesised that miRNAs are very likely to be involved in early differentiation of EC cells as they have been shown to be important in so many other cell systems.

Nullipotent 2102Ep cells are believed to have a similar differentiation mechanism to pluripotent NTera2 cells (Andrews *et al.,* 2005). However, in 2102Ep cells there is a 'lesion' or break in this differentiation mechanism (Gallagher *et al.,* 2009). As a result of this lesion, 2102Ep cells cannot fully differentiate whereas NTera2 cells can. In essence, therefore, 2102Ep cells are 'broken' NTera2 cells, a break that increases the grade of the resultant tumours. We hypothesise that the '2102Ep differentiation lesion' may involve aberrant miRNA regulation and/or expression. Once molecularly characterised, we propose that miRNAs involved in the lesion may be manipulated to force differentiation upon these nullipotent cells, thus decreasing the grade of the tumours they generate.

4.3 Chapter Aims

The aim of this study was to identify specific miRNAs whose expression patterns are differentially regulated during early differentiation of CSCs. Furthermore, by analysing pluripotent and nullipotent CSCs, we hypothesised that novel pluripotent-specific miRNAs could be identified. We also hypothesise that specific miRNAs regulate early CSC differentiation specifically. These miRNAs would then be available for functional and mechanistic analysis by our laboratory. Our overall aim is identification of targetable CSC-specific events for use in anti-cancer therapeutics, which is hampered by commonalities between normal and cancer stem cells of similar potency.

We additionally aim to identify miRNAs that are differentially regulated in NTera2 and 2102Ep cells, which may constitute a component of the '2102Ep differentiation lesion'.

4.4 Chapter material and method

Pluripotent (NTera2) and nullipotent (2102Ep) CSCs were grown in DMEM media at 37°C and 5% CO_2. Cells were harvested at time zero (T0) and three days (3D) ±RA. Total RNA (including small species RNAs) was isolated using the mirVana™ miRNA Isolation Kit (Ambion) and the quality of RNA were assessed using a NanoDrop spectrophotometer. qPCR of miRNAs was carried out using the early access miRNA TaqMan kit (Applied Biosystems), full details of which are contained in chapter 2.

All reactions were carried out in triplicate in parallel, including a non-template control (NTC) and using miR-16 and let-7a as internal controls. Data was normalised using miR-16 and let-7a for miRNAs expression. miRNAs expression fold change values were calculated using the 2^{-ddCt} method (Livak *et al.*, 2001).

TaqMan qPCR data was remarkably consistent in terms of the temporal patterns of expression: miRNA alterations occurred at the same time point and qualitative direction (upregulation or downregulation) across multiple biological replicates. The precise levels of expression, however, varied. As such, each data set is presented as a representative median data set.

4.5 Chapter Results

Both cell types Pluripotent and Nullipotent (NTera2 and 2102Ep) were grown in the undifferentiated (**undiff**) and retinoic acid (RA)-induced differentiated (**diff**) states and cells harvested at 3 days. We first tested that our normalization gene for quantitative PCR (qPCR, TaqMan) was not affected by differentiation status. Internal control miRNA (miR-16 and Let7a) expression was successfully amplified and analysed using Real Time PCR and was not affected by differentiation status (**Figure 4.1**). Non-template (NTC) controls using miR-16 and let-7a were included in all analyses. Thus miR-16 and let-7a were employed as suitable internal control miRNAs for qPCR normalization.
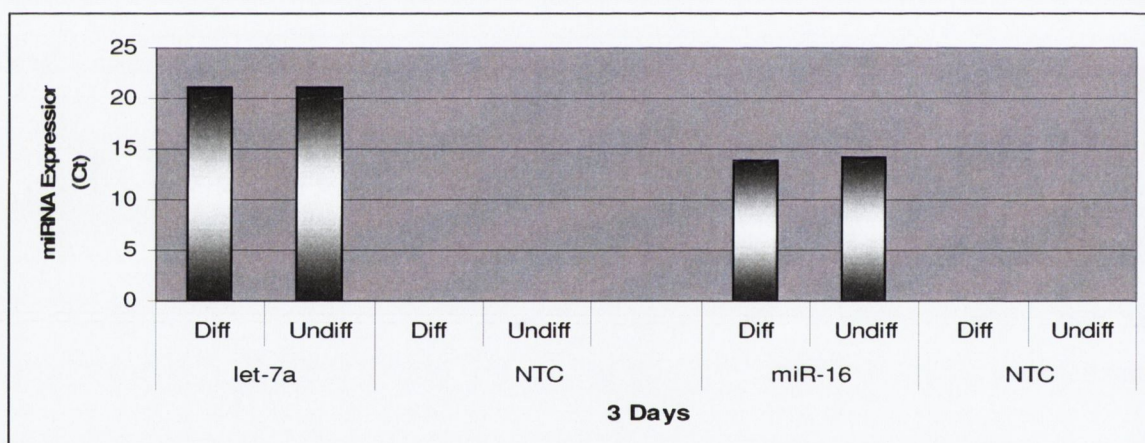
Figure 4.1 The expression of two internal control genes, Let-7a and miR-16, was assessed in undifferentiated (Undiff) and differentiated (Diff) samples and non-template controls (NTCs) using novel miRNA qPCR. Both internal genes were detected and were unaffected by differentiation status, confirming their suitability as endogenous controls.

4.5.1 Overview

Both cell lines (pluripotent and nullipotent) were used to analyse the expression of the 316 miRNAs known to exist at the start of the study. Out of 316 miRNAs, 66 miRNAs were undetectable in both cell lines in either state (self-renewing or differentiated). 199 miRNAs were expressed in both cell lines in both states. 26 miRNAs were expressed in nullipotent CSCs in both states but undetected in pluripotent CSCs. 16 miRNAs were expressed only in self-renewing NTera2 cells but in both self-renewing and differentiating 2102Ep cells. 4 individual miRNAs and one group of three miRNAs were detected in either one or both of the cell lines in only one state. MiRNAs grouped, therefore, into those expressed commonly and those expressed in specific states and cell types. This indicates a complex regulatory network, which will be detailed below.

The majority of these miRNAs (199 miRNAs) were detected in both cell lines and were unaltered during differentiation. However, sets of miRNAs that are up- or downregulated during differentiation were identified. Many of these were altered only in pluripotent cells. However, a unique set of miRNAs was altered in nullipotent cells, suggesting that they respond to differentiation specifically. Strikingly, identification of nullipotent-specific alterations in miRNAs suggests that nullipotent CSCs may posses a mechanism to facilitate avoidance of differential signals, a mechanism that may facilitate a highly malignant phenotype. The data included in this chapter has been published in the Journal of Ovarian Research (Gallagher *et al.,* 2009).

4.5.2 miRNAs undetected in both cell lines, in both states

A large group of miRNAs were undetected in this study. 20.88% (66 of 316) of miRNAs were undetermined in both cell lines, in both states. All those undetected miRNAs are catalogued in table **4.1**. These miRNAs, therefore, are not required by CSCs. By implication, their targets are regulated via transcriptional and post-translational mechanisms.

Table 4.1 miRNAs undetected during analysis in both cell lines in both states.

1	miR-128b	23	miR-US25-1	45	miR-492
2	miR-138	24	miR-US25-2-3p	46	miR-496
3	miR-144	25	miR-US25-2-5p	47	miR-506
4	miR-196a	26	miR-US5-1	48	miR-510
5	miR-208	27	miR-US5-2	49	miR-513
6	miR-216	28	miR-323	50	miR-514
7	miR-UL148D-1	29	miR-98	51	miR-432*
8	miR-UL22A-1*	30	miR-142-3p	52	miR-517c
9	miR-UL36-1	31	miR-202	53	miR-518a
10	miR-147	32	miR-215	54	miR-518f
11	miR-189	33	miR-220	55	miR-518f*
12	miR-198	34	miR-202*	56	miR-519a
13	miR-377	35	miR-217	57	miR-518d
14	miR-378	36	miR-302c	58	miR-520a
15	miR-380-5p	37	miR-330	59	miR-520d*
16	miR-381	38	miR-325	60	miR-520h
17	miR-412	39	miR-369-3p	61	miR-523
18	miR-450	40	miR-370	62	miR-524
19	miR-452	41	miR-384	63	miR-524*
20	miR-485-5p	42	miR-453	64	miR-526b
21	miR-486	43	miR-483	65	miR-526b*
22	miR-US-33-1	44	miR-488	66	miR-526c

4.5.3 Expression of miRNAs detected in both cell lines in both states

The miRNAs expressed in both cell lines, in both states, represent a high proportion of this study. 62.97% (199) of 316 miRNAs were shown to be expressed in both cell lines, in both states. In accordance with international standards, a fold change cut-off of ± 2.0 is employed in our analysis. 15.57% (31 of 199) miRNAs are downregulated in pluripotent cells and 15.07% (30) miRNAs in nullipotent cells (Appendix Table **1**). 31.65% (63 of 199) miRNAs were upregulated in pluripotent cells and 5.52% (11) in nullipotent cells (Appendix table **2**). Some of those 199 miRNAs are up or downregulated in either cell lines and up or downregulated in one line but not in the other, (data not shown). Interestingly, upregulation of miRNAs was much more pronounced in pluripotent CSCs than nullipotent CSCs, an effect much reduced in terms of downregulation, see Table **4.2**. These data indicates that upregulation of post transcriptionally regulating miRNAs are more important in pluripotent CSC cells.

Table 4.2 Numbers of miRNAs and fold change ranges in each cell line.

		Number of miRNAs out of 199	Fold Change (2ddCt)
1	miRNAs upregulated in Pluripotent cells	63	2.0 to 26.0
2	miRNAs downregulated in Pluripotent cells	31	-2.0 to -17.7
3	miRNAs with no change in Pluripotent cells	105	-1.93 to 1.96
4	miRNAs upregulated in nullipotent cells	11	2.0 to 3.79
5	miRNAs downregulated in nullipotent cells	30	-2.0 to -9.98
6	miRNAs with no change in nullipotent cells	158	-1.96 to 1.94

4.5.4 Expression of miRNAs detected in both states in only one cell type

Populations of miRNAs were determined only in one cell line. 8.22% (26) were expressed in nullipotent cells in both states but not in pluripotent while 0.31% (1) was expressed in pluripotent cells in both states but not in nullipotent (Appendix Tables **3 and 4**). 3.84% (1) of the 26 miRNAs were upregulated in nullipotent cells in both states and 15.38% (4) downregulated. miR-346, which was expressed only in pluripotent cells in both states but not in nullipotent cells, showed lower than 2 fold differential expression (-1.06 fold change). This indicates that nullipotent cells respond to differentiation by altering expression of a group of miRNAs not expressed in NTera2 cells, which has not been previously described. The potential role of these miRNAs is a key area of ongoing interest, as will be discussed later.

4.5.5 Expression of miRNAs detected only in self-renewing or differentiated NTera2 cells but in both self-renewing and differentiating states in 2102Ep cells

In this section, we demonstrated that 5.06% (16) of miRNAs were expressed in nullipotent cells in both states and expressed only in self-renewing pluripotent cells. Out of 316 miRNAs, only miR-10a was expressed in differentiated pluripotent cells and in nullipotent cells in both states. 16 miRNAs were downregulated in nullipotent cells in both states and 62.5% (10) of these 16 miRNAs had lower than 2 fold differential expression (Table **4.3A**). Strikingly, this lack of ability of nullipotent CSCs to alter expression of miRNAs differentially expressed in pluripotent CSCs may be key to malignancy.

miR-10a increased 119 fold in nullipotent cells while remaining detectable but at a level lower than the 2 fold threshold (-0.82 fold) in pluripotent cells. Therefore, the lowest level (dCt) of expression only in self-renewing pluripotent was 21.79 fold change and the highest level was 1.87 fold change (Table **4.3B**).

Table 4.3A Expression levels of miRNAs only expressed in undifferentiating NTera2 cells but in both undifferentiating and differentiating 2102Ep cells.

	NTera2	Fold Change Undiff(-dCt)		2102Ep	Fold Change (2ddCt)
1	miR-299-3p	16.23689		miR-299-3p	-38.05
2	miR-520b	13.11003		miR-520b	-9.4901
3	miR-522	14.41105		miR-522	-7.97281
4	miR-507	5.653767		miR-507	-6.85517
5	miR-490	19.07589		miR-490	-2.47681
6	miR-361	20.21413		miR-361	-2.39651
7	miR-301	21.7906		miR-301	**-1.86864**
8	miR-484	18.42621		miR-484	**-1.81975**
9	miR-7	19.24855		miR-7	**-1.64156**
10	miR-182*	7.990303		miR-182*	**-1.28517**
11	miR-373*	9.522419		miR-373*	**-1.14427**
12	miR-206	9.116274		miR-206	**1.208086**
13	miR-122a	1.875502		miR-122a	**1.283579**
14	miR-515-5p	6.150898		miR-515-5p	**1.314747**
15	miR-380-3p	8.074121		miR-380-3p	**1.394804**
16	miR-520g	13.41263		miR-520g	**1.467389**

Table 4.3B Expression levels of miRNAs only expressed in differentiating NTera2 cells but in both undifferentiating and differentiating 2102Ep cells.

	NTera2	Fold Change Diff (dCt)		2102Ep	Fold Change (2ddCt)
1	miR-10a	-0.82523		miR-10a	119.1715

4.5.6 Expression of miRNAs detected in either one or both of the cell lines (NTera2, 2102Ep) in only one state

An interesting result, 4 individual groups of miRNAs demonstrated unusual gene expression patterns. Out of 316 miRNAs, 0.31% (1) of miRNAs were detected in either one or both of the cell lines in only one state. One group of 3 miRNAs (0.94%) were only expressed in differentiating nullipotent cells but not in pluripotent cells (appendix tables **5A to 5E**). Fold changes for miRNA expression were determined using the dCt method where miRNA expression was detected only in self-renewing or differentiated states. Expression levels are increased as dCt is closer to or less than 1.0, as compared for the expression value of the internal control.

miR-142-5p was only expressed in the self-renewing state in both cell lines, while miR-137 was only expressed in undifferentiating pluripotent cells and differentiating nullipotent cells. miR-199a* was only expressed in undifferentiating pluripotent cells but not in nullipotent cells. Let-7c was only expressed in differentiating pluripotent cells but not in nullipotent cells and the last group of 3 (miR-425, miR-105 and miR-433) were only expressed in differentiating nullipotent cells but not in pluripotent cells. The level of expression of miR-142-5p decreased from a dCt of 7.19 (pluripotent) and 8.3 (nullipotent), being undetectable upon differentiation. miR-137 decreased from a dCt of 17 in the self-renewing state in pluripotent and increased to a dCt of 12 upon differentiation in nullipotent, expression being undetectable in the opposite state in each cell line: the level of expression being higher in self-renewing pluripotent cells than in differentiated nullipotent cells. The level of expression of miR-199* was 18 (dCt) in self-renewing pluripotent cells and undetectable in either state in nullipotent cells. The level of expression of let-7c was 2.4 (dCt) in differentiating pluripotent cells but not in nullipotent cells in both states. Expression levels for the last group of 3 miRNAs were shown in hierarchal order, miR-425, miR-105 and miR-433, and were 11, 2.7 and -1.1 (dCt) in differentiating nullipotent cells and undetectable in either state in pluripotent cells. Therefore, expression of miR-142-5p is similar in pluripotent and nullipotent cells: expression of miR-137 is opposite in pluripotent and nullipotent cells: a group of 3 miRNAs specifically upregulate in nullipotent CSCs in response to differentiation.

4.6 Chapter Discussion

Our analysis demonstrates the involvement of miRNAs in early CSC differentiation. We have identified that

- 66 miRNAs were undetermined in both cell lines, in both states
- 199 miRNAs were shown to be expressed in both cell lines, in both states
- 26 miRNAs were expressed in nullipotent cells in both states but not in pluripotent cells
- 1 miRNA (miR-346) was expressed in pluripotent cells in both states but not in nullipotent
- 16 miRNAs were expressed in nullipotent cells in both states and expressed only in self-renewing pluripotent cells
- 1 miRNA (miR-10a) was expressed only in differentiated pluripotent cells and in nullipotent cells in both states
- A different single miRNA (miR-142-5p) was only expressed in the self-renewing state in both cell lines
- 1 miRNA (miR-137) was only expressed in undifferentiating pluripotent cells and differentiating nullipotent cells
- Only 1 miRNA (miR-199a*) was expressed in undifferentiating pluripotent cells but not in nullipotent cells
- 1 miRNA (let-7c) was only expressed in differentiating pluripotent cells but not in nullipotent cells
- A last group of 3 miRNAs (miR-425, -105 and -433) were only expressed in differentiating nullipotent cells but not in pluripotent cells.

Our analysis clearly demonstrates that regulation by miRNAs is a key component of both cell types to differentiation. We have now characterised the involvement of specific miRNAs in EC differentiation. These miRNAs are either required by both cell types of specific cells and in specific states. These miRNAs are now available for functional analysis by the group. At the time of writing several miRNAs have been overexpressed in 2102Ep cells, based on the data described in this chapter. These analyses have already identified novel regulation of stemness (unpublished data).

Of the 316 miRNAs studied, 20% (63) of miRNAs were upregulated in differentiating pluripotent cells and 9.8% (31) were downregulated, whereas 3.5% (11 of 316) miRNAs were upregulated in differentiating nullipotent cells and 9.5% (30) were downregulated.

