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Investigation of the role of mesoderm inducing genes in a cell culture model for the progression of colorectal cancer

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Abstract

Cancer stem cells (CSC) are initiating cells in many cancer types that have similar properties like normal embryonic stem cells. Colorectal tumours can undergo epithelial to mesenchymal transition (EMT)-like processes at the invasive front, rendering them capable of invasion and metastasis. Recently, a possible link between cells undergoing EMT and cells with stem cell like properties has been identified in mammary Cancer stem cells (CSC). It is of fundamental importance to understand molecular events leading to establishment and maintenance of cancer initiating cells and how these relate to cellular transitions during tumourigenesis. However it is difficult to observe these processes *in vivo*, not least due to the transient nature of events during tumourigenesis.

We used an *in vitro* system to recapitulate changes occuring in CRC cells at the invasive front (mesenchymal-like cells) and central mass (epithelial-like cells) of tumours. We show for the first time, that the mesoderm inducer *BRACHYURY* differentially influences expression of the pluripotency gene *NANOG*, through association of BRACHYURY with upstream regulatory elements in the *NANOG* promoter in CRC cells. Binding of BRACHYURY to the *NANOG* promoter directly influences expression of *NANOG* and is predominant in mesenchymal-like cancer cells. We also demonstrate that the oncogene, β-catenin is upstream of both *BRACHYURY* and *NANOG* and influences the expression of markers of CSCs in the mesenchymal-like cells, whose presence has previously been linked to poor patient prognosis. Finally, we show that a similar pathway exists in two other cell lines (NTERA-2 and T84).

Contents – Chapters and Sections

List of figures and tables:

Abbreviations

AEBSF – 4 (2-aminoethyl) benzenesulfonyl fluoride
ALCAM - Activated leukocyte cell adhesion molecule
APC – Adenomatous Polyposis Coli
APCL – Adenomatous Polyposis Coli Like
ACF – Aberrant Crypt Foci
Ascl2 - Achaete scute-like 2
BCL - B-cell lymphoma
BMP – Bone Morphogenetic Protein
CAPS – 3-(cyclohexylamino)-1-propane sulfonic acid
CBC - Crypt base columnar
CKI – Casein Kinase I
CKIβ - Casein Kinase I beta
CCK - Cholecystokinin
ChIP- Chromatin Immunoprecipitation
CRC – Colorectal Cancer
CRM1 – Chromosome Maintenance Region 1
CSC - Cancer stem cells
DEP – Dishevelled, Eg-10, plekstin
DIC – Differential Interference Contrast
DIX – Dishevelled and Axin
DLG – Discs Large
DMEM – Dulbecco's Modified Eagle's Medium
DMSO – Dimethlysulphoxide
DNA – Deoxyribonucleic Acid
Dsh - Dishevelled
DTT – Dithiothrietol
Dvl – Dishevelled
EC – Embryonal Carcinoma
ECACC – European Collection of Cell Cultures
EDTA – (Ethylenedinitrilo) tetra acetic acid
EMT – Epithelial-mesenchymal transition
EPH - Ephrin receptors
FAP – Familial Adenomatous Polyposis
FBS – Foetal Bovine Serum
FOX – Forkhead box
GBP – GSK Binding Protein
GSK – Glycogen Synthase Kinase
GSK3β - Glycogen Synthase Kinase 3 beta
HCC - Hepatocellular carcinoma
HGF - Hepatocyte growth factor
HMBA – Hexamethylene Bisacetamide
HNPCC – Hereditary Non-Polyposis Coli Cancer
HSC – Haematopoietic stem cell
Ihh - Indian hedgehog

IL1β - Interleukin 1β
IPSC- Induced pluripotent stem cells
ISC - Intestinal stem cells
LEF – Lymphocyte Enhancer Factor
Lgr5 - leucine-rich repeat-containing G protein coupled receptor 5
LIF - Leukemia inhibitory factor
LMB – Leptomycin B
LOH – Loss of heterozygosity
LRP – LDL (Low Density Lipoprotein) Receptor Related Protein
MAPK - Mitogen activated protein kinase
MDCK - Madin-Darby Canine Kidney Cells
MCR – Mutation Cluster Region
MEK - Mitogen-activated protein kinase/extracellular signal-regulated kinase
MEM – Minimum Eagle's Medium
MET – Mesenchymal to epithelial transition
MLV - Murine leukemia virus
MMP – Matrix Metalloproteinase
MMR – Mismatch Repair
mRNA – Messanger Ribonucleic Acid
NES – Nuclear Export Sequence
NFκB - Nuclear factor κB
NLS – Nuclear Localisation Sequence
NOD/SCID – non-obese diabetes/severe combined immunodeficiency
OSE - Ovarian surface epithelial
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PDGF - Platelet-derived growth factor
PDZ – PSD-95/DLG/ZO-1
PI - Propidium iodide
PI3K - Phosphoinositide 3-kinase
PP2A – Protein Phosphatase 2A
RNA – Ribonucleic Acid
RT – Reverse Transcription
SCF – SKP-1, Cdc53, F-box
SDS-PAGE – Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
Ser – Serine
Shh - Sonic hedgehog
SHIP - SH2 domain-containing inositol phosphatase
SMAD - Mothers against decapentaplegic (MAD) + *C. elegans* protein SMA
STAT3 - Signal transducer and activator of transcription 3
TA - Transit amplifying
TAD - Transcriptional activation domain
TBM - Tubular basement membrane
TCF – T-Cell Factor
TGFβ - Transforming growth factor beta
TLE – Transducing-Like Enhancer of split

Gene names and Accession numbers

Gene name	Abbreviation	NCBI accession number
BRACHYURY	T	NM_003181
FIBROBLAST GROWTH FACTOR 4	*FGF4*	NM_002007
FIBROBLAST GROWTH FACTOR RECEPTOR 1	*FGFR1*	NM_023110
FIBROBLAST GROWTH FACTOR RECEPTOR 2	*FGFR2*	NM_000141
FIBROBLAST GROWTH FACTOR RECEPTOR 3	*FGFR3*	NM_000142
OCTAMER-4	*Oct-4*	NM_002701
NODAL	*NODAL*	NM_018055
FIBROBLAST GROWTH FACTOR 8	*FGF8*	NM_033165
GROWTH DIFFERENTIATION FACTOR-3	*GDF3*	NM_020634
ACTIVIN A	*ACTIVIN A*	NG_008004
ACTICIN B	*ACTICIN B*	NM_002193
BONE MORPHOGENETIC PROTEIN 4	*BMP4*	NM_001202
T-BOX TRANSCRIPTION FACTOR 2	*TBX2*	NM_005994
T-BOX TRANSCRIPTION FACTOR 3	*TBX3*	NM_005996
FIBROBLAST GROWTH FACTOR 19	*FGF19*	NM_005117
NUCLEAR FACTOR KAPPA-LIGHT-CHAIN-ENHANCER OF ACTIVATED B CELLS	*NFκB*	NM_003998
SNAIL	*SNA*	NM_005985
E-CADHERIN	*CDH1*	NM_004360
CAUDAL TYPE HOMEOBOX TRANSCRIPTION FACTOR 2	*CDX2*	NM_001265
CRUMBS HOMOLOG 3	*CRB3*	NM_139161
PROTEIN ASSOCIATED WITH LIN SEVEN 1	*PALS1*	AF397170
ZINC FINGER E-BOX-BINDING HOMEOBOX 1	*ZEB1*	NM_001128128
ZINC FINGER E-BOX-BINDING HOMEOBOX 2	*ZEB2*	NM_014795
WINGLESS-TYPE MMTV INTEGRATION SITE FAMILY, MEMBER 11	*WNT11*	NM_004626
EPITHELIAL V-LIKE ANTIGEN 1	*EVA1*	AF275945
INTERLEUKIN-2	*IL2*	NM_000586
INTERLEUKIN-15	*IL15*	NM_172174
ORTHOPEDIA	*OTP*	NM_032109
STRATIFIN	*14-3-3 sigma*	NM_006142
NOTCH HOMOLOG 1	*NOTCH1*	NM_017617
NOTCH HOMOLOG 2	*NOTCH2*	NM_024408
NOTCH HOMOLOG 3	*NOTCH3*	NM_000435
NOTCH HOMOLOG 4	*NOTCH4*	NM_004557
β-catenin	*CTNNB1*	NM_001904
SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3	*STAT3*	NM_139276
INTERLEUKIN-10	*IL10*	NM_000572
PLASMINOGEN ACTIVATOR UROKINASE RECEPTOR	*PLAUR*	NM_002659
BCL2L1	*BCL-2-LIKE 1*	NM_138578
SRY (SEX DETERMINING REGION Y)-BOX 2	*SOX2*	NM_003106
GATA BINDING PROTEIN 2(GLOBIN TRANSCRIPTION FACTOR 2)	*GATA2*	NM_032638
GATA BINDING PROTEIN 2(GLOBIN TRANSCRIPTION FACTOR 2)	*GATA4*	NM_002052
GATA BINDING PROTEIN 6(GLOBIN TRANSCRIPTION FACTOR 6)	*GATA6*	NM_005257
LAMIN B	*LMNB*	NM_005573
LAMIN A/C	*LMNA/C*	NM_170707
FORKHEAD BOX D3	*FOXD3*	NM_012183
C-MYC	*MYC*	NM_002467
VIMENTIN	*VIMENTIN*	NM_003380

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Chapter 1 - Introduction

Colorectal cancer, an overview:

Colorectal cancer (CRC) is the cancerous growth of the colon, rectum and appendix. It is the second most prevalent cancer in the Western world causing over 655,000 deaths per year. The tumour suppressor protein adenomatous polyposis coli (APC) is mutated in most cases of sporadic colorectal cancer; specifically mutations in *APC* have been found in 85% of the cases of non-hereditary colon cancers (Tucker and Pignetelli, 2000). *APC* has been described as 'the gene for colon cancer', describing its importance in this disease. CRC can be both hereditary (genetic) and sporadic (Non-heriditary) and 75% of CRCs are sporadic. Familial adenomatous polyposis (FAP) and hereditary non-polyposis coli cancer (HNPCC) are two non-sporadic forms of CRC. 95% of colorectal cancers are carcinomas, of which 95% are adenocarcinomas. Adenocarcinomas are generally considered to arise from adenomas. Adenomatous polyps are benign tumours which become malignant following malignant transformation. (Fenoglio *et al.*, 1974; Morson *et al.*, 1974; Shinya *et al.*, 1979). There are many risk factors associated with colorectal cancer including family history of colon cancer, high fat diet (Potter *et al.* 1986), sedentary lifestyle (Slattery *et al.* 1988; White *et al.*, 1996; Friedenreich *et al.*, 2001) and smoking (Chao *et al.*, 2000). The major signalling pathway altered in the event of colon cancer is the canonical Wnt signalling pathway and *β-catenin* is the key oncogene in this pathway. The canonical Wnt signalling pathway is described in section 1.2 of this chapter.

1.1 The normal colon

1.1.1 Structure and function of the colon

The colon, also known as the intestine is located in the lower abdomen in humans and is an essential part of the gastrointestinal tract. The function of the colon is predominantly to absorb food and nutrients, to reclaim water and to store waste products before excretion. Finger-like projections, known as villi, protrude out of the walls of the small intestine with additional extensions, called the

microvilli, which serve to increase the surface area of the intestine and increase its absorptive activity (Figure 1.1A). In the intestinal epithelial lining, glands called the crypts of Lieberkühn (or intestinal glands or intestinal crypts) are found which secrete many enzymes.

1.1.2 Cell types in the small intestine

The epithelial lining of the small intestine consists of many distinct cell types to maintain normal structure and function of the tissue. The relative number and position of the cells are precisely regulated via exchange of signals between cells within the tissue. The common cell types of the intestinal epithelium (Figure 1.1B) are as follows:

Enterocytes or absorptive cells: These tall, columnar cells with microvilli are situated at the basal nucleus and are involved in the transport of substances. These cells are attached to each other and other cell types by junctional complexes (zonula occludens or tight junction, zonula adherens, and macula adherens). These cells are also involved in the absorption of amino acids, monosaccharides, monoglycerides and fatty acids across the microvilli membranes. These cells are continuously generated in the crypts to replace the cells at the tip of the villi. The enterocytes have a lifespan of about 5-6 days.

Goblet cells: These cells are mucus-secreting and absorptive; and are the second most abundant epithelial cells, found interspersed among the other cell types. The base of these cells holds the nucleus and organelles. These cells have lifespan of 5-6 days.

Paneth cells: These cells are located at the base of the crypts of Lieberkuhn. They have an oval basal nucleus and large, acidophilic granules at their apical end. The granules contain the antibacterial enzyme lysozyme, glycoproteins and zinc which are required for correct functioning of many enzymes. Paneth cells regulate the intestinal flora and have a lifespan of approximately four weeks.

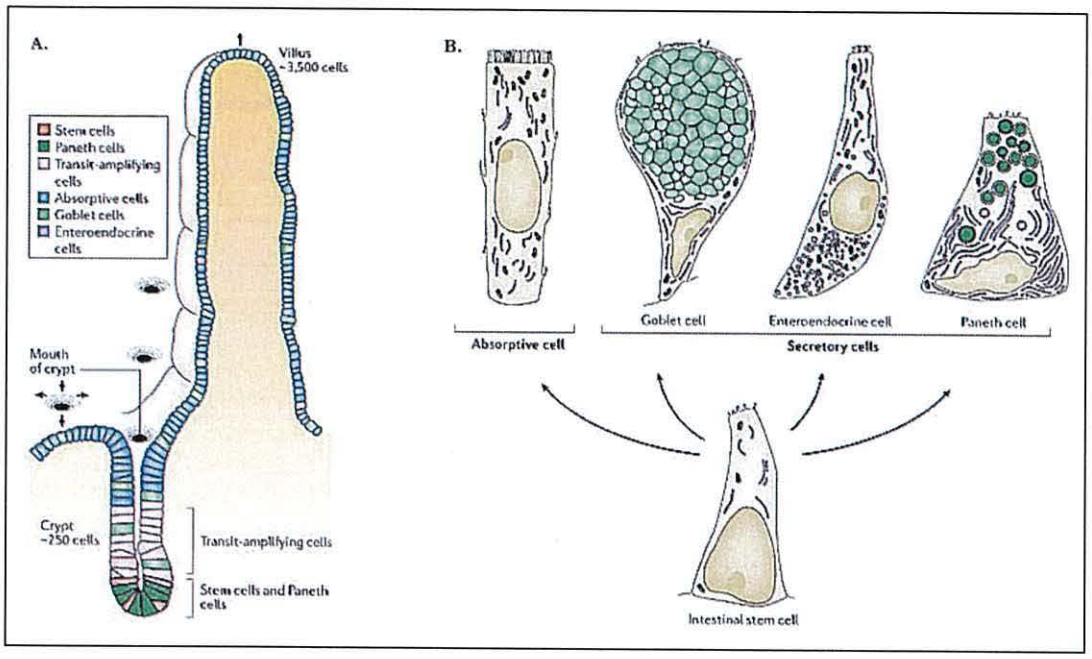


Image taken from Nature Reviews, Genetics, 2006, vol7, 5

http://www.nature.com/nrg/journal/v7/n5/images/nrg1840-f1.jpg

Figure 1.1

Intestinal villus and its cell types. A) Structure of the intestine in the crypt villi axis responsible for the renewal of its epithelium. Arrows show the upwards flow of cells out of the crypts. Stem cells lie at the crypt base. It is still not confirmed whether they are mixed with, or just above, the Paneth cells. Above the stem cells are the dividing progenitor cells, the transit-amplifying cells. At the top, lie the post-mitotic differentiated cells (which are the absorptive cells, goblet cells and enteroendocrine cells). B) Four types of terminally differentiated cells. Absorptive cells have a brush border and are at the apical surface. The other three types are all secretory and their apical cytoplasm is generally mucus-filled secretory granules.

Enteroendocrine cells: These cells are mostly found in the lower part of the crypts but can occur at all levels of the epithelium. They produce cholecystokinin (CCK), which stimulates pancreatic enzyme secretion and gall bladder contraction. They also secrete secretin to stimulate pancreatic bicarbonate secretion and gastric inhibitory peptide, which inhibits gastric acid secretion.
M or microfold cells: These epithelial cells are found over Peyer's patches and other large lymphatic aggregations. They are flat shaped and their surface has many folds. They transport antigens to the nearby lymphoid cells where immune response to foreign antigens is regulated.
Undifferentiated stem cells: The crypt of the colon consists of a monolayer of epithelial cells. At the crypt base the stem cells are located which give rise to the progenitor cells which occupy the lower third of the crypt. Epithelial cells at the surface are renewed frequently as the progenitor cells migrate towards the surface where they differentiate. Differentiation to a goblet cell or enterocyte takes about 2 additional divisions and these cells then migrate from the crypt to the villus. At the tip of the villus, these epithelial cells finally shed off into the lumen by apoptosis and are replaced by the differentiating cells moving up the crypt. The entire process of renewal of the epithelial cell takes 3-5 days (Potten & Loeffler, 1990).

In addition to these cell types, the middle part of each villus contains a dense population of mesenchymal cell types which comprise of the stroma with pericytes, fibroblasts, machrophages and endothelial cells.

1.2.3 Localisation of APC and β-catenin in normal human colon

APC is localised to the cytoplasm in differentiated epithelial cells above the crypt, and is localised to the nuclei of cells towards the base of crypts (Rosin-Arbesfeld *et al.*, 2003). Nuclear APC at the crypt base is related to the activation of the Wnt pathway in the proliferative cells of the crypt (van de Wetering *et al.*, 2002). Adenocarcinomas with truncating mutation of *APC* and loss of heterozygosity show nuclear localisation of APC (Rosin-Arbesfeld *et al.*, 2003). Studies show APC in the intestinal epithelium to be localised to the apical

membrane (Miyashiro *et al.*, 1995; Midgley *et al.*, 1997; Reinacher-Schick & Gumbiner, 2001; Anderson *et al.*, 2002).; although the specificity of the antibodies used in these studies may lead one to question this apical localisation. Later, in 2005, *Brocardo* and co-workers confirmed that the polyclonal antibody used in 2003 by Rosin-Arbesfeld and co-workers was not reliable.

β-catenin is mostly found in the nuclei of cells within the proliferating compartment at the bottom third of crypts, with nuclear staining being strongest at the crypt base where cells are active for Wnt signallig (van de Wetering *et al.*, 2002). TCF-4, a TCF family member, is widely expressed in the intestinal epithelium and is required for the crypt stem cells (Korinek *et al.*, 1997). The β-catenin/TCF-4 complex is the key switch that controls proliferation versus differentiation in normal and malignant intestinal epithelial cells (van de Wetering *et al.*, 2002).

1.2 Wnt signalling pathway

The Wnt signalling pathway is of fundamental importance in development, promotion and maintenance of stem cells and also in disease; specifically, components of the canonical Wnt signalling pathway are critically altered in CRCs (Willert *et al.*, 2002). APC is a tumour suppressor protein, central to this pathway and β-catenin is a key oncogene in this pathway.

1.2.1 APC

Functions of the *APC* gene and its protein product include controlling the Wnt pathway, apoptosis, cell migration and adhesion and chromosomal segregation during mitosis (Fodde and Clevers 2001). The *APC* gene, located on the long arm of chromosome 5, consists of 15 exons and encodes a 312 kDa protein consisting of 2843 amino acids (Groden *et al.*, 1991; Kinzler *et al.*, 1991, Nishisho *et al.*, 1991). The central part of APC contains three repeats of 15 amino acids, and seven distinct repeats of 20 amino acids, both of which can bind

independently to β-catenin (Rubinfeld *et al.*, 1993; Rubinfeld *et al.*, 1995; Su *et al.*, 1993) but only the 20 amino acid repeats are involved in phosphorylation of β-catenin (Munemitsu *et al.*, 1995). APC can be directly phosphorylated by GSK-3β (Rubinfeld *et al.*, 1996) by binding to axin (Ikeda *et al.*, 2000). Phosphorylation of APC is important for degradation of β-catenin (Rubinfeld *et al.*, 1996; Rubinfeld *et al.*, 1997). APC is a key player controling the degradation, subcellular localisation and transcriptional activity of β-catenin (Rosin-Arbesfeld *et al.*, 2003). APC is localised to kinetochores in mitotic cells (Kaplan *et al.*, 2001; Fodde *et al.*, 2001) and also in centrosomes in cancer cells (Tighe *et al.*, 2001) where it has a role in chromosome segregation. Truncated *APC* leads to increased abnormal mitoses and chromosomal aberration in embryonic stem cells (Fodde *et al.*, 2001) and many cancer cells (reviewed in Toncheva and Nacheva, 1998).

In a cell, when a gene allele loses its normal function in which the other allele was already inactivated, is known as loss of heterozygosity (LOH). In oncogenesis, after one allele of a tumour suppressor gene is inactivated, the mutation is then passed on to the zygote resulting in progeny that are heterozygous for that allele. LOH on chromosome 5q occurs for APC (Rosin-Arbesfeld *et al.*, 2003; Levy *et al.*, 1994; Lamlum *et al.*, 1999). LOH on chromosome 17p (17p3.1) occurs for the tumour suppressor protein, T53 and is found in 75% of CRCs (Forslunder *et al.*, 2001). Loss of the Smad family member 4, SMAD4 on chromosome 18q (18q21) is associated with mis-signalling of growth-suppressing transforming growth factor β (TGF-β) pathway. Smad4 mutations are found in 50-60% of LOH in CRC (Woodford-Richens *et al.*, 2001).

1.2.2 β-catenin

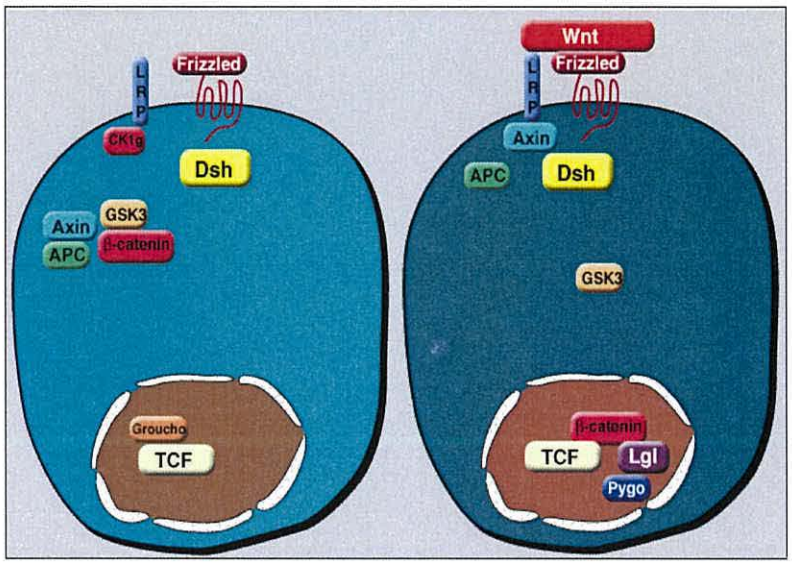
β-catenin has role in intercellular adhesion alongside its function of transducing the Wnt signal. Its structure comprises of a 130 amino acid N-terminal domain, an armadillo repeat region of 12 imperfect repeats of 42 amino acids and a 100 amino acid C-terminal domain. The N-terminal domain is

involved in regulating its stability through a number of phosphorylation sites (three serines sites at S33, 37, 45 and one threonine site, T41) within this region. Deletions and certain point mutations of this region that alter these phosphorylation sites lead to activation of *β-catenin* (Yost *et al.*, 1996; Munemitsu *et al.*, 1996; Barth *et al.*, 1997).

In the absence of a Wnt signal, free cytoplasmic β-catenin is phosphorylated by CK1 and GSK3β in a multi-protein complex known as the degradation complex, consisting of axin (or its homologue conductin), GSK-3β (□□Glycogen synthase kinase 3 beta) and APC (reviewed in Bienz, 2002) (Figure 1.2A). The complex of axin and casein kinase (CK1) induces phosphorylation of β-catenin at Ser45 which initiates or primes phosphorylation of β-catenin at the other Wnt-dependent phosphorylation sites (Amit *et al.*, 2002; Hagen & Vidal-Puig, 2002; Liu *et al.*, 2002; Sakanaka, 2002) and GSK-3β phosphorylation sites at Ser33, Ser37 and Thr41 (van Noort *et al.*, 2002; Sadot *et al.*, 2002). Once phosphorylated, β-catenin then interacts with the F-box protein□ β-TrCP (Slimb in *Drosophila*) (Hart *et al.*, 1999; Winston *et al.*, 1999; Liu *et al.*, 1999). The WD40 repeat domain of□ β-TrCP interacts specifically with the phosphorylated□ β-catenin and not with the unphosphorylated□ β-catenin which makes the phosphorylated β-catenin ubiquitinated by this complex and this targets proteosomal degradation of β-catenin by 26S proteasome (reviewed in Maniatis 1999). Due to this degradation, β-catenin is not available to enter the nucleus, instead, it is mainly membrane bound as part of the cell adhesion complex with E-cadherin. In the absence of a Wnt signal, TCF/Lefs act as transcriptional repressors (Brannon *et al.*, 1997; Bienz, 1998; Riese *et al.*, 1997).

In the presence of a Wnt signal, β-catenin is stabilized and the destruction complex mentioned above is not formed. Rather, a complex is formed between dishevelled, GBP/Frat1, axin and Zw3/GSK-3 (Li *et al.*, 1999; Salic *et al.*, 2000; Farr *et al.*, 2000). β-catenin is released from this complex escaping phosphorylation and is therefore not targeted for proteosomal degradation (Salic

A.



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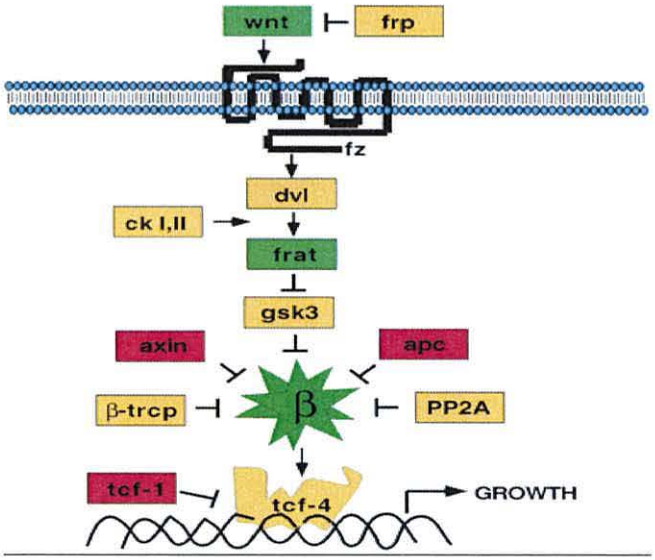


Figure 1.2

Simplified diagram of the Wnt signalling pathway. A) In the absence of a Wnt signal, β-catenin is phosphorylated by casein kinase Iβ (CKIβ) and Glycogen synthase kinase-3 (GSK-3β), which enables its ubiquitination and proteasomal degradation. When a Wnt signal is present, DISHEVELLED proteins inhibit the phosphorylation of β-catenin which leads to nuclear entry of dephosphorylated β-catenin and activation of Wnt pathway target genes. B) Oncogenes and tumor suppressors of the Wnt pathway. Arrows or bars indicate activating or inhibitory effects, respectively. Green and red indicate proto-oncogenic and tumor suppressive activity, respectively, in human cancer.

et al., 2000). The Wnt signal is initiated when Wnt ligands bind to Frizzled receptors, resulting in inhibition of the degradation complex via dishevelled (Cadigan & Nusse, 1997; Dale, 1998). Dishevelled proteins can directly interact with Axin (Li *et al.*, 1999) and phosphorylate it. The phosphorylation of Axin is reduced when there is a Wnt signal and this leads to decrease in the binding affinity of β-catenin to Axin which releases β-catenin from the degradation complex and leading to the levels of free β-catenin becoming raised in the cytoplasm (Willert *et al.*, 1999; Jho *et al.*, 1999). Dishevelled can also interact with casein kinase Iβ (Peters *et al.*, 1999; Sakanaka *et al.*, 1999), which then acts to inhibit phosphorylation of β-catenin at Ser45. This dephosphorylation of β-catenin enables it to enter the nucleus (Staal *et al.*, 2002) and interact with TCF/Lefs. This interaction of β-catenin with its partner proteins, TCF/Lefs, allows them to act as transcriptional activators (reviewed in Bienz, 1998; Roose & Clevers, 1999) and leads to activation of Wnt pathway target genes (Figure 1.2).

1.2.3 Wnt and Wnt target genes

So far, there are 19 Wnt genes identified in humans, 19 genes in mouse, 17 in Xenopus, 7 in Drosophila and 15 Wnt genes in Zebrafish (data taken from Wnt homepage, http://www.stanford.edu/~rnusse/wntwindow.html) discovered. In a microarray study in 2002 (Willert *et al.*), more than 50 genes were shown to be upregulated in response to Wnt signal, in human teratocarcinoma cells. These genes include *Cyclin D1*, *Ubc4/5E2*, *CBP/P300*, *MYC*, *ID2*, *β-TRCP*, *TLE/Groucho, MSX1, MSX2*, *Nucleophosmin, Frizzled, Follistatin* and *REST/NRSF* (Willert *et al.* 2002). Almost all Wnt target genes have putative TCF binding sites in their promoter region. Many of these target genes also respond to Bone morphogenic pathway (BMP) signaling pathway components along with Wnt pathway components. Wnt signaling is critical for activation of genes that promote stem cell fate and inhibit cellular differentiation and regulates a remarkable number of genes involved in its own signaling system (Willert *et al.*, 2002).

1.2.4 Wnt pathway in development

The Wnt family of proteins is a group of highly conserved secreted signalling molecules. The Wnt pathway plays a key role during development of the embryo where these proteins regulate cell-to-cell interactions and also inhibit cellular differentiation. Mutations in Wnt genes or Wnt pathway molecules can lead to many developmental defects. In mouse, mutation in *Wnt-1* leads to loss of midbrain and cerebellum, mutation in *Wnt-2* leads to defects in the placenta, mutation in *Wnt-3A* enables loss of tailbud, mutation in *Wnt-4* and mutation of *Wnt-7A* gives rise to ventralization of limbs. In *Drosophila*, mutation in *wingless*, affects limb development and mutation in *Dwnt-2* gives rise to defects in muscles and testis development. In *C. elegans, lin-44* mutation leads to defects in asymmetric cellular division and mutation in *mom-2* enables defects in endoderm induction and spindle orientation (reviewed in Andreas Wodarz and Roel Nusse, 1998). Thus, Wnt signaling is fundamental to embryogenesis. However, as mentioned earlier, this pathway is also involved in many forms of cancer, including colorectal cancer.

1.2.5 Wnt signalling in normal colon

The cells of the intestinal epithelium continuously self-renew and thereby act as a distinct model for the study of cell proliferation, differentiation, cell migration and also of cancer progression. At the bottom of the intestinal crypts, several Wnt factors are produced (Gregorieff *et al.*, 2005) which generate a morphogen-like gradient of Wnt signals along the crypt-villus axis. The Wnt signalling pathway drives the transcription of genes related to proliferation in the intestine such as *Sox9*, an intestinal crypt transcription factor (Blache *et al.*, 2004) and critical in maintaining the epithelial stem cells and early progenitor cells. The Wnt pathway also acts with other pathways, such as the Notch signalling to maintain the gut homeostasis and progenitor cells (reviewed in Fre *et al.*, 2009). There is evidence that the proliferating transit amplifying (TA) cells in the crypts are dependent on Wnt pathway stimulation and the accumulation of

nuclear β-catenin by the progenitor cells at the crypt bottom indicates this stimulation (Van de Wetering *et al.*, 2002). Furthermore, complete proliferation loss due to overexpression of SKK-1 (Wnt inhibitor) and removal of Tcf4 and β-catenin further serve as evidence for active Wnt pathway in the gut (Korinek *et al.*, 1998; Pinto *et al.*, 2003; Kuhnert *et al.*, 2004). Hyper proliferation of the epithelium occurs following *APC* mutation (*APC* is a negative regulator of Wnt) or oncogenic *β-catenin* expression (Harada *et al.*, 1999; Romagnolo *et al.*, 1999; Smits *et al.*, 1998; Sansom *et al.*, 2004). Wnt signalling is also required for the terminal differentiation of the Paneth cells (Andreu *et al.*, 2005). Also, it is required to drive the expression of EphB2 and EphB3 (cell-sorting receptors) where β-catenin and TCF inversely control the receptors and their ligand ephrin-B1 along the crypt-villus axis (Batlle *et al.*, 2002). Recently, it was shown that *Achaete scute-like 2* (*Ascl2*), which is transcription factor and Wnt target gene can alter the gut stem cell program; specifically, deletion of *Ascl2* can completely remove the stemness of stem cells (Van der Flier *et al.*, 2009).

1.2.6 Wnt pathway in cancer and other diseases

Cancers exhibit many independent genetic defects. Many of the components of the Wnt signaling pathway are shown to be associated with human cancers or experimental cancer models. For example:

- Wnt-1 is a proto-oncogene activated by Mammary Tumor Virus in murine breast cancer (Nusse and Varmus, 1982).
- APC acts as a tumor suppressor gene in human CRC (reviewed in Polakis 1997).
- In human, mutations in β-catenin are found in colon cancer and in melanomas (Morin *et al*, 1997).
- The Drosophila gene legless is a homolog of *Bcl-9* (part of Wnt pathway), a gene is involved in B cell malignancies (Kramps, 2002).
- In human, mutations in *AXIN1* are found in liver cancers (Satoh *et al*, 2000; reviewed by Clevers, 2000).

- In mouse, *Tcf*1 can act as a tumor suppressor gene (Roose, 1999), and its mutation may lead to adenomas in the gut and mammary glands.
- A Wnt gene, *Wnt5A*, is mutated in lymphoid malignancies of mouse (Liang *et al*, 2003).

Other than cancer, mutation in Wnt genes may also lead to other diseases. Eg: mutation in *LRP5* leads to bone density defects (Gong, 2001; Little, 2002; Boyden, 2002), mutation in *LRP6* leads to early coronary diseases (Mani et al., 2007) and late onset of Alzheimer (De Ferrari, 2007), Wnt 10B mutation relates to obesity (Christodoulides 2006), *Axin2* mutation is involved in tooth agenesis (Lammli *et al*, 2004), *WTX2* relates to Wilm's tumour (Major, 2007, Rivera, 2007), *VANGL1* mutation leads to neural tube defects (Kibar, 2007) and *Tcf4* mutation may lead to TypeII diabetes (Grant 2006, Florez 2006, O'Rahilly 2006). The oncogenes and tumour suppressors of the Wnt pathway are demonstrated in Figure 1.2B. Thus, we see that components of the Wnt pathway are not only related to normal development of an organism but also critically involved in human cancers and many other diseases.