Our data showed nearly similar numbers of downregulation in pluri- and nullipotent CSCs. However, different percentages of expression were observed between the pluripotent CSCs and nullipotent CSCs in upregulation terms. While the percentage of miRNAs downregulated was similar in both pluri-nullipotent CSC cells, that of upregulating miRNAs in pluripotent CSC cells was four times more than the nullipotent CSCs. These data indicates that upregulation of post-transcriptionally regulating miRNAs is more important in pluripotent CSCs. The bias ultimately results in nullipotency. Anti-cancer therapies could be developed towards pushing nullipotent CSCs beyond this 'differentiation lesion'. 66 miRNAs undetermined in both cell lines are not necessary for CSCs while their targets are not post-transcriptionally regulated in pluri- and nulli-CSCs. Analysis of the targets of these miRNAs may identify processes that are constitutively required in CSCs.

In accordance with international standards, a fold change cut-off ±2.0 is employed in our analysis. 20% (63 of 199) miRNAs were upregulated in pluripotent cells and 3.5% (11) in nullipotent cells. Pluripotent stem cells have the ability to differentiate while nullipotent cells are poorly-differentiating. Therefore, post-transcriptional regulation via miRNAs is more important to differentiation in pluripotent cells than nullipotent cells. Pluripotent cells upregulate miRNAs, whereas nullipotent cells cannot. It is likely that consistent expression of these miRNAs contributes to the nullipotent phenotype. Downregulation of miRNAs in nullipotent cells that are upregulated in pluripotent cells may stop nullipotent cells from differentiating while pluripotent CSC differentiation proceeds. Downregulation of miRNAs in pluripotent cells that are expressed in nullipotent cells may allow expression of target proteins in pluripotent cells, permitting differentiation, and downregulation of these proteins in nullipotent cells, which may stop differentiation. Downregulation of miRNAs in both cell lines in both states suggests that the pluripotent differentiation process is active in nullipotent cells but that a 'differentiation lesion' exists. This lesion permits expression of some early miRNAs (those expressed in the same way in both cell types) while not allowing changes in others (those altered in pluripotent CSCs but unaltered in nullipotent CSCs). The lesion ultimately results in nullipotency. Anti-cancer

therapies could be developed towards pushing nullipotent CSCs beyond this differentiation lesion.

The primary role of miRNAs if the regulation of specific target genes, which was detailed in chapter 1 (**section 1.6**). The absence of 26 miRNAs allows expression of their target proteins in the pluripotent cells to facilitate differentiation. miR-346, which was expressed only in pluripotent cells in both states but not in nullipotent cells was below threshold (had no change). This miRNA and the 26 miRNAs may be useful as specific targets to identify whether cancer cells are pluripotent and nullipotent. No validated gene targets have been described for miR-346. Predicted targets can be identified using the miRGEN resource, which combines the power of five different miRNA targeting algorithms (http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html). Predicted targets for miR-346 include key signaling genes such as cellular proliferation regulator bruton tyrosine kinase (Btk) and differentiation regulator plasminogen precursor (Plg), suggesting a stem cell role.

Differentiated pluripotent cells do not require 16 miRNAs. These 16 miRNAs may play a specific post-transcriptional regulation role in self-renewal of pluripotent cells while their downregulation facilitates differentiation of pluripotent cells in response to retinoic acid. So the presence of these 16 miRNAs allows the pluripotent cells to self-renew and the absence of these 16 miRNAs allows expression of target proteins in the pluripotent cells, allowing differentiation. Out of 16 miRNAs, 6 miRNAs were downregulated in nullipotent cell lines in both states. These 6 miRNAs acted in parallel in both cell lines. In nullipotent cells, these were downregulated, whereas in pluripotent cells expression dropped from detectable to undetectable levels upon differentiation. Therefore, these 6 miRNAs may be controlled by one or more of the same signal transduction or transcriptional regulators. This data suggests that these miRNAs may be affected by signaling or feed-back mechanisms leading to downregulation in nullipotent cells. Additionally, this suggests the presence of an early-acting lesion in pluripotent cells when both cell lines are stimulated to differentiate. An early mechanism exists in pluripotent cells that facilitates differentiation via downregulation of miRNAs. There is a lesion in this mechanism in nullipotent cells: no downregulation of these miRNAs is seen in nullipotent cells, perhaps stopping differentiation of these cells. miR-10a was expressed in differentiated pluripotent cells and in nullipotent cells in both states. The pluri-nullipotent cells do not require miR-10a in the self-renewing state, whereas in the differentiated state this miRNA plays a specific post-transcriptional regulation role in facilitating differentiation, perhaps as a regulator of

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downstream pathways and/or miRNAs. Upregulating the expression of miR-10a may be useful as a therapy in poorly-differentiated tumours to change them from dangerous malignant cells (self-renewal cells) to less malignant cells (differentiating cells). miR-10a has been shown to control hypoxia-related HoxA1 expression, in line with a role in malignancy (Garzon *et al.,* 2006).

The level of expression of miR-142-5p was similar in the self-renewing state in both cell lines and was undetermined in differentiating states in both cell lines. Therefore, miR-142-5p acted in parallel in both cell lines in both states and may be controlled by the same signal transduction or transcriptional regulators. This data suggests that this miRNA is not necessary for differentiated CSCs and that their targets are not post transcriptionally regulated in differentiating pluri- and nulli-CSCs. Lastly, this miRNA (miR-142-5p) may play a specific post-transcriptional regulation role to maintenance of self-renewal in both cell lines and is likely to be an early mechanism in both cells response to differentiation. Predicted targets for miR-142-5p include angiogenesis regulator Bai3 and Aprin 2, a negative regulator of cell proliferation linked to androgen sensitivity in prostate cancer, indicating a role in malignancy (miRGEN).

Expression of miR-137 worked in an opposite way in the two cell lines: expressed only in self-renewing pluripotent cells and expressed only in differentiated nullipotent cells. miR-137 is not required either for differentiating pluripotent cells or self-renewing nullipotent cells. Interestingly, this miRNA may play a specific post-transcriptional regulation role in maintenance of self-renewal of both cell lines, though regulated through different mechanisms. Upregulation of miR-137, which is downregulated in pluripotent cells to facilitate differentiation, may occur in response to differentiation in nullipotent cells as a differentiation-avoidance mechanism. Notably, miR-137 is predicted to target leukaemia gene Hlf, indicating a role in malignancy (miRGEN).The identification of such a mechanism may be vital to our understanding of the role of CSCs in highly malignant tumours and requires further future study.

As neither miR-199* nor Let-7C are expressed in nullipotent cells, these miRNAs are not required for nullipotent cells in either state. Both these miRNAs may be regulated by similar signal transduction or transcriptional regulation mechanisms acting after the nullipotent differentiation lesion. miR-199a* is not required in the differentiated state in either cell lines, whereas Let-7C is not required in the undifferentiated state in either cell

lines. This suggests that miR-199a* is necessary for maintenances of the self-renewal state in pluripotent CSCs, whereas, let-7C is necessary for differentiation in pluripotent CSCs. In fact, the Let-7 family of miRNAs has a long association with caner (reviewed by Boyerinas *et al.,* 2010) while miR-199a* is a validated regulator of the Met proto-oncogene (Kim *et al.,* 2008), demonstrating roles in malignancy. Therefore, over-expression of let-7C may be useful as a therapy, forcing self-renewing CSCs to differentiate, thus decreasing their malignant potential.

A group of 3 miRNAs (miR-425, miR-105 and miR-433) and the previous 26 miRNAs may be controlled by one or more of the same signal transduction or transcriptional regulators in the pluripotent cell lines. The group of 3 miRNAs acted in parallel in both cell lines in both states and they are not necessary for pluripotent cells. The absence of these miRNAs in self-renewing nullipotent cells permits expression of the target protein to maintain the self-renewal state. Whereas the lack of expression of regulatory genes in nullipotent cells demonstrates a lesion in the differentiation process, the expression of additional nullipotent CSC-specific miRNAs indicates that these cells respond to differentiation specifically: a response that may permit avoidance of differentiation. miR-433 is a validated regulator of Fgf signaling, which is linked to cancer stemness (Wang *et al.,* 2008). miRs-103 and -425 are predicted to target multiple signaling molecules linked to cellular proliferation (miRGEN).

4.7 Comparison of CSC with hES cells

As hES cells are not used by our group but are a non-malignant comparator for our CSC model, we carried out a literature-based comparison of our data with hES data. A list of 150 differentially expressed miRNAs in differentiated and self-renewing hES cells, has recently been described and was supplied to the group by the authors (Laurent *et al.,* 2008). This data was used to compare with our data. Exact levels of expression were not comparable as different technologies were used to generate the two data sets. Of the 316 miRNAs in our study, 20.25% (64) of the miRNAs were upregulated in differentiating pluripotent cells and 9.8% (31) were downregulated, whereas 5.06% (16 of 316) of the miRNAs were upregulated in differentiating nullipotent cells and 12.65% (40) were downregulated. In the hES cells, out of 700 miRNAs 10.5% (74) of miRNAs were expressed higher in differentiated hES cells and 10.85% (76) of miRNAs were expressed higher in self-renewing hES cells. This data showed similar levels of upregulation and

downregulation in hES cells. Different percentages of expression were observed between the CSCs and hES cells. While the percentage of miRNAs downregulated was similar in both pluripotent cell types, that of CSCs was twice that of hES cells in terms of upregulated genes. Conversely, while levels of downregulation were similar in hES and nullipotent CSCs, the percentage of upregulated miRNAs in nullipotent cells was half that of hES cells, supporting the nullipotent lesion hypothesis. Therefore CSCs are characterised by twice as much upregulation as downregulation of miRNAs whereas in hES cells similar numbers of miRNAs are up and downregulated.

We also identified miRNAs regulated in CSCs but not in hES cells. The miRNAs and the relative expression levels in these cells are shown in appendix tables **6A-D**. 4 miRNAs downregulated in pluripotent cells and unaltered in nullipotent cells were unchanged in hES cells (Appendix Table **6A**). 37 miRNAs upregulated only in pluripotent cells and had no change in nullipotent cells and were unaltered in hES cells (Appendix Table **6B**). 10 miRNAs downregulated in nullipotent cells and unaltered in pluripotent cells were unchanged in hES cells (Appendix Table **6C**). 6 miRNAs upregulated in nullipotent cells and unaltered in pluripotent cells also were unaltered in hES cells (Appendix Table **6D**). The expression levels of the 4 miRNAs were -3 to -9 fold. 37 miRNAs were 2 to 26 fold, 10 miRNAs were between -2 and -9 fold and the remaining 6 miRNAs were altered 2 fold (Appendix Tables **6A to 6D**). Therefore these data suggested that these 4 miRNAs are CSC-specific miRNAs that are downregulated in pluripotent cells to allow expression of target proteins to facilitate differentiation. The 37 miRNAs may be regulated only in pluripotent EC cells to facilitate differentiation. The 10 miRNAs may be downregulated in only nullipotent cells to allow expression of target proteins to maintain self-renewal. Altering expression of these miRNAs may be useful in changing the cells from self-renewing to differentiating cells. The last group of 6 miRNAs upregulated only in nullipotent cells could also be termed self-renewal CSC miRNAs. Upregulation of these miRNAs only in nullipotent cells suggest that they may play a specific post-transcriptional regulation role in the maintenance of self-renewal. Therefore downregulating these miRNAs may be useful as a therapy, forcing self-renewing CSCs to differentiate, thus decreasing their malignant potential.

4.8 Conclusion

MiRNAs are key post-transcriptional regulation molecules that have been described in numerous biological processes. In this study, we demonstrate that miRNA expression is tightly regulated during EC self-renewal and differentiation. Specifically, we have characterised the expression of each of 316 miRNAs, identifying miRNAs that are highly and specifically state and cell-specific. These miRNAs are now available for functional analysis for the group. These are actively being used in an attempt to remove stemness from EC cells.

Chapter Five

Overexpression and Knockdown

of Tgf-ß-R2

5.1 Chapter introduction

5.1.1 General overview

The Transforming growth factor, beta (Tgf-ß) pathway is a family of cytokines that includes a diverse group of locally acting signaling molecules that act through related local release and receptor mechanisms to direct pre and post natal differentiation of tissues. Transforming growth factor, beta receptor II (Tgf-β-R2) is a membrane-bound receptor for the Tgf-ß pathway (**Figure 5.1**). The type II receptor phosphorylates the type I receptor (Tgf-β-R1) through a kinase domain. The phosphorylated type I receptor then catalyzes phosphorylation of a cytoplasmic agonist/receptor complex specific receptor smad. The receptor smad then forms a complex and is translocated to the nucleus for interaction with promoter sites of target genes (Miyazawa *et al.*, 2002 and Clark *et al.*, 2009). Tgf-ß cytokines are sequestered to the extracellular matrix in a latent form that can be locally activated by several mechanisms that allow autocrine and paracrine control of cellular responses.
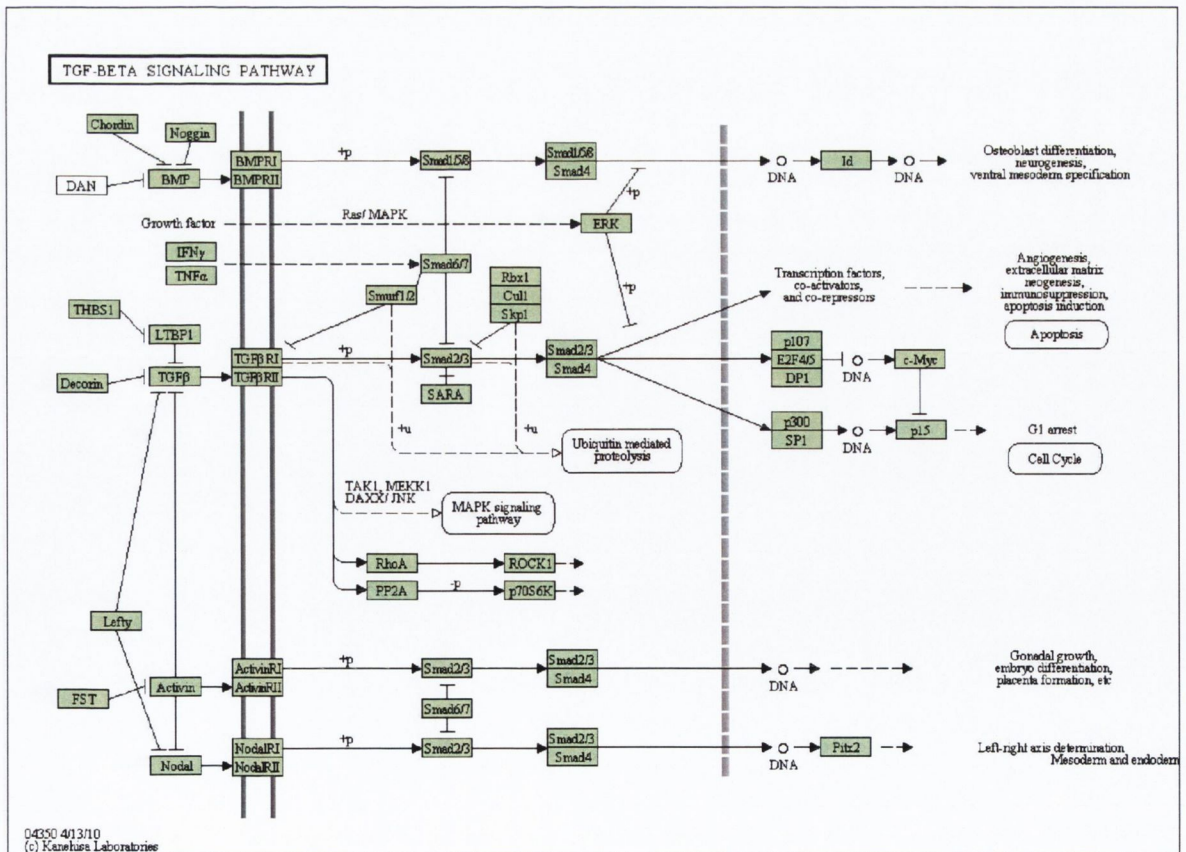
Figure 5.1 The TGF-ß signaling pathway showing gene locations and signal directions. (Tutorials Pathway analysis: www.genome.jp).

Gene knockdown and overexpression are a genetic technique facilitating inactivation or over-activating of specific genes in order to determine function. Knockdown involves insertion of a siRNA, which degrades transcripts of the gene of interest. Over-expression involves insertion of the sequence of a gene of interest into a plasmid containing the machinery to constantly produce high levels of the protein. By studying the organism with over- or under-expression of the gene, scientists can determine the function of the gene.

As it was described in the introduction chapter, the mechanism of Tgf-β signaling may be important for the development of new approaches to treatment of various clinical diseases in which Tgf-β signaling is involved. Tgf-β signaling begins on the basement membrane when binding of receptor induces phosphorylation and activation of the receptor Tgf-β-R1 by the receptor Tgf-β-R2. The activated receptor Tgf-β-R1 phosphorylates SMAD2 and SMAD3, which bind to the SMAD4 mediator to move into the nucleus and form complexes that regulate transcription (Roberts *et al.*, 2001).

5.2 Chapter Hypotheses

Analysing pluripotent and nullipotent CSCs identified specific pathways whose expression patterns are differentially regulated during early differentiation of CSCs. That analysis identified that TGF-β was up-regulated in differentiated pluripotent NTera2 cells and downregulated in differentiation of nullipotent 2102Ep cells.

We hypothesised that knockdown of TGF-betaR2 in pluripotent NTera2 cells could impact on their ability to differentiate.

We propose that nullipotent 2102Ep cells do not differentiate due to a lesion in the differentiation mechanism or apparatus. We hypothesised that TGF-betaR2 may be a component of this 'differentiation lesion'. As such, we hypothesised that overexpression of TGF-betaR2 in nullipotent cells could permit them to differentiate spontaneously or in response to retinoic acid.

Finally, we hypothesise that the mechanism of TGF-beta signaling may be different in EC cells that reported for non-malignant cells.

5.3 Chapter Aims

The aim of this chapter was to functionally assess whether TGF-betaR2 was necessary and/or sufficient for self-renewal and/or differentiation of EC cells. We aimed to initially knockdown TGF-betaR2 in NTera2 cells and assess their ability to maintain the self-renewal state and differentiate in its absence.

We have previously described how 2102Ep nullipotency may be due to a 'differentiation lesion'. We aimed to assess the involvement of TGF-beta signaling in 2102Ep cells. We aimed to over-express TGF-betaR2 in 2102Ep cells to assess whether this was sufficient to induce spontaneous or RA-induced differentiation.

Finally, we aimed to assess whether TGF-beta signaling in EC cells differed to that reported for non-malignant cells.

5.4 Chapter Materials and Methods

Full details of methods used are described in chapter 2. To aid this description, an overview flow diagram of the approach is shown in Figure **5.2**. Briefly, CSCs (NTera2 and 2102Ep) were harvested from a T-75 flask and two suspensions were diluted with DMEM media and Trypan Blue 0.4% stain to count the cells in a haemocytometer under the light microscope. A quantity of 168,000 cells/3.2mls were plated in 6-well plates and incubated at 37°C and 5% CO_2 for 24hrs. Before the start of this project, optimal conditions for transfection of NTera2 and 2102Ep cells were identified by our group. Following this optimised protocol, transfection of plasmid DNA and siRNA in 6-well plates was performed using two different transfection agents: Lipofectamine™ 2000 was used to overexpress TGF-beta-R2 in 2102Ep cells and Lipofectamine™ RNAiMAX was used to knockdown TGF-beta-R2 in NTera2 cells. Non-Transfected Control (NTC) cells, Mock-transfected (Moke) cells and 'Silencer Negative control (Ng) number 1'-transfected cells were used as controls to assess the specificity of overexpression and knockdown. The overexpressed and knocked-down cells were collected and total RNA and Protein isolated using the *mir*Vana ™ PARIS ™ Kit. The quality of RNA was assessed using a NanoDrop spectrophotometer. RNA and protein samples were stored at –80°C. Single-stranded cDNA synthesis performed using the High capacity cDNA Archive kit. The RT-PCR experiments were performed using Gene Amp PCR System (Applied Biosystems, USA). Thermocycling conditions were as follows: 25°C for 10 minutes and 37°C for 120 minutes. mRNA expression analysed using TaqMan® Universal PCR Master Mix and the 7500 Real Time PCR System or the 7900HT Fast Real-Time PCR System. All reactions were carried out in triplicate and B2M was used as the internal control to normalise gene expression data. The 2^{-ddCt} method was used to calculate gene expression fold change values. An overview of this procedures shown in Figure **5.2**. Overexpression and Knockdown values of Non-Transfected Control cells, Negative Control cells and Mock-transfected cells were used to calculate fold change values for overexpression and knockdown analysis. Non-Transfected Control and Negative Control were equated to 100% and expression in overexpressed and mock samples presented relative to this. This was achieved via the calculation % Expression $=100(100-2^{-ddCt})$. Western Blot analysis was used to validate biochemically TGF-beta-R2 overexpression in 2102Ep cells and knockdown in NTera2 cells and to confirm the real-time PCR data. Protein concentrations were assessed by BCA™ assay protocol. Protein samples and markers were separated by

acrylamide gel electrophoresis at a constant current of 120mV. The resolved proteins were transferred to a membrane using a wet transfer system, incubated with primary and secondary antibodies and imaged via the Luminescent Image Analyzer (General Electric).
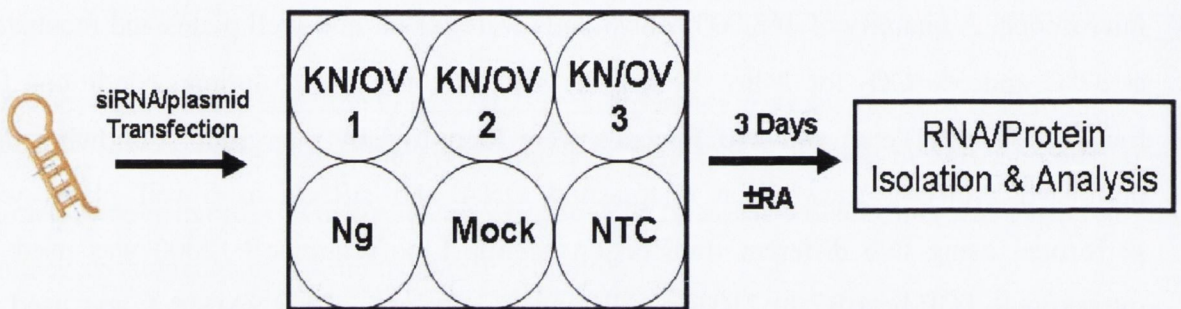
Figure 5.2 Overview of the transfection procedure. This figure shows the procedure for siRNA-induced knockdown (KN) and plasmid-induced overexpression (OV) in EC cells. siRNAs or plasmids were transfected into NTera2 and 2102Ep cells using Lipofectamine RNAiMAX or 2000 respectively. Negative control siRNA (Ng), mock-transfected (Mock) and untreated 'non-trannsfected control' (NTC) cells were used as controls. Cells were incubated for three days in ± retinoic acid (±RA), which induced differentiation. Cells were subsequently harvested and RNA and protein isolated for qPCR and western analysis respectively.

5.5 Chapter Results

5.5.1 Expression of key stemness genes and differentiation markers is altered by overexpression and knockdown of Tgf-beta-R2 in cancer stem cells

Expression of key stemness and cancer pathways (Wnt5a, Notch, Snail, Pten, Shh and Tgf-β) during RA-induced differentiation of pluripotent and nullipotent cells over a period from 3 days to 2 weeks was studied in chapter three. These pathways have been demonstrated to play an important role in stemness and to be co-ordinatedly regulated to a high level of expression after 1 week differentiation. Expressions of all pathways were up-regulated in differentiated pluripotent cells. Inclusion of the early differentiation data demonstrates that TGF-beta signaling is required during early and late differentiation. An unexpected result, TGF-β was up-regulated in differentiated pluripotent NTera2 cells and downregulated or not altered in differentiation nullipotent 2102Ep cells. These data indicate that TGF-β signaling is upregulated in pluripotent and not altered in nullipotent cells. Our expectation was that up-regulating the TGF-beta pathway in nullipotent cancer stem cells (2102Ep) may enable the nullipotent cells to differentiate and that knocking down the TGF-beta pathway from pluripotent cancer stem cells (NTera2) may self-renew the pluripotent cells. Our group's microarray data shows that the highest upregulated genes in the Tgf-β pathway were Tgf-beta-R2, Noggin and Lefty2 during early differentiation of pluripotent NTera2 cells (**see Chapter 3**). Therefore, these three genes were chosen to assess the function of differentiation and self-renewal of the CSC cells in both positive and negative fashion (Retinoic acid -treated and Non Retinoic acid-treated). The Tgf-β-R2 knockdown and overexpression techniques were developed in this study and the Noggin and Lefty2 knockdown and overexpression techniques will be developed in future work.

Lipofectamine™ 2000 Transfection Reagent and a full ORF expression cloned plasmid DNA (victor pDEST26) (**Figure 5.3**) was used to overexpress the TGF-beta-R2 in 2102Ep cells. Non-Transfected Control (NTC) cells and mock-transfected cells were used to monitor the specificity of the experiments. Lipofectamine™ RNAiMAX Transfection Reagent and Silencer Select siRNAs was used to knockdown TGF-beta-R2 in NTera2 cells. Silencer Negative control siRNAs (Negative), which do not target any gene products, were used to monitor the specificity of the experiments. B2M was used as an internal control to assess gene expression.
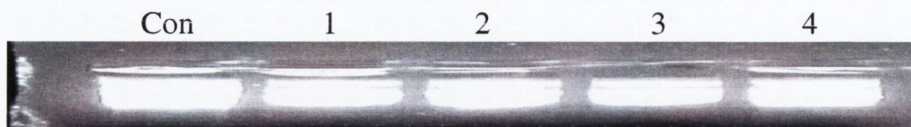
Figure 5.3 Plasmid DNA profile analysis of Tgf-β. Plasmid DNA was prepared from cultures grown in LB broth. Control (Con) had been purified by imaGenes (Berlin, Germany) and four samples of Tgf-β-R2 plasmid DNA were purified (1, 2, 3 and 4).

Expression of key stemness genes (Oct4 and Nanog) was assessed after TGF-beta-R2 had been overexpressed in nullipotent CSC cells and knocked-down in pluripotent CSCs cells. In this chapter, data is presented as a percentage expression compared to NTCs: the expression in NTCs is expressed as 100% and is presented proportional to this for all treatments. The expression of stemness genes Oct4 and Nanog in each cell type ±RA was shown in chapter three and was presented as relative expression (2^{-ddCt}). This data is replicated here and presented as percentage expression (**Figure 5.4**). The result shows that Oct4 and Nanog are expressed higher in nullipotent CSC than in pluripotent CSC (**Figure 5.4**). In contrast, TGF-betaR2 expression is much lower in 2102Ep cells (**Figure 5.5**). TGF-beta-R2 expression increased rapidly after three days differentiation in NTera2 cells and decreased rapidly after three days differentiation in 2102Ep cells (**Figure 5.5**).
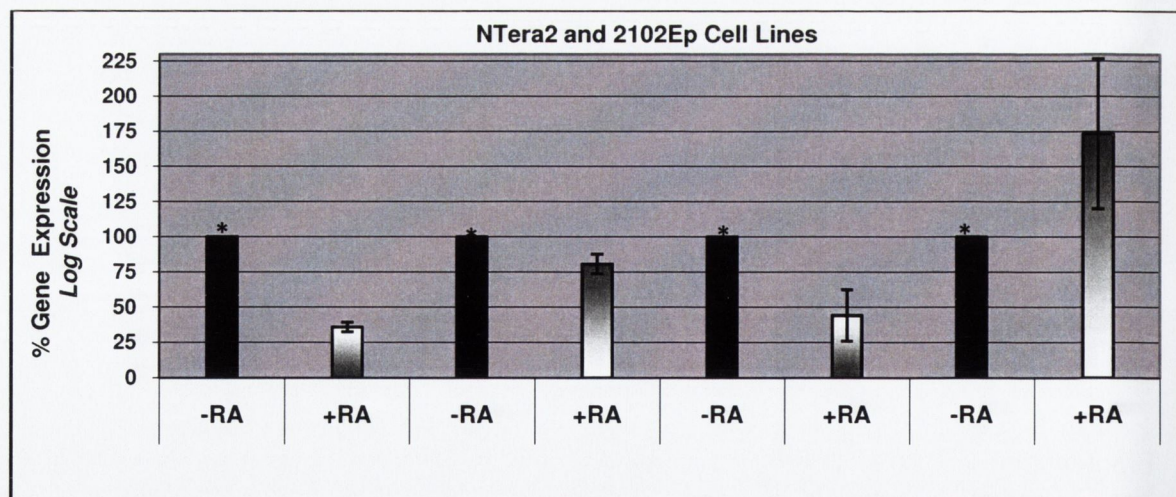
Figure 5.4 Expression of Key Stemness Genes (Oct4 and Nanog) During Differentiation in both cell lines. Non RA-treated cells (–RA) were equated to 100. Oct4 and Nanog decrease in both cell types following RA treatment. However, the levels of each gene remain higher in 2102Ep cells compared to NTera2.
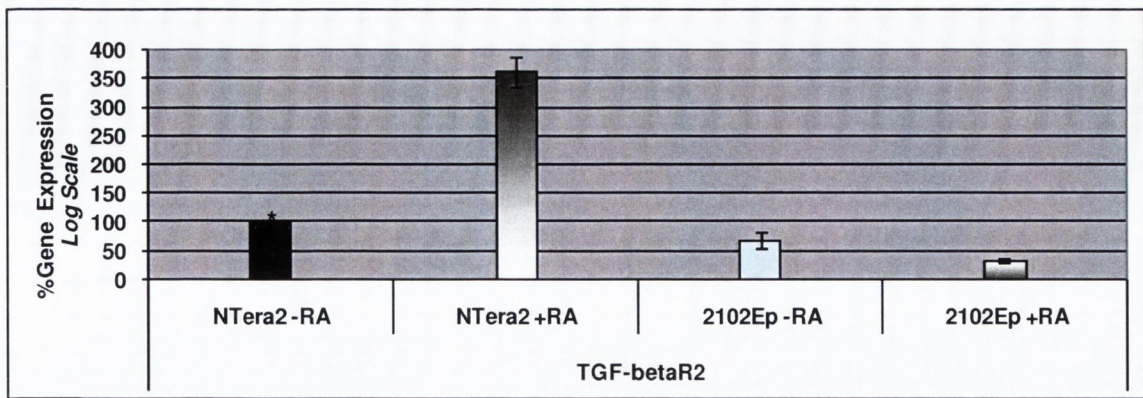
Figure 5.5 Expression of TGF-beta-R2 in non RA-treated (-RA) and RA-treated (+RA) in both cell lines NTera2 and 2102Ep CSCs. RA-treated cells were relative to non RA-treated cells. Non RA-treated NTera2 cells were equated to 100%.

Initially, transfections were carried out for 24 hours and the impact assessed. Three biological replicates of TGF-beta-R2 overexpression in 2102Ep cells and knockdown in NTera2 cells were successfully achieved in both states (differentiated and undifferentiated) after 24 hrs transfection. Overexpression of TGF-beta-R2 was 270% in non Retinoic Acid-treated and approximately 230% in Retinoic Acid-treated nullipotent (2102Ep) CSC cells (data not shown). 80% knock-down of TGF-beta-R2 in non Retinoic Acid-treated cells was achieved and 70% in Retinoic Acid-treated pluripotent (NTera2) CSC cells (data not shown). The expression of differentiation markers (Eno3, Ncam1 and Gata6) and key stemness genes (Oct4 and Nanog) were assessed in three biological replicates after TGF-beta-R2 was overexpressed and knocked-down for 24 hrs. The 24 hrs of overexpressing and knocking-down TGF-beta-R2 was not enough to show significant changes in the expression of key stemness genes and differentiation markers in cancer stem cells (data not shown). Therefore, three biological replicate of TGF-beta-R2 overexpression and knock-down in 2102Ep and NTera2 cells respectively were assessed for 3 days.

Following another group member having found that 48 hours transfections were unsuitable for our analysis, we next carried out transfections for 3 days. The 3 days transfection agent data showed 260% TGF-beta-R2 overexpression was successfully achieved in non Retinoic Acid-treated nullipotent (2102Ep) CSC cells and approximately 190% in Retinoic Acid-treated (**Figure 5.6**). The overexpressed cells were relative to Mock (mock-transfected cells treated only with Transfection Reagent) and Non-Transfected Control cells (NTC).

70% knockdown of TGF-beta-R2 was successfully achieved in non Retinoic Acid-treated pluripotent (NTera2) CSC cells and approximately 80% in Retinoic Acid-treated pluripotent (NTera2) CSC cells (**Figure 5.7**). The knocked-down cells were relative to

Mock cells, NTC and Negative control cells (Cells transfected with Silencer Negative control siRNA, termed 'Ng').