1.4 Colorectal cancer, the disease

CRC initiates through a number of genetic alterations giving rise to genetic instability and which eventually leads to series of histological changes. The earliest stage of colon cancer is observed as aberrant crypt foci (ACF): two mutations in APC are present at this stage (Powell *et al*., 1992; Jen *et al*., 1994; Smith *et al*., 1994a). These ACF are seen microscopically and consist of abnormally formed crypts containing cells of normal or abnormal morphology. The ACF may then lead to the formation of a polyp which is a benign mass that protrudes into the lumen of the colon. The Adenomatous polyps are also called adenomas and have abnormal cellular organisation whereas, the hyperplastic polyps have normal architecture and morphology. As colorectal cancer progresses, mutations in oncogenes and tumour suppressor genes occur and the chromosomal instability increases (Fodde and Clevers, 2001) (Figure 1.3).

For colorectal adenomas to progress towards malignancies, loss of response to TGF-β is a key event (Polyak, 1996). TGF-β signaling can inhibit the growth rate of the epithelial cells, for example human bronchial epithelial cells (Masui *et al.*, 1986), and renal tubular epithelial cells (Fine *et al.*, 1985). TGF-β ligands are mostly found in the villi of the intestines (Oshima *et al.*, 1997).

Colorectal cancer can be studied under two major categories as follows:

- Sporadic colon cancer
- Hereditary colon cancer

1.4.1 Sporadic colon cancer

Most cases of colon cancer are sporadic and are related to many factors such as diet, ethnicity, hormonal factors and environmental exposures. Interaction of some or all these factors may lead to increased risk of sporadic colon cancer. This type of colon cancer does not have a hereditary component and appears by random occurrence.

1.4.2 Hereditary colon cancer

Around 10% of colon cancers fall under the category of being hereditary. If members of a family over a few generations are affected with colon cancer, there is an increased likelihood that family has a genetic mutation that can lead to hereditary colon cancer. The two main types of hereditary colon cancer are as follows:

- Familial adenomatous polyposis (FAP)
- Hereditary non-polyposis colorectal cancer (HNPCC)

1.4.2A Familial adenomatous polyposis (FAP):

FAP is an uncommon hereditary colon cancer syndrome and is associated

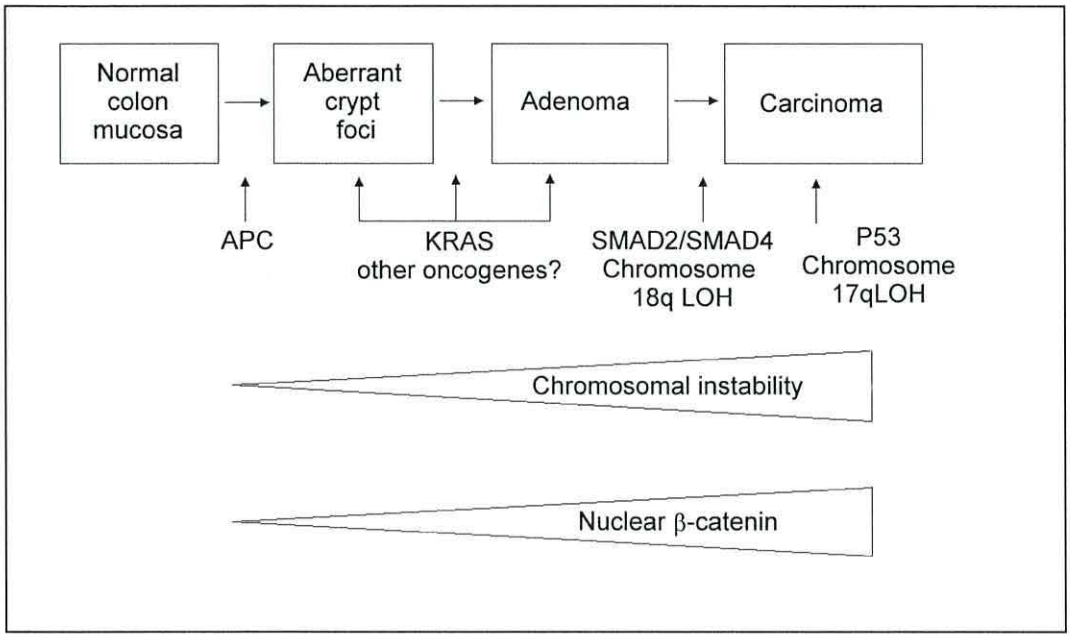


Image taken from Fodde and Clevers, 2001

Figure 1.3

The progression of colorectal cancer. Mutation in APC is observed in the earliest stage of CRC. Some aberrant crypt foci may lead to the formation of polyps (a benign mass which protrudes into the lumen), adenomatous (dysplastic) polyps (adenoma) may then progress to carcinoma of the colon. As CRC progresses, further mutations of oncogenes and tumour suppressor genes, and an increase in chromosomal instability are observed.

with a very high lifetime risk of colon cancer. The incidence of FAP is approximately 1 in 8000, 25% of which are novel germline mutations (Bisgaard *et al.*, 1994). This condition leads to the formation of hundreds of polyps in the colon in the second or third decade of life, which has the ability to become malignant. [Note: A colon polyp is an area of tissue overgrowth in the colon or rectum and can be benign or malignant]. FAP patients may also be exposed to an increased risk for other types of cancers such as cancers of the small intestine, skin, thyroid and bile ducts (summarised in the Stanford cancer centre webpage, http://cancer.stanford.edu/information/geneticsAndCancer/types/fap.html). Most FAP patients have germline mutations in *APC* between codons 200 and 1600 and leading to truncated *APC* (Cottrell *et al.*, 1992; Nagase & Nakamura, 1993; Laken *et al.*, 1999). Colorectal tumours from FAP patients have been found to contain either additional somatic mutations in the second *APC* allele, or loss of heterozygosity at the *APC* locus (Miyoshi *et al.*, 1992a; Solomon *et al.*, 1987; Ichii *et al.*, 1993; Levy *et al.*, 1994; Lamlum *et al.*, 1999). The location of the germline mutation in *APC* determines the type of mutations which eventually affects the second allele (Lamlum *et al.*, 1999).

1.4.2B Hereditary non-polyposis coli cancer (HNPCC)

Non-polyposis means that individuals, who have this syndrome, develop only a few polyps, maybe one or two, though, these polyps can become malignant or of the type that have the potential to become malignant. This hereditary disease develops due to germline mutations in the DNA mismatch repair (MMR) complex (Kinzler & Vogelstein, 1996; Kolodner, 1996; Markowitz, 2000). More than 90% of the cases of HNPCC involve mutations in *hMSG2* and *hMLH1*, two components of the mismatch repair complex (Yan *et al.*, 2000). Patients with this disease have 80% lifetime risk of developing colon cancer and also a greater risk of developing gastric and endometrial and ovarian cancers (Vasen *et al.*, 1990).

In HNPCC, *APC* is mutated in 21% of cases (Konishi *et al.*, 1996) and *β-catenin* is mutated in 43% of the cases (Miyaki *et al.*, 1999). Onset of this disease starts in a person who is in his/her early forties (Hodgson and Maher 1993).

1.4.3 Genes of colon cancer

There are a number of key genes involved in the initiation and maintenance of colon cancer. Since, *APC* is a major tumour suppressor gene, its mutation is common in most CRCs. Inactivation of *APC* leads to genetic instability leading to genetic alterations thereby initiating colon cancer and maintaining its progression. Other tumour suppressor genes, *SMAD4* and *TP53* and the oncogene *KRAS* are major targets of the genetic alteration. Mutations in other genes, such as *KRAS*, have been seen in 50% of colon cancer patients, out of which 5% have mutations in *BRAF* (gene responsible for cell growth and cell signalling). *GLUT1* is a gene found to be in high amounts in cells with mutations in *KRAS* and *BRAF* (Fodde and Clevers 2001). *GLUT1* gene is a potential marker of hypoxia in patient with CRC (Chung *et al.*, 2009). *GLUT1* glucose transporter is associated with neoplastic progression in colon and also is a marker of poor prognosis (Haber *et al.*, 1998).

1.4.4: APC and β-catenin mutation in other forms of cancer

As already mentioned in section 1.1.2, mutations in *APC* and *β-catenin* are also found in cancers other than CRC. High levels of free *β-catenin* were seen in 7 out of 26 human melanoma cell lines (Rubinfeld *et al.*, 1997), 6 of which had mutations of the *β-catenin* gene. Aggressive fibromatosis (desmoid tumours) have also been shown to involve mutations in APC and point mutations in *β-catenin* at either codon 41 or 45 (Tejpar *et al.*, 1999). Hepatocellular carcinoma (HCC) also involves mutation of *β-catenin* in 20% of cases (Huang *et al.*, 1999). *β-catenin* mutation is also common in hepatoblastoma, an embryonic liver tumour which occurs mainly in children under 2 years of age (Wei *et al.*,

2000). Miscoding mutations of both *APC* and *β-catenin* mutations have been observed in sporadic medulloblastomas (Huang *et al.*, 2000).

Thus, from the above sections, we have seen that there are various genes mutated in CRC and pathways like the Wnt signalling pathway are highly altered during this disease. Another important phenomenon associated with CRC is metastasis, by which a primary tumour invades the surrounding tissue and migrates to a secondary site in the body leading to a secondary tumour. Well differentiated carcinomas often lose epithelial differentiation during invasion at the invasive edge of the tumour and gain a mesenchymal-like phenotype by a process known as epithelial-mesenchymal transition (EMT); and then re-gain an epithelial phenotype following invasion by mesenchymal-epithelial transition (MET). Thus, this shows that malignancy in CRC is not only dependent on genetic alterations, but also on the tumour microenvironment since the stromal signals act in various combinations that can activate the EMT program (Weinberg 2007).

1.5 Epithelial-mesenchymal transition (EMT)

EMT is a vital and fundamental process in the morphogenesis of organisms. This process is also activated in cancer progression and also in many other diseases; and it occurs in response to extracellular signals (Gibbons *et al.*, 2009). EMT leads to a change in the shape of a cell and also may increase the cell's mobility as a cell transitions from an epithelial to a mesenchymal phenonotype. This transcriptional reprogramming process is associated with changes in epithelial cells which start expressing proteins that are associated with normal mesenchymal cells (reviewed in Thiery and Sleeman, 2006). Some characteristics of Epithelial-mesenchymal transition are as follows:

- The epithelial polarity is lost
- Cell:cell contact is lost/ disruption of intercellular contacts
- Aberration of cell substrate adhesion
- Basal membrane is degraded

- Cell invasion, migration and proliferation is increased
- Increase in cell motility

EMT is predominantly induced by stromal signals in the microenvironment (Weinberg, 2007). The major transcription factors involved in inducing EMT are *Zeb1/TCF8* (reviewed in Aigner *et al.,* 2007), *Zeb2* (Vandewalle *et al.,* 2005), *Snail* (reviewed in Usami *et al.,* 2008), *Slug* (reviewed in Kang and Massague, 2004), *E12/E47 FOXC2* (Mani *et al.*, 2007), and *Twist* (reviewed in Kang and Massague, 2004).

1.5.1 Growth factors regulating EMT

Several growth factors (eg TGFβ, EGF (epithelial growth factor), etc) are known to induce EMT in epithelial cell lines by disrupting the tubular basement membrane (TBM). These factors can recognize the tyrosine kinase surface receptors and activate the Ras ((Lowenstein *et al.*, 1992) pathway (observations in both *in vitro* and *in vivo* studies). [Note: Ras pathway is shown to be important in EMT]. MEK and Rac are downstream components of Ras that are shown to induce EMT in bladder carcinoma lines (Edme *et al.*, 2002). Both the mitogen activated protein kinase (MAPK) pathway and the PI3K pathway can induce tumourigenicity. EMT was shown to be dependent on the activation of the MAPK pathway, though the PI3K pathway was related to scatter and cell proliferation (reviewed in Theiry 2003). Activation of c-met (a proto-oncogene that encodes mesenchymal-epithelial transition factor) induces inositol 5'-phosphatase SHIP-1 which is important in branching morphogenesis. Overexpression of SHIP induces EMT in MDCK cells (Mancini *et al.*, 2002). Thus, SHIP-1 is needed for EMT even though MAPK was activated. Chicken tumour virus no.10 regulator of kinase (Crk) is an adaptor protein that can also induce EMT in MDCK cells by being overexpressed in the absence of hepatocyte growth factor (HGF) (Lamorte *et al.*, 2002).

Transforming growth factor β (TGFβ) is involved in EMT both during embryogenesis and tumourigenesis. TGFβ has three isoforms in humans (TGFβ1, TGFβ2, and TGFβ3); all three can regulate EMT. TGFβ was first shown to induce EMT in mammary epithelial cells (NMuMG cells) (Miettinen *et al.*, 1994) where the epithelial cells were shown to differentiate into fibroblastic phenotype by the addition of TGFβ1. TGFβ along with extracellular matrix protein Laminin-5 (Ln-5) can also induce EMT (Giannelli *et al.*, 2005). TGFβ is known to be an inducer of EMT in association with the Ras pathway. In mouse, if the Ras pathway is active, the mammary epithelial cells undergo complete EMT (Janda *et al.*, 2002). In MDCK cells, EMT is induced by TGFβ and Ras and and also by Smad3, which is involved in growth arrest, which becomes downregulated in these cells (Nicolas *et al.*, 2003). Re-expression of Smad3 cannot reverse EMT in MDCK cells (Nicolas *et al.*, 2003). TGFβ signaling is also related to the activation of the proto-oncogene Src (Kim and Joo, 2002). Src is also involved in formation of a mesenchymal phenotype in colon cancers, though it cannot actually cause carcinoma (Shimizu *et al.*, 2002).

Cytokine Interleukin 1β (IL1β) is another growth factor regulating EMT and this was first shown in mesothelial cells, LP-9 cells which undergo EMT in response to IL1β and this was marked by changes in expression of EMT markers (*Vimentin, Twist, Snail, N-cadherin* and *cytokeratin*) (Masoud *et al.*, 2009). Two key hallmarks of cells undergoing EMT are reduction in levels of E-cadherin and nuclear localization of the oncogene, *β-catenin* (reviewed in Thiery 2002).

1.5.2 EMT and development

EMT is a fundamental process in normal human development. The earliest event of EMT in development is during grastulation, when the mesenchymal cells and mesoderm are formed. Formation of placenta, neural crest and heart valve arise through EMT (reviewed in Polyak and Weinberg , 2009). The changes associated with EMT enable the cells of a developing embryo to respond

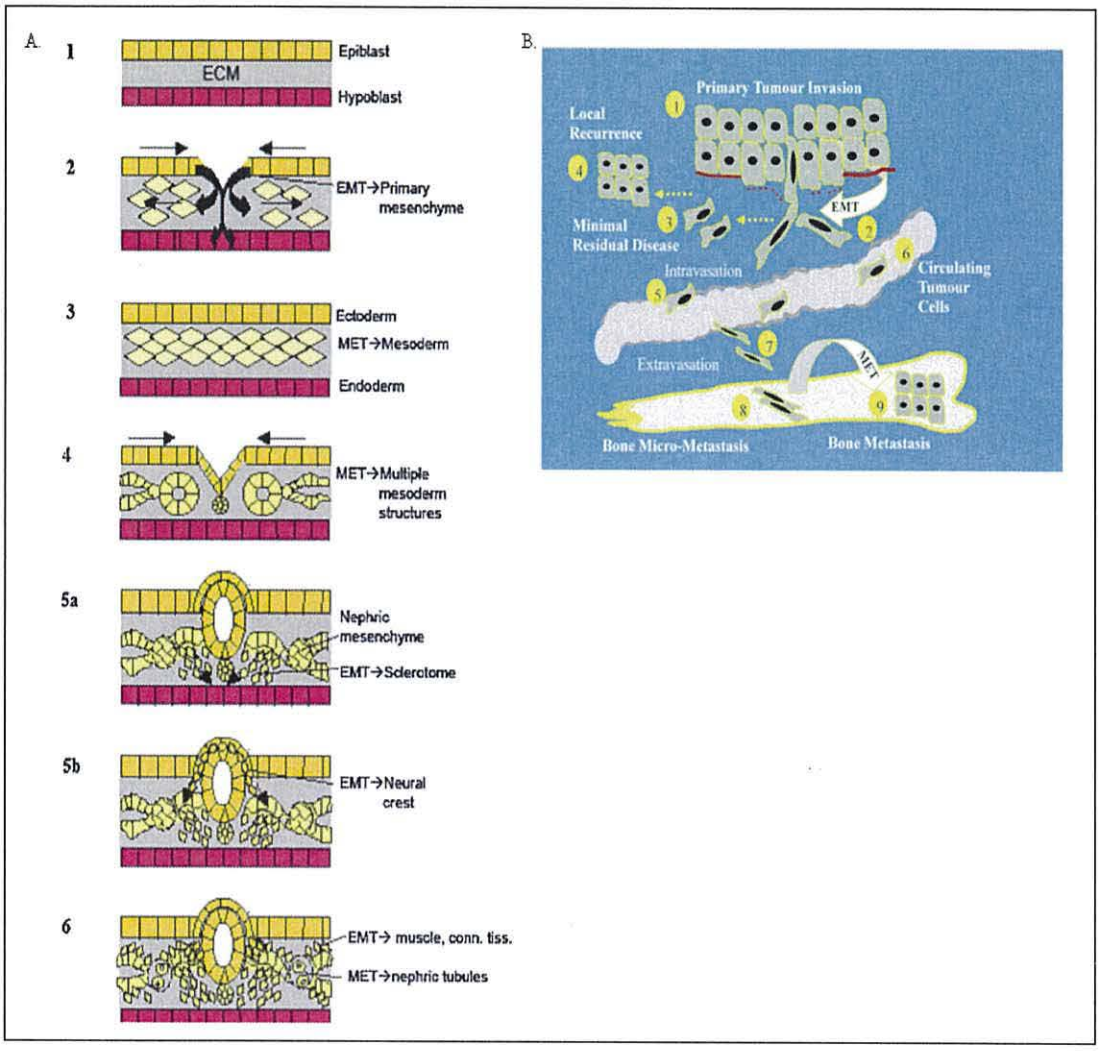
to changes in the environment. Developmental stages involving EMT can be described as follows:

- During EMT in development, upper epiblast epithelium forms the primary mesenchyme. This leads to three layered blastocyst with ectoderm, mesoderm (primary mesenchyme) and the endoderm (Figure 1.4A steps 2 and 3).
- The first somite is formed by MET. Lateral mesenchyme forms the mesodermal epithelia of the body wall (Figure 1.4A step 4).
- Another EMT event is formed which makes the neural crest cells which then forms the nervous system (Figure 1.4A step 5) (Huang and Saint-Jeannet, 2004; Newgreen and McKeown, 2005).
- More steps of EMT then forms dorsal parts of the somite to form muscles and skin (Figure 1.4A step 6), peripheral neurons and glia, and melanocytes (reviewed in Hugo *et al.*, 2007).

EMT also occurs in ovaries of women. The mammalian ovary is covered with ovarian surface epitheliums (OSE) from which ovarian carcinomas arise. Normal OSE in the ovary undergoes EMT to maintain ovarian homeostasis (Auersperg *et al.*, 2001).

1.5.3 EMT and cancer

EMT is seen in many forms of cancers such as colon cancer, prostate cancer, breast cancer and lung cancer. Most solid tumours arise from the epithelia, and the loss of epithelial-cell markers (e.g. E-cadherin and gamma catenin) is related to disease progression and tumour metastasis (Figure 1.4 B). Cancer cells can de-differentiate through EMT and attain mesenchymal-cell characteristics (e.g. expression of *Vimentin*, *Fibronectin*). Mesenchymal-Epithelial Transition (MET) is a reversible process, whereby mesenchymal tumor cells attain epithelial-like phenotype. MET enhances cell proliferation and growth of epithelial tumour cells at a secondary tumour site. Metastatic and invasive carcinoma is associated with loss of epithelial cell polarity and differen-



Reference: Hugo *et al.*, 2007, review

Figure 1.4

EMT and MET events in development and cancer. A) EMT and MET in early development. B) EMT and MET progression during carcinogenesis. Cancer cells undergo EMT escaping the environment (1, 2), create new malignancy (4). Mesenchymal cells intravasate (5), remain in circulation (6), extravasate (7). Persist as micrometastasis in bone marrow (8) and revert to epithelial cells via MET (9).

tiation. Data suggests that EMT has a role in carcinoma in micro metastatic tumour cells in bone marrow and lymph nodes (Pierga *et al.*, 2003). Sarcomatoid carcinoma may also have a possible EMT and MET balance although this has to be further explored.

During tumour progression, non-invasive benign tumour cells can gain the potential to become invasive and infiltrate surrounding tissues and migrate to distant sites leading to secondary tumours. Many EMT regulators can enhance the formation of a tumour (Thiery, 2002), eg. Expression of Snail1 increases the virulence of breast tumors, and high Snail1 expression relates to poor survival rates in human breast cancer (Moody *et al.*, 2005). Three transcriptional factors TWIST, GOOSECOID (homeobox transcriptional repressor), and *FOXC2* (transcription factor of the FOX family of forkhead helix-turn-helix DNA-binding proteins), that regulate developmental EMT also have important functions in enhancing metastasis. Twist expression is shown to be sufficient to induce *in vitro* EMT in breast cells (Yang *et al.*, 2004). Twist and Goosecoid regulate *FOXC2* that regulates EMT and development of organs (Carlsson and Mahlapuu, 2002). *FOXC2* also up-regulates mesenchymal gene transcription, rather than intiating EMT through E-cadherin repression.

Thus, we have seen that EMT is a fundamental process not only associated with embryo development and tissue regeneration but also in other forms of disease including various forms of cancer where, the invasion and metastasis is driven by the transition from a stable epithelial phenotype to a migratory, mesenchymal phenotype (Thiery and Sleeman, 2006). Recently, a possible link between cells undergoing EMT and cells with properties of cancer stem cells (CSCs) has been described in breast epithelial cells where breast cells from normal mouse and human mammary tissue were cultured and were induced to undergo an EMT, the cells acquired characteristics of stem cells (Mani *et al.*, 2008). The fundamentals of stem cells are described in section 1.6

1.6 Stem cells:

Stem cells have distinct potential to give rise to the many different cell types in the body during both embryogenesis and tissue specific growth. In a blastocyst (3- to 5-day-old embryo), the stem cells in the inner walls give rise to the entire body of the organism along with other specialized cell types and organs. The special properties of stem cells are also required for tissue repair to enable renewal of cells during an individual's lifetime. For example, as discussed previously, in the gut, adult stem cells divide regularly to replace the worn out/ damaged cells. Here, we will discuss the different types of stem cells and the factors required for the promotion and maintenance of stem cells.

1.6.1 Types of stem cells

Predominantly, there are two categories of stem cells, embryonic stem cells and somatic or adult stem cells. A new type of stem cell, called induced pluripotent stem cells (iPSCs), has also been characterized recently.

- Embryonic stem cells are the stem cells derived from four or five day old embryos (blastocyst) that develop from a fertilized egg. [Note: the blastocyst has the trophoblast which is the layer of cells that surrounds the blastocoel, a hollow cavity inside the blastocyst and the inner cell mass which is a group of cells that develop into the embryo]. Embryonic stem cells were first derived from early mouse embryos in 1981 (Evans and Kaufman) and first the human embryonic stem cells were derived and grown in 1998 (Thomson *et al.* 1998).

- An adult stem cell is an undifferentiated cell among differentiated cells in a tissue or an organ. These cells have the ability to renew themselves and differentiate into the specialized cells of that organ or tissue. Thus, these adult stem cells maintain and repair the tissue they are associated with. The area where they are found is called a "stem cell niche" which is

important in providing environmental cues and signals required for the maintenance of stem cells (reviewed in Scadden 2006). Both embryonic and adult stem cells each have advantages and disadvantages for their use in cell-based regenerative therapies (Hmadcha *et al.*, 2009). One major difference between these two types of stem cells is that, embryonic stem cells are pluripotent (ability to give rise to all cell types of the body) and can differentiate to form all cell types of the body; whereas, differentiation of adult stem cells is limited to the cell types of the same tissue or organ (NIH stem cell website). Also, embryonic stem cells are easily grown in culture compared to adult stem cells which are rare in mature tissues (NIH stem cell website). Some organs have both embryonic and adult stem cells. e.g. the bone marrow contains two kinds of stem cells, hematopoietic stem cells that form all the types of blood cells in the body; and bone marrow stromal stem cells (mesenchymal stem cells) that can give rise to bone, cartilage and fat cells that support the formation of blood, and fibrous connective tissue (Rubin and Strayer, 2007).

- Induced pluripotent stem cells (iPSCs) are derived from somatic adult cells and are obtained by genetic reprogramming of a fibroblast cell to an embryonic stem cell–like cell. This is done by forced expression of four genes, *Oct-3/4, Sox2*, *c-myc*, and *Klf4*, all of which are factors important for maintaining embryonic stem cells (Takahashi &Yamanaka, 2006). Mouse iPSCs were first reported in 2006 (Takahashi and Yamanaka) as having characteristics of pluripotent stem cells and expressing stem cell markers. Human iPSCs were first reported in 2007 (Takahashi *et al.*, 2007) as cells which expressed stem cell markers and which could also generate cells characteristic of all three germ layers. iPSCs are already in use for drug development and disease treatment (Wu *et al.*, 2008; Shizuru *et al.*, 2005; Rubin 2008; Rossi *et al.*, 2008; Nelson *et al.*, 2009). Recently, viruses have been used to introduce reprogramming factors into adult cells; however, this use of virus in treatment might itself be a cause of cancers. Thus, non-viral delivery strategies are currently being worked

on e.g. recently, virus free ESCs are made for Parkinson patients (Soldner *et al.,* 2009). Mouse iPS cells were also generated without using viral vectors (Okita *et al.*, 2008).

1.6.2 Properties of stem cells:

Stem cells can divide to generate two daughter cells, one rapidly cycling more specialized differentiated cell type and one stem cell that replace the parent stem cells (reviewed in Barker *et al.*, 2008). The former undergoes limited cell division and then terminally differentiates. The two important characteristics of stem cells are as follows:

- Stem cells are not specialized cells and can renew themselves by cell division.
- Stem cells can differentiate and induce tissue- or organ-specific cells with specialized functions

1.6.3 Leukemia inhibitory factor (LIF) and stem cells

Not much is known about the signaling pathways that regulate the human embryonic stem cells. LIF is a molecule involved in the maintenance of self-renewal and pluripotency of mouse ES cells (Smith *et al.*, 1988; Williams *et al.*, 1988). LIF was found to maintain mouse ES cells in the absence of a feeder layer. However, LIF was not the only factor to maintain stem cell identity in mouse as it was shown that null mutants deficient in LIF/ Stat3 signaling did not show any defect in establishing the stem cell niche (Smith *et al.*, 2001). Also, LIF was shown to be incapable of maintaining human ES cell identity (Thomson *et al.*, 1998; Sato *et al.*, 2001). It was therefore suggested that other signaling pathways might also be involved in stem cell maintenance, such as FGF4, TGFβ, and Wnt. Some of these pathways are discussed below in terms of stem cell maintenance.

1.6.4 NANOG and maintenance of stem cells

One of the fundamental downstream effectors, required for promotion of pluripotency in both mouse and human ES cells, is the homeodomain transcription factor NANOG (Chambers *et al*, 2003; Mitsui, Tokuzawa *et al*, 2003; Hart AH, Hartley *et al*, 2004, Pan and Thompson, 2007). *Nanog* is involved in self-renewal and maintaining pluripotency of embryonic stem cells (Mitsui *et al*., 2003; Chambers *et al*., 2003). NANOG has also been shown to be expressed in tissue specific stem cells, such as the pancreas (Koblas *et al*, 2008), germ cell tumours (reviewed in A.T. Clark, 2007) and some cancers, such as prostate (Kasper 2006), breast cancer (Ezeha *et al*. 2007) and Ewings sarcoma (Suva *et al*, 2009).

In human, NANOG is a 305 amino acid protein and is primarily located in the nucleus of a cell. It works along with two other transcription factors OCT4 and SOX2 to maintain pluripotency. [Note: Key transcription factors for maintaining murine stemness are Nanog, Oct3/4 and Sox2 (Nichols *et al*., 1998; Niwa *et al*., 2000; Boiani *et al*., 2005)]. Over- expression of *NANOG* results in increased cell growth and transformation of cells (Piestun *et al*., 2006). NANOG was also shown to be over-expressed in cervical cancers thereby aiding their progression (Ye *et al*., 2008) and also in oral cancer along with OCT4 (Chiou *et al*., 2008). NANOG has also been suggested as a marker for testicular cancer (Hoei-Hansen *et al*., 2005).

Deletion of *Nanog* leads to embryonic lethality in mice (Mitsui *et al*., 2003) and its' expression is related to autonomous self renewal of embryonic stem cells (Chambers *et al*., 2003). It is needed for germ cell formation and construction of the inner cell mass (Chambers *et al*., 2007). Cells are prone to differentiation on *Nanog* downregulation but in the permanent absence of *Nanog*, ES cells can self-renew (Chambers *et al*., 2007). *Nanog* expression is not continuous in the embryonic pluripotent cells since it is present in the blastocyst during formation of pluripotent cells, downregulated during implantation (Chambers *et al*., 2003) and expressed again in the posterior region (Hart *et al*.,

2002). Thus, Nanog is primarily involved in the formation of the inner cell mass and germ cell states. Nanog also stabilizes embryonic stem cells in culture by resisting or altering gene expression states (Chambers *et al.*, 2007).

1.6.5 Intestinal stem cells

The epithelial cell lining of the gut proliferates very rapidly, representing one of the most rapidly renewing tissues in the human body; and stem cells are responsible for this phenomenon. The differentiated cells are renewed due to continuous supply of multipotent progenitor cells that originate from stem cells of the intestinal crypts. Stem cells are responsible for the proliferation of crypt cells in the intestine (Bjerknes *et al.*, 2006; Marshman *et al.*, 2002). The epithelium in the mouse small intestine renews every five days (reviewed in Laurens, *et al.*, 2009). The stem cells at the bottom of the crypt enhance vigorous proliferation. The TA (transit- amplifying) cells or the 'rapidly cycling daughter cells' are easily distinguishable at the crypt and divide every 12-15 hour and this leads to 300 cells each day, per crypt (Marshman *et al.*, 2002). These TA cells differentiate in an irreversible manner at the crypt-villus junction, and can divide up to 6 rounds of differentiation while going up the villi (Marshman *et al.*, 2002). 3 days after the terminal differentiation, the epithelial cells undergo apoptosis at the top of villi (reviewed in Laurens *et al.*, 2008). It is now known that every crypt has 4-6 stem cells (Potten *et al.*, 1990). Intestinal stem cells (ISC) play an important role in the rapid expansion of the gut during development and also during tissue regeneration on injury, surgery or cancer. The stem cells normally undergo apoptosis in response to toxic stimuli thereby reducing the chances of acquiring mutation that could result in a tumour. It has been demonstrated that a single mutated stem cell can give rise to colorectal tumours (Simon *et al.*, 2000). Colonic stem cells are defined by a number of markers, of which LGR5 (leucine-rich repeat-containing G protein coupled receptor 5)/ GPR49 is one. This marker is used to define the location of intestinal stem cells at the crypt base of crypts (Barker *et al.*, 2007; Sato *et al.*, 2009).

1.6.6 A) Intestinal stem cell microenvironment

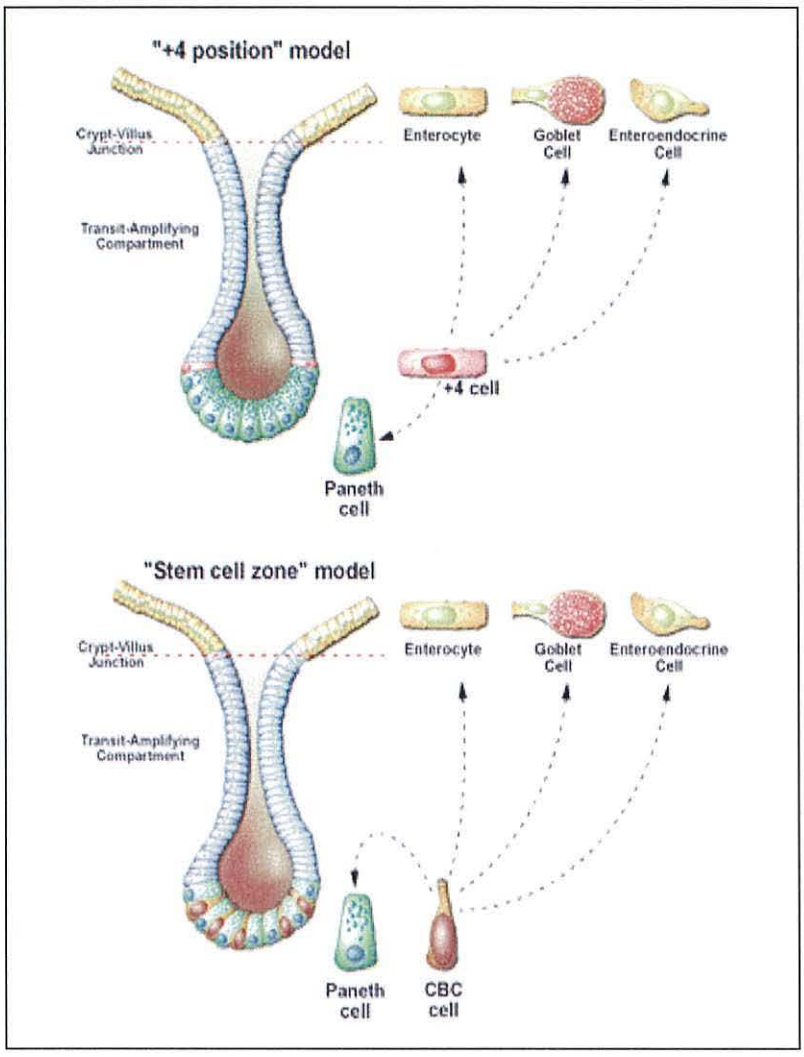
The tightly junctioned intestinal fibroblasts are responsible for the formation of the intestinal epithelial niche (Mills *et al.*, 2001). Here, the cells secrete growth factors and cytokines to enable the cells to proliferate (Powell *et al.*, 1999). In the crypt, BMP acts as a negative regulator of crypts and is active in the villus mesenchyme (Haramis *et al.*, 2004; Hardwick *et al.*, 2004). Intestinal epithelial cells express Sonic hedgehog (Shh) and Indian hedgehog (Ihh) ligands, inhibition of which leads to reduction in villi size and highly proliferative epithelium (Ramalho-Santos *et al.*, 2000).

1.6.6 B) Two models for intestinal stem cells

The two models of the intestinal stem cells are as follows:

i) +4 model (Classical model):

Understanding from previous literature suggests that the crypts in the small intestine are bound by Paneth cells from below and behave like a tube of proliferating cells. The +4 model was suggested in late 1950s (Quastler and Sherman, 1959); according to which the intestinal stem cells are based at the +4 position relative to crypt bottom and the Paneth cells occupy the first 3 positions (Cairnie *et al.* 1965). Experimental support for this model was suggested in 1974 (Potten *et al.*, 1974) where label-retaining cells were observed at the +4 position in the crypt. Recently, this model was modified when the stem cells found among the ring of cells immediately above the Paneth cells, at position 4, where they occupy a stem cell microenvironment (Potten *et al.*, 2002) (Figure 1.5 top panel). Potten and Roberts (1974) observed that the +4 cells are radiation sensitive and this sensitivity protects the stem cells from genetic damage/ alteration so that they do not acquire longer term changes. In this model, the first two to three generations of TA cells replace the damaged stem cells, enabling the stem cells to regain their stem cell property and occupy the +4 position again. Due to lack of knowledge of the cellular nature of these +4 cells, this model had constraints.