The results in this chapter will demonstrate that overexpression and knock-down of TGF-beta-R2 changes the expression of key stemness genes and differentiation markers in cancer stem cells.
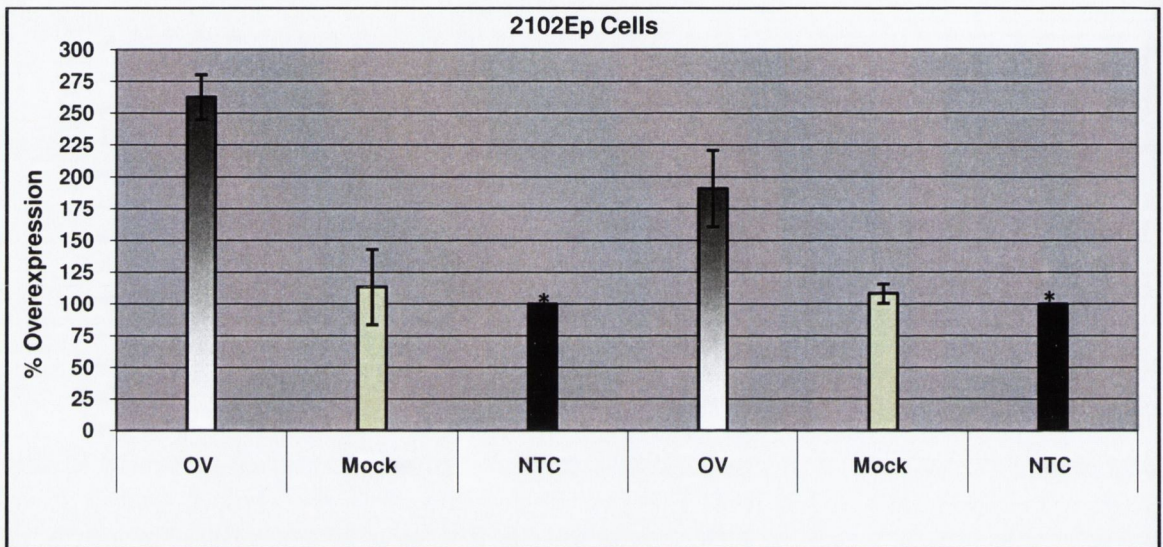
Figure 5.6 Percentage Overexpression (OV) of TGF-beta-R2 in non RA-treated and RA-treated cells relative to Mock cells and Non-Transfected Control cells (NTC cells). 260% overexpression of TGf-beta-R2 achieved in non Retinoic Acid-treated and 190% in Retinoic Acid-treated in nullipotent (2102Ep) CSC cells.
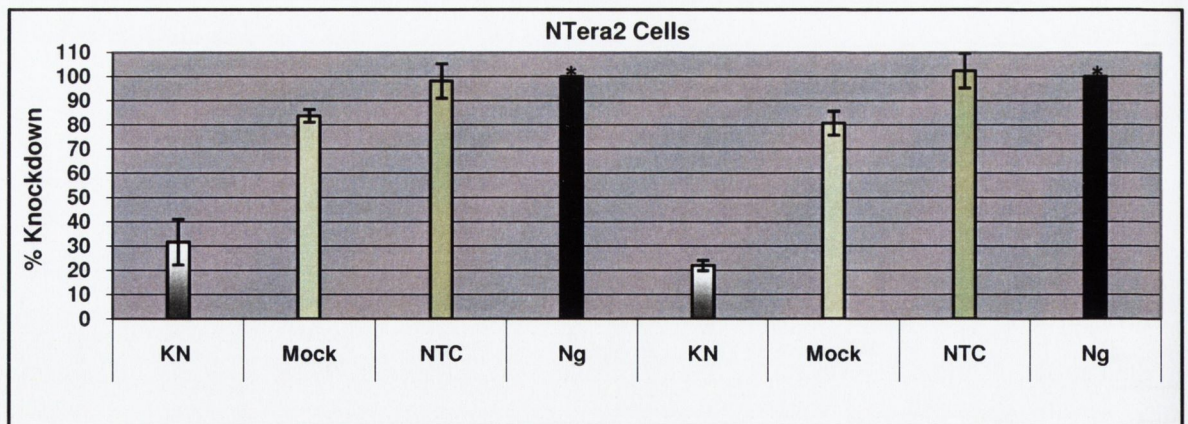
Figure 5.7 Percentage Knockdown (KN) of TGF-beta-R2 in non RA-treated and RA-treated cells relative to Mock cells, Non-Transfected Control cells (NTC cells) and negative control cells (Ng) in pluripotent (NTera2) CSC cells. 70% knockdown of TGF-beta-R2 achieved in non Retinoic Acid-treated and 80% in Retinoic Acid-treated pluripotent (NTera2) CSC cells.

We further validated biochemically the three biological replicate of TGF-beta-R2 overexpression in 2102Ep cells and knockdown in NTera2 cells using Western Blot analysis. This validation study demonstrated that the Western Blot was a suitable method to confirm the real-time PCR data. Overexpressing TGF-beta-R2 was achieved in 2102Ep cells relative to Mock-treated NTC (**Figure 5.8**). Gapdh was used as a loading control and was not affected by the overexpression of TGF-beta-R2 in 2102Ep cells (**Figure 5.8**).

Knock-down of TGf-beta-R2 in pluripotent (NTera2) CSC cells also was achieved and relative to Mock, negative-siRNA (Ng) and NTC cells, as confirmed by western blot (**Figure 5.9**). Gapdh was used as a loading control and was not affected by the knockdown of TGF-beta-R2 in NTera2 cells (**Figure 5.9**).
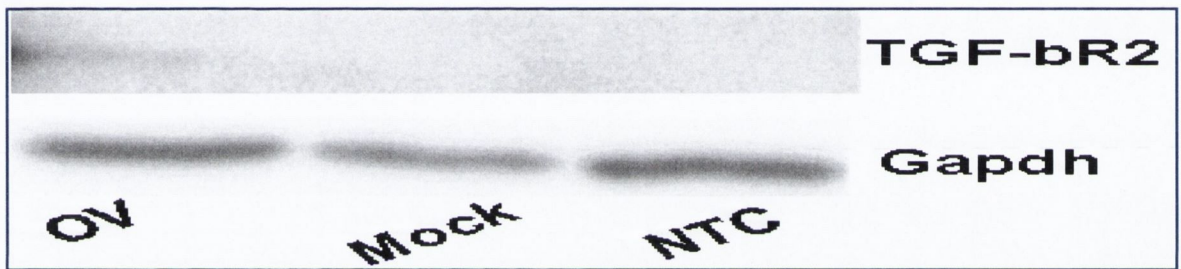
Figure 5.8 The expression of TGF-beta-R2 protein in TGF-beta-R2 overexpressed (OV), mock-transfected (mock) and non-treated 2102Ep cells (NTC). Western blot shows the overexpression of TGf-beta-R2 achieved in nullipotent (2102Ep) CSC cells but not in Mock cells and NTC cells. Gapdh expression was detected as a loading control and was unaffected by overexpression, confirming their suitability as a control.
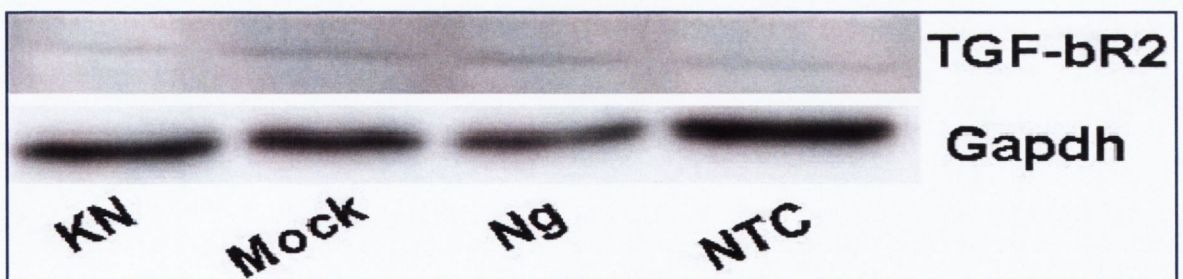
Figure 5.9 Knocking-down (KN) TGF-beta-R2 in NTera2 cells relative to Mock cells, negative control cells (Ng) and Non-Transfected Control cells (NTC cells). Western blot analysis shows that knocking-down TGF-beta-R2 were achieved in pluripotent (NTera2) CSC cells but not in Mock cells, negative control cells and NTC cells. Control Gapdh were detected and were unaffected by knocking-down of TGf-beta-R2, confirming their suitability as a control.

During our analysis, we noted that TGF-beta-R1 protein expression was affected by TGF-beta-R2 over-expression or knockdown. Historically, in normal cells TGF-beta-R2 phosphorylates and activates TGF-beta-R1, which then results in the phosphorylation of intracellular messengers, the SMADs (Miyazawa *et al.*, 2002, Kaklamani *et al.*, 2004 and Clark *et al.*, 2009). Studies suggest that TGF-beta-R1 may contribute to the development of a large proportion of common forms of cancer and may become a target for cancer chemoprevention (Kaklamani *et al.*, 2004).

Our results demonstrated that TGF-beta-R1 protein expression was downregulated by TGf-beta-R2 overexpression in nullipotent (2102Ep) cells (**Figure 5.10**). In concordance with 2102Ep results, TGF-beta-R2 knock-down caused overexpression of TGf-beta-R1. TGf-beta-R1 was promoted and showed a strong band when TGF-beta-R2 is knocked down in pluripotent (NTera2) CSC cells (**Figure 5.11**). This is the first report of the negative regulation of TGF-beta-R1 by TGF-betaR2. This interesting result will be investigated in future work.
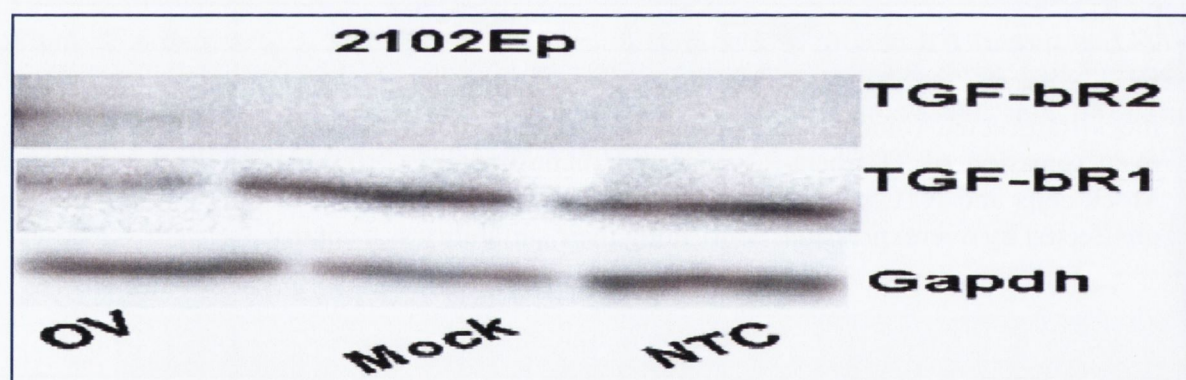
Figure 5.10 The expression of TGF-beta-R1 and TGF-beta-R2 protein in TGF-beta-R2 overexpressed (OV), mock-transfected (mock) and non-treated 2102Ep cells (NTC). Western blot analysis shows the overexpression of TGF-beta-R2 was achieved in nullipotent (2102Ep) CSC cells but not in Mock and NTC cells. TGF-beta-R1 was downregulated when TGF-beta-R2 was overexpressed in nullipotent (2102Ep) cells. Control Gapdh expression was detected as a loading control and was unaffected by overexpression, confirming their suitability as a control.
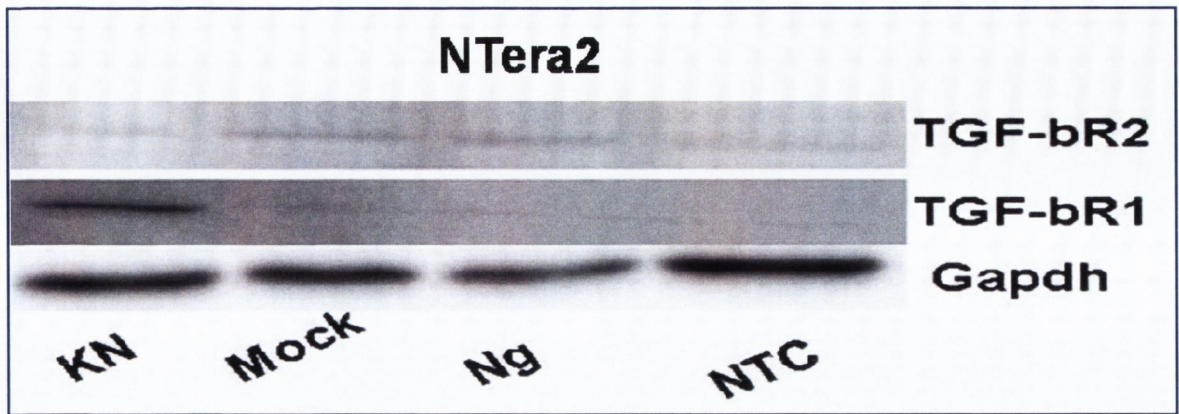
Figure 5.11 Knocking-down (KN) TGF-beta-R2 in NTera2 cells relative to Mock cells, negative control cells (Ng) and Non-Transfected Control cells (NTC cells). Western blot analysis shows the knockdown of TGF-beta-R2 achieved in pluripotent (NTera2) CSC cells but not in Mock cells, negative control cells and Non-Transfected Control cells (NTC cells). TGF-beta-R1 is promoted when TGF-beta-R2 is knocked-down in pluripotent (NTera2) cells. Control Gapdh were detected and were unaffected by knockdown of TGf-beta-R2, confirming their suitability as a control.

5.5.2 Knock-down and Overexpression of TGF-beta-R2 changed the expression of differentiation marker genes (Eno3, Ncam1 and Gata6) in 2102Ep and NTera2 CSCs

In chapter three, data shows that expression of differentiation marker genes Eno3 (mesoderm) and Ncam1 (ectoderm) was up-regulated in early differentiation of pluripotent CSCs cells and not altered in nullipotent CSCs cells. In contrast, the endoderm marker gene (Gata6) was up-regulated in both CSC lines.

Knocking-down TGF-beta-R2 reduced expression of Eno3 and Gata6 in non RA-treated pluripotent CSC cells (NTera2) and had no change in Ncam1 expression (**Figure 5.12**). TGF-betaR2 regulates mesoderm and endoderm marker genes but dose not regulate the ectoderm marker gene in non RA-treated pluripotent CSC cells (NTera2). As expected, differentiation markers genes (Eno3, Ncam1 and Gata6) increased during differentiation (+RA) of control pluripotent cells (Mock and Negative and Non-Transfected Control cells) relative to non RA-treated Non-Transfected Control cells (**Figure 5.13 and 5.14**).

Knocking-down TGF-beta-R2 had no effect on expression of Eno3 and was observed to reduce Ncam1 in RA-treated pluripotent CSC cells (NTera2) relative to Mock and Negative and Non-Transfected Control cells (**Figure 5.13**). Expression of endoderm Marker Gene (Gata6) decreased about 30% relative to the average of Mock and Negative and Non-Transfected Control cells in RA-treated pluripotent cells (**Figure 5.14**). TGF-

betaR2 regulates ectoderm and endoderm marker genes but dose not regulate the mesoderm marker gene in RA-treated pluripotent CSC cells (NTera2).
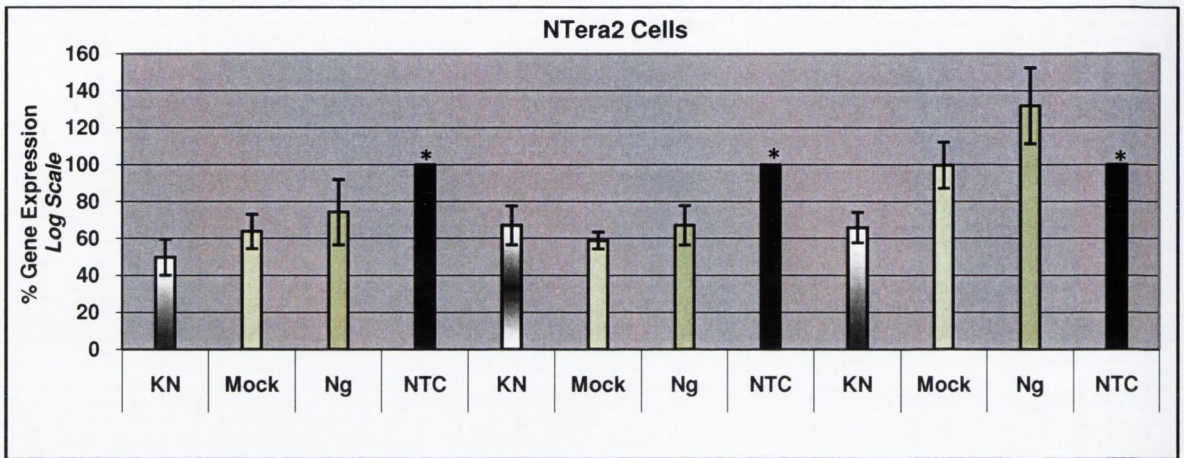
Figure 5.12 Percentage Expression of Differentiation Marker Genes (Eno3, Ncam1 and Gata6) after TGF-beta-R2 had been knocked-down (KN) in non RA-treated pluripotent CSC cells (Ntera2). Knocked-down cell values are relative to Mock cells, Negative control cells (Ng) and Non-Transfected Control cells (NTC), which are equated to 100%. Expression of Eno3 and Gata6 reduced in non RA-treated pluripotent CSC cells when, TGF-beta-R2 knocked-down and had no change in Ncam1.
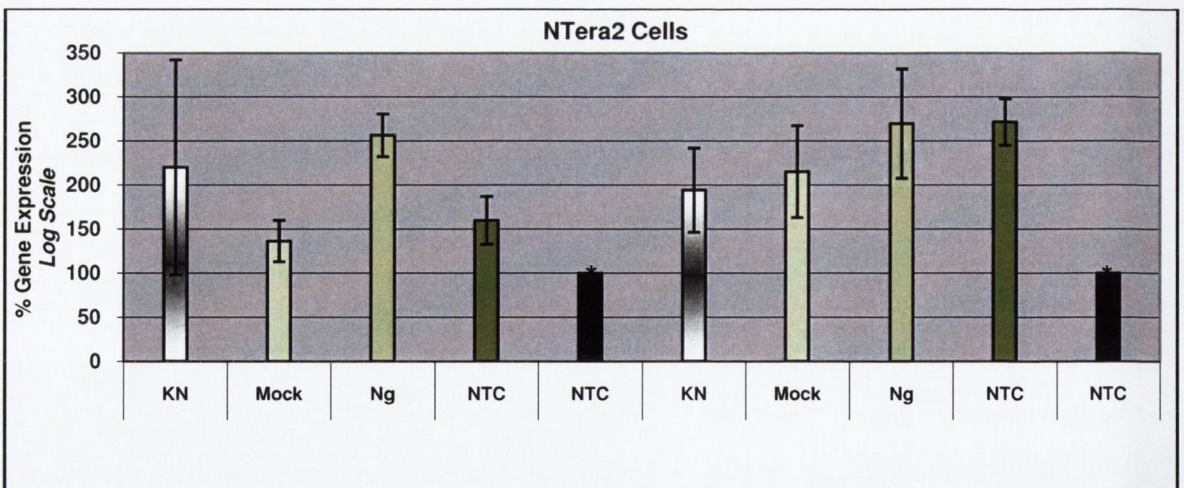
Figure 5.13 Percentage Expression of Differentiation Marker Genes (Eno3 and Ncam1) after TGF-beta-R2 had been knocked-down (KN) in RA-treated Ntera2 CSCs. Knocking-down cell values were relative to Mock cells, Negative control cells (Ng), Non-Transfected Control cells (NTC) and non RA-treated Non-Transfected Control cells, which are equated to 100%. Eno3 and Ncam1 increased during differentiation (+RA) of control pluripotent cells (Mock and Negative and Non-Transfected Control cells) relative to non RA-treated of Non-Transfected Control cells.
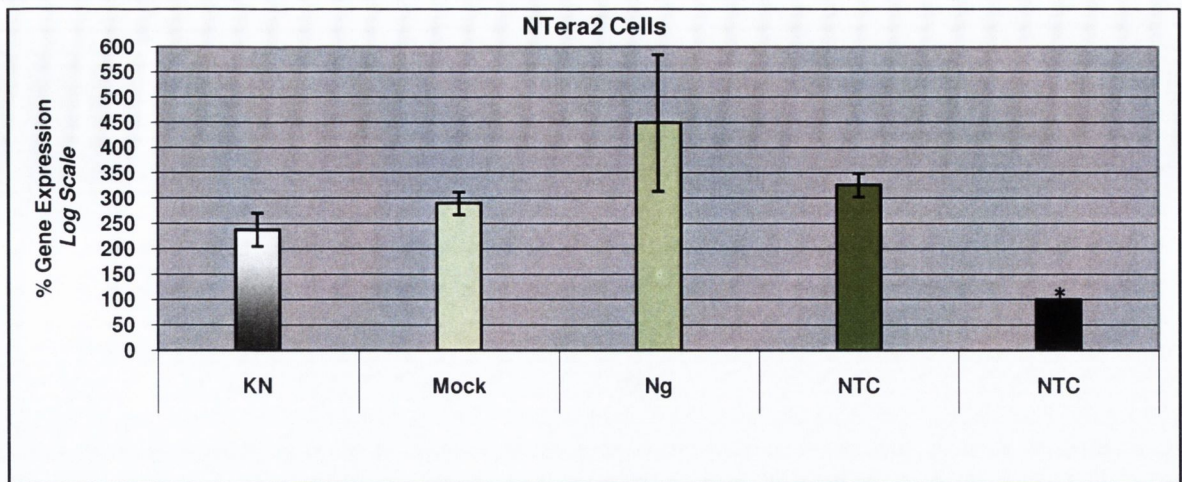
Figure 5.14 Percentage Expression of Endoderm Marker Genes (Gata6) after TGF-beta-R2 had been knocked-down (KN) in RA-treated Ntera2 CSCs. Knock-down cell values are relative to Mock cells, Negative control cells (Ng), Non-Transfected Control cells (NTC) and non RA-treated Non-Transfected Control cells which, are equated to 100%. Gata6 increased during differentiation (+RA) of control pluripotent cells (Mock and Negative and Non-Transfected Control cells) relative to non RA-treated of Non-Transfected Control cells.