Reference: Barker *et al.*, 2008

Figure 1.5

The two models of intestinal stem cells: +4 position model (top), stem cell zone (bottom). In +4 position model, it was assumed that that the crypt base consists mainly of terminally differentiated Paneth cells and the stem cells were located just above the Paneth cells at the +4 position. Stem cell zone is a more recent model stating that small, undifferentiated, cycling cells (crypt base columnar cells) with the Paneth cells are the intestinal stem cells.

Apart from this, small and undifferentiated cells were identified hidden between the Paneth cells which were referred to as the crypt base columnar (CBC) cells (Cheng *et al.*, 1974a, b). This gave rise to the second model of the intestinal stem cells.

ii) Stem cell zone model:

This model is based on Crypt Base Columnar (CBC) cells which are small and undifferentiated cells found between the Paneth cells (Cheng and Leblond 1974a, b; Bjerknes and Cheng 1981a, b, 1999). In this model, the CBC cells are considered to be the true stem cells and the short lived multipotent ''mix'' cells represent the direct derivative of the CBC cells. The common 'origin of differentiation' sites directly above the Paneth cells are occupied by the Mix cells. The maturing Paneth cells migrate downwards where the oldest cells are at the crypt base. The cells other than the Paneth cells move up the crypt-villi axis (Figure 1.5 bottom panel). Due to lack of distinct stem cell marker at the time, identification of these cells was not possible and this model suffered a setback. An attempt to mark these stem cells was tried using Musashi-1 and β-integrin, but, since they lacked specificity, the identification of stem cells was not possible (Kayahara *et al.*, 2003, Spangrude *et al.*, 1998). Some specific markers of +4 cells are phosphor-PTEN and phosphor-AKT, Dcamkl1, Sox4 and SFRP5 (He *et al.*, 2004; Giannakis *et al.*, 2006). But, their positional information remains inadequate.

Recently, the CRC stem cell marker LGR5, shows the expression of stem cells is restricted to CBC cells (Sato *et al.*, 2009). Another stem cell marker, *BMI 1*, that encodes a chromatin remodeling protein shows the expression of the intestinal stem cells at the +4 position (Sangiorgi and Capecchi 2008). Thus, it might be possible that there are different activation states of the stem cells in the intestinal tract. However, this debate came to an end in 2009, with the conclusion that the CBC cells within the stem cell zone at the crypt base is the actual site of the intestinal epithelial stem cells (van der Flier *et al.*, 2009) where induced

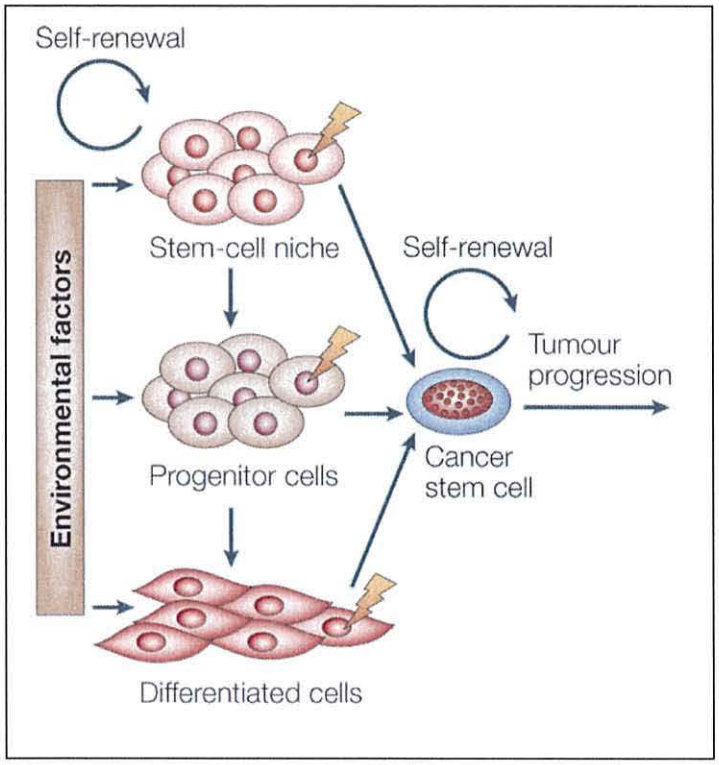
deletion of the transcription factor Achaete Scute-Like 2 (ASCL2) leads to removal of LGR5 stem cells because ASCL2 binds to the LGR5 promoter.

1.6.7 Cancer stem cells

It is well known that both tumour cells and normal stem cells can proliferate extensively and can lead to abnormal tissues. Both have a combination of cells with multiple phenotypes, proliferative capacities and different degrees of differentiation (Fidler *et al.*, 1997; Fidler *et al.*, 1982; Heppner *et al.*, 1984; Nowell *et al.*, 1986). Most tumours contain a heterogenous population of cancer cells with both tumourigenic and non-tumourigenic cancer cells. Cancer stem cells (CSCs) are cancer cells found in tumours or hematological cancers, which have similar properties to normal stem cells, i.e ability to develop into all the cell types in a cancer sample and to self renew (Reya *et al.*, 2001). CSCs are therefore tumourigenic (tumour-forming) and can produce tumours through self-renewal (Clarke *et al.*, 2005, 2006) and differentiation into various cell types. Such cells reside in a tumour as a population which can cause metastasis to give rise to a new tumour at a secondary site in the body. Thus, the CSCs are the rare cells in a tumour that are 'immortal' and produce 'normal' cancer cells those can divide many times to form a tumour, and also can give rise to new cancer stem cells.

It is already known that cancer results from various genetic mutations in a single cell may be over a period of many years (Fearon *et al.*, 1990). The stem cells are the only the long-lived cells in many tissues. Thus, it is stem cells in which these early mutations might have been accumulated. Mutations in stem cells along with/ or mutation in the progenitor cells can lead to the formation of cancer stem cells (reviewed in Bjerkvig *et al.*, 2005) (Figure 1.6). It might be considered that a tumour can be like an abnormal organ whose activity is induced by a cancer cell that acquired the capacity to proliferate indefinitely through mutation (Morrison *et al.*, 1997; Kummermehr *et al.*, 1997).

According to the stochastic cancer stem cell model, all tumour cells can form the entire tumour and the growth of the tumour depends on the genetic events, proliferation and cell death. But, according to the new model for cancer



Clarke *et al.*, 2005

Figure 1.6

Factors giving rise to cancer stem cells. Environmental factors may give rise to the progenitor, differentiated or stem cell niche. These can further lead to the formation of cancer stem cells and hence initiate tumour progression.

stem cells, only a subset of cancer cells, which are long-lived cancer stem cell, can sustain the tumour growth *in vivo* and form the entire tumour; and the short-lived differentiated cells in a tumour cannot maintain this tumour growth. This tumour growth depends on the stem cell renewal mechanisms. The origin of cancer stem cells is still not clear. But, it is widely accepted that they might result from specific mutations in normal or early stem cell progenitors. Also, there is a possibility that they are obtained from differentiated cells (Bjerkvig *et al.*, 2005).

The first evidence for CSCs was in 1997 where a sub-population of leukaemic cells was isolated and enriched, which expressed a specific surface marker CD34, not CD38 marker. Thus, it was established that the CD34+/CD38- subpopulation is capable of initiating tumors in NOD/SCID (nonobese diabetic/severe combined immunodeficient) mice (Bonnet and Dick, 1997). [Note: NOD/SCID is a mouse model, efficient for human cells to engraft and also to proliferate and differentiate (Ito *et al.*, 2002)]. Furthermore, Spangrude and co-workers (1998) identified the hematopoetic stem cells from bone marrow (Spangrude *et al.*, 1998). Since then there has been evidence of cancer stem cells in many tumours including breast (Al-Hajj *et al.* 2002), colon (O'Brien *et al.*, 200)], brain [Singh *et al.*, 2003], ovary (Zhang *et al.*, 2008), pancreas (Li *et al.*, 2007), prostate (Maitland *et al.*, 2008, Lang *et al.*, 2009). Identification of cancer stem cells in solid tumours was done by several other groups in later years (Singh *et al.*, 2004; Dalerba *et al.*, 2007a). One of the most fascinating works done in this field was by Visvader and co-workers when they grew an entire mammary gland from single isolated stem cells (Visvader *et al.*, 2006). Prior to this, single stem

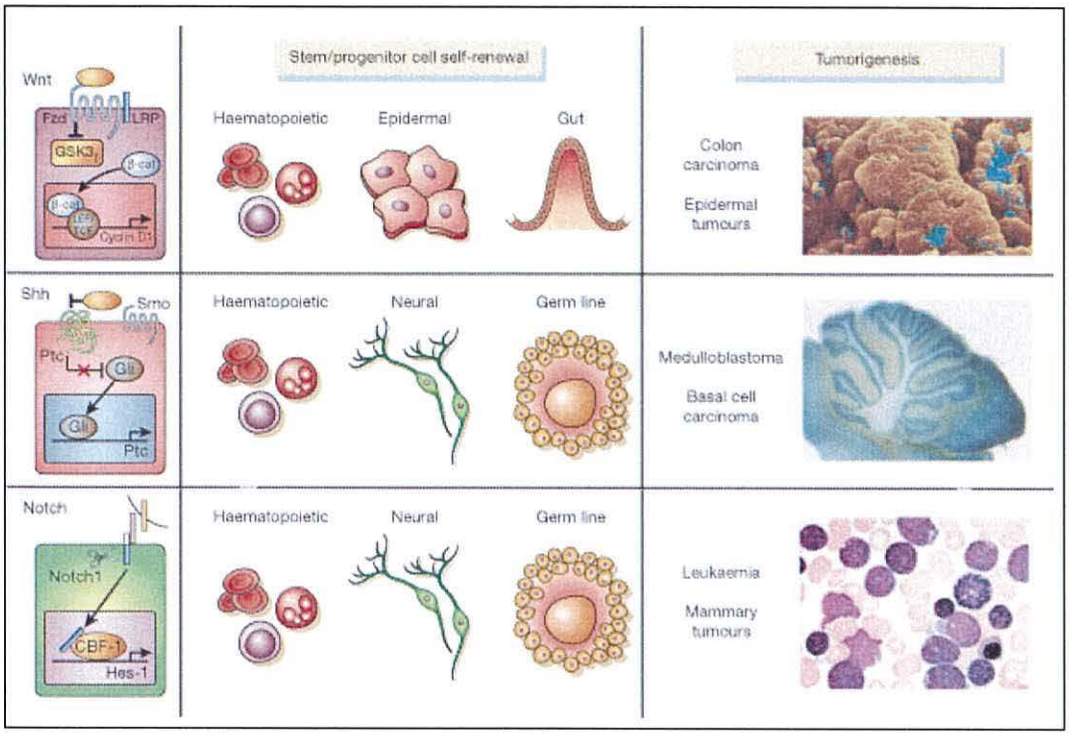
cells were shown to give rise to epidermis, hair follicles and sebaceous gland (Blanpain *et al.*, 2004; Claudinot *et al.*, 2005). Genetic marking of the stem cells to visualize the modified stem cells has also been explored. One such example is the expression of a progesterone-responsive Cre recombinase enzyme in cells of

bulge hair follicle regions. This was done by a transgenic keratin-15 promoter (Morris *et al.*, 2004) in mouse.

1.6.8 Regulatory pathways of stem cells and cancer cells

Since, both stem cells and cancer stem cells can self renew, one might guess that there are similarities in mechanisms of self-renewal in both ''normal'' stem cells and ''cancer'' stem cells. Evidence shows that many of these pathways that are cancer related may also regulate normal stem cells. The transformation of normal stem cells to cancer stem cells are controlled by various signaling pathways which includes Bmi-1 (active in cancer stem cells in brain tumours) (Dontu *et al.*, 2004), Notch pathway (demonstrated in neural and mammary stem cells (Dontu *et al.*, 2004), Sonic hedgehog and the Wnt pathway (Figure 1.7). Cell death also has a role in regulating homeostasis in hematopoietic stem cells (HSCs) (Domen, *et al.*, 1998, 2000). Shh, Notch and Wnt are also associated with oncogenes and thus may regulate stem cell self-renewal in CSCs (Taipale *et al.*, 2001). The role of these pathways is outlined below:

A) Notch pathway in stem cell maintenance: Notch signaling is involved in both normal embryonic development (Gridley *et al.*, 2003) and also in the regulation of self-renewal of stem cells along with other pathways such as Wnt and hedgehog pathways (Molofsky *et al.*, 2004). The Notch ligand, Notch-1, has a critical role in regeneration of the HSCs from the hematogenic endothelial cells during the early embyogenesis (Dzierzak *et al.*, 2003; Kumano *et al.*, 2003; Hadland *et al.*, 2004., Robert-Moreno *et al.*, 2004). When the Notch pathway is activated in HSCs, it increases the progenitor activity *in vivo* and *in vitro* indicating its role in maintaining multipotency (Varnum-Finney *et al.*, 2000). In genetically modified mice, the osteo-blastic cells were shown to produce high levels of Jagged 1 (Notch ligand) and to increase the numbers of HSCs on activation of Notch-1, *in vivo* (Calvi *et al.*, 2003). Notch receptors are also



Reference: Reya *et al.*, 2001

Figure 1.7

Signalling pathways regulating self-renewal mechanisms during normal stem cell development and transformation. Shh and Notch pathways contribute to the self-renewal of stem cells and/or progenitors in organs such as haematopoietic and nervous systems. These pathways can also lead to oncogenesis when dysregulated and can be associated with a human tumours such as colon carcinoma and epidermal tumours (Wnt), medulloblastoma and basal cell carcinoma (Shh), and T-cell leukaemias (Notch).

the gut (Schroder *et al.*, 2002). Active Notch signalling can also be seen in the muscle stem cells where activation of the Notch pathway can reinstate the regenerative potential in the aged mouse muscles (Conboy *et al.*, 2002; Conboy *et al.*, 2005).

B) Shh in stem cell regulation: The Shh pathway increases mesenchymal cell proliferation and regulates the differentiation of smooth muscle progenitor cells in mouse kidney (Yu *et al.*, 2002). Populations highly enriched for human HSCs exhibit increased response of self-renewal following Shh stimulation *in vitro* (Bhardwaj *et al.*, 2001). In neural cells, Shh signaling can regulate the number of stem cells and can also control the proliferation of early neuron production (Palma and Ruiz, 200; Palma *et al.*, 2005). Shh also has an important role in regulating epidermal stem cells of the skin (Kameda *et al.*, 2001) by maintaining a stem cell population for the proliferation of human putative epidermal stem cells. Studies also show that Shh is involved in the proliferation and cell-fate specification of mesenchymal stem cells (Kondo *et al.*, 2005).

C) Wnt in regulating stem cells: When ES cells self renew and maintain their pluripotency to differentiate into other cell types, many signal transduction pathways are involved, β-catenin/ Wnt pathway being one such pathway. Unknown factors in conditioned medium or Wnt activation by 6-bromoindirubin-3'-oxime (BIO), a GSK3β inhibitor, can also maintain the undifferentiated state of hESCs (Sato *et al.*, 2004). The role of β-catenin in stem cell maintenance and early differentiation of embryonic stem cells was studied and the loss of β-catenin in ES cells was shown to alter the expression of stem cell markers (Anton *et al.*, 2007). It was also concluded that the effect of Wnt signalling on ES cells varies according to the time-point of treatment. Despite the above mentioned evidence suggesting a role for β-catenin in stem cell maintenance, the actual mechanism is still not clear. Wnt3a conditioned medium, which is an inhibitor of the GSK3β, is shown to maintain murine and human stem cells (Sato *et al.*, 2004). Wnt genes were shown to inhibit differentiation by upregulating Stat3 in mouse (Hao *et al.*, 2006). However, a different study showed that Wnt3a conditioned medium had no effect on Stat3 phosphorylation (Ogawa *et al.*,

mechanism is still not clear. Wnt3a conditioned medium, which is an inhibitor of the GSK3β, is shown to maintain murine and human stem cells (Sato *et al.*, 2004). Wnt genes were shown to inhibit differentiation by upregulating Stat3 in mouse (Hao *et al.*, 2006). However, a different study showed that Wnt3a conditioned medium had no effect on Stat3 phosphorylation (Ogawa *et al.*, 2006). It was also suggested that there is no direct cross-reactivity between the Wnt and Lif pathways (Ogawa *et al.*, 2006). Wnt3a has been shown to maintain the self renewal of hematopoietic cells (Reya 2002), however, recombinant purified Wnt3a does not stop the differentiation of mouse ES cells (Ogawa *et al.*, 2006; Singla *et al.*, 2006).

D) TGFβ in regulation of hESCs: The activation of TGFβ/ activin/ nodal through SMAD2/3 was shown to be required for the maintenance of the undifferentiated state of hESCs (Daylon *et al.*, 2005). It has been shown that in a mouse embryo that lacks *Smad* (*Smad* modulates the TGFβ ligands) expression, results in defect of epiblast proliferation and also delays the growth of the inner cell mass (Sirard *et al.*, 1998). Also, mouse which lack ALK-3BMP type IA receptor can decrease the cell proliferation in the epiblast confirming that BMP signaling pathway is also important in the maintenance of mouse ES cells (Mishina *et al.*, 2002). BMP-4 is also shown to be important for the maintenance of ES cell self-renewal (Qi *et al.*, 2004).

1.6.9 Colon cancer stem cell markers

CD133/ Prominin 1: CD133 is a 5-transmembrane domain molecule with a molecular weight of 120 kDa (Miraglia *et al.*, 1997) and has been shown to be located on the apical plasma membrane in embryonal epithelial cells (Weigmann *et al.*, 1997; Corbeil *et al.*, 1999) and the plasma membrane of CRC cells (Ricci-Vitiani, *et al.*, 2007; reviewed in Corbeil *et al.*, 2000).
Colon cancer cells were earlier demonstrated to have a subpopulation of $CD133^+$ cells in tumours. These were shown to initiate the tumour growth and were called colon-cancer-initiating cells or colon cancer stem cells (CSCs) (O'Brien *et al.*,

2007, Ricci-Vitiani *et al.*, 2007). On the other hand, $CD133^-$ cells showed none of these characteristics (Ricci-Vitiani *et al.*, 2007, Matilde *et al.*, 2007). CD133 is presently considered to be a potential marker of colon cancer stem cells though its expression is much wider (reviewed in Shmelkov *et al.*, 2008) such as in differentiated kidney and breast (reviewed in Montgomery and Shivdasani, 2009). A sub-population of tumour cells without CK20 (a differentiation marker) expression was shown to have strong CD133 expression *in situ* and the expression of CD133 in colon cancer is associated with low patient survivals (Horst *et al.*, 2008). Other than this evidence, CD133 localization *in situ* is not well studied.

LAMIN A: LAMIN A is a type V filamentous protein mostly localised to the inner nuclear membrane (Hutchison 2002). In 2008, LAMIN A was shown to be a critical biomarker for colon cancer stem cells (Weichert *et al.*, 2004). About one third of the patients with aggressive bowel cancer express LAMIN A. LAMIN A expression is altered in many forms of cancer such as lung tumours (Rowlands *et al.*, 1994), testicular germ cell tumours (Machiels *et al.*, 1997) and Hodgkins disease (Jansen *et al.*, 1997).

CD166: Activated leukocyte cell adhesion molecule (ALCAM) alternatively known as CD166, was shown to be over expressed in colorectal cancers where 58.6% of the cases were shown to have cytoplasmic expression of CD166 and 30.6% of the cases showed its membranous expression (Weichert *et al.*, 2004). It was previously shown to be expressed in melanoma (Léon *et al.*, 2000) and also in prostate cancer (Kristiansen *et al.*, 2003). CD166 is differentially expressed in normal and diseased colonic epithelium which makes it a good marker for colon cancer stem cells (Dalerba *et al.*, 2007b).

1.7 SW480 colorectal cancer cells transitioning between low and high density, an *in vitro* model to study CRC

It is not easy to study the events surrounding EMT during progression of cancer, partly due to the very transient nature of this process *in situ*. Thus, we used CRC cells from the cell line SW480, grown to low and high density, as a model for cells transitioning between the mesenchymal-like and epithelial-like cell states respectively (Brabletz *et al*, 2001; Davies *et al*, 2004). SW480 cells are human colorectal cancer cells derived from human colorectal adenocarcinoma. It is derived from lymph node metastasis (Leibovitz *et al*., 1976) and has single base pair mutation at codon 1338 of *APC* and a complete loss of the second allele. This mutation results in a stop codon and truncation at 1337 amino acids (Nishisho *et al*., 1991). In 2001, Brabletz and co-workers grew SW480 cells at low density with fibroblastoid phenotype with protruding lamellipodia. These cells resembled the mesenchyme-like cells at the invasive fronts of a colon tumour. With increasing density, these cells changed their phenotype to epithelial-like cells similar to those found at the central mass of a tumour. This phenotypic change was associated with β-catenin translocation from the nucleus to the cytoplasm and membranous co-localization of E-cadherin. There are several genes overexpressed at the invasive front of CRC, one of which is nuclear factor κB (NF-κB) (Horst *et al*., 2009). This dimeric transcription factor has role in immune response, cell growth control and survival of the cell. In our laboratory too, this transition of SW480 cells has been shown (Davies *et al*., 2004) where high amount of nuclear β-catenin were shown in low density SW480 cells whereas in high density SW480 cells, β-catenin was predominantly located at the cell membrane. As the SW480 cells are grown from low to high cell density, levels of E-cadherin expression was shown to increase and that the total level of APC expressed was decreased. Interaction of β-catenin and E-cadherin expression is also increased at high cell density and that between β-catenin and APC decreased. Thus, there has been an established series of evidence for the use of the human CRC cell line, SW480 grown at low and high density, as an *in vitro* model to study the CRC disease progression. An *in vitro* system that would mimic the changes in colorectal cancer cells during EMT would be of great importance to us in studying these changes further. Using this system, we show that the founding member of the T-box transcription factor family of genes, *BRACHYURY*, is differentially expressed in low density

(mesenchymal-like) cells. BRACHYURY, which we show to be regulated by β-catenin, acts directly to maintain levels of the pluripotency determinant, NANOG and to maintain a CSC phenotype.

1.8 Aims and overview

Cancer stem cells (CSC) are initiating cells in many types of cancer that have similar properties to normal embryonic stem cells. Colorectal tumours also have the ability to undergo an epithelial to mesenchymal transition (EMT)-like processes at the invasive front, rendering them capable of invasion and metastasis. Recently, a possible link between these two processes, that is, cells undergoing EMT and cells with stem cell like properties, has been identified in mammary cells induced to undergo EMT, where such cells acquired SC characteristics. It is of importance to understand the molecular components leading to promotion or maintenance of cells involved in these disease causing processes; however since it is difficult to observe EMT in cancer due to the transient nature of these transition events during tumourigenesis, we use the colorectal cancer cell line, SW480, at low and high cell density as a model for mesenchymal-like cells at the invasive front and epithelial-like cells in the central mass of a colorectal tumour, respectively.

The initial aim was to carry out a limited screen for a set of developmental genes which are differentially normally expressed early on in mesodermal development, to determine whether they are differentially expressed in low and high density SW480 cells. We considered those genes that were expressed in low and high density SW480 cells might be involved in mesenchymal transitions in these cells and as such might be important in the progression of colorectal tumours. Our aim was to identify such differentially expressed genes and then to determine their possible role in inducing transitions between mesenchymal and epithelial cell types in SW480 cells. One such gene that we isolated in this screen was *BRACHYURY*, a developmental mesoderm

inducing gene that is highly expressed in low density SW480 cells in contrast to expression levels in high density SW480 cells which are reduced to below 50%. In the first instance, our aim was to investigate whether *BRACHYURY* is involved in EMT; but if not, to determine its' possible role in processes such as cell migration, cell proliferation and/or other signalling pathways that might be relevant to the progression of colorectal tumours. It was our aim to then extend our finding in SW480 cells to address whether *BRACHYURY* was expressed similarly in other tumour cell lines.

Chapter 2 - Materials and Methods

2.1 Source of cell lines

Cell lines (SW480, HCT116, LoVo, HT29, SW626 and T84) were obtained from the European Collection of Cell Cultures (ECACC). NTERA-2 (clone D1) cells were a gift from P.W. Andrews (University of Sheffield).

2.2 Routine cell culture

SW480 and NTERA-2 cell lines were cultured in Dulbecco's modified Eagle's medium 1X (DMEM+GlutaMAX™-I) (Invitrogen, GIBCO 61965) supplemented with 10% (v/v) foetal bovine serum (FBS) (Invitrogen, GIBCO 10270-098). HCT116 and HT-29 were cultured in 1X McCoy's 5A medium+GlutaMAX™-I, (Invitrogen, GIBCO 36600) supplemented with 10% (v/v) FBS. LoVo cell were cultured in Ham's F-12 medium (Sigma, N6658) with 10% (v/v) FBS and T84 cells needed both DMEM with 10% (v/v) FBS and Ham's F-12 medium (Invitrogen, GIBCO N6658) with 10% (v/v) FBS in 1:1 ratio to grow. SW626 was maintained in Leibovitz L15 medium (Invitrogen, GIBCO 5520) with 10% (v/v) FBS. L-Glutamine (Invitrogen, GIBCO 25030, 200mM stock) is added if medium does not contain Glutamine. All cells were maintained in a 37°C incubator 5% CO_2 (apart from NTERA-2 cells which were maintained in 37°C incubator 10% CO_2 and SW626 cells which do not require CO_2). For low density cells, SW480 cells were grown for 24 hours and for high density, cells were grown for 24 hours after they are confluent. The description of the origin and mutations of the cell lines are mentioned in table 2.1.

2.3 Freezing cells

Cells were grown in T75 flasks, washed with PBS and trypsinized. Medium was added after the cells started to detach and after a centrifugation of 5 minutes at 2000 rpm, cells from each flask were resuspended in 1ml freezing medium (1%

DMSO (w/v), 90% (v/v) FBS). Tubes were then transferred to a tub containing isopropanaol and incubated at -80°C for overnight. Next day, cells were transferred and stored in liquid nitrogen with proper passage numbers.

Table 2.1 Description of the origin and mutations of the cell lines used

Cell line	**Age of patient (years)**	**Sex of patient**	**Site**	**Differentiation**	**APC mutation 1**	**APC mutation 2**	**β-catenin mutation**
SW480	50	male	colon	moderate	1338	-	-
HCT116	/	male	colon	/	-	-	codon 45
LoVo	56	male	colon, distant lymph node	/	1114	1430	-
HT29	43	female	colon	moderately well	853	-	-
T84	76	male	colon, lung metastasis	/	1488	-	-
NTERA-2	22	male	Testis, lung metastasis	/	/	/	/
SW626	46	female	Ovarian, colon metastasis	/	frameshift at codon 2941	inframe termination at codon 9	/

/ = data not available; - = no mutation

2.4 Phenol-Chloroform extraction of DNA

First of all, chloroform equilibrated with isoamyl alcohol (24:1) was made. To the experimental samples, equal volumes of phenol and chloroform equilibrated with isoamyl alcohol were added (25:24:1). The mixture was nicely mixed by hand, followed by a spin of 13000 rpm for 10-15 minutes. The supernatant was collected in a separate tube and a same volume of chloroform was added. The supernatant was again collected in another tube, followed by a 13000 rpm spin for 5 minutes. The DNA was now ethanol precipitated.

2.5 Ethanol precipitation of DNA

To the pellet, 2.5 to 3times 100% (v/v) ethanol were added and then 3M sodium acetate of $1/10^{th}$ the final volume was also added. This was left at -80°C for about 1hour followed by a 13000 rpm spin for 10 minutes. The pellets were washed with 70% (v/v) ethanol, spun for 5-10 minutes and air dried. Finally, the DNA was resuspended in an appropriate volume of TE.

2.6 Cell counting by Haemocytometer

Cells were grown as required, medium aspirated, washed with PBS and trypsinized. The trypsin (Sigma, T3924) was deactivated by adding some media to it. 10µl of this were added to the end of the coverslips on a Hemocytometer. Under a microscope, the number of cells was counted on both the grids of the Haemocytometer. For each grid, cells were counted on the four corner squares.

Then, the average was taken for both the grids, divided by 4 (since we counted four corner squares) and multiplied by 25 (since, there were 25 squares in total). This total value was multiplied by 10^4 to get the number of cells per ml. The number of cells per flask can now be counted depending on how many ml there was in the flask.

2.7 Total RNA isolation

Cells were grown in T75 flasks or in 6-well plates. Aspirate medium and wash the cells with PBS. 7.5ml of Trizol Reagent (Invitrogen, Cat#15596-026) was added to each T75 flask or 1ml Trizol to each well of 6-well plate. After pipetting up and down a few times, cells were transferred to separate tubes and incubated at room temperature for 5 minutes. To every 1ml, 0.2ml chloroform was added and shaken for 15 seconds in hand followed by 2-3 minutes incubation at room temperature. Cells were then centrifuged at 12,000g for 15 minutes at 4°C. The colourless upper

aqueous phase was transferred to a separate tube. To precipitate the RNA, 0.5 ml of isopropanol was added to per 1ml of this upper aqueous phase, incubated for 10 minutes at room temperature and then centrifuged at 12000g for 10 minutes at 4°C. The pellet was the collected and the RNA was washed with 1ml of 75% (v/v) ethanol and centrifuged at 7500g for 5 minutes at 4°C. The pellet was then dried on bench after which it was resuspended in 500µl RNAse free water and then incubated at 55-60°C for 10 minutes. Finally, the absorbance was measured using 1µl of RNA on a nanodrop (ND_1000). The RNA isolated was run on 1% agarose gel to check the quality of the RNA.

2.8 cDNA synthesis by MLV method

To a sterile PCR tube, 1µl of oligo d(T) (500µg/ml), 1µg of RNA and 1µl of dNTP mix (10mM) were added and made upto 12µl with distilled water. This was mixed gently by pipetting and then heated to 65°C for 5 minutes, followed by a brief chill on ice. To this, 4µl of 5X first strand buffer, 2µl 0.1M DTT and 1µl of RNaseOUT were added, mixed gently and incubated at 37°C for 2 minutes. 1µl of M-MLV Reverse Transcriptase was added to gently mixing and 1µl of sterile water was added to the –RT experimental control. After incubating at 37°C for 50 minutes, followed by a 70°C heating for 15 minutes to deactivate the reaction, the sample was diluted with 130µl sterile water. The cDNA can be stored at -80°C for long term or can be used immediately for PCR. The cDNA made can be tested on a 2-3% (w/v) agarose gel.

2.9 cDNA synthesis by SuperscriptIII

The Superscript III 1st Strand Synthesis Kit (Invitrogen, Cat #18080-051) was used. 1µg of total RNA per 20µl of cDNA was used. Initial primer/ RNA mix was made by adding 1µl oligo d(T) primers, 10mM dNTP mix, 1µg RNA and made upto 3µl with DEPC treated water. This was heated to 95°C for 5 minutes. The enzyme mix was made by adding 2µl 10XRT buffer, 4µl of 25mM MgCl2, 2µl of

0.1 M DTT, 1µl RNaseOUT and 1µl SuperscriptII in the mentioned order. This was incubated at 50°C for 50 minutes followed by 85°C for 5 minutes. 1µl of RNaseH was then added and incubated for 20 minutes at 37°C. The cDNA can now be stored at -20°C.

2.10 Real time PCR using Biomix

In a sterile PCR tube, 25µl of BioMix™ Red (Bioline-Cat#BIO-25005) was added along with 1µl each of the primers, 2µl of CDNA and 21µl of distilled water. The samples were run on a PCR machine on a suitable program. The initial heating was at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing for 30 seconds at suitable temperature for the primer set and the extension at 72°C for 30 seconds. The number of cycles was usually 30-35. Final elongation was done at 72°C for 10 minutes. The samples were then stored at 4°C. Refer table 2.2 for RT-PCR primer details.

Table 2.2: RT-PCR Primers

Primers	Sequence	Primer base region	Primer length (bp)	Tm (°C)
FGF4 F	CTG CGG CGG CTC TAC TGC	566	18	62.8
FGF4 R	GAGGAAGTGGGTGACCTTCAT GG	929	23	64.2
FGFR3 F	CTC ATC ACT CTG CGT GGC TGG	1305	21	63.7
FGFR3 R	GCC CGG TCC TTG TCA ATG C	1747	19	61.0
FGFR2 F	ATG ACA CCGATG GTG CGG	1051	18	58.2
FGFR2 R	CCA CGT GCTTGA TCCACTGG	1870	20	61.4
FGF8 F	GAG TCC GAG GAG CCG AGA C	430	19	63.1
FGF8 R	GCC AGG CAG CAC CTA TCG	914	18	60.5
GDF3 F	GGC CAG GCA GTC CAA TTT CA	91	20	59.4
GDF3 R	CAG CAC CTT GTG GCC ATG G	222	19	61.0
ActivinA F	GGA TGT ACC CAA CTC TCA G	93104	20	56.7
ActivinA R	CCT AAC GGA CAC TCG TCA	93328	18	56.0
ActivinB F	TCG GCT CGA CTC GGC TCG	133	18	62.8
ActivinB R	CAGGTAAAG CCA CAG GCTGGCC	456	22	65.8
BMP4 F	CTG CAG CTT CCC TGAGCC TTT C	324	22	64.0
BMP4 R	GAGGAG ATCGCCTCGTTCTCA GG	884	23	66.0
NODAL F	GCAGTA CAACGCCTATCGCTG TG	849	23	64.2

NODAL R	CTT GGC CAG ACT CCA CTG AGC	1271	21	63.7
BRACHYURY F	GGA GCC ACC GGT CCT CAC C	1341	19	65.3
BRACHYURY R	CTG GGA CAG CAC CGC TAC TGC	1851	21	65.7
TBX3 F	CAA GGT GCA CCT GGA GGC	1273	18	60.5
TBX3 R	CT CTT CGG CCA TTT CCA GTG	1839	20	59.4
TBX2 F	CCA GTT CCACAA GCTAGGCAC G	629	22	64.0
TBX2 R	CAC GTA GGT GCG GAA GGT GC	1044	20	63.5
FGF19 F	AGC TGC TTC CTG CGC ATC C	435	19	61.0
FGF19 R	CCA TGC TGT CGG TCT CCA GG	1056	20	63.5
FGFR1 F	CCA ACC GTG TGA CCA AAG TGG	919	21	61.8
FGFR1 R	GGT GAC CCT CCT TCA GCA GC	1160	20	63.5
NFkB F	GCT TGC AAA GAG GCA TGC C	1991	19	58.8
NFkB R	CAG CAG CAG CAA ACA TGG C	2481	19	58.8
SNAIL F	GCG GGA AGG CCT TCT CTA GG	624	20	63.5
SNAIL R	CCT CTT CCA GAG CCC AGG C	1064	19	63.1
Oct F	CTG GTG CCG TGA AGC TGG AG	410	20	63.5
Oct R	CGAGAGGAT TTT GAG GCT GCTG	848	22	62.1
E-cad F	ATT GTG GGC ATG AGC TGC	3762	18	56.0
E-cad R	ATC CTC AGC ATC AGT TTG C	4193	19	54.5

Note: Tm = melting temperature.
This was calculated using the formula 2[A+T] + 4[G+C], where, A= Adenine, T=Thymine, G=Guanine, C= Cytosine. Here, Tm was calculated using automated web based program (Oligoperfect designer, Invitrogen) based on the formula mentioned above (http://tools.invitrogen.com/content.cfm?pageid=9716).
The annealing temperature was given by the lowest Tm for the PCR program + 3°C.
Please refer to 'Gene names and Accession number' on page XI for the NCBI accession numbers of the genes. The gene nomenclature is followed according to MGI guideline (Eppig and Levan, 2009).

2.11 Quantitative Real time RCR

After the cDNA was made by the MLV method, 1.1X cDNA loading mix was made by adding 1.5µl cDNA and 2.5µl distilled water in a sterile PCR tube. 1.1X Sybr Master mix for each primer set was made by adding 10µl 2X Sybr green PCR mastermix (Applied Biosystem, Cat#4309155, Lot#0803295), 2µl of the forward primer, 2µl of the reverse primer and 2µl of distilled water in a sterile tube. 4µl of the cDNA loading mix was added to the wells of 96-well plates in triplicate followed by addition of 16µl Sybr Master Mix to the cDNA. The 96-well plates were sealed by optically clear sealing film. Plates were either left in 4°C for few hours or directly loaded to AB7900HT machine. The program used on this machine was SDS v2.3 (an Applied Biosystem software used to get the Ct values of the samples from

the AB7900HT machine) and the Ct values were collected and transferred to MS-excel sheets. The primers for the quantitative PCR are in table 2.3.

Note: The Ct (cycle threshold) value is the number of cycles required for the fluorescent signal to cross the threshold (which is sets by the user). δCt gives the difference of the experimental and control Cts. The levels of the Ct value are inversely proportional to the amount of target nucleic acid in the sample. This would suggest that the lower the Ct level the greater the amount of target nucleic acid in the sample (Wisconsin diagnostic laboratory webpage, http://www.wvdl.wisc.edu//PDF/WVDL.Info.PCR_Ct_Values.pdf)

Cts < 29 = abundant target nucleic acid in the sample
Cts of 30-37 = positive reactions with moderate amounts of target nucleic acid
Cts of 38-40 = weak reactions with minimal or no amounts

Table2.3: qRT-PCR Primers

Primers	Sequence	Base no.	Tm (°C)	Product length (bp)	Primer length (bp)
CRB3 F	tctcccttcattgctgtgtg	588	59.83	73	20
CRB3 R	taagcactgggtggatctga	660	59.24		20
PALS1 F	gacacgtcgcagaatgagaa	82	59.99	97	20
PALS1 R	gttgcttgatggactgctga	178	59.99		20
EVA1 F	gggacagatgctcggttaaa	242	60.07	142	20
EVA1 R	tgggttggaagggatctatg	383	59.74		20
ZEB1 F	gcacaaccaagtgcagaaga	1832	60.03	146	20
ZEB1 R	actttgcctggttcaggaga	1977	59.84		20
ZEB2 F	gacctggacgtgaaggaaaa	4743	60.09	107	20
ZEB2 R	ggcacttgcagaaacacaga	4849	60.03		20
NANOG F	ttccttcctccatggatctg	377	60.00	60	20
NANOG R	ctttgggactggtggaagaa	436	60.08		20
WNT 11F	catggagctctgcttgtgaa	1271	60.14	72	20
WNT 11R	gcttccaagtgaaggcaaag	1342	59.99		20
IL-2 F	gaatcccaaactcaccagga	211	59.90	65	20
IL-2 R	tcagttctgtggccttcttg	275	59.01		20
IL-15 F	gaagccaactgggtgaatgt	984	59.97	116	20
IL-15 R	actttgcaactggggtgaac	1099	60.01		20
Orthopedia F	ctctgtgcccttggttgttt	2273	60.15	147	20
Orthopedia R	gaccagcacatgcagaaaga	2419	59.99		20
CDX2 F	tcttggggctgatgaagaag	1884	60.33	70	20
CDX2 R	agaccaacaacccaaacagc	1953	60.01		20
NOTCH1 F	tttctggggaaagacactgc	8566	60.23	102	20

NOTCH1 R	t g t g t t g c t g g a g c a t c t t c	8667	59.99		20
NOTCH2 F	c c t g g a a g t c a t t g c c a a c t	367	60.11	127	20
NOTCH2 R	c t g c t t t c c c a a a c a g c t t c	493	59.99		20
NOTCH3 F	a t g c a g g a t a g c a a g g a g g a	5975	59.80	86	20
NOTCH3 R	a a g t g g t c c a a c a g c a g c t t	6060	59.91		20
NOTCH4 F	c c c a g g a a t c t g a g a t g g a a	4848	60.00	85	20
NOTCH4 R	c c a c a g c a a a c t g c t g a c a t	4932	59.91		20
FGFR1 F	c g a t g t g c a g a g c a t c a a c t	1119	60.02	137	20
FGFR1 R	t g c t g g t t a c g c a a g c a t a g	1255	60.04		20
FGFR2 F	a t g c t g c t g t c a g a c g a t t g	3928	60.02	125	20
FGFR2 R	t c c a g t t a g a c g t t g c g t t g	4052	59.90		20
FGFR3 F	a c c t g a a g a t g g g a g c c t t t	2999	60.07	145	20
FGFR3 R	t c c g t t g t a c c a g c c t t t t c	3143	60.11		20
TBX2 F	c g a t t g c c g c t a t a a g t t c c	764	59.70	85	20
TBX2 R	g t g g a t g t a c a t g c g t t t g g	848	59.85		20
14-3-3 σ F	t t g t g g c t g a g a a c t g g a c a	1150	60.44	104	20
14-3-3 σ R	t c a a t c t c g g t c t t g c a c t g	1253	59.98		20
FGF 8 F	g g a c a c c t t t g g a a g c a g a g	440	59.84	127	20
FGF 8 R	a a t c t c c g t g a a g a c g c a g t	566	59.87		20
BRACHYURY F	t t g t g g c t g a g a a c t g g a c a	800	59.85	142	20
BRACHYURY R	t c a a t c t c g g t c t t g c a c t g	941	60.18		20
TBX3 F	t t t t c a g a g c c g c t g t a g g t	3734	60.02	136	20
TBX3 R	c a c t g a t t t g a g g g c a a a g g	3869	60.63		20
SNAIL F	t t t c a g c c t c c t g t t t g g t g	1395	61.20	93	20
SNAIL R	c c g a c a a g t g a c a g c c a t t a	1487	59.72		20
E-cad F	c t g g a c a g g g a g g a t t t t g a	1766	60.04	135	20
E-cad R	g g c g t t g t c a t t c a c a t c a g	1900	60.12		20
β-catenin F	a c c t t t c c c a t c a t c g t g a g	927	59.93	90	20
β-catenin R	a a t c c a c t g g t g a a c c a a g c	1066	59.97		20
LAMINA/C F	t t c t c c t c c c t t c c t t t t c c	2358	59.62	125	20
LAMIN A/C R	g g g c a g a a a a g c a g a a g c t a	2482	59.73		20
NF-KB F	c t g g a a g c a c g a a t g a c a g a	927	59.98	89	20
NF-KB R	c c t t c t g c t t g c a a a t a g g c	1015	59.98		20
STAT3 F	c c t t t g g a a c g a a g g g t a c a	1974	59.96	146	20
STAT3R	c c t t c t c c a c c c a a g t g a a	2119	60.08		20
IL-10 F	c c t t t g g a a c g a a g g g t a c a	1158	59.90	90	20
IL-10 R	c t c c c a a a g t g c t g g g a t t a	1247	60.07		20
VIMENTIN F	g a g a a c t t t g c c g t t g a a g c	6255	60.00	144	20
VIMENTIN R	c t c a a t g t c a a g g g c c a t c t	6398	60.07		20
PLAUR F	c c t g g a g c t t g a a a a t c t g c	839	59.96	105	20
PLAUR R	c g g c a g t c a a t g a g g a a a g t	943	60.26		20
BCL2L1 F	t g g t c c c t t g c a g c t a g t t t	1207	59.96	79	20
BCL2L1R	a t t c t g a g g c c a a g g g a a c t	1285	60.26		20
SOX2 R	c t g t a g c c c c a a a t c g g a t a	8265	59.92	61	20
SOX2 F	g t t g c c t g g c t t c t c t t t t g	8325	59.99		20
OCT-4 R	a g c g a t c a a g c a g c g a c t a t	911	60.15	118	20
OCT-4 F	t a g c c t g g g t a c c a a a a t g	1028	59.82		20
GATA2 R	a c c g g a a g a t g t c c a a c a a g	1271	59.97	81	20
GATA2 F	t c t c c t g c a t g c a c t t t g a c	1351	59.99		20
GATA4 R	a c g g g t c a c t a t c t g t g c a a	780	59.17	52	20
GATA4 F	t g c c g t t c a t c t t g t g g t a g	831	59.72		20
GATA6 R	t t g g c t t g g g a c t t g a g a g t	3440	59.84	149	20
GATA6 F	g a a c t c c c c g a c t g a c a a a a	3588	60.09		20

FOXD3 F FOXD3 R	a c t c t g c c t c t c c c c a a t t t t t t g c t c g a c c a g c t t a g g t	1551 1645	60.07 60.02	95	20 20
VIMENTIN F VIMENTIN R	g a g a a c t t t g c c g t t g a a g c t c a a t g t c a a g g g c c a t c t	6255 6398	60.00 60.07	144	20 20
MYC F MYC R	a g g g a t c g c g c t g a g t a t a a t g c c t c t c g c t g g a a t t a c t	118 191	59.83 59.98	74	20 20

Note: Tm = melting temperature.
Tm was calculated using the formula 2[A+T] + 4[G+C], where, A= Adenine, T=Thymine, G=Guanine, C= Cytosine. Here, Tm was calculated using automated web based program (Oligoperfect designer, Invitrogen) based on the formula mentioned above (http://tools.invitrogen.com/content.cfm?pageid=9716).
The annealing temperature was given by the lowest Tm for the PCR program + 3°C.
Please refer 'Gene names and Accession number' on page XI for the NCBI accession numbers of the genes. The gene nomenclature is followed according to MGI guideline (Eppig and Levan, 2009).

2.12 Selection of control genes for qRT-PCR:

LAMIN C and *β-catenin* were used as control genes for the qRT-PCR experiments. The levels of expression of these two genes (*LAMIN C* and *β-catenin)* were very similar in low and high density cells in all the cell lines used (Figure 3.2, Chapter 3) and also throughout the different cell lines (Figure 3.3, Chapter 3). [Note: Housekeeping genes such as *β-actin* varies in low and high density SW480 cells. So, it was not used as a control for our experiments].

Also, *LAMIN C* and *β-catenin* expression levels remain similar even after knocking down *BRACHYURY* in low density SW480 cells (Figure 3.2 and 3.3, Chapter 3). Therefore, since these genes do not change their expression levels on *BRACHYURY* RNAi, we used *LAMIN C* and *β-catenin* for RNAi experiments too.

2.13 A worked example for analyzing qRT-PCR data using $2^{-\delta\delta C}{}_T$ method

Reference: Livak and Schmittgen, 2001; Dharmaraj 2006; Hunt 2009; Michael, 2001
Note: This is an example. Refer to table 2.4

Table 2.4 qRT-PCR data analysis

	β-catenin			*SNAIL*						2^-δδCt
Sample	**Ct**	**Mean Ct**	**S.D.**	**Ct**	**Mean Ct**	**S.D.**	**δCt**	**δδCt**	**S.D.**	**Fold increase (relative expression)**
SW626 sub	21.26	21.66	0.92	27.72	27.72	0.322	6.063	0	0.98	1
	22.71			27.35						
	21.00			27.94						
SW626 super	22.37	22.150	0.561	27.69	27.78	0.07	5.631	-0.43	0.565	1.348
	21.51			27.82						
	22.56			27.82						

Abbreviations: sub = sub-confluent cells; super = super-confluent cells; Ct = cycle threshold; S.D. = standard deviation.
δCt = The difference between the mean Ct of the experimental and reference gene
δδCt = The difference between the δCt of the condition we are comparing and the condition to which we are comparing.
Fold increase is calculated using the formula 2^- δδCt. This formula (2^- δδCt) was derived by Applied Biosystems in 1997 by ABI prism 7700 sequence detection system)

Here, expression of *SNAIL* is analysed in sub- and super-confluent SW626 cells. The data was compared to *β-catenin* and the sub-confluent data was set to 1 to which super-confluent data was compared. Each sample has a triplicate value. The Mean Ct will give the average of those three Ct value. So, we have Mean Ct for the control gene (here, *β-catenin* Mean Ct) and Mean Ct of the experimental gene (here, *SNAIL* Mean Ct). δCt will give the difference of the Mean Cts of the experimental and control gene. δδCt will give the difference between the δCt of the condition we are comparing (here, δCt of *SNAIL* in super-confluent SW626 cells) and the condition to which we are comparing (here, δCt of *SNAIL* in sub-confluent SW626 cells). Here, δδCt of the *SNAIL* in sub-confluent cells is made to 0 by subtracting its own value. This gave the value for $2^{\wedge -\delta\delta C}$t for *SNAIL* expression in sub-confluent SW626 cells as 1. Then *SNAIL* expression in super-confluent SW626 cells was compared to 1 and was calculated using $2^{\wedge -\delta\delta Ct}$. This gave the expression to be 1.3 times stronger than *SNAIL* expression in sub-confluent SW626 cells (which is set as 1). The final standard deviation is given by standard deviation if the β-catenin and

standard deviation of *SNAIL* Ct value. A graph can now be plotted for *SNAIL* expression in sub-confluent SW626 cells as 1 and its expression in super-confluent SW626 cells as 1.3 and the final standard deviations will give the error bars. Note: When we have done multiple repeats of the experiment, we calculate the fold difference ($2^{\wedge-\delta\delta Ct}$) for each experiment, and the take an average of all the $2^{\wedge-\delta\delta Ct}$ values from all repeats to plot the graph. The final standard deviation (SD) is given by the calculating the SD of all repeats, which gives the error bars.

2.14 Western blot

Cells were grown as required, washed twice with 1X PBS and scraped to detach from the flask surface/ 6-well plate surfaces using a scraper. Then the cells were centrifuged at 2000g for 5 minutes and resuspended in lysis buffer (50mM Tris-HCl pH7.4, 200mM sodium chloride, 0.5% (v/v) Triton X-100, 1mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride) and one complete, mini, EDTA-free protease inhibitor cocktail tablet (Roche- cat#11836170001) per 10ml of lysis buffer. Each tablet contains 0.02 mg/ml Pancreas-extract, 0.0005 mg/ml Thermolysin, 0.002 mg/ml Chymotrypsin, 0.02 mg/ml Trypsin, 0.33 mg/ml Papain. An equal volume of 2X (loading) Laemmli buffer (20% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 4% (w/v) SDS (sodium dodecyl sulphate), 200mM DTT (Dithiothreitol), 100mM Tris-HCl pH6.8) was added and the lysates were boiled for 5-10 minutes. The lysates were then loaded on 1mm thick 4-12% (w/v) Tris glycine precast gels (Invitrogen- cat#EC60352BOX) or 0.75mm thick 7.5% (w/v) SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis). Precision Plus Protein Dual colour standards (BioRad- cat#161-0374) were also run as marker to detect the protein sizes. Gels were run at 125 volts for 2-3 hours. Proteins were transferred to methanol wet Immobilon-P PVDF membrane (Millipore-cat#IPVH00010) at 500mA for 15-18 hours in 2x Towbin buffer (380mM Glycine, 50mM Tris) with(only in case of APC transfers) or without 0.02% (w/v) SDS. After transfer, the membrane was blocked in 10% (w/v) milk overnight at 4°C or 1hour at

room temperature. Immunodetection was performed in 10% (w/v) milk in PBS with 0.1% (v/v) Tween 20 (Sigma- cat#D-1379) for monoclonal primary antibodies and 0.5% (v/v) Tween 20 for polyclonal primary antibodies. Incubation with primary antibody solutions was at room temperature for 1 hour or 4°C overnight, after which a 15 minutes wash was done in milk solution at room temperature. Incubation with secondary antibodies was again at room temperature for 1 hour or 4°C overnight. Antibody details can be seen in tables 2.5 and 2.6. Following incubation with secondary antibody membranes were washed for 10 minutes in milk solution, followed by two 10 minutes washes in PBS/0.1% (v/v) Tween 20 (for monoclonal primaries) or PBS/0.5% (v/v) Tween 20 (for polyclonal primaries). Bands were gained on Kodak X-OMAT AR film with Super signal west pico chemiluminesent ECL substrate (Pierce- cat#34080).

Table 2.5 Primary Antibodies

Antibody	**Clone no. / Cat no.**	**Lot no.**	**Source**	**Host**	**Clonality**	**Stock conc.**	**IF**	**Weste-rn**	**IP**
Rel A	Ab1604	LV1447 918	Chemicon	rabbit	polyclonal	1mg/ ml	-	1/2000	-
CD133	Ab19898	447711	Abcam	rabbit	polyclonal	1mg/ ml	1/510	1/500	-
VIMENTIN	V9/ Ab8069-1	05430	Abcam	mouse	monoclonal		-	1/1000	-
BRACHYURY	AF2085	KQP010 7011	R&D systems	goat	polyclonal	0.2m g/ml	-	1/1000	-
NANOG	Ab21624	478537	Abcam	rabbit	polyclonal	0.2m g/ml	1/150	1/300	-
LAMIN B	M-20/ sc-6217	E0208	Santa-Cruz	goat	polyclonal	200µ g/ml	-	1/1000	-

NANOG	NNG-811/ Ab62734	505711	Abcam	mouse	monoclonal	0.5mg/ml	1/520	-	-
BRACHYURY	Ab57480	476734	Abcam	mouse	monoclonal	1mg/ml	1/250	-	-
BRACHYURY	C-19/ sc-717745	K2106	Santa Cruz	Goat	polyclonal	200µg/ml			
BRACHYURY	N-19/ sc-17743	G1006	Santa Cruz	mouse	monoclonal	200µg/ml			
14-3-3σ	C-18/ sc-7683	I12061	Santa Cruz	Goat	polyclonal	200µg/ml			
β –catenin	H-102/ sc-7199	I2105	Santa-Cruz	rabbit	polyclonal	200µg/ml	1/75	1/1000	
E-CADHERIN	36		Transduction labs.	mouse IgG_{2a}		250µg/ml	1/250	1/2500	-
Ki-67	PP-67		Sigma	mouse IgM		-	1/500	-	-
LAMIN A+C	636/ sc-7292	H2007	Santa-Cruz	mouse	monoclonal	200µg/ml	-	1/750	-
CD166/ ALACAM	105902/ MAB6561	DXM01	R & D	Mouse	monoclonal	500 ug/ml	-	1/250	-
NANOG	AF1997	KKJ03	R & D	Goat	polyclonal	0.1 mg/ml	-	1/ 100	-
Oct-4	Ab19857	641990	abcam	rabbit	polyclonal	0.1 mg/ml	5ug/ml	1ug/ ml	-

Table 2.6 Secondary Antibodies

Antibody	**Conjugate**	**Stock conc.**	**Use**	**Source**
Goat anti-mouse	AlexaFlour 488	2mg/ml	IF (ML) 1/400	Molecular Probes
Goat anti-mouse	AlexaFluor 568	2mg/ml	IF (ML) 1/400	Molecular Probes
Goat anti-rabbit	AlexaFluor 488	2mg/ml	IF (ML) 1/200	Molecular Probes

Goat anti-rabbit	AlexaFlour 568	2mg/ml	IF (ML) 1/200	Molecular Probes
Goat anti-mouse	HRP	400µg/ml	W 1/1000	Santa-Cruz
Goat anti-rabbit	HRP	400µg/ml	W 1/2000	Santa-Cruz
Bovine anti-goat	HRP	200µg/ml		Santa-Cruz
Donkey anti-rabbit	HRP		W 1/25000	Jackson Immunoresearch
Donkey anti-mouse	HRP		W 1/30000	Jackson Immunoresearch

Abbreviations for tables 2.5 and 2.6: "IP": Immunoprecipitation "W": Western "IF": Immunofluorescence "-": not known/not used

"ML": Suitable for multiple labelling (Highly cross-adsorbed) "HRP": Horseradish peroxidase.

2.15 Immunofluorescence

Cells were washed in 1X phosphate buffered saline (PBS) then fixed with 4% (w/v) paraformaldehyde for 20 minutes at room temperature. Cells were then washed in 1X PBS and then permeabilised in 0.2% (v/v) Triton-X-100 for 15 minutes at room temperature. Cells were again washed with 1X PBS followed by blocking in PBS-5% (v/v) FBS for 1 hour at room temperature/ 4°C overnight. Cells were then incubated with primary antibodies (see table 2.5) diluted in 5% (v/v) FBS in PBS for overnight at 4°C. Cells were then rinsed in PBS five times for 5 minutes each and then incubated for 30 minutes at 37°C in secondary antibodies (see table 2.6) diluted in 5% (v/v) FBS in PBS. Following secondary antibody incubation, cells were again washed three times in PBS and stored in PBS until they were imaged using a Zeiss Axioplan 2 confocal microscope. For propidium iodide staining, 1µg/ml of propidium iodide was added to the PBS in the second wash step after secondary antibody incubation.

2.16 Immunoprecipitation

Cells were grown as required, washed with PBS and detached from the flask surface using a cell scraper and collected in 10ml PBS. Cells were then centrifuged at 2500g for 3 minutes and resuspended in 200-500µl lysis buffer (50mM Tris-HCl pH7.4, 200mM sodium chloride, 0.5% (v/v) Triton X-100, 1mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride) and one complete, mini, EDTA-free protease inhibitor cocktail tablet (Roche- cat#11836170001) per 10ml of lysis buffer. Each tablet contained 0.02mg/ml Pancreas-extract, 0.0005mg/ml Thermolysin, 0.002mg/ml Chymotrypsin, 0.02mg/ml Trypsin, 0.33mg/ml Papain). Cells to be lysed with beadbeater were mixed with 200 µl of glass beads and lysed in a beadbeater (FastPrep FP120, Bio101) for 30 seconds on speed 5000 rpm. Whereas, the cells lysed without beadbeater were kept on ice for 30 minutes. All cells were then centrifuged at 15,000g at 4°C for 20 minutes. Supernatants were collected to fresh tubes. Around 15µl of the supernatant were kept separately and 15µl of 2X Laemmli buffer was added to load directly on the gel. Rest of the supernatant was divided into IP (to add the antibody) and IgG controls (negative controls to which normal IgG from the relevant species was added in place of the antibody, details in table 2.7). Both IP and IgG were precleared by adding 25µl raw Protein G linked to agarose beads (Upstate, Cat#16-266, and Lot #0610042813) and incubated for 1hr at 4°C with shaking, after which they were centrifuged for 2 minutes at 13000rpm. The supernatant was collected and the respective antibody (20µg/ml) and IgG were added. The lysates were then incubated at 4°C for 2-3 hours. Again 25µL Protein G linked to agarose beads was then added to the lysates, which were then incubated at 4°C with shaking for 1 hour. Following incubation with the lysate/antibody mix the protein G beads were washed three times in the lysis buffer. The pellets containing the Protein G beads were finally resuspended in 20µl Laemmli buffer. Following boiling for 5-10 minutes at 100°C, samples were loaded onto SDS-PAGE gels.

For western blotting, protocol is described in section 2.14. Bands of interest were cut from the gel using a scalpel and transferred in 1.5 ml eppendorf tubes which were then sent for mass spectrometry.

Table 2.7: Normal IgGs

Normal IgG	**ID no.**	**Lo no.**	**Source**	**Stock conc.**
Normal mouse IgG	Sc-2025	B0606	Santa Cruz	200µg/ 0.5ml
Normal rabbit IgG	Sc-2027	I1306	Santa Cruz	200µg/ 0.5ml
Normal goat IgG	Sc-2028	L0905	Santa Cruz	200µg/ 0.5ml

2.17 RNAi *(BRACHYURY, β-catenin, E-CADHERIN, TBX-2, TBX-3, FGF8)*

Cells were grown in T75 flasks till confluent. Cells were then split 1in 4 dilution and 0.6ml of the cells were added to 12ml of fresh medium (with 10% (v/v) FBS). 2ml of this cell and media mixture was added to each well of 6 well plates and/or 0.5ml to each well of 24-well plates. The plates were incubated in 37°C incubator with 5% CO_2. Meanwhile, the RNAi mixture was prepared by adding 6µl of siRNA which was diluted 1/10 in serum free medium, 94 µl of serum free medium and 6µl of Hiperfect Transfection Reagent (Qiagen, Cat#301705, Lot#130172730) for each sample for the 6-well plates. The RNAi mixture for the 24-well plates was prepared by adding 1.5µl of siRNA which is diluted 1 in 10 in serum free medium, 98.5µl of serum free medium and 3µl of Hiperfect Reagent. Control RNAi mixture was prepared with negative control siRNA. These mixtures were then vortexed, centrifuged and incubated at room temperature for 20-25 minutes, after which the mixtures were slowly added to the plates incubated in the 37°C incubator by mild rotating and mixing. The cells were grown at 37°C for 1 day (subconfluent), 2 days (mid-point between subconfluent and confluent), 3 days (confluent) and 4 days (superconfluent cells). Cells were then fixed, permeabilised

and blocked for Immunofluorescence and/or used for western blots. Details of the siRNA can be found in table 2.8.

2.17A: Mechanism of RNAi:

RNAi (RNA interference) works by introducing a gene- specific dsRNA into a cell that can degrade the homologous mRNA (Cottrell and Doering 2003). It is a post- transcriptional gene silencing mechanisms (Ullu and Tschudi 2000).
For our experiments, RNA is made in a test tube by adding phage RNA polymerases that bind to phage promoters that are within expression vectors. The cDNA is transcribed through PCR and the sense and antisense strands are annealed to give double stranded RNA, which is then cut into 21-25 by small interfering RNAs (siRNA) by an enzyme, RNase II. RNase II also has a helicase section, which helps in unwinding of the dsRNA necessary for the binding the RNA to the target RNA that we want to inhibit the translation of (Shuey, McCallus, and Giordano 2002).

Table 2.8: siRNAs

siRNA	**Name**	**Cat#**	**siRNA type**	**Stock conc.**
BRACHYURY	Hs_T_1	SI00738255	Predesigned	5nMol
TBX2	Hs_TBX2_2	SI00740782	Predesigned	5nMol
TBX3	Hs_TBX3_3	SI00083496	Predesigned	5nMol
FGF8	Hs_FGF8_1	SI00145593	Predesigned	5nMol
β-catenin	Hs_CTNNB1_5	SI02662478	Predesigned	5nMol
E-CADHERIN	Hs_CDH1_13	SI02654029	Functionally validated	5nMol
Negative control	Alexa Fluor 488	1022563	Control	5nMol

Note: All siRNA constructs were purchased from Qiagen.

2.18 ChIP (Chromatin Immunoprecipitation)

Approximately $1X10^6$ cells were grown. The medium was aspirated and washed with PBS. Histones were cross-linked to DNA by adding formalydehyde to a final concentration of 1% (v/v) and incubated at room temperature for 20 minutes. To quench the reaction, Glycine was added directly to the medium to a final concentration of 125mM, shaken a bit and incubated at room temperature for 5 minutes. The medium was then aspirated and the cells were washed 2X with PBS. The cells were then scrapped and collected in a tube and centrifuged for 2000rpm for 4 minutes. The cells were then resuspended in 500µl SDS lysis buffer with protease inhibitors and kept on ice for 10 minutes. Chromatin was sheered by sonicating the cells on ice for 5 pulses of 10 secconds each at 8µm amplitude. To reverse the cross-linking, 8µl of 5M NaCl was added and incubated at 65°C for 4-5 hours. The DNA was recovered by phenol-chloroform extraction and ethanol precipitation. The samples were then run on 1% (w/v) agarose gel to optimize the sonication conditions.

Once the sonication condition has been optimized, the above protocol was repeated till the sonication step and then centrifuged for 10 minutes, 13000 rpm at 4°C. The supernatant was diluted 10 fold in Chip dilution buffer with protease inhibitors. Around 20 µl of supernatant was kept separate as the 'Input' for the PCR later. Rest of the supernatant was divided into IP and IgG and was precleared with protein G for 1 hour at 4°C with agitation, followed by a brief centrifugation. Respective antibodies and IgGs were added to the supernatant and incubated rotating for overnight at 4°C.

Next day, protein G agarose was again added to the samples and kept rotating at 4°C for 1 hour. After a gentle centrifugation (700-1000 rpm, 4°C) for about 1 minute, the pellets were washed once with low salt immune complex buffer, once with high salt immune complex buffer, once with LiCl immune complex buffer and twice with TE buffer. The samples were now protein G/ Histone/ DNA

complex. For Immunoprecipitaion/ Immunoblot experiment, they were loaded on a gel added with Laemmli buffer and heated for 5-10 minutes at 100°C.

For PCR, to amplify the DNA bound to immunoprecipitated histones, the protein G/ Histone/ DNA complex was eluted by adding 250µl $NaHCO_3$+SDS elution buffer, vortexed briefly and incubated at room temperature for 15 minutes with rotation. Agarose was the centrifuged and the supernatant was transferred to a separate tube and the elution step was repeated. After combining the eluates of both the elution steps, 5M NaCl was added and heated to 65°C for 4 hour to reverse the crosslinking. The details of the buffers are described in table 2.9.

Table 2.9 ChIP assay buffers:

Buffers	Contents	Conc.	pH
SDS lysis buffer	SDS EDTA Tris.	1% (w/v) 10 mM 50 mM	8.1
ChIP dilution Buffer	SDS Triton X-100 EDTA Tris-HCl NaCl	0.01% (w/v) 1.1% (v/v) 1.2 mM 16.7 mM 167 mM	8.1
Low salt immune complex wash buffer	SDS Triton X-100 EDTA Tris-HCl NaCl	0.1% (w/v) 1% (v/v) 2 mM 20 mM 150 mM	8.1
High salt immune complex wash buffer	SDS Triton X-100 EDTA Tris-HCl NaCl	0. 1% (w/v) 1% (v/v) 2 mM 20 mM 500 mM	8.1
Licl immune complex wash buffer	LiCl IGEPAL-CA630 Deoxycholic acid EDTA Tris.	0.25 M 1% (v/v) 1% (w/v) 1 mM 10 mM	8.1
TE buffer	EDTA Tris-HCl	1 mM 10 mM	8.0
$NaHCO_3$+SDS elution buffer	SDS $NaHCO_3$	1% (w/v) 0.1M	

Table 2.10 Primer sequences for ChIP assay

Primer	Sequence	Length	GC content	Tm
CH1 F	gtgtgggagcttttgagcatc	21mer	52.38%	66.2°C
CH2 R	ccatcacgcctggctaattttg	22mer	50.00%	69.4°C
CH3 F	cctcaagtgatccacgtgcctc	22mer	59.09%	70.4°C
CH4 R	tgcacgtctgtggtcccagc	20mer	65.00%	72.5°C

Note: Primer pair 1 was used for site 1 and gave a product length of 193bp and primer pair 2 was used for site 2 and gave a product length of 249bp. APC primers used were APC15a (ttagttttacaccgggggatgata) and APC15 (gcagaagacgacgcagatg) with Tm = 50°C.

2.19 DNA cloning and Luciferase assay

SW480 cells were grown to low density and the genomic DNA was extracted using Wizard SV Genomic DNA Purification System (Cat. Number A2360, Promega). The genomic DNA was stored at -20°C for future use. Primers were designed for the Brachyury binding sites (refer chapter 5 for the sites) in the Nanog promoter region. Details of the primers can be found in section 2.19. PCR was carried out using 2µl of 1/50 dilution of the genomic DNA prepared, 25µl BioMix™ Red (Bioline-Cat#BIO-25005), 1µl of each primer ser diluted 1/10 and 21µl distilled water in a 25µl PCR reaction. The annealing temperature was maintained at 56°C and the extension time was set accordingly (30 seconds per kilo base) and the PCR reaction was set for 40 cycles. PCR products were run on a 1% (w/v) agarose gel and the product bands were cut and purified using Geneclean kit (Cat. Number 1001-200, Q-biogene) and the DNA was eluted in TE buffer. DNA digestion reaction was set up at 37°C for 2 hours using 10µl DNA, 0.5µl KpnI restriction enzyme (Cat. Number R0142S, NEB), SacI restriction enzyme (Cat.Number R0156S, NEB), 2µl NEbuffer1 from 10X stock (Cat. Number B7001S, NEB), 0.2µl BSA from 100X stock (Cat.Number B9001S, NEB) and 6.8µl distilled water in a 20µl DNA digestion reaction. A vector digest was set up alongside 1µl PGL3 basic

vector of 20µg stock of concentration 1µg/ µl (E175A, 19205606, Promega), thus using 0.05µg vector in a 20µl reaction. Buffer1, BSA and the restriction enzymes were used with same concentration mentioned above and 15.8µl of distilled water is used to make up the volume of the reaction to 20µl. The digestion reactions (both experimental and vector digests) were purified using High Pure PCR Purification kit (Cat. Number 11732676, Roche). [Note: Heat inactivation of the restriction enzymes was not possible because KpnI is not feasible for heat inactivation, although SacI can be heat inactivated]. A small amount of the digested products were run on 1% (w/v) agarose gel to get an estimate of how much vetor: digest ratio is needed for ligation. Ligation mix was made up using 1µl of digested PGL3 basic vector with 1µl T4 DNA ligase (Cat. Number M0202S, NEB), 1.5µl T4 DNA ligase buffer 10X stock (Cat. Number B0202S, NEB). The amount of DNA digest used varied accordingly based on the fact that we used 1:3 ratio of vector: DNA digest, respectively. The reaction was made up to 15µl with ultrapure water. The ligation reactions were incubated at 4°C for overnight. Next day, 5µl ligation mix was then transformed to LB-Ampicillin plates with 50µl competent cells (One shot top 10, Invitrogen). [For transformation, the ligation mix and competent cells were incubated on ice for 5-30 minutes, heated at 42°C for 30 seconds to 1 minute, recovered with 250µl LB, incubated at 37°C for 1 hour, shaking and the transformed to LB-Amp plates]. The plates were incubated for overnight at 37°C. Next day, colonies were picked up and were grown in 5ml LB for overnight at 37°C. Miniprep was made from 1ml of the overnight culture using miniprep kit (Cat. Number 27104, Qiagen). Rest of the overnight culture can be used for making glycerol stock (i.e 80% (v/v) sterile glycerol is added to the overnight culture in the ration 1:1 and stored at -80°C). The miniprep was digested again for 1 hour at 37°C using 5µl miniprep DNA with 0.1µl BSA, 1µl NEbuffer1, 0.5µl KpnI, 0.5µl SacI and the reaction was made up to 10µl with ultrapure water. The digested products were then run on 1% (w/v) agarose gel to check whether the insert and vector bands are of the expected size.