Overexpressing TGF-beta-R2 also showed changes in the expression of differentiation markers in nullipotent CSCs. In non RA-treated nullipotent CSC cells (2102Ep), mesoderm differentiation marker (Eno3) was reduced. In contrast, ectoderm (Ncam1) and endoderm (Gata6) markers were observed to increase relative to mock and non-Transfected Control cells (**Figure 5.15**).

As expected, differentiation markers genes (Eno3, Ncam1) had no change during differentiation of nullipotent control cells (Mock and Non-Transfected Control cells) relative to non RA-treated Non-Transfected Control cells. However, Gata6 increased 2500% on average in mock and non-transfected control cells relative to non RA-treated non-transfected control cells (**Figure 5.16 and 5.17**). Overexpressing TGF-beta-R2 increased Eno3 about 200% and Ncam1 about 160% whereas, Gata6 decreased about 2000% in RA-treated nullipotent cells relative to mock and non-transfected control cells (**Figure 5.16 and 5.17**). TGF-betaR2 is involved in regulation of mesoderm, ectoderm and endoderm marker genes in both states RA and non RA-treated nullipotent CSC cells (2102Ep).
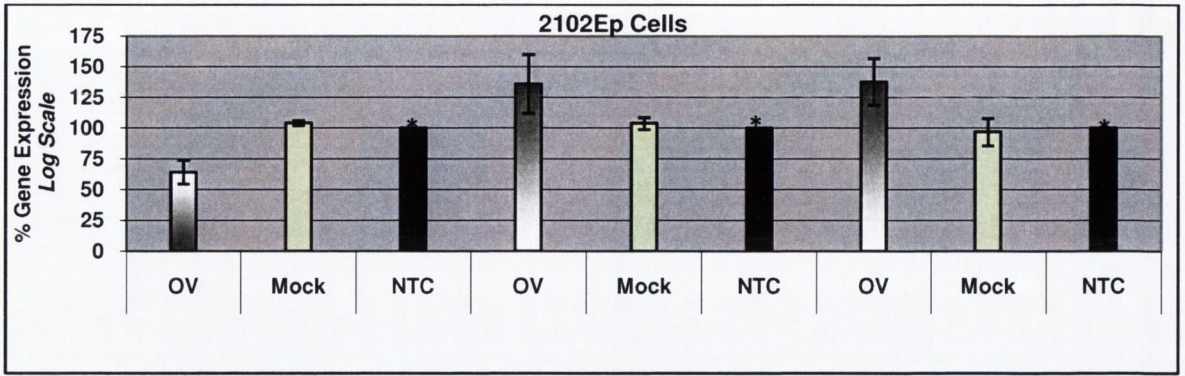
Figure 5.15 Percentage Expression of Differentiation Marker Genes (Eno3, Ncam1 and Gata6) after TGF-beta-R2 overexpressed (OV) in non RA-treated 2102Ep CSCs. Overexpressed cell values are relative to Mock cells and Non-Transfected Control cells (NTC), which was equated to 100%. Overexpressing TGF-beta-R2 showed changes in the expression of differentiation markers in nullipotent CSCs. In non RA-treated 2102Ep CSCs, Eno3 reduced 30%, Ncam1 and Gata6 markers were observed to increase relative to mock and non-Transfected Control cells.
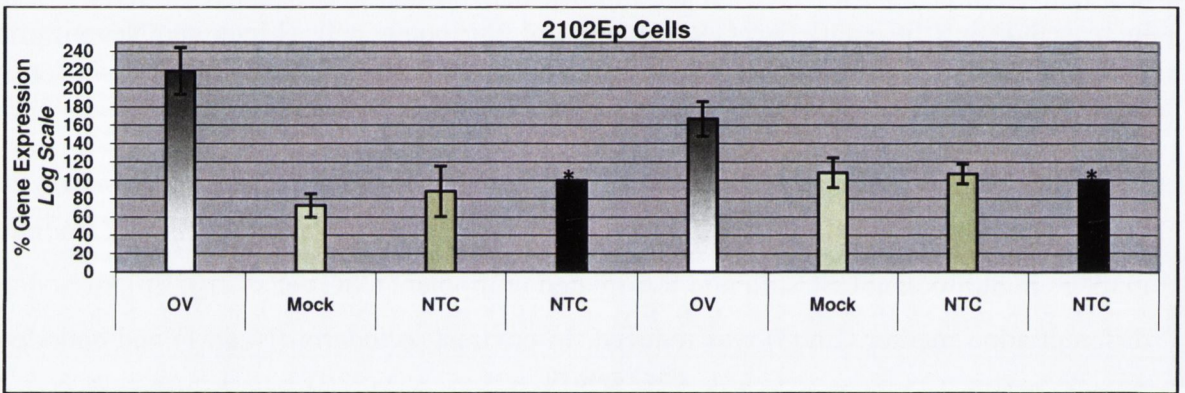
Figure 5.16 Expression of Differentiation Marker Genes (Eno3 and Ncam1) after TGF-beta-R2 overexpressed (OV) in RA-treated 2102Ep CSCs. Overexpressed cell values are relative to Mock cells and Non-Transfected Control cells (NTC). Overexpressing TGF-beta-R2 increased the expression of Eno3 and Ncam1 in RA-treated 2102Ep CSCs relative to mock and non-transfected control cells.
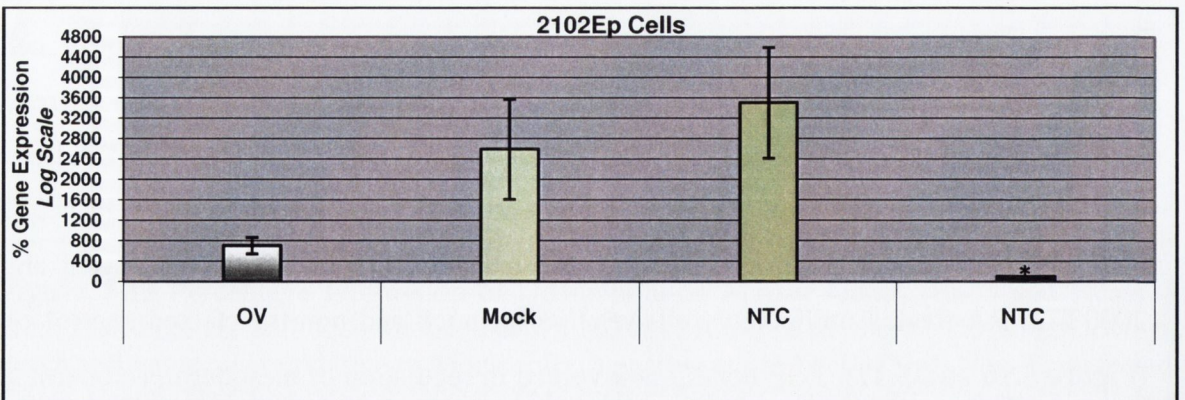
Figure 5.17 Expression of Differentiation Marker Genes (Gata6) after TGF-beta-R2 overexpressed (OV) in RA-treated 2102Ep CSCs. Overexpressing TGF-beta-R2 decreased the expression of Gata6 in RA-treated 2102Ep CSCs relative to RA-treated mock cells and non-transfected control cells. Gata6 up-regulated in differentiation of nullipotent cells (RA-treated of Mock and Non-Transfected Control cells (NTC)) relative to non RA-treated Non-Transfected Control cells (Non RA-treated), which are equated to 100%.

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5.5.3 Knocking-down and Overexpressing TGF-beta-R2 changes the expression of key stemness genes (Oct4 and Nanog) in NTera2 and 2102Ep CSCs

Our previous data in chapter three, shows that regulation of Oct4 and Nanog is achieved through down-regulation early in differentiation (only after 3 days) in pluripotent cells and is unaffected in nullipotent cells. As previously shown (**Figure 5.4**), the expression of these key stemness genes (Oct4 and Nanog) is higher in 2102Ep cells than NTera2. Oct4 and Nanog are downregulated during differentiation. In this section, we see that these regulatory mechanisms are altered by TGF-betaR2 knock-down or over-expression.

In pluripotent CSC cells (NTera2) knocking down TGF-beta-R2 did not affect Oct4, but reduced the expression of Nanog (**Figure 5.18**). When these cells were stimulated to differentiate, knocking down of TGF-beta-R2 also had no affect on Oct4 but the Nanog gene was observed to increase relative to mock cells, Non-Transfected Control cells and non RA-treated Non-Transfected Control cells (**Figure 5.19**). As expected, key stemness genes (Oct4 and Nanog) were down-regulated in differentiation of pluripotent cells (RA-treated of Knockdown, Mock, Negative control and Non-Transfected Control cells) relative to non RA-treated Non-Transfected Control cells (**Figure 5.19**). TGF-betaR2 is needed for normal regulation of Oct4 and Nanog in pluripotent CSC cells (NTera2).
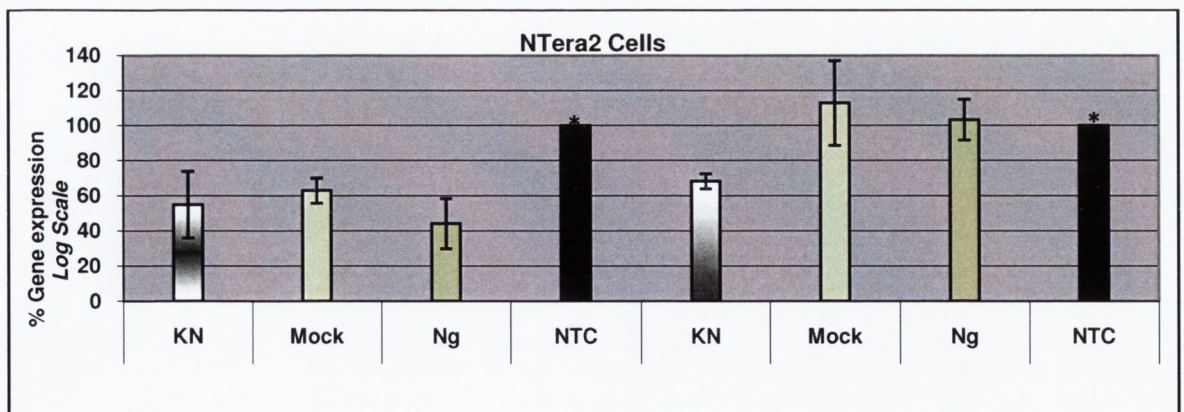
Figure 5.18 Percentage Expression of key stemness Genes (Oct4 and Nanog) after TGF-beta-R2 had been knocked-down (KN) in non RA-treated NTera2 CSCs. Knocked-down TGF-beta-R2 showed no affect in the expression of Oct4, but reduced the expression of Nanog. Knocked-down cell values are relative to Mock cells, Negative control cells (Ng) and Non-Transfected Control cells (NTC), which are equated to 100%.
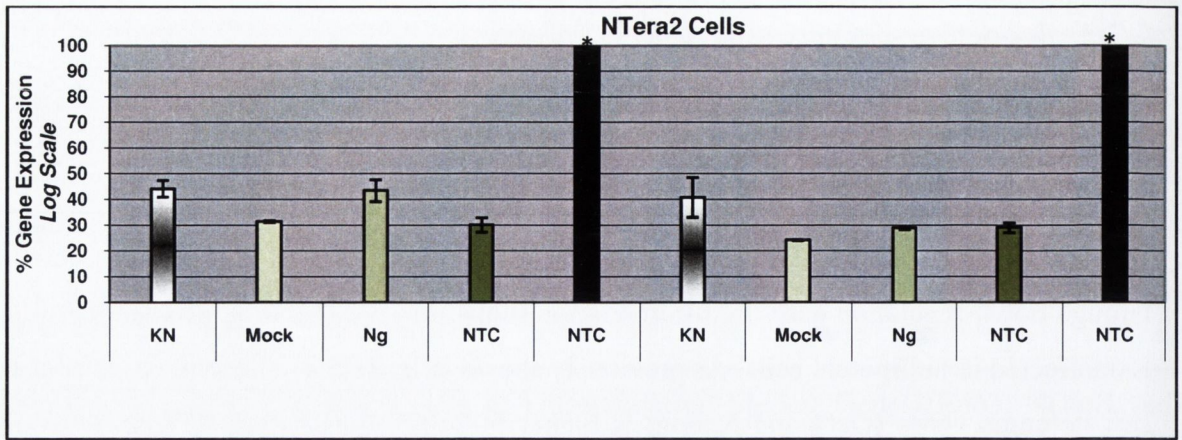
Figure 5.19 Expression of Key Stemness Genes (Oct4 and Nanog) after TGF-beta-R2 had been knocked-down (KN) in RA-treated NTera2 CSCs. Knocked-down TGF-beta-R2 showed no effect in the expression of Oct4 but the expression of Nanog gene was observed to increase relative to RA-treated mock cells, Negative control cells (Ng) and Non-Transfected Control cells. Oct4 and Nanog down-regulated in differentiation of pluripotent cells (RA-treated of Knockdown (KN), Mock, Negative control (Ng) and Non-Transfected Control cells (NTC)) relative to non RA-treated Non-Transfected Control cells (Non RA-treated) which are equated to 100%.