Luciferase assay: $2x10^5$ SW480 cells were seeded and grown at a low density in 96 well plates (0.33cm^2 area). When the cells were ready, the transfection complex was prepared using 5µg of DNA (clone) by diluting it in serum free DMEM to a total volume of 30µl and 0.1µg of Renilla vector, PGL4.7 [hRluc/SV40] (E6911, Promega) as a control vector. [Note: at this step, both experimental and control vectors should be added to the cells. Since, the insert already has PGL3 basic vector, thus only Renilla control vector is added here]. 2.5µl of Superfect Transfection Reagent (Cat. Number 301305, Qiagen) was added to transfect the DNA to the SW480 cells. This complex with DNA, experimental and control vectors and the transfection reagent was incubated at room temperature for 10-15 minutes. Meanwhile, the medium from the 96 well plates were aspirated and washed with 1X PBS. After 10-15 minutes incubation, 1ml of DMEM with serum was added to the transfection complex and this mix was added to the cells and incubated at 37°C incubator for 2-3 hours. After 2-3 hours, the medium was removed from the plates, washed with 1X PBS and then the fresh medium with serum was added to the cells. Dual-Glo Luciferase assay system (E2920, Promega) was used to measure the luciferase activity of the DNA. This was done by adding a volume of Luciferase Reagent equal to the culture medium volume to each well of the 96 well plate. The plates were incubated for 10 minutes in room temperature and then the luminescence was measured for the experimental samples using luminometer (Wallace Victor, 1420 multilabel counter). This gave the result for the luminescence of the clones (experimental). Next, Dual Glo stop and glo reagent was added directly to the plate equal to the original culture volume and incubated at room temperature for 10 minutes and measured the luminescence again. This gave the luminescence for the control (Renilla). This experiment was performed in triplicates for each sample. The data was then normalized using the formula:

Change in fold activity = [Average (F/R) from one sample]/ [Average (F/R) from another sample], where F= Experimental luminescence obtained; R= Control luminescence obtained.

Since, we analysed our values statistically, we plotted our F/R values on a scale rather than plotting the fold increase. The error bars were obtained using the standard deviations and the cut-off for significance was given by the p-values. P-values <0.05 were not considered statistically significant.

[Note: *Renilla* luciferase is a 36 kDa protein obtained from sea pansy (*Renilla reniformis*) which requires coelenterazine and oxygen to produces a blue light of 480nm. This enzyme is used in dual-reporter assays due to their differences in substrate requirements and light output from the experimental vector].

2.20 P-value and t-test:

P-value is statistical hypothesis testing which gives the probability, with a value ranging from zero to one. It tells that what would be the probability that the random sampling will show a difference between sample means as large as observed. P-values can be two-tailed (i.e. what is the probability that the randomly selected samples will give means as far apart as observed with either group having the larger mean); or one-tailed (i.e. what is the probability that the randomly selected samples will give means as far apart as observed with the specified group having the larger mean). We used 2-tailed t-test since we were looking for a difference in gene expression. We did not use 1-tailed t-test since it is for a difference only in one direction.

P-value is obtained from Student's t-test which is statistical test to find if the difference between two sample means is statistically significant. To calculate the p-value, from a t-test, MS-Excel was used, the syntax for which is t-test (array_1, array_2, tails, type). Array_1 and _2 are the data sets; tail value is 1 for one-tailed distribution and 2 for two-tailed distribution. The type will give the type of t-test to be performed.ie. Type of 1 for a paired t-test, type of 2 for a two-sample equal (homoscedastic) variance and type of 3 is for a two-sample unequal (heteroscedastic) variance (Microsoft office website, TTEST). As mentioned before,

P-values ≤0.05 is considered significant. We used type 3 test since, which is independent TTEST where we compared three repeats of the controls genes on a plate to three repeats of experimental gene on the same plate then it is 3 repeats of each and we cannot say that 1 well of one lot is equivalent to 1 well of another. So, we used independent TTEST. [Note: We have used paired t-test where there is definite relationship between the two data sets.]

Table 2.11: Clone details for the luciferase assay

Clone number	**Primer combination**	**BRACHYURY binding sites**	**Expected product size**
33	L2a + LR1	Sites 1 and 2	2271 bp
52	L2C + LR1	Site 1	2312 bp
53	L0F + LR1	No sites	634 bp

Note: The clone numbers are the clone reference numbers (arbitrary). The primers combinations are the name of the primers used (arbitrary) to get the respective clones. The BRACHYURY binding sites are the sites included in the respective clones. The expected product size is the size of the product obtained by using the respective primer pairs.

The primers are as follows:

LR1: attcca gagctc ctggtggtaggaagagtaaagg
L0F: attcca ggtac gagacagtctggttctgttgtcacgc
L2C: attcca ggtac gtcaggagt tcgagaccag cctc
L2a: attcca ggtac gtgtgggagcttttgagcatc

attcca = random base pairs added on each site

2.21 Mitotic index count

Cells were grown in 24 well plates with coverslips. Immunoflouresence protocol was followed (section 2.14) and the coverslips were mounted on a slide and imaged using a Zeiss Axioplan 2 confocal microscope. The number of cells in any

stage of mitosis was counted against cells not in mitosis and thus percentage of cells undergoing mitosis was calculated for each sample.

2.22 Cell Viability Asssay (Promega CellTiter-Glo™)

SW480 cells were grown in 24 well plates (1.65 cm^2) for 1,2,3,4 and 5 days (1 being subconfluent and 4 being superconfluent). For each day, the medium was taken out from the plates, washed with PBS and 250µl trypsin was added to detach the cells. 750 µl medium was added to this and number of cells per ml was counted by haemocytometer. Cells were now transferred to another 24 well plate with a few different dilutions which were needed for drawing a standard curve later. The plates were incubated at 37°C for 3-4 hrs, giving enough time for cells to start adhering to the base. Later, the medium was taken out from these plates and equal volume of medium and CellTiter-Glo™ Reagent (Promega, Cat#G7571, Lot#258909) was added to the cells and was incubated at room temperature for 2 minutes. Equal volume of this mixture was transferred to opaque 96 well plates (0.37 cm^2) in triplicates, mixed on shaker for 2 minutes and incubated at room temperature for 10 minutes. Reading was taken by a luminometer (Wallace Victor, 1420 multilabel counter) which gave luminescence against number of viable cells. [Note: Viable cells are checked by adding Trypan blue (Sigma) stain to the cells. The cells that are viable do not pick up the stain, since they have intact cell membrane].

2.23 Cytoselect cell migration assay

This assay was run using Cytoselect 24-Well cell migration assay (Cell Biolabls, Cat. number CBA-101). Pore size used was 8µm (suitable for SW480 cells). Assay plates stored at 4°C was warmed to room temperature for 10 minutes. Cell suspension was prepared in serum free medium (1.2 X 10^6 cells/ ml). 500µl of medium with serum was added to the lower well of the migration plate. 300µl of cell

suspension was added to each insert in the assay wells. 2.5 µg/ ml of mitomycin C was added to the cells at this atge. Cells were grown in 37°C incubator. One time point analysed was 12 hours and the 2nd time point was 24 hours. After the required time, the plates were taken out of the incubator and medium was taken out from each insert. The inserts were then transferred to clean wells containing 225µl Cell Detachment Solution and incubated for 30 minutes at 37°C. The cells were completely dislodged from the underside of the membrane by tilting the inserts gently. Meanwhile, 4X Lysis Buffer/ CyQuant GR dye solution was prepared by diluting the dye 1:75 in the lysis buffer. 75µl of this buffer/ dye solution was added to each well with 225µl cell detachment solution and incubated for 20 minutes at room temperature. 200 µl of this mixture was then transferred to a 96-well plate and the fluorescence was measured at 480 nm/ 520 nm.

2.24 Conditioned media experiment

Cells were grown to a high density (4 days for SW480) at 37°C at 5% CO_2. Medium from the high density cells were collected and then centrifuged to remove the debris. This medium is either snap frozen and stored at -80°C for long term use or is used immediately for growing fresh cells.

2.25 Protein purification

A) Amicon Ultra-15 spin column (Chemicon/ Millipore, Cat. Number UFC9 050 96): This was centrifuged for 30 minutes at 4000g at room temperature. This contains a 10kDa filter that separates components with more than 10kDa size in the upper column and less than 10kDa in the lower column.

B) HiTrap Blue affinity column (GE healthcare, Cat. Number 17-0413-01): The dye ligand in this column is Cibacron Blue F3G-A which is covalently attached to the matrix by the triazine part of the dye molecule. Few proteins may interact biospecifically with the dye due to its structural similarity with nucleotide cofactors.

In this column, albumin and interferon can bind in a less specific manner by electrostatic and/or hydrophobic interactions with the aromatic anionic ligand.

C) MonoS cationic exchange column (GE healthcare): This column was used at pH 6 and is negatively charged. Thus, it would bind the positively charged components.

D) MonoQ anionic exchange column (GE healthcare): This column was used at pH 8 and is positively charged. Thus, it would bind the negatively charged components.

Note: At the end of final run using any of the above mentioned columns, the columns are washed with elution buffer, binding buffer, water and 20% (v/v) ethanol of 20-25 ml each, in the order mentioned. [The details of binding and elution buffers are mentioned in chapter 7].

Chapter 3 –a) The mesoderm inducer *BRACHYURY* is differentially expressed in colorectal cancer cells

3.1 Introduction:

Cancers may arise from disruption of normal cell proliferation, differentiation and survival. The initiation and maintenance of cancer often involves the aberrant expression of genes that are associated with normal development. As mentioned previously (section 1.5), the process of EMT (or similar), a fundamental feature of normal development is also inappropriately involved in the progression of CRC. As such, the invasive fronts of colorectal tumours are characterized by epithelial de-differentiation, loss of intercellular adhesion and an increase in cell migration. This is associated with the activation of transcription factors related to epithelial-mesenchymal transition (EMT) (Brabletz *et al.*, 2005).

It is not easy to study the events involved in EMT during the progression of cancer and an *in vitro* system that re-capitulates this process would be invaluable to the study of these transition events. We used CRC cells from the cell line SW480, grown to low and high density, as a model for cells reversibly transitioning between the mesenchymal-like and epithelial-like cell states, respectively (Brabletz *et al.*, 2001; Davies *et al.*, 2004)

Our initial aim was to carry out a directed RT-PCR screen on a range of mesoderm inducing genes that may be candidates for inducing a transition from epithelial to mesenchymal cell types in high and low density SW480 cells. Specifically, we wanted to look for any differential expression of these genes in low density cells (mesenchymal-like) compared to high density cells (epithelial-like) SW480 cells. The following candidate genes were studied to determine if they are differentially expressed in low and high cell density SW480 cells. *E-CADHERIN, ACTIVIN A, ACTIVIN B, FGFR1, FGFR2, FGFR3, SNAIL, NFκB, BRACHYURY, NOTCH 1, NOTCH 2, NOTCH 3, NOTCH 4,TBX2, TBX3, FGF-4, FGF-19, BMP4, FGF-8, GDF3, NODAL, Oct-4* (refer to table 3.1).Alongside this study in SW480 cells (which have single copy of *APC* with a mutation at codon 1338 resulting in a stop codon and therefore a truncated protein of 1337 amino acids, Nishisho *et al.*, 1991), we also determined the expression of some of these mesoderm inducers in other human colorectal cancer cell lines including

Table 3.1: Summary of function of the developmental genes in early development we used in our screen.

Gene	Role	Reference
E-CADHERIN	E-cadherin is a tumor suppressor gene involved in calcium dependent cell-cell adhesion. It is downregulated in cells undergoing EMT.	Semb *et. al.*, 1998; Wong *et al.* 2003
Activin	Activin is involved in early mesoderm formation in *Xenopus*. Involved in cell proliferation, cellular differentiation, apoptosis, metabolism, homeostasis, immune response, wound repair and endocrine function.	Dyson *et al.*, 1997 Chen *et al.*, 2006; Sulyok *et al.*, 2004
FGFR1 (Fibroblast growth receptor 1)	Expressed during vey early neural development. Involved in limb induction, mammalian liver development, inner ear development in mouse.	Walshe and Mason 2000;Jung *et al.* 1999; Pirvola *et al.* 2002
FGFR2 (Fibroblast growth receptor 2)	Expressed in early neural development. Involved in retinal development.	Walshe and Mason 2000 Moore *et al.* 2004
FGFR3 (Fibroblast growth factor receptor 3)	Expressed in early neural development. Inhibits cell proliferation by interfering with ribosomal biogenesis. Roles in nonsyndromic cleft lip and palate	Walshe and Mason 2000 Riley *et al.*, 2007; Antoine *et al.*, 2005
SNAIL (SNAI1)	Induces EMT during development of mouse. Downregulates the ectodermal gene expression in the mesoderm	Ethan *et al.*, 2001 Twigg *et al.*, 1999
NFκB	Involved in B cell development during organogenesis. Role in regulating the immune response to infection. Involved in synaptic plasticity and memory.	Shaffer *et al.* 1997 Gilmore *et al.*, 2006; Albensi *et al.*, 2000
BRACHYURY	It is a transcription factor. In mouse, it is needed for mesoderm formation and anteroposterior axis extension in the mammalian embryo . Involved in formation and differentiation of posterior mesoderm and axial development in	Chesley 1935; Gluecksohn-Schoenheimer 1944; Spiegelman 1976 Naiche *et al.*, 2005

	vertebrates.	
NOTCH family	Notch family includes 4 receptors (Notch 1-4). Notch is important for cell-cell interaction involved in gene regulation which controls cellular differentiation during embryogenesis.	Tanigaki *et al.* 2001; Gaiano *et al.*, 2002; Uyttendaele *et al.*, 2001
TBX2, TBX3	In development of the atrioventricular canal. Involved in cell proliferation during heart development.	Ribeiro *et al.*, 2007
FGF-4	Function in bone morphogenesis and limb development and secreted during early mesoderm induction	Powers *et al.*, 2000
FGF-19	Initiates inner ear during early development and involved in embryonic development, cell growth and morphogenesis.	Nishimura et al., 1999
FGF-8	Secreted in midbrain and limb development, organogenesis, left-right axis determination, embryo gastrulation	Powers *et al.*, 2000
NODAL	Secreted during mesoderm formation and axis specification during development.	Gebbia *et al.*, 1997
BMP4 (Bone morphogenetic protein 4)	Mediates dorsal/ ventral patterning during *Xenopus* early embryogenesis, ventralises mesoderm, involved in EMT during tooth development.	Friedle *et al.*, 1998 Vainio *et al.*, 1993 Hee *et al.*, 2009
GDF3 (Growth differentiation factor-3)	Regulates cell fate of the embryo during early development and is present throughout early embryogenesis in mouse. Active during during embryonic development and also in adults during the development of the brain, thymus, spleen, bone marrow and adipose tissue	Ariel et al., 2006 Chen *et al.*, 2006; Hexige *et al.*, 2005
Oct-4	Essential for early embryo development and is present during the the preimplantation period. Critical for self-renewal of undifferentiated embryonic stem cells	Rosner et al., 1990. Niwa *et al.*, 2000

Table 3.1: This table lists the names and known developmental functions of the genes we screened to check for their differential expression in low and high density SW480 cells.

HCT116 cells (which has functional *APC*, but a mutation leading to constitutively active *β-catenin*, Kitaeya *et al.,* 1997), LoVo (double *APC* mutation at 1,114 amino acid and 1,430 amino acid, Rowan *et al.*, 2000), HT29 (double *APC* mutation at 853 amino acid and 1555 amino acid positions, Brocardo *et al.*, 2005), T84 (APC mutation at 1,488 position, Rowan *et al.*, 2000) and the germ cell tumour EC cell line NTERA-2.

3.2 Validation of SW480 cell line for use as a model to study EMT:

SW480 cells grown to low and high density were previously shown to exhibit features of cells undergoing EMT (Brabletz *et al.*, 2001; Davies *et al.*, 2004). E-CADHERIN (both expression levels and co-localisation) and β-catenin (localisation) are key hallmarks (reviewed in Thiery 2002) of epithelial-to-mesenchymal transition and have been shown to change in SW480 cells with cell density. In summary, in low density SW480 cells (mesenchymal-like), the cells have low levels of E-CADHERIN located in cytoplasm and nuclear β-catenin. In high density SW480 cells (epithelial-like), the cells exhibit higher levels of E-cadherin, mainly membrane associated, and predominantly membrane bound β-catenin.

In accordance with previous literature, we were able to provide further evidence that SW480 cells grown at different densities might indeed be a good *in vitro* model to study the molecular events involved in EMT. Nuclear NFκB has previously been shown to be associated with induction and maintenance of EMT (Huber *et al.*, 2004). Figure 3.1 shows that one of the NFκB family members, REL A (Nuclear factor, NFκB p65 subunit), which is the most abundant form of NFκB, changes localisation in SW480 indicative of cells undergoing EMT. As the SW480 cells progress from low to high density, REL A translocates from the nucleus to the cytoplasm, respectively. The change in localization of REL A is in accordance with the changes in localization of β-catenin and E-CADHERIN and further validates the use of SW480 cells grown to different densities as a system to study EMT.

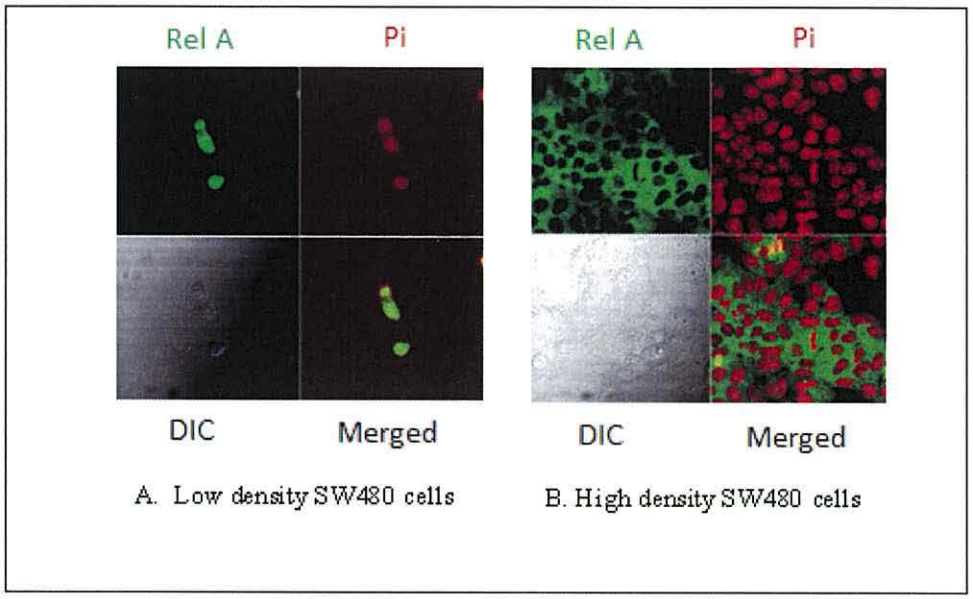


Figure 3.1

Confocal immunofluoresence microscopy showing localization of REL A in SW480 sub and superconfluent cells. A) Low density SW480 cells were stained with REL A primary antibody (green) which localizes to the nucleus of the low density SW480 cells. Nuclei are marked by staining with Propidium iodide (Pi) (red). B) High density cells where REL A (green) is located to the cytoplasm and the Pi (red) to the nucleus. The DIC image (in both A and B) shows the cells without any stain and the merged image shows the REL A and Pi staining together, giving a relative co-localisation. This translocation is indicative of EMT. [Note: The images were taken under 63X objective.]

3.3. Screen of the mesoderm inducing genes by RT-PCR:

Based on the observations above, we set up a small scale and directed screen to determine if a selected group of predominantly mesoderm inducing genes, that are key inducers during development might be differentially expressed in low and high density SW480 cells. Those genes identified from this screen would then be studied to determine if they play a role in inducing EMT in SW480 cells. This screen was also extended to other cell lines to see if they behaved similarly.

The cells from the different cell lines were grown to low and high densities (as described in materials and methods section 2.2) and the cDNA synthesised (as described in section 2.9) Primers were designed for all the genes (refer to table 2.1) and PCR was carried out (as described in section 2.10). Table 3.2 summarizes the RT-PCR results illustrating the expression levels of various genes in the different CRC cell lines. *Brachyury* was found to be differentially expressed in SW480 cells, but was not expressed in other CRC cell lines studied. As expected, *E-CADHERIN* levels increase in SW480 high density cells. Other candidates were also differentially expressed in SW480 cells (*TBX2, TBX3, FGF-8, FGFR1, 2* and *3*), but the expression patterns were not consistent between cell lines.

3.4 qRT-PCR quantification:

In order to gain more detailed quantitative expression for these genes we also carried out qRT-PCR based on our RT-PCR screen results. For this, we followed the method described in materials and methods section 2.11 using Sybr green and analysed the data using $2^{-\delta\delta C}{}_T$ method, as mentioned in section 2.13 of materials and methods. A worked example of how the raw data is converted into relative quantities is shown in section 2.13 of materials and methods. Section 2.20 of materials and methods describes how the p-values were calculated to check the significance of the data obtained.

Gene	SW480 sub	SW480 super	HCT 116 Sub	HCT 116 super	LoVo sub	LoVo super	HT29 sub	HT29 super	T84 Sub	T84 super
BRACHYURY	+++	+	-	-	-	-	-	-	-	-
E-CADHERIN	++	+++	+++	+++	++	++	+++	+++	+++	+++
ACTIVIN A	-	-	-	-	-	-	-	-	/	/
ACTIVIN B	-	-	-	-	-	-	-	-	/	/
FGFR1	++	+++	+++	+++	+	++	-	-	/	/
FGFR2	+	-	++	+++	+	-	+	-	/	/
FGFR3	++	+	+	+	-	-	-	-	/	/
SNAIL	+	+	+++	+++	-	-	-	-	/	/
NFκB	+++	+++	+++	++	-	-	-	-	/	/
NOTCH 1			+++	+++	-	+	+++	+	/	/
NOTCH 2	-	-	-	-	-	-	-	-	/	/
NOTCH 3	-	-	-	-	-	-	-	-	/	/
NOTCH 4	-	-	-	-	-	-	-	-	/	/
TBX2	+++	++	-	+	-	-	-	-	/	/
TBX3	+++	+++	+++	++	-	-	+	-	/	/
FGF-4	-	+	-	-	-	-	-	-	/	/
FGF19	+++	+++	+++	+++	-	-	-	-	/	/
BMP4	+++	+++	+++	+++	-	-	-	-	/	/
FGF8	++	+	+++	+++	-	-	-	-	/	/
GDF3	+	-	-	-	-	-	-	-	/	/
NODAL	+	-	+++	++	-	-	-	-	/	/
Oct4	+	-	/	/	/	/	/	/	/	/
β-catenin	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Abbreviations:
/ = not checked, - = no expression, + = low expression, ++ = moderate expression, +++ = high expression, Sub = subconfluent cells, Super = superconfluent cells.

Table 3.2:
Summary of the expression of genes in low and high density CRC cell lines as assessed by RT-PCR. The experiment was performed 5 times (N=5). *β-catenin* was used as a control since the level of *β-catenin* is high and similar in all the cell lines used. This table demonstrates the presence and absence of the genes found by running them on 1% agarose gel.

In general, the data for SW480 cells show gene expression patterns that resemble cells undergoing EMT, for example, high density SW480 cells have high E-CADHERIN levels, low SNAIL and ZEB1 expression relative to low density cells (Table 3.2). Table 3.4 is obtained by comparing low-density data to '1' and comparing high density data to '1'. None of the other cell lines follow such a distinct pattern nor do they display changes in localisation of β-catenin and E-CADHERIN (Brabletz *et al.*, 2001; Davies *et al.*, 2004). These observations further agree with SW480 cells being a good model in which to study EMT, when grown to different densities.
Note: Data for qRT-PCR was compared to either to *LAMINC* or *β-catenin*. Section 2.12 of material and methods and Figure 3.2, 3.3 shows that both *LAMINC* and *β-catenin* are proper controls for analysing the qRT-PCR data since their expression levels do not change with cell densities.

From table 3.2, we observe that *BRACHYURY* is differentially expressed in low and high density SW480 cells (low in high density cells and high in low density cells). *Brachyury* is the founding member of the T-box transcription factor family of genes and is involved in early mesoderm development (Wilson and Conlon, 1990). It has a conserved DNA-binding domain (Papaioannou *et al.,* 1998). Apart from its role in cellular differentiation and mesoderm formation (Willison, 1990), *Brachyury* has recently been identified as a possible target for T-cell mediated cancer immuno-therapy and was associated with a number of tumour cell types, including colorectal tumour cell lines (Palena *et al.*, 2007). This complies with our finding of *Brachyury* being expressed in SW480 cells. *Brachyury* has also been shown to be expressed in chordomas and in the embryonal carcinoma (EC) cell line NTERA2, where it was found to be expressed in the absence of mesodermal differentiation (Vujovic *et al.*, 2006; Gokhale *et al.*, 2000). However, to date no role of *Brachyury* in cancer has been described. Results from RT-PCR and qRT-PCR analysis in SW480 cells, showed that *BRACHYURY* was differentially expressed in SW480 cells. The qRT-PCR data we obtained for SW480 cells indicates that *BRACHYURY* expression is decreased by 56% as the cells progress from low to high cell density (Figure 3.4). As mentioned earlier, we also analysed the expression of the panel of genes by qRT-PCR in other colorectal cancer cell lines; the results are summarised in Figures 3.5 (HCT116), Figure 3.6 (LoVo) and Figure 3.7 (HT29).

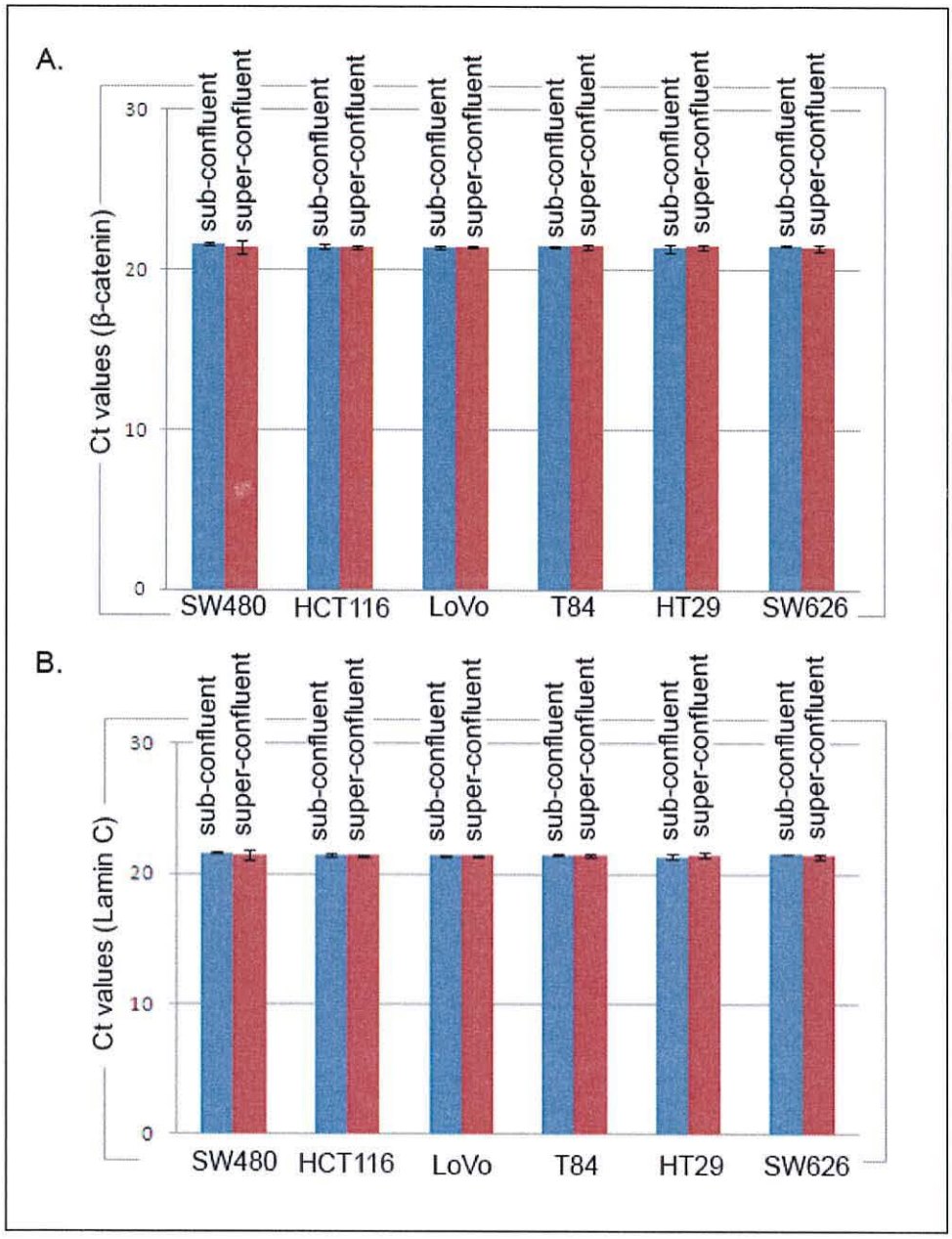


Figure 3.2: ***LAMIN C* and *β-catenin* expression levels in SW480, HCT116, LoVo, T84, HT29 and SW626 cell lines.** A) *β-catenin* expression levels are similar in all the cell lines mentioned and also in low and high density of each cell line mentioned above. The graph is obtained from Table 1A of Appendix. B) Expression levels of *LAMIN C* are also similar in all cell lines used and also in low and high density cells in each line. The graph is obtained from Table 1B of Appendix.
The error bars are obtained from the standard deviations. Each experiment was done in triplicate. The X-axis plots the different cell lines used and the Y-axis plots the average Ct values obtained from Tables 1A and B of Appendix.

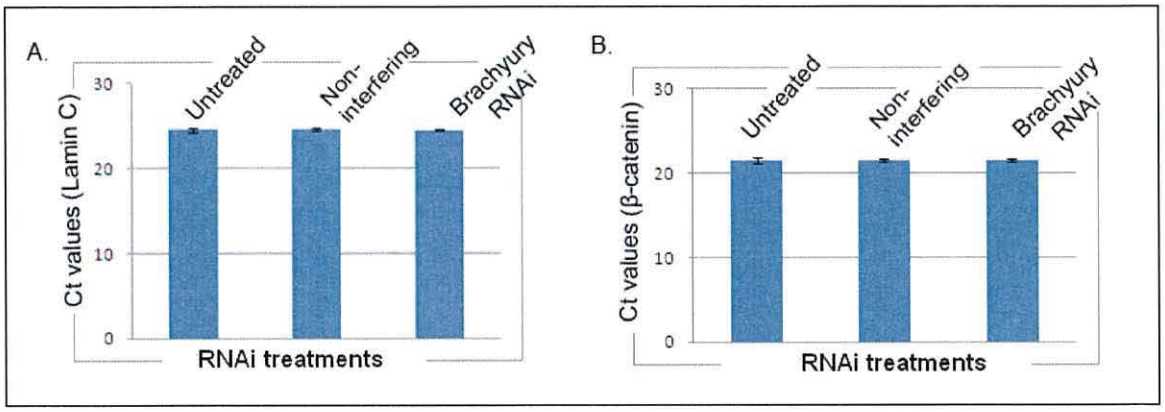


Figure 3.3: ***LAMIN C* and *ß-catenin* expression levels on *BRACHYURY* RNAi in SW480 low density cells.** A) *LAMIN C* expression levels are similar in SW480 low density cells with no RNAi treatment, cells treated with negative siRNA and on knocking down *BRACHYURY.* The graph is obtained from Tables 3.3A. B) Expression levels of *ß-catenin* are also similar in SW480 low density cells with no RNAi treatment, cells treated with negative siRNA and on knocking down *BRACHYURY.* The graph is obtained from Tables 3.3 B.
The error bars are obtained from the standard deviations. Each experiment was done in triplicate. The X-axis plots the different RNAi treatment and the Y-axis plots the average Ct values obtained from Tables 3.3 A and B. The error bars were obtained from the final standard deviations. [Note: Final SD is obtained by the formula [{(X-Y)^2+(X-Z)^2)/2}^0.5], where X is the average of two standard deviations; Y and Z are the two standards deviations]. Each experiment was done in triplicate.

Table 3.3 A

	LAMIN C	Mean Ct	S.D.
Untreated	24.437		
	24.395	24.5323	0.20
	24.765		
Non-interfering	24.53		
	24.74	24.5683	0.15
	24.435		
***BRACHYURY* RNAi**	24.37		
	24.475	24.465	0.09
	24.55		

Table: 3.3 B

	β- catenin	Mean Ct	S.D.
Untreated	21.356		
	21.2861	21.5293	0.36
	21.946		
Non-interfering	21.267		
	21.7304	21.5108	0.23
	21.535		
***BRACHYURY* RNAi**	21.3435		
	21.768	21.5168	0.22
	21.439		

Table 3.3:

qRT-PCR data to show that both *LAMIN C* and *β-catenin* were used as good controls. A) The table indicates that expression levels of *LAMIN C* does not change in SW480 cells treated with *BRACHYURY* siRNA compared to untreated cells, therefore a good control gene. B) The table indicates that expression levels of *LAMIN C* does not change in SW480 cells treated with *β-catenin* siRNA compared to untreated cells, therefore a good control gene.

A.

	SW480 subconfluent (relative expression)	S.D.	SW480 superconfluent (relative expression)	S.D.
E-CADHERIN	1	0.08	6.45	0.22
BRACHYURY	1	0.07	0.44	0.20
TBX2	1	0.05	0.49	0.14
TBX3	1	0.12	0.35	0.17
SNAIL	1	0.07	0.42	0.20
FGF8	1	0.12	0.63	0.13
NOTCH1	1	0.15	0.95	0.14
FGFR1	1	0.11	1.04	0.19
FGFR2	1	0.15	1.00	0.27
FGFR3	1	0.12	1.05	0.15
NANOG	1	0.21	0.49	0.19
ZEB1	1	0.10	0.26	0.15
CDX2	1	0.16	0.54	0.22

B.

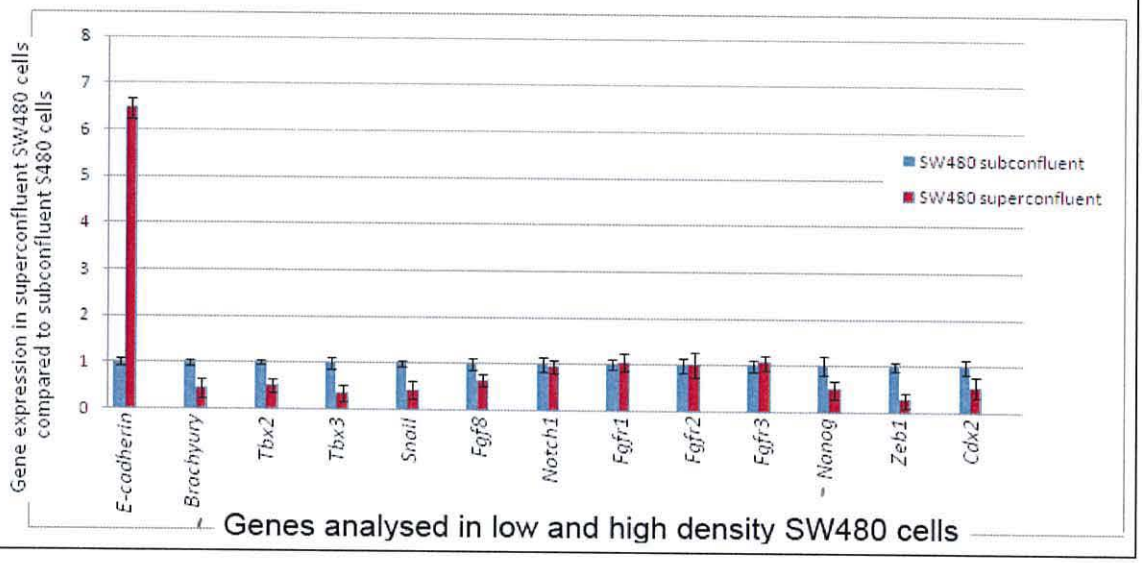


Figure 3.4
qRT-PCR data obtained for genes in SW480 cells. A) Table illustrating the qRT-PCR data for selected mesoderm inducing genes in SW480 cells grown to low and high densities. Data is represented in terms of fold increase or fold reduction compared to sub-confluent cells (taken as 1). The data was compared to LAMINC and experiment was repeated 3 times (N=3), each time in triplicate. The table shows that *TBX2, TBX3, BRACHYURY, E-CADHERIN, FGF8, NANOG, ZEB1, SNAIL* and *CDX2* are differentially expressed in low and high density SW480 cells. B) Graphical representation of fold changes in qRT-PCR values in high density SW480 cells compared to low density cells. The error bars are obtained from the standard deviation from Figure 3.2A. The raw data with along with the data working is shown in appendix Table 2. Refer appendix Table 26 for the statistical significance data of the fold differences obtained for each gene in low and high density cells.

A.

	HCT116 Subconfluent (relative expression)	S.D.	HCT116 superconfluent (relative expression)	S.D.
E-CADHERIN	1	0.18	3.44	0.12
TBX2	1	0.20	5.06	0.11
TBX3	1	0.26	0.71	0.07
SNAIL	1	0.25	0.49	0.08
FGF8	1	0.18	2.03	0.10
NOCTH1	1	0.18	1.01	0.16
FGFR1	1	0.17	0.32	0.13
FGFR2	1	0.18	0.98	0.09
FGFR3	1	0.23	1.08	0.07
ZEB1	1	0.19	1.90	0.24

B.

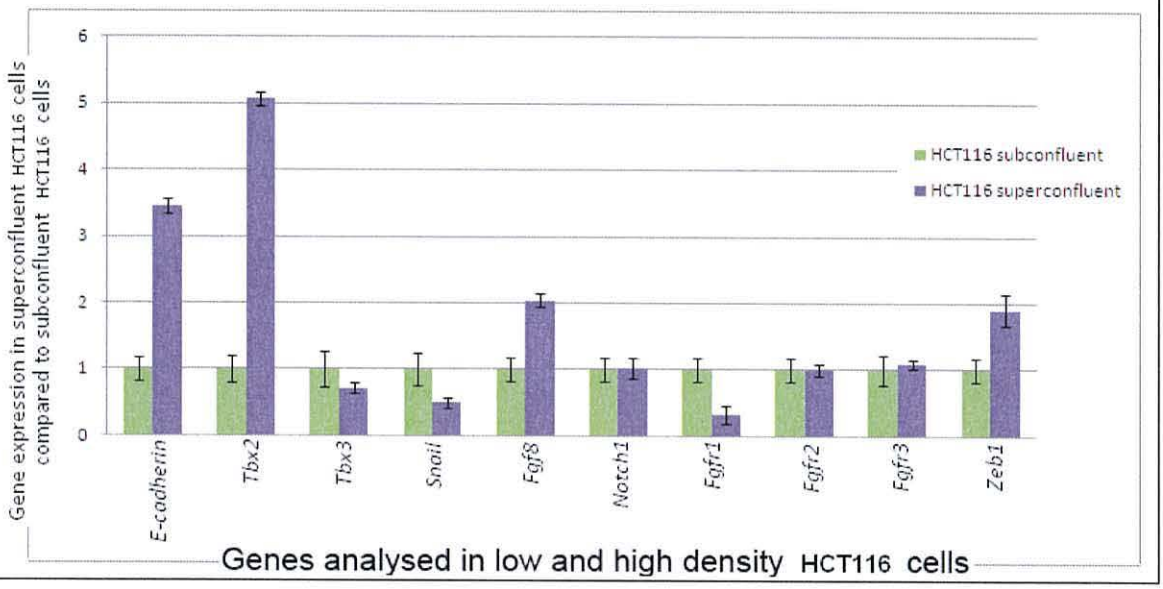


Figure 3.5

qRT-PCR data obtained for genes in HCT116 cells. A) Table illustrating the qRT-PCR data for selected mesoderm inducing genes in HCT116 cells grown to low and high densities. Data is represented in terms of fold increase or fold reduction compared to sub-confluent cells (taken as 1). The data was compared to LAMINC and experiment was repeated 3 times (N=3), each time in triplicate. *BRCAHYURY* was not examined in this screen since it was found to be absent in HCT116 cells. Genes such as *FGFR1, SNAIL, FGF8, E-CADHERIN, ZEB1, TBX2* and *TBX3* are found to be differentially expressed in low and high density HCT116 cells. B) Graphical representation of fold changes in qRT-PCR values in high density HCT116 cells compared to low density cells. The error bars are obtained from the standard deviation from Figure 3.3A. The raw data with along with the data working is shown in appendix table 3. Refer appendix Table 26 for the statistical significance data of the fold differences obtained for each gene in low and high density cells.

A.

Genes	LoVo Sub-confluent (relative expression)	S.D.	LoVo super-confluent (relative expression)	S.D.
E-CADHERIN	1	0.19	2.04	0.18
TBX2	1	0.21	2.02	0.24
NOCTH1	1	0.15	1.06	0.18
FGFR1	1	0.15	2.01	0.19
FGFR2	1	0.12	1.00	0.19
FGFR3	1	0.21	5.07	0.24

B.

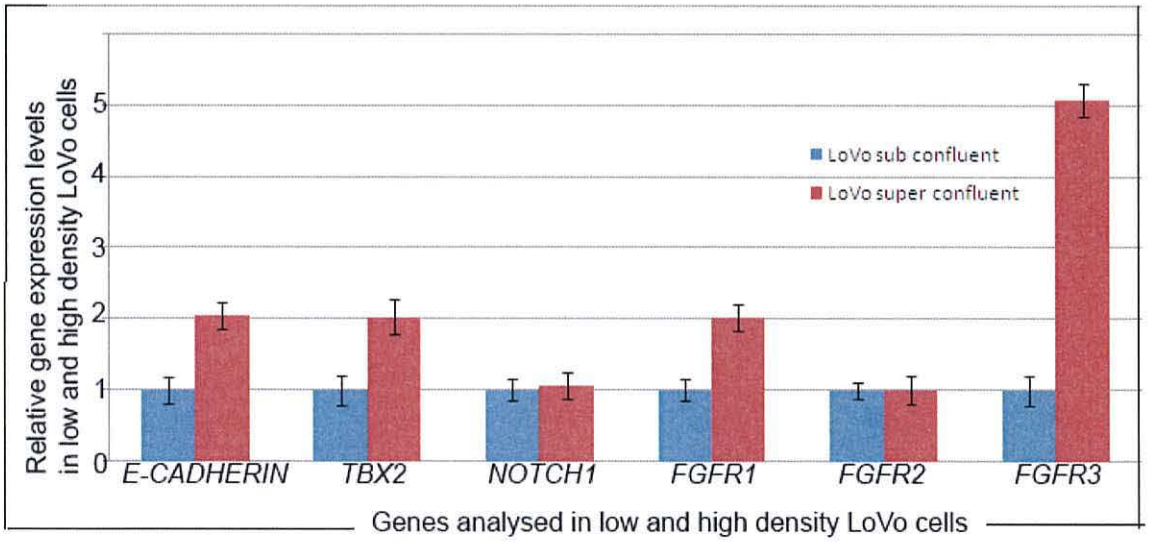


Figure 3.6

qRT-PCR data obtained for genes in LoVo cells. A) Table illustrating the qRT-PCR data for selected mesoderm inducing genes in LoVo cells grown to low and high densities. Data is represented in terms of fold increase or fold reduction compared to sub-confluent cells (taken as 1). The data was compared to LAMINC and experiment was repeated 3 times (N=3), each time in triplicate. Genes such as *FGFR1, FGFR3, E-CADHERIN* and *TBX2* are found to be differentially expressed in low and high density LoVo cells. B) Graphical representation of fold changes in qRT-PCR values in high density LoVo cells compared to low density cells. The error bars are obtained from the standard deviation from Figure 3.4A. The raw data with along with the data working is shown in appendix table 4. Refer appendix Table 26 for the statistical significance data of the fold differences obtained for each gene in low and high density cells.

A.

Genes	HT29 Subconfluent (relative expression)	S.D.	HT29 Superconfluent (relative expression)	S.D.
E-CADHERIN	1	0.17	2.09	0.08
TBX2	1	0.18	4.42	0.21
NOCTH1	1	0.22	0.94	0.10
FGFR1	1	0.20	3.70	0.10
FGFR2	1	0.24	2.11	0.12
FGFR3	1	0.18	0.99	0.08
ZEB1	1	0.19	0.63	0.13

B.

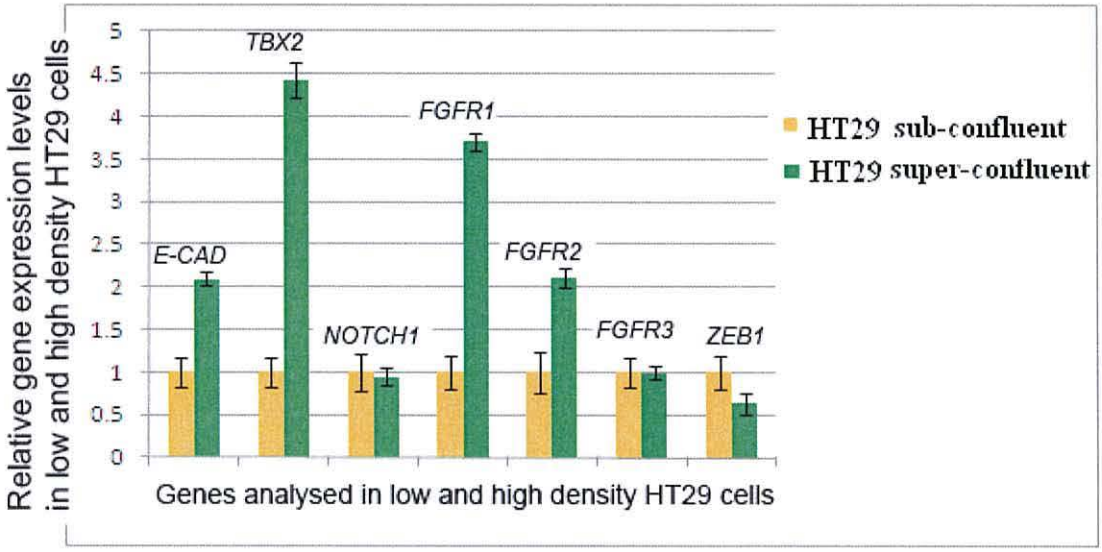


Figure 3.7

qRT-PCR data obtained for genes in HT29 cells. A) Table illustrating the qRT-PCR data for selected mesoderm inducing genes in HT29 cells grown to low and high densities. Data is represented in terms of fold increase or fold reduction compared to sub-confluent cells (taken as 1). The data was compared to LAMINC and experiment was repeated 3 times (N=3), each time in triplicate. Genes such as *FGFR1, FGFR2, E-CADHERIN, ZEB1*and *TBX2* are found to be differentially expressed in low and high density HT29 cells. B) Graphical representation of fold changes in qRT-PCR values in high density HT29 cells compared to low density cells. The error bars are obtained from the standard deviation from Figure 3.5A. The raw data with along with the data working is shown in appendix table 5. Refer appendix Table 26 for the statistical significance data of the fold differences obtained for each gene in low and high density cells.

b) Localization of BRACHYURY in SW480 cells

We focused our further studies on *BBRACHYURY* and determining its possible role in EMT in CRC cells. This was based on the following: 1) *Brachyury* had not previously been shown to be expressed in CRCs, its expression had been shown in chordomas and germ cell tumours. However, during this study, another group, Palena and co-workers (2007) showed that *Brachyury* was expressed in some of the CRC cell lines. The study by Palena and co-workers therefore further validated our screen. 2) *Brachyury* is a key mesoderm inducing gene during development and might have a possible role in inducing EMT during cancer. 3) From our RT-PCR data, we observed that SW480 cells were the only CRC cell line that expressed *Brachyury.* Also, SW480 cells were the only cell line we studied that shows features of cells undergoing EMT with respect to its cell density variation.

Knowledge of the localization of proteins in a cell is often key to our understanding of its functions. Futhermore, co-localization patterns allow us to determine the likelihood of one protein interacting with another. To understand the role of *Brachyury* in inducing EMT, we used indirect immunofluorescence (IF) in SW480 cells grown to different densities (see section 2.15 for IF protocol). Figure 3.8 shows the localization of BRACHYURY in these cells as determined by confocal microscopy. Since, BRACHYURY is a transcription factor, it is expected to be localised to the nucleus of the cells. Figure 3.6 shows that BRACHYURY is located in the nucleus of low density SW480 cells. However, in high density SW480 cells, very few cells are positive for BRACHYURY and those cells that are positive also have nuclear localization. These immunofluorescence results correlate with the qRT-PCR data showing high expression in low density cells but low expression of *BRACHYURY* in high density SW480 cells.

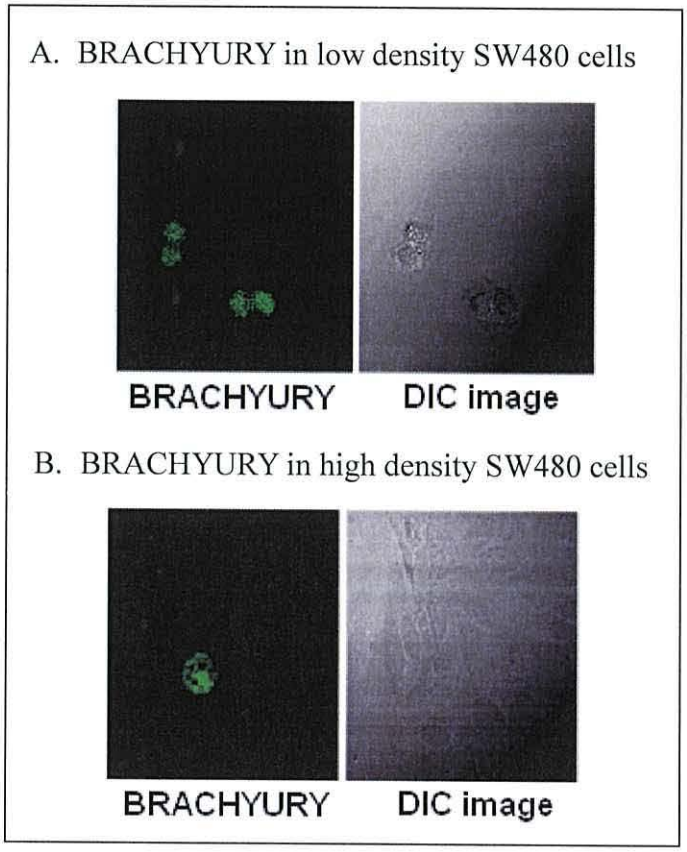


Figure 3.8

Confocal microscopy image of BRACHYURY localization in low and high density SW480 cells. A) BRACHYURY is located to the nucleus (green) of almost all cells in low density SW480 cells, confirming its nuclear localization and therefore, a transcription factor. B) In high density SW480 cells, very few cells positive for BRACHYURY (green). The DIC image shows the cells without any staining. This shows the differential expression of BRACHYURY in low and high density SW480 cells.

3.5 Discussion:

A common theme has emerged over the last few years concerning genes that give rise to cancer, that is, many of these genes involved in initiation or maintenance of cancer are actually developmental genes that become mis-expressed. One of key developmental processes involved in normal embryogenesis is EMT. But, it has also been shown that EMT is also active with the similar phenomenon in many forms of cancer, colorectal cancer being one of them. Our study and this chapter in particular, aimed to identify genes, from a limited group of genes that are involved in embryogenesis that might be involved in regulating the process of EMT in CRC. Changes in the cell density of the CRC cell line SW480 resemble the cells transitioning between mesenchymal-like and epithelial-like states. Our data show that mesoderm inducer, *BRACHYURY*, is differentially expressed in low and high density SW480 cells. *BRACHYURY* is highly expressed in the low density SW480 cells which are mesenchymal-like and representative of cells that are found at the invasive fronts of a tumour. Conversely, expression of *BRACHYURY* is very low in the high density SW480 cells which are epithelial-like and representative of cells at the central mass of a tumour. We found a number of other developmental genes which were also differentially expressed in SW480 and other CRC cell lines. We focused our studies on *BRACHYURY* because it plays a critical role in embryogenesis and it is not been extensively studied in cancer. Recent studies also showed (Palena, 2007) that *Brachyury* is present in SW480 cells, further validating our results. Human *BRACHYURY (T)* is located on human chromosome 6q27. This also makes it important to study because chromosome 6 is associated with colorectal cancers (Ezaki 2003). Presumably this should be mutation of or deletion of chromosome 6. Also, since chromosome 6 represents 5-6% of the DNA of cells, it is a possible site for many significant genetic alterations.

IF studies showed that BRACHYURY is present in the nucleus of all low density SW480 cells, but is present at low frequency in high density SW480 cells. We tested 4 CRC cell lines in total, but only one, SW480, expressed *BRACHYURY* leading us to suggest that expression of BRACHYURY is

associated with the ability of the cells to undergo EMT. Furthermore, SW480 cells showed a gene expression profile that was representative of cells undergoing EMT (in contrast to the other cell lines studied). The following chapters will focus on the possible role of *BRACHYURY* in colorectal cancer.

Further studies related to this screen, might involve work on the candidates *TBX2* and *TBX3* genes. The expression of transcription factor *TBX2* is amplified in pancreatic (Chen *et al.,* 2008) and breast cancer (Sinclair *et al.*, 2002). Its related factor TBX3 is also over-expressed in breast cancer. TBX2 is known to maintain proliferation in melanomas (Vance *et al.*, 2005). TBX3 is found to be a downstream target of the Wnt pathway and also shown to be expressed in the human CRC cell lines SW480 and HCT116 (Renard *et al.*, 2007). It is also involved in maintaining proliferation in liver cancers (Suzuki *et al.*, 2008).

CDX2 (caudal related homeobox-2) could be another interesting member to be studied in SW480 cells. *CDX2* is often mutated in rare cases of CRC and it is also a marker of CRC. It is critical for maintenance of intestinal epithelial cells (Duluc *et al.,* 1997; Lorentz *et al.,* 1997). It also has tumourigenic potential in CRC cell lines SW480 and LoVo (Dang *et al.*, 2006).

As mentioned earlier, we were searching for developmental genes those are differentially expressed in low and high densities of SW480 and other colon cancer cell lines. Therefore, differential expression of such genes (mentioned in this chapter) would be interesting to study further which might be involved in EMT and thus in progression of cancer.

Chapter 4 - Determining the role of *BRACHYURY* in SW480 cells

a) Does *BRACHYURY* play a role in inducing EMT in the colorectal cancer cell line SW480?
b) Role of *BRACHYURY* in regulating *NANOG* and maintaining a putative cancer stem cell population
c) A role for *BRACHYURY* in regulating the NFκB pathway?
d) A role for *BRACHYURY* in cell proliferation?
e) A role for *BRACHYURY* in cell migration?

a) Does *BRACHYURY* play a role in inducing EMT in the colorectal cancer cell line SW480?

In Chapter 3, we have identified the differential expression of a key mesoderm inducing gene, *BRACHYURY,* from a screen of expressed developmental genes in the CRC cell line SW480. Specifically *BRACHYURY* was found to be relatively highly expressed in the low density mesenchymal-like cells (resembling the invasive fronts of a tumour) whereas its expression was very low in high density epithelial-like cells (resembling the central mass of a tumour). In this chapter, we further studied *BRACHYURY* to elucidate its function in SW480 cells.

4.1.1 A role for *BRACHYURY* in inducing EMT?

EMT is an important process for growth of a tumour and metastasis (Boyer *et al.*, 2000). During this phenomenon, the cells undergo a transition from the epithelial phenotype to a highly motile mesenchymal phenotype (Huber *et al.,* 2005). E-CADHERIN is a member of the classic cadherin family and is a key component of the cell-junction adhesion complex which is critically altered during EMT (Tomita *et al.,* 2000, Wang *et al.,* 2006). Most inducers of EMT found to date are transcription factors that repress the cell-junction adhesion complex, E-CADHERIN, for example, *Snail* (Bolos *et al.,* 2003*), Slug* (Barrallo *et al.*, 2003) and *Twist* (Karreth *et al.,* 2004). A reduced level of E-CADHERIN also has been seen in various cancer metastases (Huber *et al.,* 2005).

Loss of E-CADHERIN expression is a hallmark of EMT along with the translocation and accumulation of β-catenin in the nucleus of cells (Hirohashi *et al.,* 2003). β-catenin is a central component of the Wnt signalling pathway and is also a subunit of the cadherin–adhesion complex. β-catenin has shown to be involved in EMT in normal development (such as in development of the heart)

(Liebner *et al.*, 2004) and also in the development of many cancers such as prostate cancer (Jiang *et al.,* 2007).

Based on our qRT-PCR data (Chapter 3), we predicted that *BRACHYURY* might play a crucial role in the process of EMT in SW480 cells. Therefore, we investigated whether *BRACHYURY* might be a direct inducer of EMT. For this, we investigated the following features of SW480 cells, following RNAi induced knock-down of *BRACHYURY.*

i) Localisation of β-catenin and E-CADHERIN
ii) mRNA levels of *E-CADHERIN*

<u>i) Localisation of β-catenin and E-CADHERIN in response to *BRACHYURY* knock-down</u>

We performed a transient knock-down of *BRACHYURY* in SW480 cells (protocol in materials and methods section 2.17), followed by immunofluorescence (according to the protocol in Materials and Methods section 2.15) and the results were viewed by confocal microscopy. RNAi sequence for *BRACHYURY* knock-down is described in Table 2.10 of materials and methods.

If BRACHYURY is a direct inducer of EMT we might expect that knocking down *BRACHYURY* expression would affect the two key indicators of EMT, that is β-catenin localisation (a nuclear to cytoplasmic shift would be expected following RNAi in low density SW480 cells), and localisation of E-CADHERIN would be expected to change (E-CADHERIN re-localisation to membrane following *BRACHYURY* RNAi). The levels of *BRACHYURY* mRNA and protein were low following *BRACHYURY* RNAi (62% knock-down) as determined by qRT-PCR and Western blot analysis, respectively (Figure 4.1), confirming the success of the knock-down. [**Note**: Figure 4.1A is derived from Table 6 of appendix]. We found no change in β-catenin localisation from cytoplasm to the nucleus of the SW480 cells, nor in the localization and levels of E-CADHERIN following *BRACHYURY* knock-down; suggesting that *BRACHYURY* might not be a direct inducer of EMT in SW480 cells (Figure 4.2).

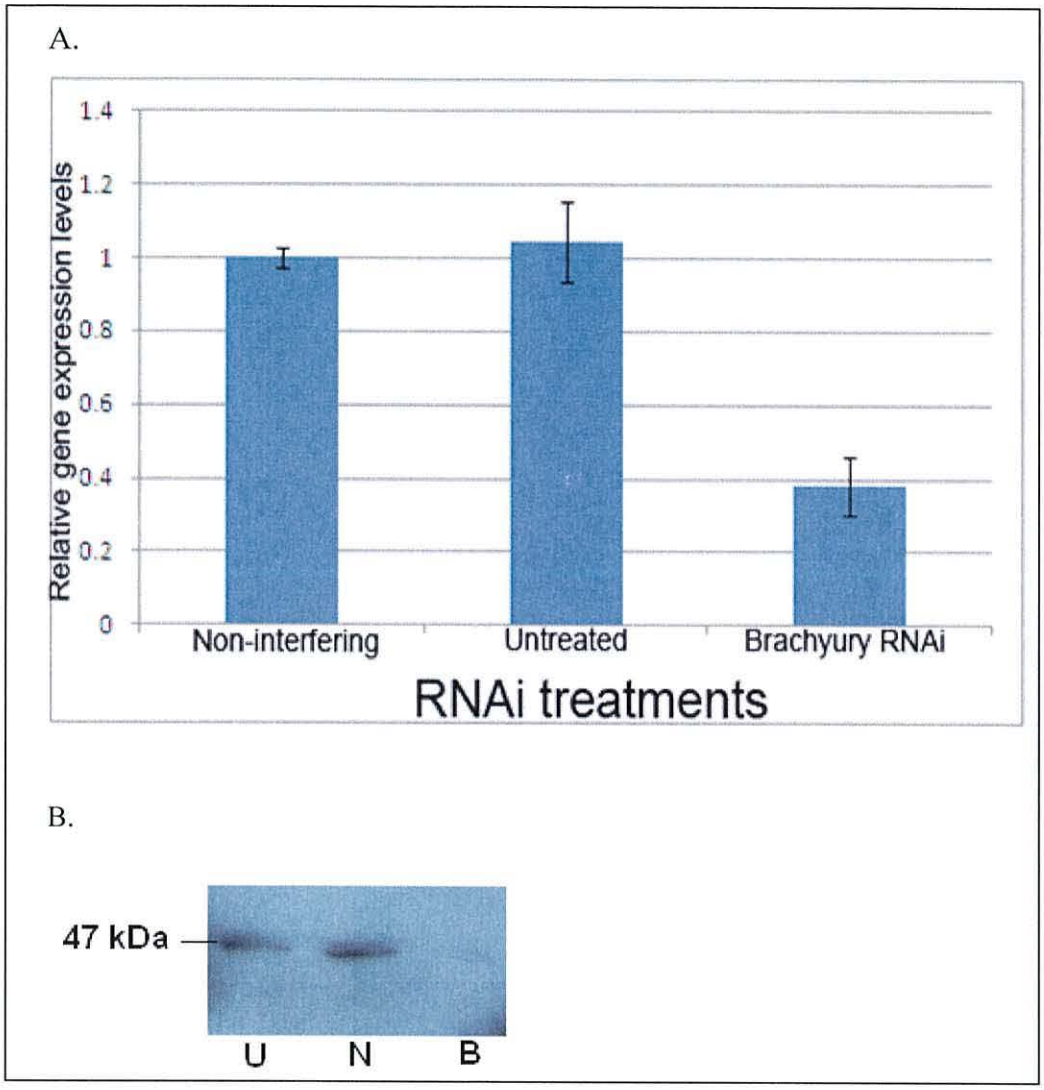


Figure 4.1:

BRACHYURY RNAi in SW480 cells. A) Graph showing knock-down of *BRACHYURY* by qRT-PCR. Knock-down level was 62% (calculated by qRT-PCR). Raw data is detailed in appendix table 6. The X-axis gives the different RNAi conditions and the Y-axis gives the relative expression levels. B) Western blot analysis showing the reduction of BRACHYURY protein confirming the knock-down. U= cells not treated with siRNA; N=cells treated with non-interfering siRNA; B=cells treated with BRACHYURY RNAi.

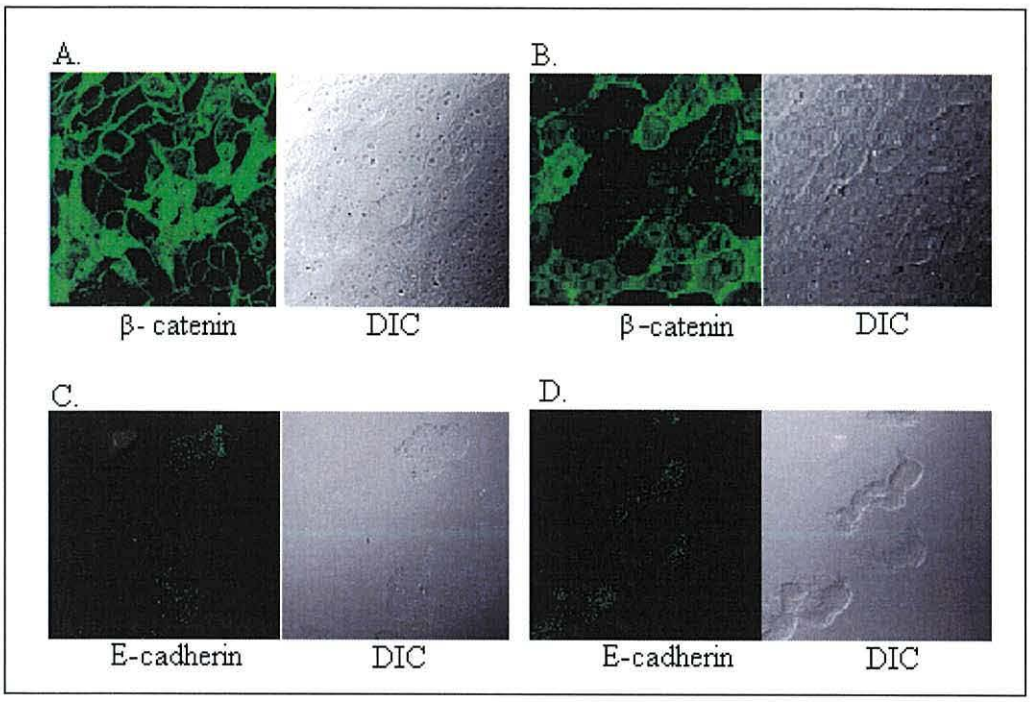


Figure 4.2:

Confocal microscopy showing β-catenin and E-CADHERIN localisation following *BRACHYURY* knock-down. A) Localistaion of β-catenin (green) is mostly at the cell membranes following *BRACHYURY* knock-down in super-confluent SW480 cells. B) Membrane localisation of β-catenin in control SW480 cells that have not been treated with RNAi BRACHYURY. C) In low density SW480 cells, E-CADHERIN levels (green) are low following *BRACHYURY* knock-down and is localised to the cell junctions. (D) E-CADHERIN localised to the cell junctions in low levels in sub-confluent SW480 cells not treated with *BRACHYURY* siRNA. Therefore, this figure shows no change in sub-cellular localisation of E-CADHERIN and β-catenin in response to *BRACHYURY* RNAi, confirming that *BRACHYURY* is not directly involved in EMT.
Note: Levels of *BRACHYURY* knock-down was determined by qRT-PCR to be 69%. The DIC images show the cells without any staining.

ii) Effect of *BRACHYURY* knock-down on expression of *E-CADHERIN* by qRT-PCR in SW480 sub-confluent cells

In the section above, we showed that *BRACHYURY* does not have an effect on two important hallmarks of EMT (β-catenin and E-CADHERIN localisation). We next determined a further indicator of EMT, the expression levels of *E-CADHERIN* following *BRACHYURY* RNAi, as assessed by qRT-PCR in SW480 sub-confluent cells. We show that *BRACHYURY* knock-down does not alter the expression of *E-CADHERIN* when compared to control cells treated with non-interfering siRNA and also untreated cells (Figure 4.3). LAMIN C was used as a control to compare the data (refer section 2.13 of materials and methods for its validation). Final data for Figure 4.3A is obtained in a similar manner as mentioned in material and methods section 2.13. Thus, we concluded that the levels of *E-CADHERIN* gene expression do not change effectively following *BRACHYURY* knock-down. [Note: *BRACHYURY* knock-down level is 64%]. Together, these results suggest *BRACHYURY* does not have a direct effect on inducing EMT in SW480 CRC cells. We, therefore explored the possible effects of *BRACHYURY* on four different categories of genes in low density SW480 cells, as described in the following sections.

4.1.2: The qRT-PCR screen to study the possible effects of *BRACHYURY* on four different categories of genes:

Data so far suggests that *BRACHYURY* may not be a direct inducer of EMT since knock-down of *BRACHYURY* does not have a direct effect on either E-CADHERIN or β-catenin expression and localisation. Therefore, as a next step to elucidate the possible function of *BRACHYURY* in SW480 cells, we studied the effect of *BRACHYURY* on the expression of following four categories of genes:

- Markers of epithelial cell polarity
- *CDX2* (marker of intestinal epithelium)
- Known gene targets of *BRACHYURY*
- Developmental genes which are inducers of EMT

A.

RNAi conditions	*E-CADHERIN (Relative expression)*	*BRACHYURY (Relative expression)*
Non-interfering	1±0.06	1±0.06
Untreated	1.05±0.12	1.06±0.12
Brachyury RNAi	0.98±0.30	0.36±0.15

B.

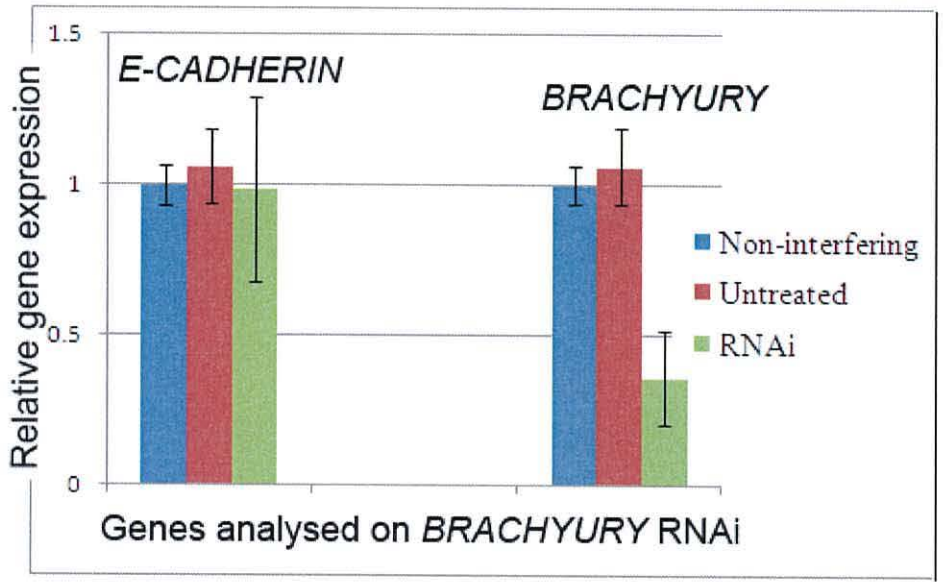


Figure 4.3

E-CADHERIN and *BRACHYURY* expression following *BRACHYURY* knock-down in SW480 sub-confluent cells by qRT-PCR. A) Table showing expression of *E-CADHERIN* and *BRACHYURY* following knock- down of *BRACHYURY* as assessed by qRT-PCR and compared to LAMIN C. *BRACHYURY* RNAi does not show any effective change on levels of E-CADHERIN. *BRACHYURY* knock-down level obtained is 64%. B) Graphical representation of Figure 4.3A showing that *BRACHYURY* RNAi has no effect on expression of *E-CADHERIN* (2% reduction). The Y-axis gives the relative expression levels and the X-axis mentions the genes analysed under different RNAi conditions. Raw data and the calculations are shown in Table 7 of appendix. Experiment was repeated 3 times (N=3), each experiment was done in triplicate. After ±, the final standard deviation is mentioned that gives the error bars in the graph.