In nullipotent CSC cells, overexpressing TGF-beta-R2 reduced the expression of Oct4 and had no effect on Nanog relative to Mock and Non-Transfected Controls (**Figure 5.20**). When the nullipotent CSC cells were stimulated to differentiate, overexpressing TGF-beta-R2 kept the expression of the two key stemness genes high (Oct4 and Nanog) relative to Retinoic acid-treated Mock and Non-Transfected Control (**Figure 5.21**). TGF-betaR2 is needed for normal regulation of Oct4 and Nanog in nullipotent CSC cells (2102Ep).
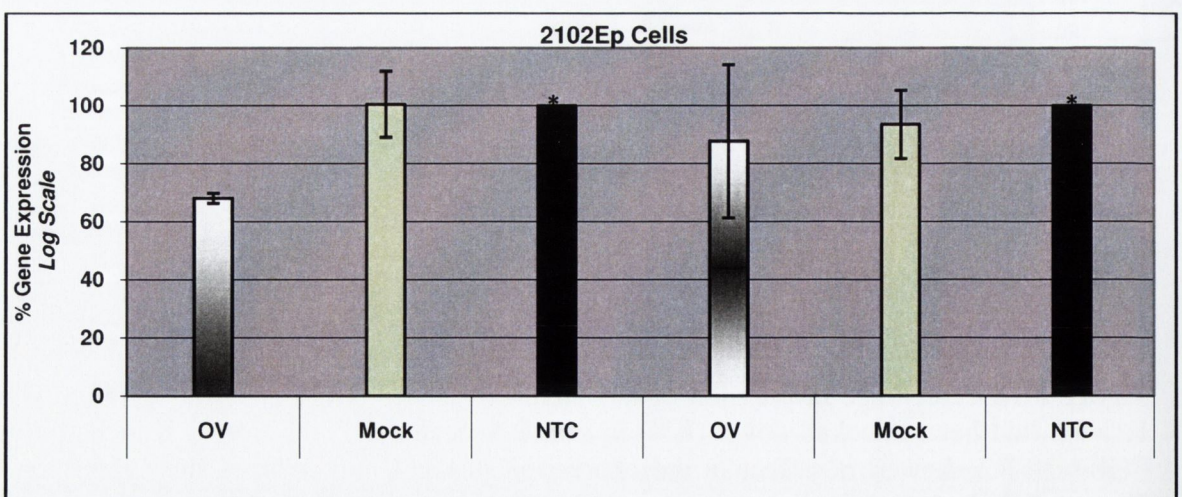
Figure 5.20 Expression of key stemness Genes (Oct4 and Nanog) after TGF-beta-R2 had been overexpressed (OV) in non RA-treated 2102Ep CSCs. Overexpressing TGF-beta-R2 reduced the expression of Oct4 and had no effect on Nanog relative to Mock and Non-Transfected Controls cells (NTC)) which are equated to 100%.
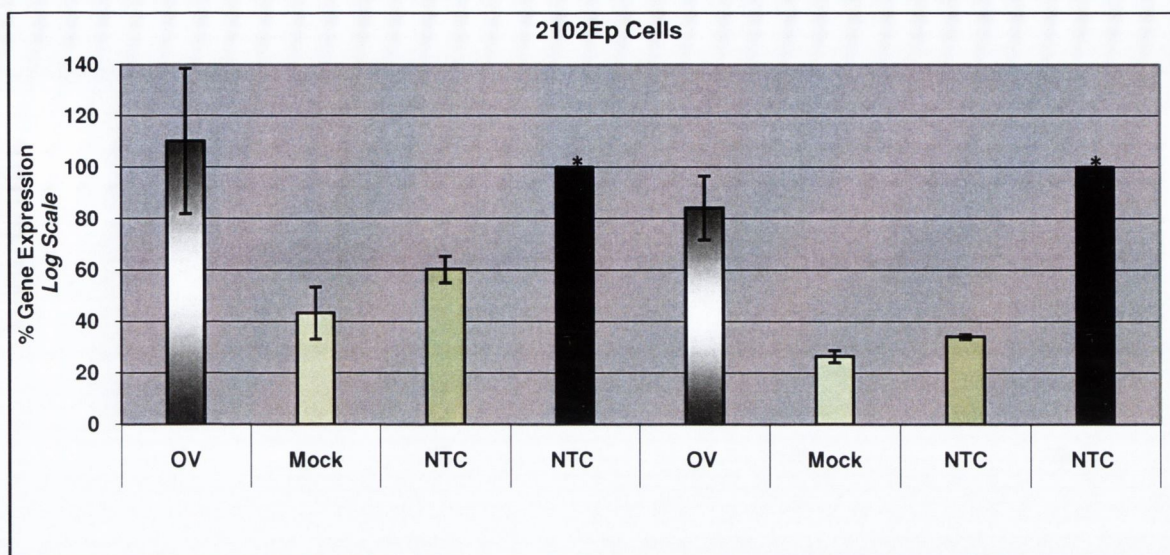
Figure 5.21 Expression of Key Stemness Genes (Oct4 and Nanog) after TGF-beta-R2 had been overexpressed (OV) in RA-treated 2102Ep CSCs. Overexpressing TGF-beta-R2 kept the expression of Oct4 and Nanog genes high relative to RA-treated mock cells and Non-Transfected Control cells (NTC).

In overview, our results describe the role of TGF-beta signaling in EC cells. TGF-betaR2 is upregulated by NTera2 cells upon differentiation. 2102Ep cells decrease TGF-betaR2 expression following RA-treatment. This represents a considerable difference between the cell types. On further analysis, we demonstrated that precise levels of TGF-betaR2 expression are required by EC cells. If overexpressed or suppressed, altered TGF-betaR2 affected the normal regulation of Oct4, Nanog, Ncam1, Eno3 and Gata6. Over-expression and knockdown of TGF-betaR2 did not lead to opposite effects on stemness genes as might be predicted. This indicates that TGF-betaR2 regulation of stemness is achieved through different mechanisms in NTera2 and 2102Ep cells, as will be discussed in the next section.

5.6 Chapter Discussion

Ongoing analysis by our group has indicated involvement of the TGF-beta signaling pathway in early NTera2 RA-Induced differentiation. This signaling pathway has long associations with differentiation and development from pluripotent cells (Pardo *et al*, 2010). Additionally, mutations in TGF-beta receptors and Smads are linked to several cancers (reviewed in Massague *et al.*, 2000). Specifically, TGF-betaR2 is known as a tumor suppressor as its expression is negatively associated with tumorigenesis and has been suggested as a potential gene therapy target in altering aberrant TGF-beta signaling (Chung *et al.*, 2002 and Paterson *et al.*, 2002).

In Chapter three we demonstrated that TGF-beta signaling was involved in early differentiation of EC cells. TGF-beta expression increased rapidly after only three days differentiation in NTera2 cells and decreased rapidly after three days differentiation in 2102Ep cells (Chapter 3 **Figure 3.4A and B**). Collectively, TGF-β appears to be required for pluripotency, playing an early regulatory role in cancer pathways in malignant cells. Additionally, its down-regulation in nullipotent malignant cells may allow maintenance of the self-renewal state. TGF-beta-R2 was one of the highest upregulated genes in the TGF-β pathway during early differentiation of pluripotent (NTera2) cells in our group microarray data (manuscript in prep). This was validated using qPCR and was presented in Chapter 3. Mechanistically, TGF-beta-R2 expression increased rapidly after three days differentiation in NTera2 cells and decreased rapidly after three days differentiation in 2102Ep cells. Both TGF-beta-R2 and TGF-β pathway acted in parallel and were up-regulated in pluripotent cells and down-regulated in nullipotent cells over time after being stimulated to differentiate.

We postulated that
- Up-regulating TGF-beta-R2 in nullipotent cancer stem cells might change the signalling of the TGF-β pathway to enable the nullipotent cells to differentiate.
- Knocking-down TGF-beta-R2 in pluripotent cancer stem cells may change the signalling of the TGF-β pathway to remove their differentiation potential.

Addressing these, in this chapter we altered the levels of TGF-beta-R2 using genetic techniques to knockdown and overexpress TGF-betaR2 in NTera2 and 2102Ep cells respectively.

In this chapter, we knocked down TGF-betaR2 in NTera2 cells and over-expressed TGF-betaR2 in 2102Ep cells. Following this we assessed these manipulations as follows

- Effects on differentiation marker genes Ncam1, Eno3 and Gata6.

- Effects on pluripotency master genes Oct4 and Nanog.

Each of these will now be discussed in detail. In overview, TGF-betaR2 manipulation altered the normal regulation of Oct4, Nanog, Ncam1, Eno3 and Gata6 in EC cells. However, this impact was limited. Thus we can state that TGF-beta signaling appears to be an important component of EC cell differentiation. However, differentiation is clearly governed by other pathways and process, which can still largely function around TGF-beta signaling.

5.6.1 TGF-beta-R2 is required for regulation of differentiation in 2102Ep and NTera2 CSCs

We initially found that manipulation of TGF-betaR2 affected the expression of differentiation markers Ncam1, Eno3 and Gata6 to varying extents in EC cells. TGF-betaR2 knockdown reduced the expression of Eno3 in –RA NTera2 cells (**Figure 5.11**). The expression of Eno3 was not affected by TGF-betaR2 in +RA NTera2 cells (**Figure 5.12**). This mechanism was different in 2102Ep cells. Overexpression of TGF-betaR2 in –RA 2102Ep cells caused a reduction in Eno3 expression (**Figure 5.14**). In +RA 2102Ep cells, TGF-betaR2 overexpression increased Eno3 expression (**Figure 5.15**). Thus TGF-betaR2 is required for regulation of mesodermal differentiation mechanisms in EC cells. This is in concordance with studies in normal mouse development (Goumans *et al.,* 1999). In NTera2 cells, TGF-betaR2 is required for normal regulation in –RA cells but not in +RA cells. However, TGF-betaR2 regulated ±RA 2102Ep cells. This is a significant difference between the cells types and suggests that the 'differentiation lesion' hypothesis only partially explains nullipotency. In terms of the differentiation lesion hypothesis, our results suggest that 2102Ep cells are attempting to differentiate along the mesoderm lineage, which is thwarted by low levels of TGF-betaR2 expression.

A similar difference between NTera2 and 2102Ep cells was observed for Gata6 endodermal differentiation signaling. TGF-betaR2 knockdown reduced Gata6 expression in ±RA NTera2 cells (**Figure 5.13**). In concordance with this, overexpression of TGF-betaR2 upregulated Gata6 expression in −RA 2102Ep cells (**Figure 5.15**). However, when TGF-betaR2 was overexpressed in +RA 2102Ep cells, Gata6 expression was reduced (**Figure 5.16**). Thus TGF-betaR2 is a functional requirement for normal endodermal differentiation in NTera2 cells. This was previously similarly reported for hES cells (Shiraki *et al.,* 2005). In contrast, TGF-betaR2 negatively regulates endodermal differentiation signaling in 2102Ep cells. Thus, we observe more evidence for independent 2102Ep mechanisms as well as 'differentiation lesion' mechanisms.

Similar EC mechanisms were observed for Ncam1 ectodermal signaling. Our results show that ectodermal differentiation in +RA NTera2 cells was compromised by TGF-betaR2 knockdown (**Figure 5.12**). In concordance with this, overexpression of TGF-betaR2 permitted upregulation of Ncam1 in 2102Ep cells treated with RA (**Figure 5.14**). These specific changes were not observed in control treatments but were not sufficient to stop +RA NTera2 differentiation or stimulate +RA 2102Ep differentiation. Thus TGF-betaR2 appears to be a requirement for normal ectodermal differentiation signaling in EC cells. This is in concordance with data from hES cells (Mahmood *et al.,* 2010). This is more evidence for the differentiation lesion hypothesis. However, TGF-beta signaling is imposing a limited regulation on these cells, which indicates the involvement of other molecular processes governing EC differentiation.

Having determined that TGF-betaR2 is involved in the regulation of EC differentiation, TGF-betaR2 regulation of pluripotency regulators Oct4 and Nanog was next assessed. Sox2 gene expression is not altered by RA treatment of EC cells. Thus, when NTera2 cells increase TGF-betaR2 expression in response to RA, Sox2 is unaltered. As such, time and resources were not invested in assessing Sox2 as an output from this experiment. In parallel with differentiation marker genes, TGF-betaR2 was shown to regulate Oct4 and Nanog in EC cells. The regulation of Oct4 and Nanog by TGF-beta signalling has been previously described. In the presence of ligand, Oct4 expression is activated by Smad4 while loss of Smad2 leads to decreased Oct4 expression (Waldrip *et al.,* 1998 and Puceat, 2007). TGF-beta expression is required for Oct4 expression in the early embryo (Puceat, 2007). Nanog is the direct target of Smad 2/3 and can block TGF-beta signaling by binding

Smad1 (Suzuki *et al.,* 2006 and Greber *et al.,* 2008). However, our demonstration of regulation of Oct4 and Nanog by TGF-betaR2 specifically is the first such description.

Oct4 expression was unaltered by TGF-betaR2 knockdown in ±NTera2 cells (**Figures 5.17 and 5.18**). In contrast, Oct4 was reduced by TGF-betaR2 overexpression in –RA 2102Ep cells (**Figure 5.19**). This indicates that TGF-betaR2 is not a functional requirement for Oct4 regulation in NTera2 cells. However, TGF-betaR2 appears to negatively regulate Oct4 in 2102Ep cells. This indicates that specific Oct4 regulation mechanisms are active in 2102Ep cells that are not present in NTera2 cells, which disagrees with the 'differentiation lesion' hypothesis. Mechanistically, it appears that low levels of TGF-betaR2 in 2102Ep cells play a role in the maintenance of high Oct4 expression. Once again, this is a limited role that is not sufficient for full differentiation of EC cells.

The level of Nanog expression was particularly sensitive to TGF-betaR2 manipulation in EC cells. Nanog was reduced upon knockdown of TGF-betaR2 in –RA NTera2 cells (**Figure 5.18**). In contrast, Nanog was increased by TGF-betaR2 knockdown in +RA NTera2 cells (**Figure 5.19**). Thus TGF-betaR2 is required for Nanog expression in –RA NTera2 cells and negatively regulates Nanog expression in response to RA. Nanog expression was not affected by TGF-betaR2 overexpression in ±RA 2102Ep cells (**Figure 5.20 and 5.21**). Thus it appears that TGF-betaR2 plays a complex role in Nanog regulation in EC cells. This role is dependent upon the differentiation status of NTera2 cells. TGF-betaR2 has a specific but limited function in Nanog regulation, suggesting the presence of additional molecular mechanisms. This role appears to be non-functional in 2102Ep cells, in concordance with the 'differentiation lesion' hypothesis.

Collectively, the Oct4, Nanog, Ncam1, Eno3 and Gata6 data suggest that TGF-betaR2 is required for the normal regulation of EC cells. Certain aspects of this mechanism are similar in both cell types and others are functional in only NTera2 cells: as such they are likely to be components of the 2102Ep differentiation lesion. Some aspects of TGF-beta signaling in 2102Ep cells act independently of NTera2 cells: as such they represent an alternative mechanism. It is tempting to postulate that this may be a 'differentiation-avoidance' mechanism, which will be assessed in future work.

During our analysis we observed that TGF-beta-R1 is negatively regulated by TGF-betaR2 in EC cells (**Figure 5.10 and 5.11**). This is in contrast to non-malignant cells. In non-malignant cells the pathway is activated upon ligand detection by TGF-betaR1 and R2 (Shi *et al.,* 2003). TGF-betaR2, the more dominant receptor, activates TGF-betaR1, which facilitates activation of Smad modulators (Shi *et al.,* 2003 and Clark *et al.,* 2009). Smads can then enter the nucleus of the cell to influence transcription (Ten Dijke *et al.,* 1994, Miyazawa *et al.,* 2002 and Massague *et al.,* 2005). We have noted that TGF-beta-R1 protein expression was affected by TGF-beta-R2 overexpression or knockdown and this is the first report of the negative regulation of TGF-beta-R1 by TGF-betaR2. As such, our data represents a significant difference between normal and cancer cells. This requires comprehensive analysis, which will be carried out by our group.

To briefly conclude, the expression of differentiation marker genes (Eno3, Ncam1 and Gata6) and key stemness genes (Oct4 and Nanog) was functionally related to TGF-bR2 in EC cells. However, the ultimate differentiation capacity of each cell type was unaltered by TGF-bR2 manipulation. TGF-bR2 appears to be one component of a complex network of differentiation regulation in EC cancer stem cells. The elucidation of further components of this network will be assessed by future work and may hasten CSC targeting, our ultimate aim.

Chapter Six

General Discussion

6.1 General Discussion

At the start of this project the EC model of cancer stemness had been established by the group. Cells were routinely cultured in the undifferentiated state and stimulation of differentiation had been optimised. The initial goals of this project were to take this model system for cancer stemness and characterise the genes and miRNAs involved. We assayed genes via microarray analysis. This microarray data was mined and several stemness genes, pathways and modulators were highlighted. These subsequently required validation and characterisation. This was achieved in chapter 3. In parallel, the expression patterns of approximately 300 miRNAs known to exist at the start of this study were generated. This was achieved in chapter 4. In a classic methodology, specific genes, pathways and miRNAs of interest have been selected by the group for further analysis. TGF-beta signaling was selected based on four parameters:

1) Upregulation during NTera2 differentiation:
2) Downregulation by 2102Ep cells in response to RA treatment:
3) Relevance of the pathway to stem cell biology:
4) Relevance of the pathway to cancer biology.

The functional assessment of TGF-beta signaling was described in chapter 5. Thus the work described in this thesis has provided gene and miRNA information that will provide a platform for the group for many years to come. Additionally, the work detailed in this thesis has identified a novel cancer stemness regulation mechanism. This is a significant contribution to the group's analysis of cancer stemness.

Referring to this project specifically, we set out to assess whether subtle differential expression of key downstream genes and pathways could be detected early in differentiation. Pluripotency studies have generally assessed gene expression changes at approximately one week differentiation or later, while characterisation of earlier events in differentiation has received less attention. This is largely due to the fact that most stem cell models do not spontaneously differentiate (Andrews 2002, Andrews *et al*, 2005 and Josephson *et al*, 2007). The EC model is unique: EC cells uniformly differentiate as soon as a morphogen is added. Differentiation can essentially be processed at any time, so long as molecular differences can be detected by the technologies available in the lab. By assaying early differentiation, we are opening up an ocean of mechanisms that facilitate the early differentiation of cancer stemness, an approach that has not been previously possible.

At its most basic level it permits us to look backwards along a molecular pathway. This is not usually possible. In molecular biology we can knockdown a gene such as Oct4 or Nanog and look downstream to identify all the genes regulated by these genes (Hyslop *et al.*, 2005 and Fong *et al.*, 2008). The opposite is not possible: we cannot look backwards to assess what genes regulate Oct4 and Nanog. The early differentiation approach was likely to contain upstream regulators of Oct4, Sox2 and Nanog. The work presented in chapter 5 and ongoing analysis in our group shows that many genes and miRNAs identified by expression patterns during early differentiation are upstream regulators of Oct4, Sox2 and Nanog (manuscript in prep). A comprehensive screen of all these genes and miRNAs is being undertaken by the group, which should yield a significant paper, as described in section 6.2. Without the work described in chapters 3, 4 and 5 of this thesis, the significance of early differentiation would not have been fully appreciated.