4.1.2A: Exploring a role for *BRACHYURY* in influencing the expression of epithelial cell polarity markers:

As previously mentioned, during the process of EMT, epithelial cells lose their cell polarity and cell junction molecules and obtain a fibroblastic phenotype.

Zeb1 is a key transcription factor that is up-regulated during EMT (Liu *et al.*, 2008), that disrupts the cell polarity by suppressing the expression of tight junction molecules such as *Lgl2, Crumb3* (Crb3), *HUGL2* and *PATJ (Pals1*-associated tight junction protein), (Aigner *et al.*, 2007; Spaderna *et al.*, 2008). *Snail*, a repressor of *E-CADHERIN*, also inhibits the expression *Crumb3* and disrupts apical cell polarity complex (Whiteman *et al.*, 2008). *Eva1* (Epithelial V-like antigen. *Eva1* is an epithelial cell polarity marker and is a transmembrane glycoprotein. *Crb3* are transmembrane proteins (Bachmann *et al.*, 2001) which are linked to *Pals1*, a membrane associated guanylate kinase protein (Kamberov *et al.*, 2000). *Zeb2* (Zinc finger E-box binding homeobox 2) (also known as *SIP1*), is another marker that represses various epithelial cell–cell junction genes thereby inducing EMT (Vandewalle *et al.*, 2005).

We looked at the expression of some of these genes, as markers for cells undergoing EMT following *BRACHYURY* RNAi. We observed no change in the expression levels of these marker genes of cell polarity following knock-down of *BRACHYURY* in SW480 sub-confluent cells (Figure 4.4). **Note:** Raw data for the Figure 4.4 is presented in Table 8 of the appendix.

Data from Figure 4.4 shows that none of the cell polarity markers mentioned changes their expression levels in response to *BRACHYURY* knock-down. This suggests that *BRACHYURY* does not regulate genes involved in cell polarity and EMT and confirms our observations in the previous section. This data is graphically represented in Figure 4.4B. We also carried out similar *BRACHYURY* knock-down experiments in high density SW480 cells. In high density SW480 cells, knock-down of *BRACHYURY* resulted in a slight increase

A.

RNAi treatments	*ZEB1* (Relative expression)	*ZEB2* (Relative expression)	*EVA1* (Relative expression)	*PALS 1* (Relative expression)	*CRB3* (Relative expression)
Non-interfering	1±0.13	1±0.19	1±0.19	1±0.15	1±0.31
Untreated	1.06±0.19	1.13±0.02	1.02±0.02	1.08±0.21	1.12±0.10
Brachyury RNAi	1.20±0.06	1.02±0.18	1.11±0.18	1.16±0.07	1.19±0.04

B.

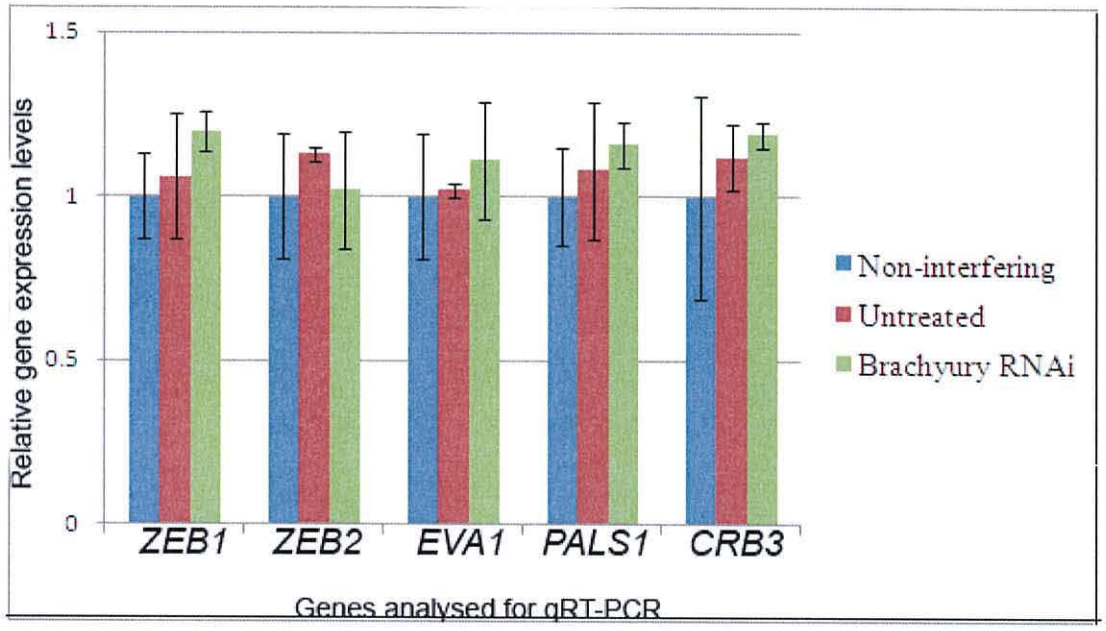


Figure 4.4

qRT-PCR showing expression of EMT markers in low density SW480 cells following knock down of *BRACHYURY*. A) Table demonstrating the expression of these markers do not alter following knock-down of *BRACHYURY*. B) Graphical representation of Table A, showing no effect of the cell polarity marker genes in response to *BRACHYURY* RNAi in low density SW480 cells. The X-axis mentions the genes analysed by qRT-PCR under different RNAi conditions and the Y-axis gives the relative gene expression levels. Experiment was repeated 3 times (N=3), each done in triplicate. All data were compared to *β-catenin*. The raw data is shown in appendix Table 8.

in expression of *ZEB1* increased by 1.2 times. But, since the p-value for this reduction was >0.05, the increase in *ZEB1* expression was not considered significant. (Refer to appendix Table 25 for the p-values). *PALS1* expression increased by 1.02 times (p-value>0.05; no significant reduction); *ZEB2* expression was increased by 1.11 times, *EVA1* by 1.16 times and *CRB3* expression increased by 1.19 times. But the p-values obtained for these expression levels were >0.05, thus were not considered statistically significant difference in gene expression. The biological significance of such changes in high density cells is likely to be complex and was not studied further since the focus of our experiments was on low density cells where *BRACHYURY* expression was high. [Note: Data for *BRACHYURY* RNAi on high density SW480 cells is not presented because, level of BRACHYURY in high density SW480 cells is very little; knocking down which will not give any effective data].

4.1.2B: Effect of *BRACHYURY* on the expression of a marker of intestinal epithelium

We next explored the expression of the key marker of intestinal epithelium (*CDX2,* Caudal type homeobox transcription factor 2) in response to *BRACHYURY* RNAi by qRT-PCR. *CDX2* is a developmental gene which is involved in the formation of the placenta in mice (Chawengsaksophak *et al.*, 2004) and is also a biomarker of gastrointestinal differentiation, especially colorectal cancer (Liu *et al.*, 2007). In 2004, De Lott and co-workers studied tissue microarrays of 71 colorectal adenocarcinomas, 71.8% of which were *CDX2* positive. 74.5% of these positive tumours were differentiated tumours and 65% were high grade tumours. In contrast, when other carcinomas were studied 1 out of 47 were positive for *CDX2* staining (lung adenocarcinoma) and 2 out of 43 were positive for *CDX2* (pancreatic carcinoma). Therefore, it was concluded that *CDX2* is a critical intestinal marker though it is not that sensitive for those colorectal carcinomas which are not well-differentiated.

Figure 4.5 shows the effect of *BRACHYURY* knock-down by RNAi on expression of *CDX2*, as assessed by qRT-PCR. We show that the expression of the intestinal epithelial marker, *CDX2,* is reduced by 74% in response to *BRACHYURY* RNAi in low density but not in high density SW480 cells.

[**Note**: Figure 4.5 is obtained from appendix table 9]. This may suggest that *BRACHYURY* may play a role in regulating *CDX2* at the invasive fronts but not at the central mass of the tumour.

Invasive fronts of CRC undergo a loss of basement membrane and activation of transcription factors related to EMT. This is the main reason for distant metastasis (Conacci-Sorrell *et al.*, 2003; Franci *et al.*, 2006; Spaderna *et al.* 2006). In contrast to what we might predict from our data, *CDX2* expression was shown to be decreased in invasive cells at the stromal front of differentiated CRC (Brabletz *et al.*, 2004). In contrast to the studies of Brabletz and co-worker, and in support of our *in vitro* studies, Gross and co-workers (2008) showed that expression of *CDX2* is high at the invasive front and its expression opposes cell migration and dissemination in colorectal tumours. We therefore thought it is relevant to investigate a possible role of *BRACHYURY* in influencing colon cancer cell migration. This is further described in section 4.5 of this chapter.

4.1.2C: Role of BRACHYURY in regulating expression of known target genes

In this section, selected known target genes of BRACHYURY were studied in response to *BRACHYURY knock-down* by RNAi. *Orthopedia (otp)* in *Drosophila* is regulated by its *BRACHYURY* homologue Brachyenteron (Byn) (Simeone *et al.*, 1994; Singer *et al,.* 1996). In *Drosophila*, Byn is involved in development of the hindgut and also to a certain extent in midgut development (Kispert *et al.* 1994; Singer *et al.,* 1996; Kusch and Reuter 1999). O*tp* was shown to be a direct target of Byn (Kusch *et al.*, 2002). The *cis*-regulatory region of *otp*

A.

RNAi treatments	Average fold difference (Relative *CDX2* expression)	Final SD
N.I.	1	0.14
Untreated	1.06	0.22
Brachyury RNAi	0.26	0.04

B.

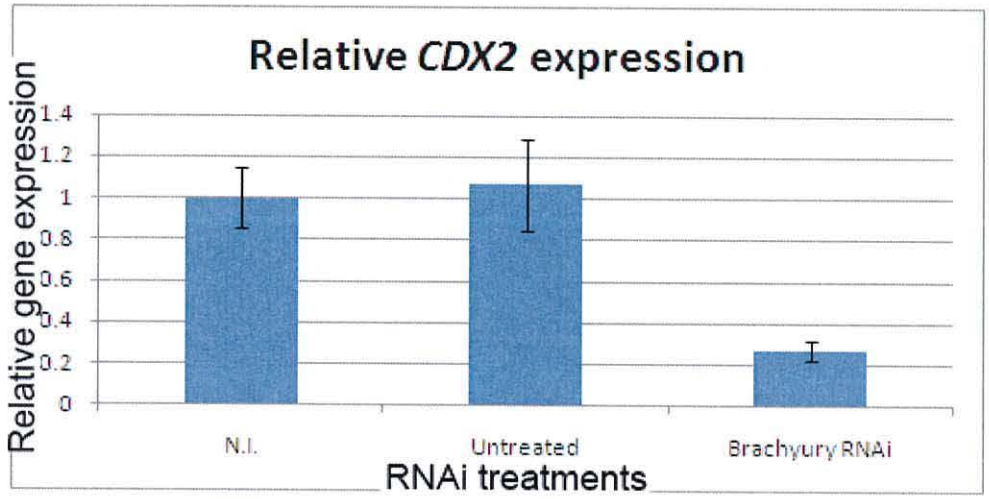


Figure 4.5

Expression of *CDX2* following *BRACHYURY* RNAi by qRT-PCR in low density SW480 cells. A) Table showing that *CDX2* expression is reduced by 74% following *BRACHYURY* RNAi in sub-confluent SW480 cells. The CT value for CDX2 was compared to CT value of *LAMIN C*. The p-value for this reduction was 0.00016, therefore may be considered a significant reduction. B) Graphical representation of Table 4.5A along with error bars (obtained from SD). The X-axis mentions different RNAi conditions for *CDX2* and the Y-axis gives the relative *CDX2* expression levels.
Experiment was done 3 times (N=3); each experiment done in triplicate.
Raw data is detailed in Table 9 of appendix.

contains multiple binding sites of high (type A) and of low (type B) affinity to *Byn*.

In *Xenopus*, *BRACHYURY* (*Xbra*) is required for mesoderm formation and notochord development (Smith *et al.*, 1991). *Xwnt11* was shown to be a direct target of Xenopus BRACHYURY (Masazumi *et al.*, 2000). Wnt11 is a non-canonical Wnt (i.e. Wnt family member activating other pathways) has been shown to have increased expression in many prostate tumours and during prostate cancer progression (Zhu *et al.*, 2004). As summarised by Katoh and Katoh (2009), Wnt11 is also expressed and up-regulated in many other human cancers such as breast cancer, gastric cancer, esophageal cancer, primary colorectal cancer, neuroblastoma and Ewing sarcoma.

Expression of *IL-2* and *IL-15* were shown to be positively regulated by *BRACHYURY,* in mouse (Baldassarre *et al.*, 2001). *IL-2* and *IL-15* (structurally similar to *IL-2*) are two cytokine immune system signalling molecules and are widely studied in cancer cells. Both *IL-2* and *IL-15* can upregulate stimulatory molecules of chronic leukaemia cells (Spaner *et al.*, 2004; Brentjens *et al.*, 2003). In human, *IL-15* is expressed in metastatic colorectal cancer, activate NK cells and put forth an anti-tumour effect. It also causes growth of colon cancer cells by metastasis and tumour progression (Kuniyasu *et al.*, 2003).

NANOG is a key gene involved in the maintenance of pluripotency in stem cells. In mouse, NANOG has *BRACHYURY* and *Stat3* binding sites *in vivo* (Suzuki *et al.*, 2006) which regulates the expression of *NANOG*. *NANOG* has been shown to be expressed in a number of cancers such as primordial germ cell tumours (where it is involved in malignant cell transformation of gonads), osteosarcoma and lung cancer (Nirasawa *et al.*, 2009). *NANOG* was also shown to be expressed in human breast adenocarcinoma cell line, MCF-7 (Hart *et al.*, 2005; Ezeh *et al.*, 2005). *NANOG* has been shown to be a direct target of Tcf3 (Pereira, 2006) and Tcf3 interacts with β-catenin in the Wnt pathway (Korinek, 1998).

In this section, we investigated the role of *BRACHYURY* in regulating selected target genes in SW480 sub-confluent cells. Knock-down of *BRACHYURY* in low density SW480 cells has the following effects (refer Figure 4.6): *WNT11* expression was reduced by 70% , *NANOG* expression reduced by 85%, *Orthopedia* by 75%, *IL2* by 74% and *IL15* by 64%. The p-value <0.05 for all these expressions; therefore these fold differences were considered statistically significant. [**Note**: p-values mentioned are significance of the gene knock-down compared to non-interfring siRNA treated cells. Refer appendix table 25 for the p-values obtained]. Based on these results, it seemed interesting to study some of these genes further. Specifically, we focussed our studied on the potential regulation of *NANOG* by *BRACHYURY* and this will be discussed in a subsequent section (section 4.2.1).

[**Note**: Figure 4.6 is obtained from appendix table 10)

4.1.2D Effect of *BRACHYURY* RNAi on inducer of EMT, *E-CADHERIN*, *SNAIL* and T-box genes, *TBX2* and *TBX3*

In this section, the expression levels of *E-CADHERIN*, *SNAIL* and the T-box developmental genes (*TBX2* and *TBX3*) were studied in response to transient *BRACHYURY* knock-down in low density SW480 cells. We analysed expression of *TBX2* and *TBX3* in response to *BRACHYURY* RNAi because *BRACHYURY* is a T-box gene and we wanted to analyse the effect of BRACHYURY in other T-box genes such as *TBX2* and *TBX3*. We analysed expression of *SNAIL* since *Snail* is a negative regulator of *E-CADHERIN*. The expression of T-box gene *TBX2* was shown to be reduced by 59% and *TBX3* expression was shown to be reduced by 57%, in response to *BRACHYURY* knock-down (Figure 4.7). P-value for these expressions were shown to be <0.05 and were considered statistically significant results.

Once again, *E-CADHERIN* expression was analysed along with the expression of *SNAIL,* which is a negative regulator of *E-CADHERIN,* both of which have shown to have no change in expression in response to *BRACHYURY* RNAi in low density SW480 cells (Figure 4.7). The p-values for these changes

A.

RNAi treatments	*WNT11* (Relative expression)	*NANOG* (Relative expression)	*ORTHOPEDIA* (Relative expression)	*IL2* (Relative expression)	*IL15* (Relative expression)
Non-interfering	1±0.30	1±0.15	1±0.15	1±0.19	1±0.30
Untreated	1.24±0.11	1.23±0.21	1.28±0.16	1.00±0.20	1.13±0.11
Brachyury RNAi	0.30±0.22	0.15±0.13	0.25±0.09	0.26±0.13	0.36±0.22

B.

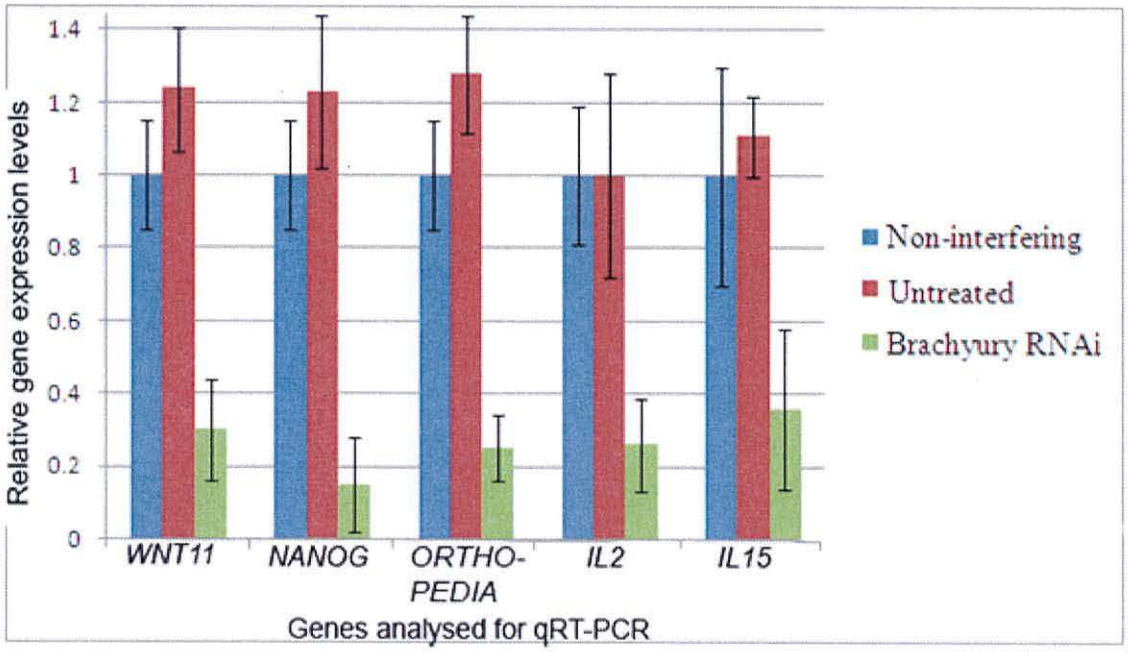


Figure 4.6

Effect of *BRACHYURY* RNAi on target genes of *BRACHYURY* in SW480 sub-confluent cells as assesed by qRT-PCR. A) Table demonstrating the relative gene expression in response to *BRACHYURY* knock-down on its target genes. B) Graphical representations for Figure 4.6A showing reduction in expression of these target genes on *BRACHYURY* RNAi. The X-axis mentions the genes analysed by qRT-PCR under different RNAi conditions and the Y-axis gives the relative gene expression levels.
All data are compared to *LAMIN C.* Experiments were done 3 times (N=3), each in triplicate. Raw data for this figure is detailed in appendix table 10.
The final SD is mentioned after ±

A.

	TBX2 (Relative expression)	*TBX3* (Relative expression)	*E-CADHERIN* (Relative expression)	*SNAIL* (Relative expression)
Non-interfering	1±0.33	1±0.11	1±0.05	1±0.15
Untreated	1.04±0.26	1.16±0.18	1.07±0.21	1.07±0.23
Brachyury RNAi	0.39±0.06	0.43±0.12	1.08±0.16	1.05±0.08

B.

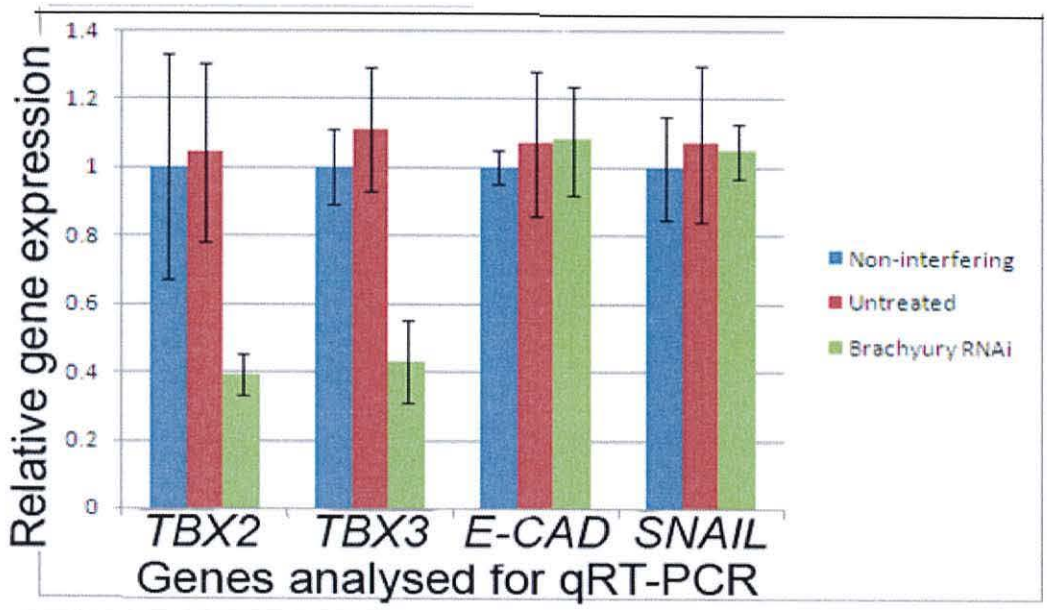


Figure 4.7:

Expression of developmental genes in response to *BRACHYURY* RNAi in SW480 low density cells: A) Table demonstrating the relative expression level of the genes. B) Graphical representations of Table A showing reduction in expression of T-box genes on *BRACHYURY* knock-down in low density SW480 cells but not alteration in expression of *E-CADHERIN* and its negative regulator, *SNAIL*. The X-axis mentions the genes analysed by qRT-PCR under different RNAi conditions and the Y-axis gives the relative gene expression levels. Data was compared to *LAMIN C*. Appendix table 11 details the raw data for this figure. Experiment was done 3 times (N=3), each in triplicate.
The final SD is mentioned after ±

were > 0.05, thus not showing any statistical significant data. [**Note**: p-values mentioned are significance of the gene knock-down compared to mock-treated SW480 cells]. These data were summarised in Figure 4.7. Figure 4.7 is obtained from appendix Table 11.

Changes in the expression of few of these genes appeared interesting, one of which was the reduction in expression of *NANOG*. Since, *NANOG* is involved in maintenance of pluripotency in stem cells; we focussed our studies on exploring a possible role for *BRACHYURY* in maintaining a cancer putative stem cell population. This is explained in part b of this chapter in full details.

b) Role of *BRACHYURY* in influencing the regulation of *NANOG* and maintaining a putative cancer stem cell population

4.2.1 The expression of *NANOG* is altered in response to changes in *BRACHYURY* in low density SW480 cells:

One of the most significant changes in gene expression following *BRACHYURY* RNAi was reduction in *NANOG* expression (shown by qRT-PCR). *NANOG* is a key inducer of pluripotency involved in maintenance of stem cells and is expressed in tissue-specific stem cells, such as the pancreas (Koblas *et al.*, 2008). This led us to suggest that *NANOG* may be maintaining a population of so called cancer stem cells for the reasons outlined below.

CD166, CD133 and LAMIN A are three colorectal cancer stem cell markers we analysed in this study. LAMIN A was shown to be a novel biomarker for colorectal cancer stem cells (Naomi *et al.*, 2008). It has recently been shown that expression of A-type lamins in CRCs is correlated with a poor prognosis due to increased tumour invasiveness (Willis *et al.*, 2008). Furthermore, in addition to LAMIN A being expressed in differentiated epithelia, it has also been shown to be expressed in cells of the colonic crypt that reside in the stem cell niche. Due to this critical link between the presence of LAMIN A and cells having stem cell character, together with the link with poor prognosis for CRC patients, we investigated whether *BRACHYURY* might also regulate the levels of LAMIN A in CRC cells. Ricci-Vitiani and co-workers have demonstrated that CD133 can be used as a colorectal cancer stem cells marker (2007). However, not all tumours display this marker nor is every $CD133^{+}$ cell a CSC (Dalerba *et al.*, 2007: LaBarge and Bissel, 2008; Shmelkov *et al.*, 2008)]. In mouse, *CD133* expression was observed in differentiated cells such as proximal renal tubule cells, neurons in adult brain and pancreas (Zhu *et al.*, 2009). This agrees with recent studies done by Shmelkov and co-workers (2009) in mouse tissues. In small intestine of mouse, $Lgr5^{+}$ stem cells were shown to express *CD133* (Zhu *et al.*, 2009). [Lgr5 marks intestinal stem cells (Barker *et al.*, 2007).]

A more specific marker for CRC-SCs has been described as CD166 (Dalerba *et al.*, 2007). Almost 60% of CRCs express strong cytoplasmic staining of CD166, and 30% have membrane staining of CD166, and expression of CD166 in CRC is associated with a poor prognosis (Weichert *et al.*, 2004).

Firstly, we investigated the endogenous protein levels of BRACHYURY, NANOG and CRC stem cell markers (CD166, CD133, LAMINA) in low and high density SW480 cells grown for 1 day and 4 days, respectively (Figure 4.8a). Protein levels decrease to almost non-detectable when the cells transition from sub- to super-confluent stages.

We next investigated the protein levels of NANOG in response to BRACHYURY knock-down in low density SW480 cells. Our results show that NANOG levels are down-regulated in response to *BRACHYURY* knock-down in low density SW480 cells (Figure 4.8b). We also studied the expression of colorectal cancer stem cell markers in response to BRACHYURY RNAi. We show that levels of CRC stem cells markers (CD166, CD133, LAMIN A) are decreased following knock-down of *BRACHYURY* (Figure 4.8b).

β-catenin is upstream of BRACHYURY in SW480 sub-confluent cells:

β-catenin is a major oncogene which drives CRC and the Wnt signalling pathway (Wnt3) has been shown to regulate expression of *BRACHYURY* (Arnold *et al.*, 2000). We therefore knocked down *β-catenin* to determine whether it might be upstream of *BRACHYURY* in these cells. Knock-down of β-catenin by RNAi results in the down-regulation of BRACHYURY as shown by western blot analysis, in low density SW480 cells (Figure 4.8c). This down-regulated the levels of NANOG and CD133 in low density SW480 cells. LAMIN B was used as loading control in this experiment (Note: Figure 4.8 shows equal loading of LAMIN B, thus as a control).

These results led us to suggest that in low density mesenchymal-like cells, signalling through β-catenin induces BRACHYURY which in turn induces NANOG which acts to maintain a population of cells with stem cell-like

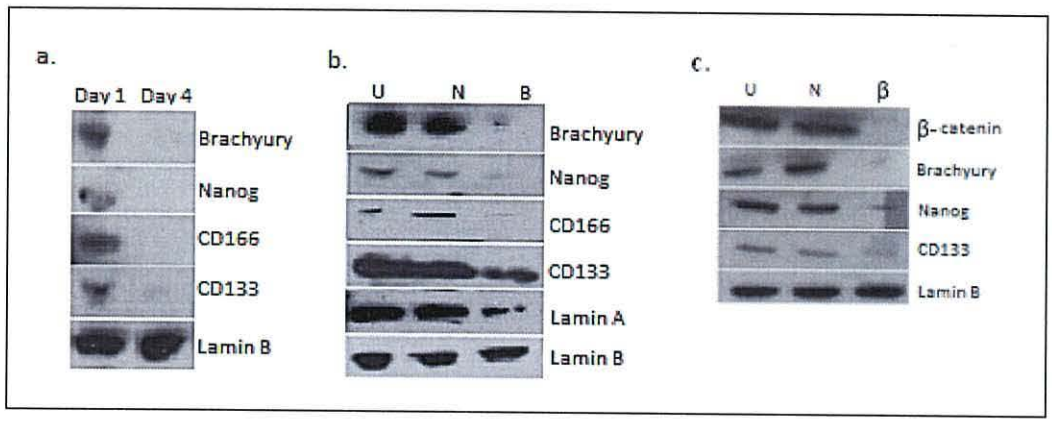


Figure 4.8

Western blot analysis of levels of BRACHYURY, NANOG and CRC stem cell markers. a) Endogenous levels of BRACHYURY, NANOG, CD166 and CD133 at day 1 (sub-confluent) SW480 cells which decreased to almost non-detectable levels on day 4 (super-confluent) SW480 cells. b) Levels of NANOG and CRC stem cell markers (CD133, CD166, LAMN A) decrease in response to *BRACHYURY* RNAi in low density SW480 cells. LAMIN B was used as a loading control. The BRACHYURY lane confirms the knock-down. c) Western blot analysis showing *β-catenin* knock-down decreases the levels of BRACHYURY suggesting that β-catenin is upstream of BRACHYURY. This inturn, reduces the levels of NANOG and CRC stem cell marker. LAMIN B in each blot was used as loading control confirming equal amount of protein loaded in each lane. U = untreated cells, N=cells treated with non-interfering RNAi, B = BRACHYURY RNAi, β = β-catenin RNAi.

properties. (At high cell density in epithelial like cells, signalling through β-catenin might be low and subsequent expression of *BRACHYURY*, *NANOG* and cells with cancer stem cell phenotype also would be low). To confirm the relationship we observed by western blot analysis, between levels of BRACHYURY, NANOG and CRC stem cell markers, we studied the localisation of BRACHYURY, NANOG and CRC stem cell markers by immunoflouresence in low and high density SW480 cells. Specifically, we wanted to determine the levels and localisation of BRACHYURY, NANOG, CD133 and CD166 (CRC stem cell marker) in low and high density SW480 cells and to determine whether cells that express BRACHYURY also express NANOG, CD133 and CD166. [**Note**: β-catenin knock-down reduces BRACHYURY levels, thus β-catenin is upstream of BRACHYURY. But, *BRACHYURY* RNAi does not reduce the levels of β-catenin, confirming that β-catenin is upstream of BRACHYURY. Therefore, β-catenin can be used a control].

We show that all SW480 cells at low density express BRACHYURY and NANOG and also CD166 and CD133 (Figure 4.9). BRACHYURY and NANOG localisation was found to be nuclear (as expected as they are transcription factors), whereas, CD133 and CD166 confirms their surface antigen localisation. Thus, there is 100% co-localization of BRACHYURY, NANOG and the CRC stem cell markers in low density SW480 cells.

In contrast to low density cells, high density SW480 cells express BRACHYURY, NANOG, CD133 and CD166 very rarely. Moreover, the rare cells that express BRACHYURY also express NANOG. Furthermore, we showed that the cells that are positive for BRACHYURY and NANOG, are also positive for CD166 in high density SW480 cells (Figure 4.9).

In high density SW480 cells, β-catenin localisation mostly membrane bound with occasional nuclear localisation (personal communication Dr. Melanie Davies). We wanted to analyse whether the rare BRACHYURY positive cells in high density SW480 cells co-localise with occasional nuclear β-catenin (nuclear

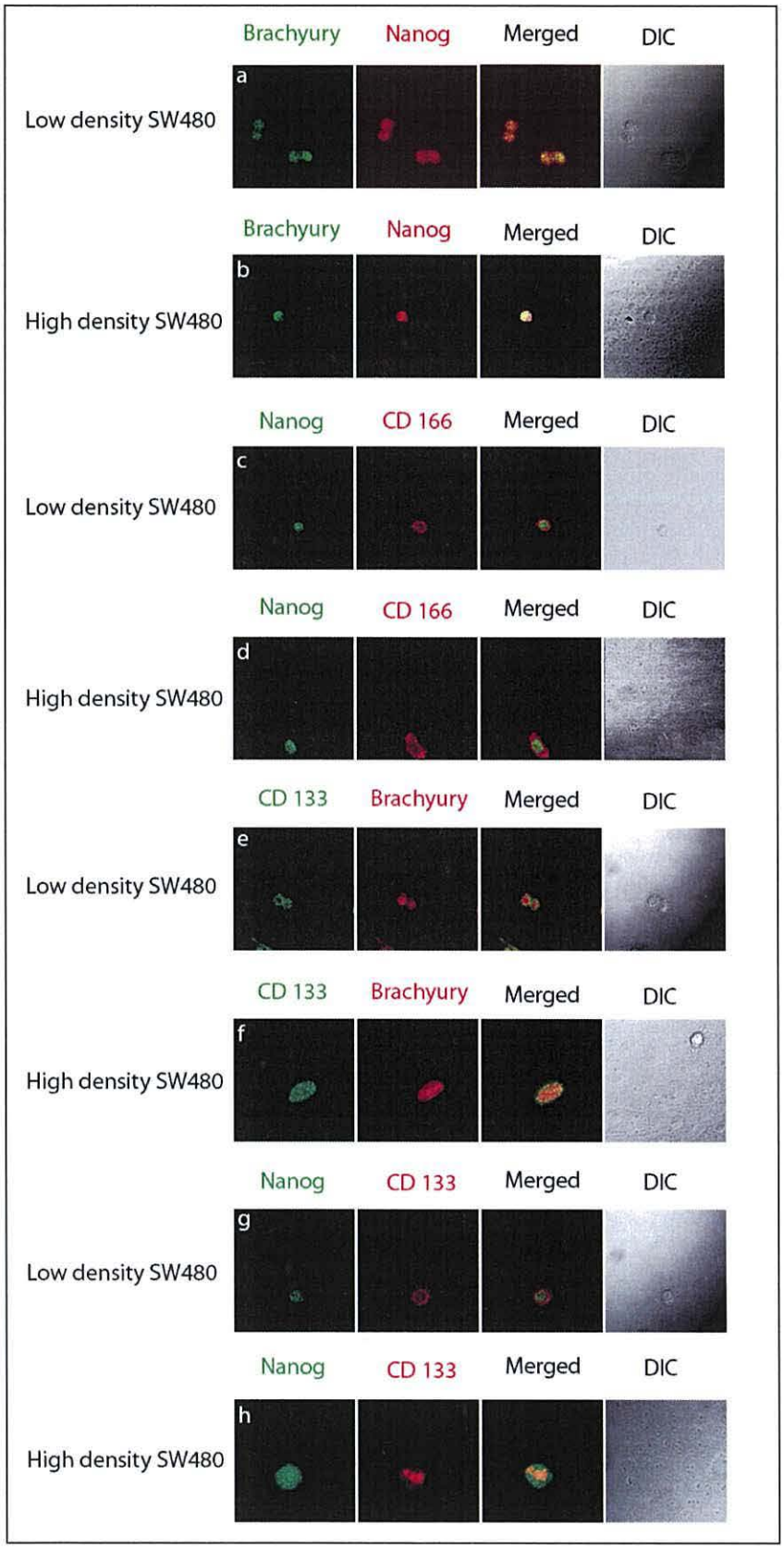


Figure 4.9 (a-h): Confocal IF images showing localisation of BRACHYURY, NANOG, CD133 and CD166 in low and high density SW480 cells.