We have conducted analysis from as early as three days differentiation in both pluripotent and nullipotent CSCs and identified genes and miRNAs regulating early differentiation. All analysis was conducted in response to RA-treatment, which has been commonly used to differentiate pluripotent cells in various studies (Andrews *et al.*, 2005 and Josephson *et al.*, 2007). Changes in expression can be detected at this early time point, where consistent co-ordinated regulation of Nanog, and Oct4 was observed upon treatment of EC cells with RA.

Our data suggests that malignant pluripotency may be characterised by earlier induction of key stemness processes than non-malignant pluripotency. This is based on the fact that many of the genes and miRNAs regulated during early differentiation of EC cells are not altered in ES cells (Lu *et al.*, 2007 and Laurent *et al.*, 2008). This is a remarkable departure from our current thinking and is a justification of our approach. For example, EC and ES cells are considered to be 'almost identical' in the undifferentiated states (Josephson *et al.*, 2007). Similarly, chapter 1 described how many of the key pathways, genes and modulators are the same for ES and EC cell differentiation (**section 1.5.3**). Whereas before this study there were few known differences between CSCs and NSCs in this model, we appear to have uncovered an area of remarkable difference. Once fully elucidated, these upstream mechanisms may provide CSC-specific targets for use in anti-cancer therapeutics without impinging upon the NSC population. Nullipotent CSCs did not respond to RA-treatment for most processes assessed. Where they do respond, the levels of expression of key genes such as Oct4 and Nanog are always maintained at higher levels than NTera2

cells. Our overall aim is identification of targetable CSC-specific events for use in anti-cancer therapeutics. We have demonstrated that key stemness genes and pathways, as well as markers of differentiation, have detectably altered gene expression early in differentiation, alterations that indicate a network of upstream regulation and reveal a hierarchal model for pluripotent CSC differentiation. Additionally, we demonstrate that highly malignant nullipotent CSCs are characterised by dampened effects in response to RA-treatment, suggesting that the lesion maintaining nullipotent CSCs in their self-renewing state affects these pathways in a non-uniform fashion. Our ongoing approach may facilitate identification of CSC-specific regulation mechanisms for use in anti-cancer therapeutics.

MicroRNAs have quickly emerged as an important group of regulatory molecules, which were detailed in chapter 1 (**section 1.6**). The ultimate aim of our group is the development of CSC-targeting as a therapeutic approach. Before this can be achieved, we must fully characterise the molecular events associated with the processes of self-renewal and differentiation. We choose to characterise miRNAs for 4 reasons:

1) miRNAs have emerged as a substantial molecular regulation mechanism
2) Different groups of miRNAs are expressed in undifferentiated and differentiated stem cells
3) Different groups of miRNAs are expressed in normal and malignant tissues
4) Many of the miRNAs linked to stem cells and malignancy have been shown to regulate key genes whose function is necessary for the processes.

The expression of 316 miRNAs was assessed by qPCR and was described in chapter 4. Profiles of miRNAs specifically or commonly required for each cell type in either state were identified, indicating that post-transcriptional regulation of target proteins is a key mechanism during early differentiation of CSCs.

Specifically, this study demonstrated that different populations of miRNAs were expressed in:

1) Undifferentiated EC cells

2) Differentiated EC cells

3) Pluripotent EC cells

4) Nullipotent EC cells.

As such this project has generated multiple sets of miRNAs that are expressed in specific aspects of CSC biology: these are now available for functional analysis by our group. Indeed, several of the miRNAs identified in chapter 4 have been functionally assessed by other members of our group. For example, miR-15a, which is upregulated in +RA NTera2 cells but downregulated in +RA 2102Ep cells, has been shown to target key cancer gene c-Jun (manuscript in prep). Our group is actively engaged in a screen of these miRNAs, which will assess roles in cancer stemness, differentiation and tumorigenesis as well as regulatory effects on Oct4, Sox2 and Nanog (**section 6.2**). This ongoing analysis would not be possible without the work described in chapter 4. Our study reiterates the importance of miRNAs in cancer stemness and characterises miRNA expression in early differentiation of EC cells, which has not been previously described.

Throughout this thesis, we have presented and evaluated the hypothesis that EC nullipotency is due to a 'differentiation lesion'. Our miRNA data supports this thesis: the majority of miRNAs in the study are altered in +RA NTera2 cells but unaltered in +RA 2102Ep cells (**chapter 4**). We proposed and published a 'four group model' of miRNAs from this study (Gallagher *et al.*, 2009). Group 1 miRNAs are regulated similarly in both EC cell types, which suggest that these miRNAs act upsteam of the differentiation lesion. Group 2 miRNAs are altered in +RA NTera2 cells but unaltered in +RA 2102Ep cells, which suggests that these miRNAs act downstream of the differentiation lesion. Groups 3 and 4, however, indicate a second mechanism, which we have termed the 'differentiation avoidance' mechanism (Gallagher *et al.*, 2009). Group 3 miRNAs are altered in an opposite fashion in EC cells, suggesting a potential counteraction of differentiation by 2102Ep cells. Additionally, Group 4 miRNAs are altered in +RA 2102Ep cells only. This indicates that 2102Ep cells are not simply 'broken NTera2' cells but can mount a specific response to differentiation. As stated in our paper, it is tempting to postulate that this may

be a differentiation avoidance mechanism (Gallagher *et al.,* 2009). This hypothesis is being actively pursued by the group and may have important implications for the understanding and targeting of CSCs.

Similar levels of upregulation and downregulation of miRNAs are reported during differentiation of hES cells (Laurent *et al.,* 2008) but this was not observed in our EC cells. Downregulation of miRNAs was similar in both pluripotent cell types. However, that of EC cells was twice that of hES cells in terms of upregulated miRNAs (**section 4.7**). Thus, CSCs appear to be characterized by alterations in the expression of miRNAs that are not altered in NSCs. miRNAs differentially expressed in either CSC cell line were not differentially expressed in hES cells (**section 4.7**). Therefore, CSCs are characterised by twice as much upregulation as downregulation of miRNAs whereas in hES cells similar numbers of miRNAs are up and downregulated: perhaps a key difference between normal and cancer stem cells. Upregulation miRNAs only in CSCs may play a specific post-transcriptional regulation role in maintenance malignancy.

In chapter 5, we functionally assessed the role of TGF-beta signaling in EC cells, advancing the observations from Chapter 3. TGF-beta signaling appears to be required for pluripotency, playing an early regulatory role in stemness pathways in malignant cells (**Chapter 3**). In contrast, TGF-beta signaling is downregulated in nullipotent ECs, and as such involved in 2102Ep maintenance of the undifferentiated state. TGF-betaR2 was functionally assessed and affected Oct4, Nanog and the differentiation marker genes (**section 5.5**). This was comprehensively discussed in Chapter 5 (**section 5.6**).

Where TGF-beta signaling had a functional effect on EC cells, this effect was limited. This clearly indicates that other molecular mechanisms are involved in early EC differentiation. While TGF-beta signaling plays a role, other molecular mechanisms appear to govern EC differentiation more powerfully. Additionally, this suggests that EC cells may be able to work around our manipulations of TGF-beta signaling to facilitate differentiation. This appears to be the case in both NTera2 cells and 2102Ep cells. It is tempting to postulate that one such governing pathway may be the Sonic Hedgehog (Shh) pathway. In Chapter 3 we described how Shh is rapidly upregulated upon RA treatment of both EC cell types (**Figure 3.4**). In both NTera2 and 2102Ep cells the pathway shows the highest upregulation of the pathways assayed (**Figure 3.4**). Indeed, a relationship between TGF-beta and Shh

signaling has been described (Gou and Wang, 2009). This will be considered by the group during future experiments.

In terms of EC differentiation, differentiation marker genes (Eno3, Ncam1 and Gata6) were up-regulated during differentiation of pluripotent CSCs cells and not altered in nullipotent CSCs cells. Knocking-down TGF-beta-R2 reduced the expression of most differentiation markers in pluripotent CSC cells in both states. Overexpressing TGF-beta-R2 increased the expression of most differentiation marker genes in nullipotent CSC cells in both states. This result suggests that pluripotent cells are avoiding self-renewal and nullipotent cells are attempting to differentiate. However, these affects are partial: manipulated TGF-beta-R2 signaling was not sufficient to maintain self-renewal in pluripotent cells or differentiate nullipotent CSC cells.

The expression of pluripotency master genes Oct4 and Nanog is altered in EC cells, although Sox2 is maintained (**section 3.3**). Knocking-down TGF-beta-R2 in NTera2 cells had a limited effect on key stemness genes in both states. Overexpressing TGF-beta-R2 in nullipotent CSC cells reduced the expression of Oct4 and Nanog in the absence of retinoic acid, whereas with addition of retinoic acid the expression of Oct4 and Nanog remained high. These results indicate that TGF-beta-R2 is not sufficient to comprehensively alter the master pluripotency genes in EC cells. TGF-betaR2 is required for the usual regulation of Oct4, Nanog and of differentiation in EC cells. Once again, this regulation is not sufficient to fully facilitate or abolish differentiation, indicating the involvement of other mechanisms.

In overview, EC cells were successfully exploited to identify genes and miRNAs that are involved in the upstream regulation of differentiation. NTera2 cells alter the expression of genes and miRNAs that are not reportedly altered in their non-malignant comparator, hES cells. 2102Ep cells appear to employ at least two methods of regulation to avoid differentiation:

      1) A passive differentiation lesion

      2) An active differentiation-avoidance mechanism

This is an exciting development in cancer stem cell biology, which will be investigated during future work by our group.

6.2 Future work

Our analysis in chapter 3 identified that TGF-β signaling was up-regulated in differentiated NTera2 cells and downregulated in RA treated 2102Ep cells (**Figure 3.4A and B**). We expanded this observation by mining our group's microarray data for TGF-beta signaling pathway genes. Comprehensive descriptions of TGF-beta signaling were presented in chapters 3 and 5. Briefly, several different classes of ligand can be intercepted by membrane-bound receptors, which activate Smad modulators, which in turn enter the nucleus of the cell to influence gene expression (**Figure 5.1**). Our microarray data indicated that TGF-beta pathway genes Noggin and Lefty2 were also altered during differentiation of NTera2 cells (manuscript in prep).

Lefty2 is a negative regulator of bone morphogenic protein (Bmp) signaling, a parallel arm of the TGF-beta signaling pathway (**Figure 5.1**). Noggin negatively regulates the pathway by blocking receptor binding of specific ligands in a mechanism that is not fully elucidated (**Figure 5.1**). Therefore, these two genes will be functionally assessed in the undifferentiated and differentiated states of both EC cell types using the protocols described in chapter 5. We postulate that combinations of knockdown and/or overexpression of these two genes may result in a more dramatic alteration of Oct4, Nanog or the differentiation marker genes. In parallel, other pathways such as Shh are being assessed by other members of the group on an ongoing basis.

Chapter 4 described the classification of miRNAs into those involved in NTera2 and/or 2102Ep cells in the undifferentiated and/or differentiated states. These are actively being screened for effects upon EC cells.

Examples of miRNAs of particular interest are:

> 1) 26 miRNAs that were expressed in nullipotent cells in both states but not in pluripotent cells
> 2) miR-10a, which was expressed only in differentiated pluripotent cells and constantly expressed in nullipotent cells
> 3) miR-142-5p, which was only expressed in the self-renewing state in EC cells

4) miR-199a*, which was expressed in undifferentiating pluripotent cells but not in nullipotent cells

5) 3 miRNAs (miR-425, -105 and -433), which were only expressed in differentiating nullipotent cells but not in pluripotent cells.

Differentiation status is being actively screened through an alkaline phosphatase (AP) method developed within the group. Alkaline phosphatase is expressed on the cell surface of pluripotent and nullipotent cells and is lost upon differentiation. MicroRNAs of interest are knocked-down or overexpressed using similar protocols to those described in chapter 5. Cells are subsequently stained for AP. Cells showing altered AP staining are assessed in detail individually in a manner similar to that developed in chapter 5 of this thesis. This approach is also being employed to screen genes of interest, particularly from those pathways highlighted in chapter 3.

The manipulation of genes and miRNAs of interest does not always alter differentiation status *per se*. As such, we are developing screens to identify genes and miRNAs that effect Oct4, Sox2 and/or Nanog expression. Within the group, luciferase expression systems representing the promoters and miRNA binding sites of Oct4, Sox2 and Nanog are being prepared. These will be screened using knockdown and overexpression of genes and miRNAs of interest. Those affecting Oct4, Sox2 and/or Nanog are assessed individually in a manner similar to that developed in chapter 5 of this thesis. We hope to model the extent of Oct4, Sox2 and Nanog master pluripotency regulators. We will ask if this trio is indeed at the top of pluripotency regulation or whether other, more powerful, stemness regulators exist.

The ultimate goal of our group is the development of CSC-targeting. As such, gene and miRNAs targets that effect the differentiation of EC cells will be further assessed in animal model experiments. Genes and miRNAs affecting differentiation will be permanently knocked-down or overexpressed in EC cells. These will be introduced into immuno-compromised mice. Targeting will be deemed to be successful where tumour size or grade is negatively affected in treated cells compared to untreated comparator cells. A licence for this work has been acquired by the group through the Bioresources facility at the University of Dublin, Trinity College.

In summary, it is important to reiterate that all the analysis suggested in this section would not have been possible without the studies described in this thesis. In time, we hope that these contributions will grow towards the ultimate goal of targeting CSCs therapeutically.

Appendices

Table 1: Relative expression of downregulated miRNAs in differentiating NTera2 and 2102Ep cells.

	NTera2	Fold Change (2ddCt)		2102Ep	Fold Change (2ddCt)
1-	miR-519e*	-17.7759	1-	miR-504	-9.985490755
2-	miR-520f	-16.54492	2-	miR-362	-8.126982757
3-	miR-519d	-6.679329	3-	miR-324-3p	-7.016709657
4-	miR-485-3p	-5.960053	4-	miR-17-3p	-6.230404509
5-	miR-519b	-5.882087	5-	miR-203	-5.383157614
6-	miR-372	-5.182336	6-	let-7g	-5.356693361
7-	miR-520e	-4.654398	7-	miR-211	-4.688162521
8-	miR-302b	-4.498036	8-	miR-302a	-4.578843745
9-	miR-96	-4.390393	9-	miR-32	-4.234091656
10-	miR-516-5p	-4.257285	10-	miR-204	-4.060953947
11-	miR-409-5p	-3.763191	11-	miR-337	-3.466413876
12-	miR-302b*	-3.526844	12-	miR-193b	-3.248406094
13-	miR-518a-2*	-3.197463	13-	miR-320	-2.943765328
14-	miR-519e	-2.88075	14-	miR-455	-2.794155381
15-	miR-93	-2.817884	15-	miR-133b	-2.753255824
16-	miR-519c	-2.71487	16-	miR-154*	-2.703336306
17-	miR-337	-2.708538	17-	miR-516-5p	-2.675084567
18-	miR-139	-2.688048	18-	miR-UL112-1	-2.481152367
19-	miR-302a*	-2.5563	19-	miR-31	-2.439200188
20-	miR-520d	-2.48773	20-	miR-365	-2.425309166
21-	miR-367	-2.450578	21-	miR-302C*	-2.246399729
22-	miR-520a*	-2.407219	22-	miR-338	-2.239857059
23-	miR-371	-2.267275	23-	miR-15a	-2.224086656
24-	miR-183	-2.242788	24-	miR-520e	-2.207849501
25-	miR-329	-2.161272	25-	miR-9*	-2.146131286
26-	miR-30e-3p	-2.152556	26-	miR-30e-3p	-2.137982187
27-	miR-518b	-2.111346	27-	miR-512-3p	-2.112918568
28-	miR-373	-2.103453	28-	miR-485-3p	-2.059379197
29-	miR-517b	-2.091162	29-	miR-34c	-2.046929385
30-	miR-190	-2.088922	30-	miR-376b	-2.009934053
31-	miR-211	-2.041269			

Table 2: Relative expression of upregulated miRNAs in differentiating NTera2 and 2102Ep cells.

	NTera2	Fold Change (2ddCt)		2102Ep	Fold Change (2ddCt)
1-	miR-328	2.025838	1-	miR-19a	2.005685517
2-	miR-148a	2.033792	2-	miR-99a	2.039296195
3-	miR-133a	2.063145	3-	miR-25	2.049654629
4-	miR-338	2.100007	4-	miR-199a	2.147022038
5-	let-7b	2.131706	5-	miR-326	2.150715209
6-	miR-28	2.132275	6-	miR-519d	2.439301119
7-	miR-191	2.133014	7-	miR-487	2.47498709
8-	miR-92	2.145691	8-	miR-199b	2.567165103
9-	miR-UL22A-1	2.148289	9-	miR-363	2.571720162
10-	miR-214	2.149107	10-	miR-129	2.956472791
11-	miR-187	2.235768	11-	miR-184	3.797441307
12-	miR-342	2.245458			
13-	miR-200a*	2.266317			
14-	miR-203	2.281561			
15-	miR-34a	2.284833			
16-	miR-148b	2.316527			
17-	miR-324-3p	2.322365			
18-	miR-218	2.385027			
19-	miR-494	2.397841			
20-	miR-331	2.485617			
21-	miR-320	2.492081			
22-	miR-146b	2.493112			
23-	miR-30a-3p	2.547571			
24-	miR-133b	2.563195			
25-	miR-505	2.563475			
26-	miR-192	2.655252			
27-	miR-145	2.793437			
28-	miR-221	2.820949			
29-	miR-302a	2.914417			
30-	miR-149	2.914516			
31-	miR-22	3.032373			
32-	miR-205	3.105236			
33-	miR-151	3.194619			
34-	miR-451	3.225695			

35-	miR-15a	3.251744			
36-	miR-146a	3.305124			
37-	miR-34c	3.373104			
38-	miR-324-5p	3.407712			
39-	miR-32	3.452129			
40-	miR-9	3.457999			
41-	miR-184	3.710261			
42-	miR-204	3.830488			
43-	miR-339	3.846054			
44-	miR-18a*	3.934668			
45-	miR-369-5p	4.136148			
46-	miR-365	4.182079			
47-	miR-134	4.339225			
48-	miR-33	4.494379			
49-	miR-382	4.571268			
50-	miR-424	4.639486			
51-	miR-340	4.690395			
52-	miR-18b	4.698068			
53-	miR-489	4.811206			
54-	miR-191*	5.38866			
55-	miR-1	5.915788			
56-	miR-140	6.050426			
57-	miR-9*	7.866198			
58-	miR-188	8.30087			
59-	miR-509	9.512494			
60-	miR-99b	11.14045			
61-	miR-219	21.89723			
62-	miR-99a	22.49141			
63-	miR-335	26.02063			

Table 3: Expression levels of miRNAs expressed in 2102Ep (undifferentiating and differentiating) cells but not in NTera2 cells.

	NTera2	Fold Change		2102Ep	Fold Change (2ddCt)
1-	miR-518C*			miR-518C*	-633.995
2-	miR-153			miR-153	-30.8909
3-	miR-431			miR-431	-2.74677
4-	miR-511			miR-511	-2.05583
5-	miR-516-3p			miR-516-3p	**-1.94979**
6-	miR-155			miR-155	**-1.76864**
7-	miR-224			miR-224	**-1.21543**
8-	miR-448			miR-448	**-1.15703**
9-	miR-517*			miR-517*	**-1.10597**
10-	miR-527			miR-527	**-1.10564**
11-	miR-379			miR-379	**-1.09075**
12-	miR-409-3p			miR-409-3p	**-1.04518**
13-	miR-525*			miR-525*	**-1.01366**
14-	miR-376a			miR-376a	**1.052978**
15-	miR-499			miR-499	**1.129508**
16-	miR-498			miR-498	**1.161566**
17-	miR-493			miR-493	**1.200589**
18-	miR-525			miR-525	**1.223735**
19-	miR-503			miR-503	**1.337221**
20-	miR-10b			miR-10b	**1.422906**
21-	miR-449			miR-449	**1.466761**
22-	miR-526a			miR-526a	**1.473795**
23-	miR-213			miR-213	**1.486117**
24-	miR-375			miR-375	**1.524057**
25-	miR-196b			miR-196b	**1.811386**
26-	miR-518e			miR-518e	2.044686

Table 4: Expression levels of miRNAs only expressed in NTera2 (undifferentiating and differentiating) cells but not in 2102Ep cells.

	NTera2	Fold Change (2ddCt)		2102Ep	Fold Change
1-	miR-346	**-1.06722**		miR-346	

Table 5A: Expression levels of miRNAs only expressed in self-renewing state in both cell lines (NTera2 and 2102Ep).

	NTera2	Fold Change U dCt		2102Ep	Fold Change U dCt
1-	miR-142-5p	7.195375		miR-142-5p	8.318299

Table 5B: Expression levels of miRNAs only expressed in undifferentiating NTera2 cells and differentiating 2102Ep cells.

	NTera2	Fold Change U dCt		2102Ep	Fold Change D dCt
1-	miR-137	17.42199		miR-137	12.31705

Table 5C: Expression levels of miRNAs only expressed in undifferentiating NTera2 cells but not in 2102Ep cells.

	NTera2	Fold Change U dCt		2102Ep	Fold Change
1-	miR-199a*	18.45135		miR-199a*	

Table 5D: Expression levels of miRNAs only expressed in differentiating NTera2 cells but not in 2102Ep cells.

	NTera2	Fold Change D dCt		2102Ep	Fold Change
1-	Let-7c	2.422794		Let-7c	

Table 5E: Expression levels of miRNAs only expressed in differentiating 2102Ep cells but not in NTera2 cells.

	NTera2	Fold Change		2102Ep	Fold Change D dCt
1-	miR-425			miR-425	11.18966
2-	miR-105			miR-105	2.711329
3-	miR-433			miR-433	-1.11199

Table 6A: miRNAs downregulated in pluripotent cells (NTera2) and had no change in nullipotent cells (2102Ep) were unchanged in hES cells.

	NTera2	Fold Change (2ddCt)	2102Ep	hES
1-	miR-372	-5.182336	No Change	Unchanged
2-	miR-409-5p	-3.763191	No Change	Unchanged
3-	miR-520d	-2.48773	No Change	Unchanged
4-	miR-190	-2.088922	No Change	Unchanged

Table 6B: miRNAs upregulated in pluripotent cells (NTera2) and had no change in nullipotent (2102Ep) were unaltered in hES cells.

	NTera2	Fold Change (2ddCt)	2102Ep	hES
1-	miR-328	2.025838	No Change	Unchanged
2-	miR-133a	2.063145	No Change	Unchanged
3-	let-7b	2.131706	No Change	Unchanged
4-	miR-28	2.132275	No Change	Unchanged
5-	miR-UL22A-1	2.148289	No Change	Unchanged
6-	miR-214	2.149107	No Change	Unchanged
7-	miR-200a*	2.266317	No Change	Unchanged
8-	miR-34a	2.284833	No Change	Unchanged
9-	miR-148b	2.316527	No Change	Unchanged
10-	miR-218	2.385027	No Change	Unchanged
11-	miR-494	2.397841	No Change	Unchanged
12-	miR-331	2.485617	No Change	Unchanged
13-	miR-146b	2.493112,	No Change	Unchanged
14-	miR-192	2.655252	No Change	Unchanged
15-	miR-145	2.793437	No Change	Unchanged
16-	miR-221	2.820949	No Change	Unchanged
17-	miR-22	3.032373	No Change	Unchanged
18-	miR-451	3.225695	No Change	Unchanged
19-	miR-146a	3.305124	No Change	Unchanged
20-	miR-324-5p	3.407712	No Change	Unchanged
21-	miR-9	3.457999	No Change	Unchanged
22-	miR-339	3.846054	No Change	Unchanged
23-	miR-18a*	3.934668	No Change	Unchanged
24-	miR-134	4.339225	No Change	Unchanged
25-	miR-33	4.494379	No Change	Unchanged
26-	miR-382	4.571268	No Change	Unchanged
27-	miR-424	4.639486	No Change	Unchanged
28-	miR-340	4.690395	No Change	Unchanged
29-	miR-489	4.811206	No Change	Unchanged
30-	miR-191*	5.38866	No Change	Unchanged
31-	miR-1	5.915788	No Change	Unchanged

32-	miR-140	6.050426	No Change	Unchanged
33-	miR-188	8.30087	No Change	Unchanged
34-	miR-509	9.512494	No Change	Unchanged
35-	miR-99b	11.14045	No Change	Unchanged
36-	miR-219	21.89723	No Change	Unchanged
37-	miR-335	26.02063	No Change	Unchanged

Table 6C: miRNAs downregulated in nullipotent cells (2102Ep) and had no change in pluripotent cells (NTera2) were unchanged in hES cells.

	2102Ep	**Fold Change (2ddCt)**	**NTera2**	**hES**
1-	miR-504	-9.985490755	No Change	Unchanged
2-	miR-362	-8.126982757	No Change	Unchanged
3-	miR-17-3p	-6.230404509	No Change	Unchanged
4-	let-7g	-5.356693361	No Change	Unchanged
5-	miR-193b	-3.248406094	No Change	Unchanged
6-	miR-455	-2.794155381	No Change	Unchanged
7-	miR-154*	-2.703336306	No Change	Unchanged
8-	miR-UL112-1	-2.481152367	No Change	Unchanged
9-	miR-31	-2.439200188	No Change	Unchanged
10-	miR-302c*	-2.246399729	No Change	Unchanged

Table 6D: miRNAs upregulated in nullipotent cells (2102Ep) and had no change in pluripotent cells (NTera2) also were unaltered in hES cells.

	2102Ep	**Fold Change (2ddCt)**	**NTera2**	**hES**
1-	miR-25	2.049654629	No Change	Unchanged
2-	miR-199a	2.147022038	No Change	Unchanged
3-	miR-326	2.150715209	No Change	Unchanged
4-	miR-487	2.47498709	No Change	Unchanged
5-	miR-199b	2.567165103	No Change	Unchanged
6-	miR-129	2.956472791	No Change	Unchanged

Table 7: A list of miRNAs assays (330) analysed in this study.

1	let-7b	51	miR-193a	101	miR-328	151	miR-137
2	let-7c	52	miR-194	102	miR-33	152	miR-140
3	let-7d	53	miR-195	103	miR-331	153	miR-142-3p
4	let-7e	54	miR-196a	104	miR-335	154	miR-153
5	let-7i	55	miR-196b	105	miR-338	155	miR-187
6	miR-100	56	miR-199a	106	miR-339	156	miR-192
7	miR-101	57	miR-19a	107	miR-340	157	miR-203
8	miR-103	58	miR-19b	108	miR-342	158	miR-204
9	miR-106b	59	miR-200a	109	miR-34a	159	miR-221
10	miR-107	60	miR-200b	110	miR-34c	160	miR-324-5p
11	miR-10a	61	miR-200c	111	miR-365	161	miR-431
12	miR-122a	62	miR-205	112	miR-433	162	miR-9
13	miR-125a	63	miR-206	113	miR-448	163	miR-99a
14	miR-125b	64	miR-208	114	miR-449	164	miR-UL112-1
15	miR-126	65	miR-20a	115	miR-92	165	miR-UL148D-1
16	miR-126*	66	miR-21	116	miR-98	166	miR-UL22A-1
17	miR-128a	67	miR-210	117	miR-99b	167	miR-UL22A-1*
18	miR-128b	68	miR-212	118	hsa-let-7g	168	miR-UL36-1
19	miR-130a	69	miR-213	119	miR-1	169	miR-US25-1
20	miR-130b	70	miR-214	120	miR-133b	170	miR-US25-2-3p
21	miR-132	71	miR-216	121	miR-148a	171	miR-US25-2-5p
22	miR-133a	72	miR-218	122	miR-149	172	miR-US33-1
23	miR-135a	73	miR-219	123	miR-15a	173	miR-US5-1
24	miR-135b	74	miR-22	124	miR-182	174	miR-US5-2
25	miR-136	75	miR-222	125	miR-188	175	miR-105
26	miR-138	76	miR-223	126	miR-189	176	miR-106a
27	miR-139	77	miR-23a	127	miR-199a*	177	miR-10b
28	miR-141	78	miR-23b	128	miR-301	178	miR-129
29	miR-142-5p	79	miR-24	129	miR-302a	179	miR-146b
30	miR-143	80	miR-25	130	miR-361	180	miR-147
31	miR-144	81	miR-26a	131	miR-375	181	miR-151
32	miR-145	82	miR-26b	132	miR-377	182	miR-154*
33	miR-146a	83	miR-27a	133	miR-378	183	miR-155
34	miR-148b	84	miR-27b	134	miR-380-5p	184	miR-17-3p
35	miR-150	85	miR-28	135	miR-381	185	miR-181d
36	miR-152	86	miR-296	136	miR-382	186	miR-182*
37	miR-154	87	miR-299-5p	137	miR-412	187	miR-18a*
38	miR-15b	88	miR-29a	138	miR-425	188	miR-18b
39	miR-16	89	miR-29b	139	miR-450	189	miR-191*
40	miR-17-5p	90	miR-29c	140	miR-452	190	miR-193b
41	miR-181a	91	miR-30a-3p	141	miR-484	191	miR-197
42	miR-181b	92	miR-30a-5p	142	miR-485-5p	192	miR-198
43	miR-181c	93	miR-30b	143	miR-486	193	miR-199b
44	miR-183	94	miR-30c	144	miR-7	194	miR-200a*
45	miR-184	95	miR-30d	145	miR-9*	195	miR-202
46	miR-185	96	miR-30e-5p	146	hsa-let-7a	196	miR-202*
47	miR-186	97	miR-32	147	hsa-let-7f	197	miR-20b
48	miR-18a	98	miR-320	148	miR-124a	198	miR-211
49	miR-190	99	miR-323	149	miR-127	199	miR-215
50	miR-191	100	miR-324-3p	150	miR-134	200	miR-217

201	miR-220	**234**	miR-379	**267**	miR-501	**300**	miR-519b
202	miR-224	**235**	miR-380-3p	**268**	miR-502	**301**	miR-519c
203	miR-299-3p	**236**	miR-383	**269**	miR-503	**302**	miR-519d
204	miR-489	**237**	miR-384	**270**	miR-504	**303**	miR-519e
205	miR-302b	**238**	miR-409-3p	**271**	miR-505	**304**	miR-519e*
206	miR-302b*	**239**	miR-409-5p	**272**	miR-506	**305**	miR-519e*
207	miR-302c	**240**	miR-410	**273**	miR-507	**307**	miR-520a*
208	miR-302c*	**241**	miR-422a	**274**	miR-508	**308**	miR-520b
209	miR-302d	**242**	miR-422b	**275**	miR-509	**309**	miR-520c
210	miR-30e-3p	**243**	miR-423	**276**	miR-510	**310**	miR-520d
211	miR-31	**244**	miR-424	**277**	miR-511	**311**	miR-520d*
212	miR-325	**245**	miR-429	**278**	miR-512-3p	**312**	miR-520e
213	miR-326	**246**	miR-432	**279**	miR-512-5p	**313**	miR-520f
214	miR-329	**247**	miR-432*	**280**	miR-513	**314**	miR-520g
215	miR-330	**248**	miR-451	**281**	miR-514	**315**	miR-520h
216	miR-337	**249**	miR-452*	**282**	miR-515-3p	**316**	miR-521
217	miR-345	**250**	miR-453	**283**	miR-515-5p	**317**	miR-522
218	miR-346	**251**	miR-455	**284**	miR-516-3p	**318**	miR-523
219	miR-34b	**252**	miR-483	**285**	miR-516-5p	**319**	miR-524
220	miR-362	**253**	miR-485-3p	**286**	miR-517*	**320**	miR-524*
221	miR-363	**254**	miR-487	**287**	miR-517a	**321**	miR-525
222	miR-367	**255**	miR-488	**288**	miR-517b	**322**	miR-525*
223	miR-368	**256**	miR-490	**289**	miR-517c	**323**	miR-526a
224	miR-369-3p	**257**	miR-491	**290**	miR-518a	**324**	miR-526b
225	miR-369-5p	**258**	miR-492	**291**	miR-518a-2*	**325**	miR-526b*
226	miR-370	**259**	miR-493	**292**	miR-518b	**326**	miR-526c
227	miR-371	**260**	miR-494	**293**	miR-518c	**327**	miR-527
228	miR-372	**261**	miR-495	**294**	miR-518c*	**328**	miR-93
229	miR-373	**262**	miR-496	**295**	miR-518d	**329**	miR-95
230	miR-373*	**263**	miR-497	**296**	miR-518e	**330**	miR-96
231	miR-374	**264**	miR-498	**297**	miR-518f		
232	miR-376a	**265**	miR-499	**298**	miR-518f*		
233	miR-376b	**266**	miR-500	**299**	miR-519a		

Table 8: ID numbers and suppliers of the TaqMan assays, antibodies and cloned Plasmid DNA used in this study.

	Assays and antibodies Symbols	ID Numbers	Companies
1	Gapdh	Hs99999905	Applied Biosystems
2	Oct4	Hs01654807	Applied Biosystems
3	Nanog	Hs 02387400	Applied Biosystems
4	Sox2	Hs00602736	Applied Biosystems
5	Ncam1	Hs00941823	Applied Biosystems
6	Eno3	Hs 00266551	Applied Biosystems
7	Afp	Hs 01040597	Applied Biosystems
8	Gata6	Hs00232018	Applied Biosystems
9	Gata4	Hs00171403 & Hs00232018	Applied Biosystems
10	Wnt5a	Hs00998537	Applied Biosystems
11	Notch2	Hs01050719	Applied Biosystems
12	Snail2	Hs00950344	Applied Biosystems
13	Shh	Hs00179843	Applied Biosystems
14	Pten	Hs00829813	Applied Biosystems
15	Tgf-β-R2	Hs01548876	Applied Biosystems
16	Lefty2	Hs00745761	Applied Biosystems
17	B2M	Hs00984230	Applied Biosystems
18	Noggin	Hs00271352	Applied Biosystems
19	Nodal	Hs01086749	Applied Biosystems
20	Smad2	Hs00183425	Applied Biosystems
21	Smad3	Hs00706299	Applied Biosystems
22	Smad4	Hs00929647	Applied Biosystems
23	Gapdh Antibody	ab8245	Abcam
24	TGF-β-R2 Antibody	ab78419	Abcam
25	Lefty2 Antibody	ab34593	Abcam
26	Noggin Antibody	ab56497	Abcam
27	TGF-β-R1 Antibody	3712	Cell Signaling
28	TGF-β-R2 Antibody	3713	Cell Signaling
29	TGF-β-R2 cloned Plasmid DNA	IOH29610	imaGenes
30	Gapdh cloned Plasmid DNA	IOH3380	imaGenes

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