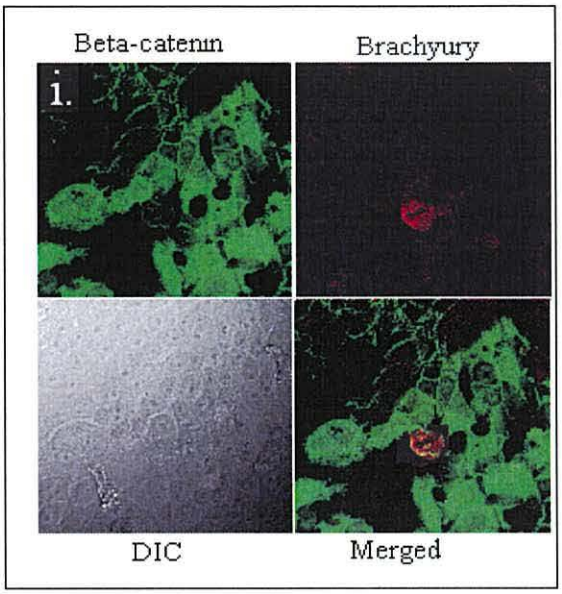


Figure 4.9h

Figure 4.9: Indirect IF using respective antibodies and confocal microscopy shows that BRACHYURY and NANOG are localised to the nucleus, whereas CD166 and CD133 are surface antigen markers. a) BRACHYURY co-localises with NANOG in low density SW480 cells. b) BRACHYURY co-localises with NANOG in high density SW480 cells. c) NANOG co-localises with CD166 in all SW480 sub-confluent cells. d) NANOG co-localises with CD166 in all SW480 super-confluent cells. e) CD133 co-localises with BRACHYURY in all low density SW480 cells. (f) CD133 co-localises with BRACHYURY in rare high density SW480 cells. g) NANOG co-localises with CD133 in all SW480 sub-confluent cells. h) NANOG co-localises with CD133 in SW480 super-confluent cells. i) Rare BRACHYURY positive SW480 cells in high density are not the cells with nuclear β-catenin.

β-catenin means active Wnt signalling). Our data show that rare BRACHYURY positive cells in high density SW480 cells do not correspond to cells with nuclear β-catenin (Figure 4.9 i).

Thus, this section of the chapter has given us important information on localisation of BRACHYURY, NANOG and CRC stem cell markers in SW480 cells and their co-localisation with each other. We have studied further possible roles of BRACHYURY in later sections.

4.2.2 Does *NANOG* regulate specific gene expression patterns in SW480 cells?

The down-regulation of *NANOG* in human ES and EC (embryonal carcinoma) cells was shown to result in differentiation of the cells along an extraembryonic lineage, as determined by the expression of genes such as *GATA2*, *GATA4*, *GATA6* and *CDX2* (Hyslop *et al.*, 2005). Specifically, knock-down of *NANOG* increased the expression of the genes *GATA1, 4, 6*, (extraembryonic endoderm genes), *CDX2* and *GATA2* (tropho-ectoderm associated genes) showing that the down-regulation of *NANOG* induced differentiation in human ES and EC cells. *NANOG* knock-down (by RNAi) also decreased the expression of *Oct-4* but did not change the expression of *SOX2* indicating that this differentiation cannot be used to measure the pluripotency in these cells. We wanted to determine whether expression of these genes altered similarly, as a result of lower expression levels of *NANOG* in SW480 cells. Therefore, we looked at the expression of *GATA2, GATA4, GATA6, Oct-4* and *SOX2* in low density SW480 cells following RNAi induced knock-down of *BRACHYURY.*

The MYC family of transcription factors play an important role in cell proliferation, differentiation and in development of the embryo and in the progression of cancer (Henrikson and Lu¨scher, 1996) and is also one of the key

transcription factors used to induce pluripotency (Takahashi *et al.*, 2006). C-MYC has been shown to be a target of Wnt signalling pathway (He *et al.*, 1998). Since MYC is an important downstream target of β-catenin, we therefore, looked at expression of this gene following knock-down of *BRACHYURY*.

FOXD3 is another embryonic stem cell transcription factor which is down-regulated during endoderm formation (Guo *et al.*, 2002). We therefore, also studied the expression of *FOXD3* in low and high density SW480 cells and also in response to *BRACHYURY* RNAi in low and high density SW480 cells.

Our results show that *GATA2, GATA6*, *MYC* and *FOXD3* do not alter in response to changes in SW480 cell density as compared to low density SW480 cells. Expression of *GATA4* was reduced by 57%, *Oct-4* by 50% and *SOX2* by 78% in SW480 high density cells compared to SW480 sub-confluent cells (Figure 4.10).

In response to *BRACHYURY* knock-down, expression of *SOX2* is reduced by 68% expression of *FOXD3* is reduced by 69% in SW480 sub-confluent cells, whereas expression of *MYC* increases by 9.8 times in low density SW480 cells (Figure 4.11).

To summarise, down-regulation of *NANOG* in SW480 cells does not induce differentiation and expression of genes associated with extra-embryonic lineages. Expression of the pluripotency genes *SOX2* and *FOXD3* is reduced, but not *Oct-4*. Furthermore, expression of *c-MYC* is increased by 9.8 fold suggesting that a complex regulatory mechanism is working in these cells.

Thus, we present data for the use of SW480 cells grown to low and high cell density as a model in which to study EMT/MET. We have shown that the expression of *BRACHYURY* is linked with the regulation of the pluripotency gene *NANOG*, with the maintenance of markers of CSCs and regulation of LAMIN A (a biomarker linked to poor prognosis) in low density SW480 cells.

A.

	SW480 subconfluent	S.D.	**SW480** superconfluent	S.D.
GATA 2	1	0.14	0.98	0.15
GATA 4	1	0.20	0.43	0.18
GATA 6	1	0.15	1.02	0.15
MYC	1	0.18	1.01	0.18
FOXD3	1	0.17	0.99	0.15
Oct-4	1	0.14	0.50	0.19
SOX2	1	0.11	0.22	0.13

B.

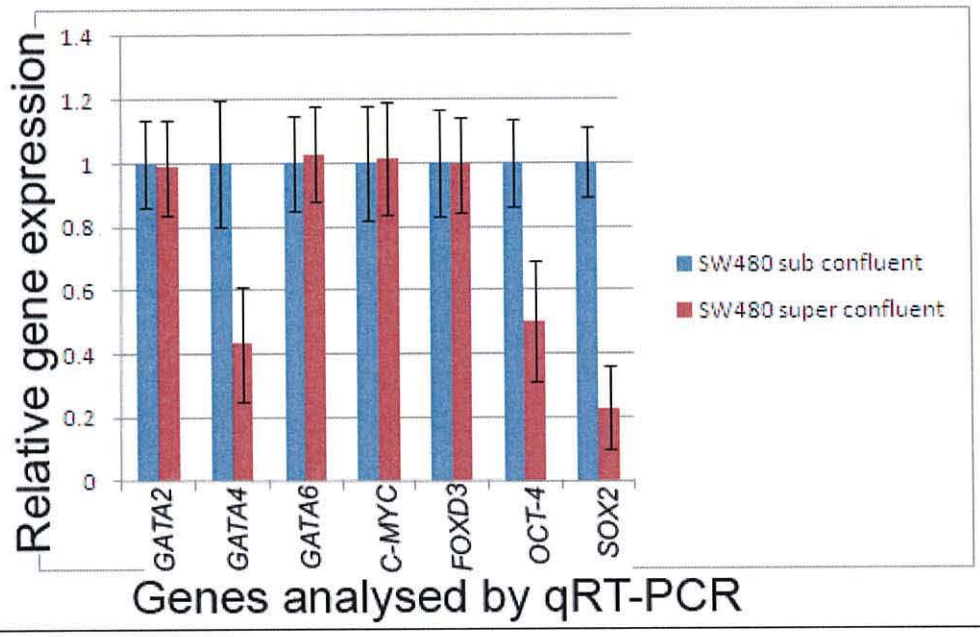


Figure 4.10

qRT-PCR expression data for *GATA* genes, pluripotency marker genes, *MYC* and *FOXD3* in SW480 cells. A) This table summarises the relative expression of the above mentioned genes in low and high density SW480 cells. [Expressions in high density cells are in relative to that of sub-confluent cells]. B) Graphical representation of Table A showing the changes in gene expression of the genes in low and high density SW480 cells. The X-axis mentions the genes analysed by qRT-PCR in low and high density SW480 cells. The Y-axis gives the relative gene expression levels. [Experiments were done 3 times (N=3), each in triplicate. All data were compared to β-catenin. This figure is derived from Table of appendix 13. Refer appendix Table 26 for the statistical significance data of the fold differences obtained for each gene in low and high density cells.

A.

	GATA2 (Relative expression)	*GATA4* (Relative expression)	*GATA6* (Relative expression)	*MYC* (Relative expression)	*FOXD3* (Relative expression)	*OCT-4* (Relative expression)	*SOX2* (Relative expression)
Non-interfering	1±0.04	1±0.03	1±0.13	1±0.03	1±0.06	1±0.04	1±0.05
Untreated	1.08±0.08	1.07±0.04	1.02±0.22	0.97±0.02	1.09±0.05	1.11±0.05	1.03±0.23
Brachyury RNAi	1.05±0.09	0.98±0.11	0.95±0.31	9.8±0.30	0.31±0.03	1.06±0.09	0.33±0.06

B.

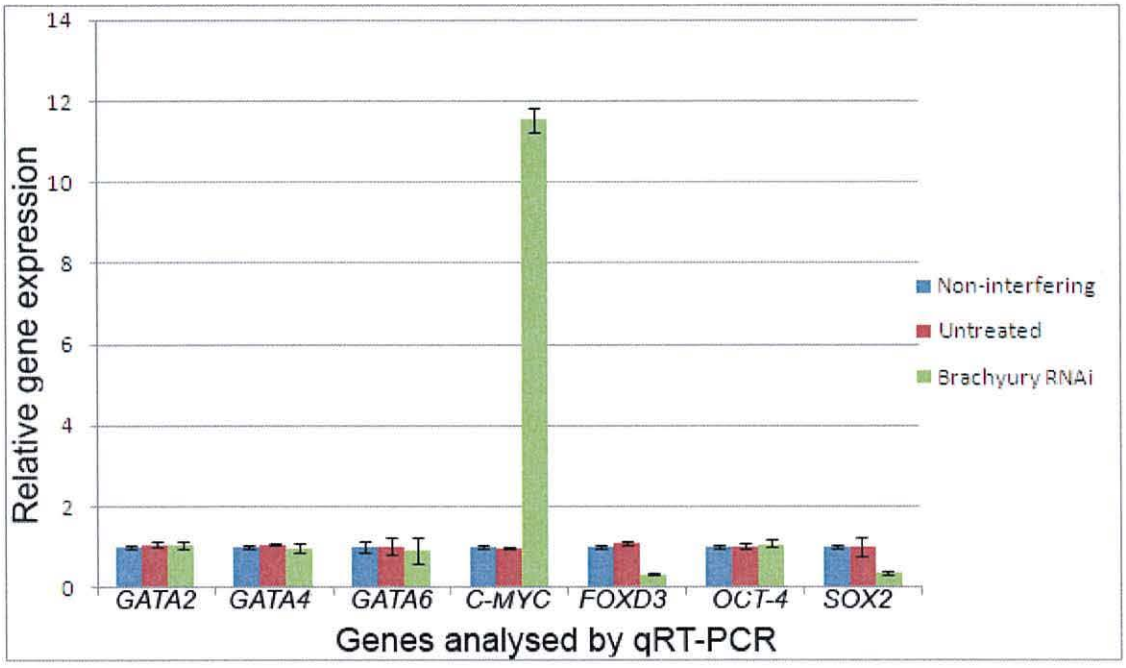


Figure 4.11:

qRT-PCR of *GATA* genes, pluripotency marker genes, *MYC* and *FOXD3* in SW480 sub-confluent cells. A) Table demonstrating the expression of these genes in low density SW480 cells on BRACHYURY RNAi, as assessed by qRT-PCR. B) Graphical representation of Table A showing no change in *GATA* genes and *OCT4* but 9.8 fold increase in *c-MYC*. The X-axis mentions the genes analysed by qRT-PCR under different RNAi conditions and the Y-axis gives the relative gene expression levels.
[Experiments were done 3 times (N=3), each in triplicate. All data were compared to *β-catenin*. This figure is derived from Table of appendix 12. *BRACHYURY* knock-down ranged from 68-80%].
The Final SD is mentioned after ±.

We also show that the oncogene of the Wnt pathway, β-catenin, which is a modulator of 'stem' signaling pathways, affects the levels of BRACHYURY showing that *BRACHYURY* may be an important factor in transducing the β-catenin signaling pathway in the maintenance of cells with a CSC-like phenotype. We continue to explore the role of BRACHYURY in SW480 cells and the next section will describe the influence of BRACHYURY on NFκB signalling.

c) A role of *BRACHYURY* in regulating the NFκB pathway?

4.3 A role for *BRACHYURY* in regulating the NFκB pathway

NFκB (Nuclear factor kappa β) refers to a family of transcription factors and has crucial roles in the immune system (Ghosh *et al.,* 1998; Li and Verma 2002; Bonizzi and Karin 2004). The activation of the NFκB pathway is also involved in many cancers such as leukemia, lymphoma, colon cancer and ovarian cancer (Rayet and Gelinas 1999) and commonly occurs through de-regulation of upstream regulators. In mammals, the NFκB family is composed of the transcription factors, p50, p52, REL A (p65), c-REL And REL B (Moynagh 2005; Hoffmann *et al.,* 2006; Hayden *et al.*, 2004; Perkins *et al.*, 2007). REL A, c-REL and REL B contain C-terminal transcriptional activation domains (TADs), which are involved in activation of target gene expression. p50 and p52 do not contain C-terminal TADs and are derived from larger precursors, p105 and p100, respectively. Their homodimers repress transcription unless they are bound to a protein containing a TAD, such as REL A, c-REL or REL B or Bcl-3 (a related transcriptional co-activator).

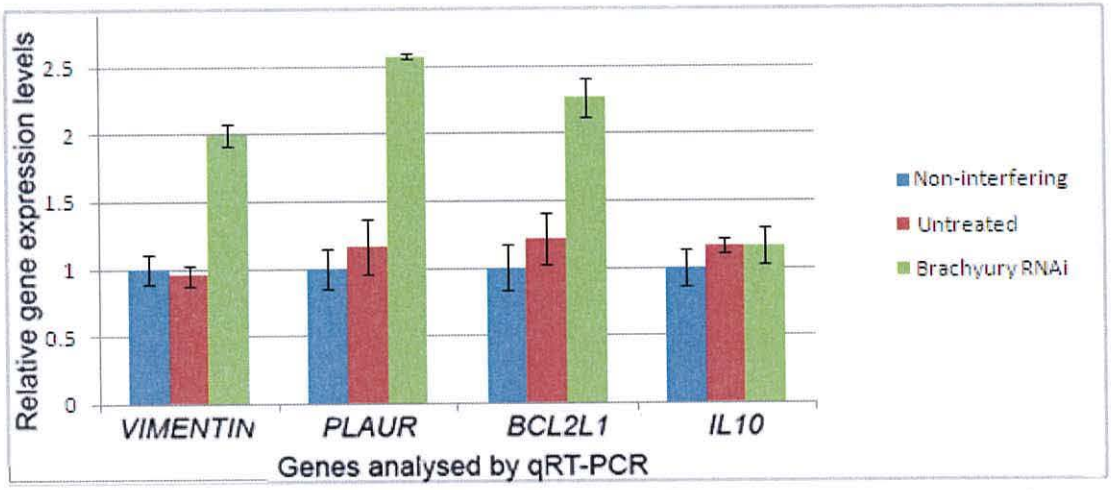
Recently, it has been shown that *NANOG* inhibits the NFκB signalling pathway in mouse ES cells, thereby maintaining pluripotency (Watt, F. and Torres, J., 2008). As mentioned above, the NFκB family consists of 5 member proteins, out of these *REL A and REL B* were extensively expressed in ES cells, the other three members being present to be a lesser amount. *NANOG* was shown to bind to REL A in ES cells where it acts to inhibit NFκB regulated genes. Expression of NFκB regulated genes occurs on differentiation with dissociation of this complex. We wanted to determine whether *NANOG* was regulating targets of NFκB genes similarly in SW480 cells. We examined the expression of NFκB regulated genes in response to *BRACHYURY* knock-down in low density SW480 cells by qRT-PCR (Figure 4.12). These values are derived from the raw data presented in Table 14 of appendix.

The following NFκB regulated genes were studied:

A.

	VIMENTIN (Relative expression)	*PLAUR* (Relative expression)	*BCL2L1* (Relative expression)	*IL10* (Relative expression)
Non-interfering	1±0.11	1±0.15	1±0.17	1±0.14
Untreated	0.95±0.08	1.16±0.21	1.22±0.19	1.16±0.05
Brachyury RNAi	1.99±0.08	2.57±0.02	2.26±0.14	1.16±0.14

B.



C.

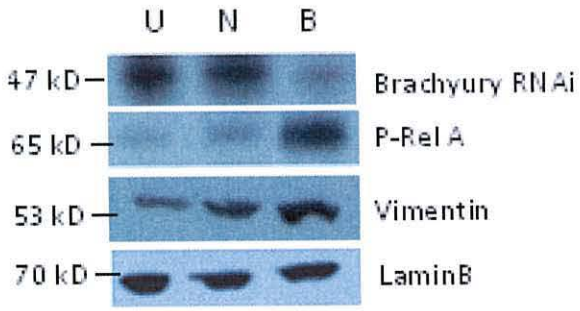


Figure 4.12
Expression of NFκB regulated genes in response to *BRACHYURY* knock-down in SW480 sub-confluent cells. A) Table demonstrating the up-regulation of *VIMENTIN*, *PLAUR*, *BCL2L1* and *IL10* in response to *BRACHYURY* RNAi in low density SW480 cells obtained by qRT-PCR. This data was compared to cells with no treatment and was also controlled with mock treated SW480 low density cells. The table was derived from Appendix table 14. B) Graphical representation of the table 4.12A along with error bars obtained from their standard deviations. The X-axis mentions the genes analysed by qRT-PCR under different RNAi conditions and the Y-axis gives the relative gene expression levels. Experiments were repeated 3 times (N=3), each done in triplicate; and data was compared to *LAMIN C*. C) Western blot analysis demonstrating the increased levels of REL A, phosphorylated REL A and VIMENTIN in response to *BRACHYURY* RNAi in low density SW480 cells. LAMIN B was used as a loading control showing equal loading for all samples. The experiment was repeated 3 times (N=3).

VIMENTIN is an intermediate filament protein and has metastatic and invasive roles in many cancers such as prostate cancer (Wei *et al.*, 2008) and colon cancer. *PLAUR* (plasminogen activator) has a role in metastasis in colon and liver cancers (Illemann *et al.*, 2009). *Il10* is an anti-inflammatory cytokine and also is critical for the development and progression of sporadic colon cancer (Tamara *et al.*, 2008). *BCL2L1* belongs to the Bcl-2 family which forms dimers and can behave as anti- and pro-apoptotic regulators in many cellular activities.

Figure 4.12 (A, B) demonstrates that the expression of NFκB regulated genes; *PLAUR*, *BCL2L1* and *VIMENTIN* are upregulated following *BRACHYURY* knock-down in low density SW480 cells (by 2 fold increase) obtained by qRT-PCR, but there was no change in expression of *IL10* (Figure 4.12 A, B). But, the p-value for the difference sin expression of *IL10* in response to the knock-down was shown to be <0.05 (appendix Table 25). Thus, this change in expression was considered significant along with the expression of *PLAUR*, *BCL2L1* and *VIMENTIN*. We then carried out Western blot analysis to confirm the levels phosphorylated REL A (Ab1604, Chemicon), the p65 subunit of NFκB and also the expression of *VIMENTIN*, in response to *BRACHYURY* RNAi in low density SW480 cells (Figure 4.12 C). Our Western blot analysis show that both phosphorylated REL A (p-REL A) and VIMENTIN are upreguated on knocking down *BRACHYURY* showing possible active NFκB signalling in low density SW480 cells in response to knock-down of *BRACHYURY*. In summary, *BRACHYURY* may inhibit NFκB signalling in low density SW480 cells, and this may occur through activation of *NANOG*.

We also examined the expression of *NFκB* and *STAT3* in low density SW480 cells on *BRACHYURY* RNAi (Figure 4.13). Stat3 is a transcription factor shown to interact with NFκB1 (Yu *et al.*, 2002). Wnt pathway is shown to upregulate Stat3 and prevents differentiation of mouse embryonic stem cells (Hao *et al.*, 2003). So, we wanted to check the expression of *STAT3* in response to *BRACHYURY* RNAi in SW480 cells to know more about a possible role of *BRACHYURY* in NFκB signalling. Our results (Figure 4.13) show that knocking down *BRACHYURY* did not have any effect on the expression levels of *NFκB* and *STAT3* in low density SW480 cells, showing no direct interaction of BRACHYURY with *NFκB* and STAT3 in SW480 cells.

A.

RNAi treatments	NFKB (relative gene expression)	Final SD	STAT3 (relative gene expression)	Final SD
N.I.	1	0.19	1	0.09
Untreated	1.08	0.10	1.09	0.29
Brachyury RNAi	0.94	0.23	1.16	0.30

B.

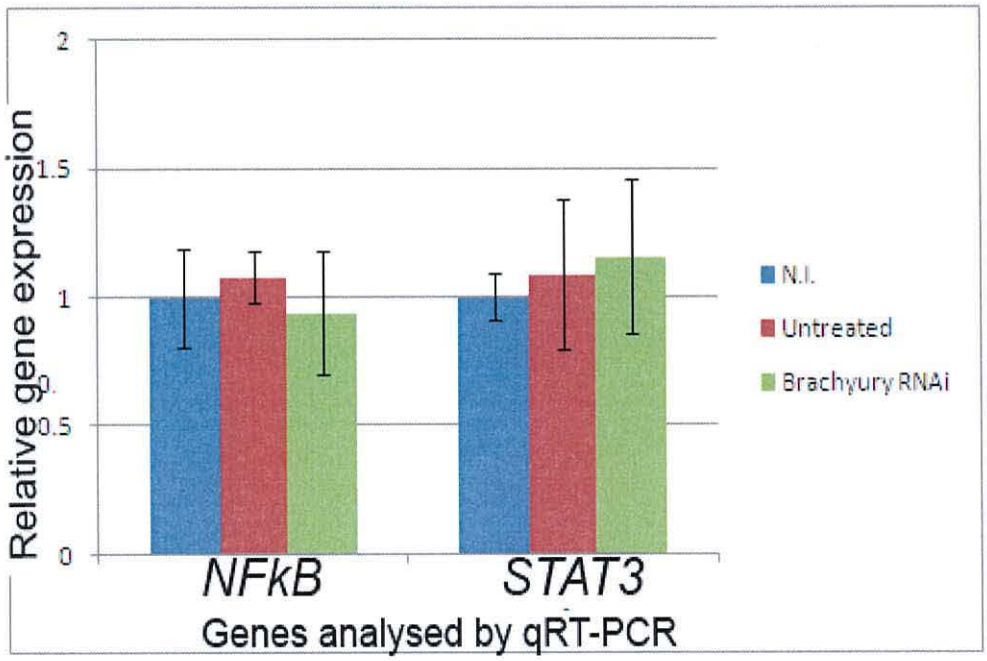


Figure 4.13

Expression of *NFκB* and *STAT3* in response to *BRACHYURY* RNAi in low density SW480 cells. A) Table demonstrating the no change in expression of these genes on knocking down *BRACHYURY* in sub-confluent SW480 cells. Raw data for table A is in Table 15 of appendix. Data on *BRACHYURY* RNAi was compared to cells treated with non-interfering siRNA. B) Graphical representation of the expression of the table A, showing reduction in *NFκB* expression was 6% and *STAT3* expression was increased by 1.16 times; p-values >0.05. Experiments were repeated 3 times (N=3), each done in triplicate; and data was compared to *LAMIN C*. The X-axis mentions the genes analysed by qRT-PCR under different RNAi conditions and the Y-axis gives the relative gene expression levels. For p-value, refer to appendix Table 25.

d) Role of *BRACHYURY* in cell proliferation

4.4.1 Cell proliferation and cancer:

Cell proliferation is usually maintained by growth factors, receptor molecules in the cell, signalling pathways and transcription factors that bind to the DNA. Mutation in any of these genes can lead to changes in the cell proliferation, as frequently occurs in cancer. In cancer, genetically altered cells can proliferate abnormally to a secondary site (neoplasia) to continue forming tumour and thus spread the disease. Cell proliferation is often characterized by loss of normal tissue organisation and cell structure. In colon cancer, this type of proliferation is caused by chronic ulcerative colitis, infectious agents, and saturated fats (Preston-Martin, 1990).

Earlier in this chapter, we have seen that expression of *NANOG* is influenced by *BRACHYURY* in SW480 cells. *NANOG* is a transcription factor required to maintain the undifferentiated state of embryonic stem cells. Recently, exogenously transcribed *NANOG* was shown to enhance the proliferation of NIH3T3 cells by enabling the cells to enter S phase (Zhang *et al.*, 2005). [Note: NIH3T3 cells are mouse embryonic fibroblast cell line]. Thus, it was predicted that *NANOG* might have a similar function in ES cells. *NANOG*'s overexpression in many forms of cancer makes it an interesting candidate to study; furthermore, the oncogenic potential of NANOG was demonstrated when it was shown to transform NIH3T3 cells and increase the cell growth rate (Piestun *et al.*, 2006). We therefore, wanted to determine whether *BRACHYURY* has an effect on cell proliferation in SW480 cells via *NANOG.*

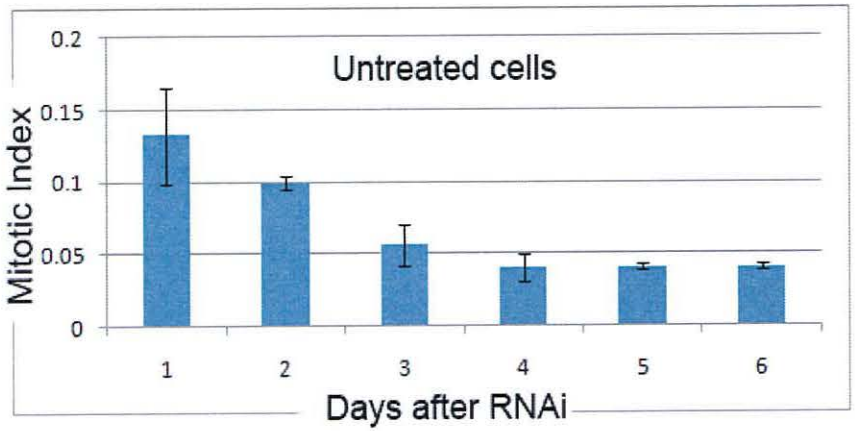
4.4.2 Cell proliferation by mitotic index count

We have shown that the regulation of *NANOG* is influenced by *BRACHYURY* in SW480 cells (section 4.2); and since *NANOG* plays an important role in proliferation in NIH3T3 cells, we investigated the possible role of *BRACHYURY* in regulating cell proliferation in SW480 cells. We knocked down the expression of *BRACHYURY* in SW480 cells by RNAi and grew the

cells for 1, 2, 3, 4, 5 and 6 days (1 day = sub-confluent, 3 days = confluent, 4 days = super-confluent). We counted the number of Propidium iodide stained cells in all stages of mitosis to calculate the number of proliferating cells. [Mitotic index is a measure of the proliferating cells in a population, and is expressed as the ratio between the number of cells in mitosis and total number of cells]. This experiment was controlled by counting the cells in mitosis in cells without any treatment and also cells with non-interfering siRNA. Data in Figure 4.14 shows that the number of cells in mitosis decreases as SW480 cells grew from low to high density. On treating SW480 cells with *BRACHYURY* siRNA, the number of proliferating cells increase on day 4 (super-confluent) and day 5 (24 hours after super-confluency) as compared to cells with no treatment. (Raw data for these graphs are in Table 16 of appendix; and the data for each condition was normalised to medium without any cells). We applied the two tailed t- test to measure the statistical significance of this data (Table 17, appendix). Thus, we were able to show that the higher rate of proliferation of SW480 cells treated with *BRACHYURY* siRNA, 4 days after RNAi is statistically significant (Table 17, appendix). Furthermore, to determine whether the knock-down is active over the period of 6 days after *BRACHYURY* RNAi, we analysed the knock-down levels for each day of the experiment. We show that the levels of *BRACHYURY* knock-down remain high for days 1 (56%), 2 (72%), 3 (57%) and 4 (66%). On day 5 (38%) and 6 (38%) after *BRACHYURY* RNAi the knock-down efficiency starts to decrease (Figure 4.15).

In summary, there is no change in mitotic index in *BRACHYURY* knocked down cells for the first 3 days after RNAi; however after 4 days of *BRACHYURY* RNAi there is a small but statistically significant increase in number of cells in mitosis following *BRACHYURY* knock-down (Figure 4.14). The next day (day 5 after RNAi) showed a further increase in number of proliferating cells in response to *BRACHYURY* RNAi. But, Figure 4.15 shows that the knock-down levels of BRACHYURY for day 5 (38%) is not as high as the first 4 days after RNAi. Thus, the mitotic index result for day 5 is not considered. Day 6 after *BRACHYURY* RNAi shows no increase in number of proliferating cells on knocking down the expression of *BRACHYURY*.

A.



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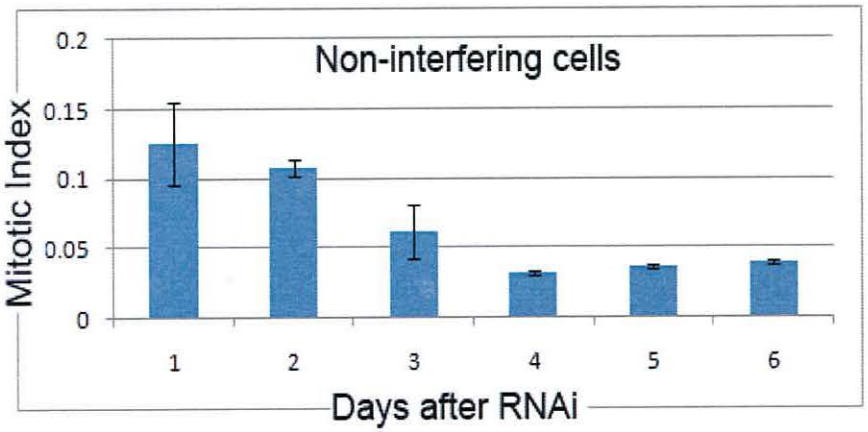

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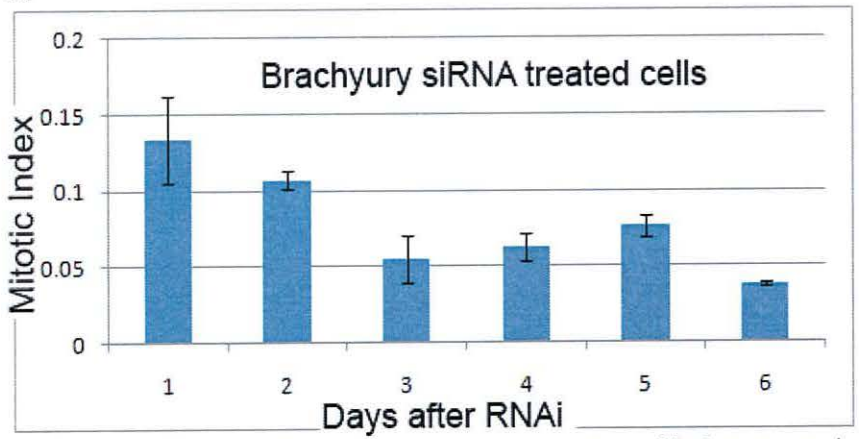

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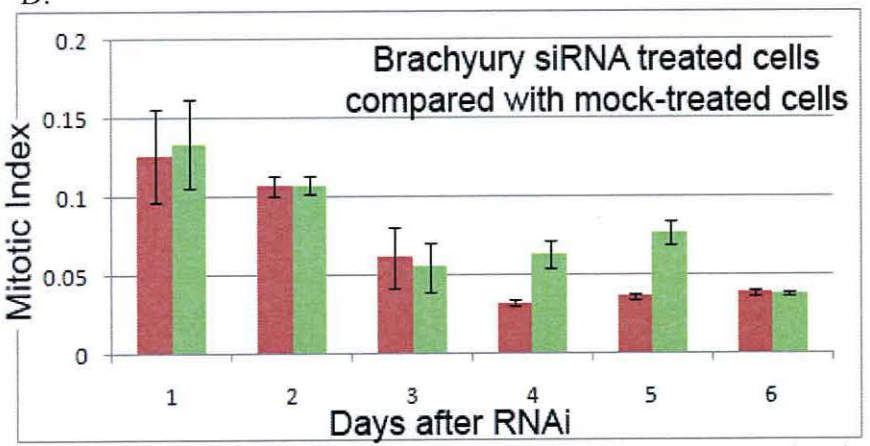


Figure 4.14: Mean mitotic index count of SW480 cells. Graphical representation of mitotic index count of A) untreated, B) non-interfering siRNA, C) *BRACHYURY* knocked down SW480 cells over a period of 6 days after RNAi. D) This graph represents a comparison of the mitotic index count of cells treated with *BRACHYURY* siRNA (green) in comparison to mock-treated cells (red). X-axis = no. of days cells were grown after RNAi, Y-axis = Mitotic index count. Raw data for this figure is detailed in appendix Table 16. N=4 (for days 1 to 4) and N= 3 (for days 5 and 6).

A.

	1 day after RNAi (relative expression)	2 days after RNAi (relative expression)	3 days after RNAi (relative expression)	4 days after RNAi (relative expression)	5 days after RNAi (relative expression)	6 days after RNAi (relative expression)
Non-interfering	1	1	1	1	1	1
Untreated	1.03	0.98	0.97	1.04	1.06	0.99
Brachyury RNAi	0.44	0.28	0.43	0.34	0.62	0.62

B.

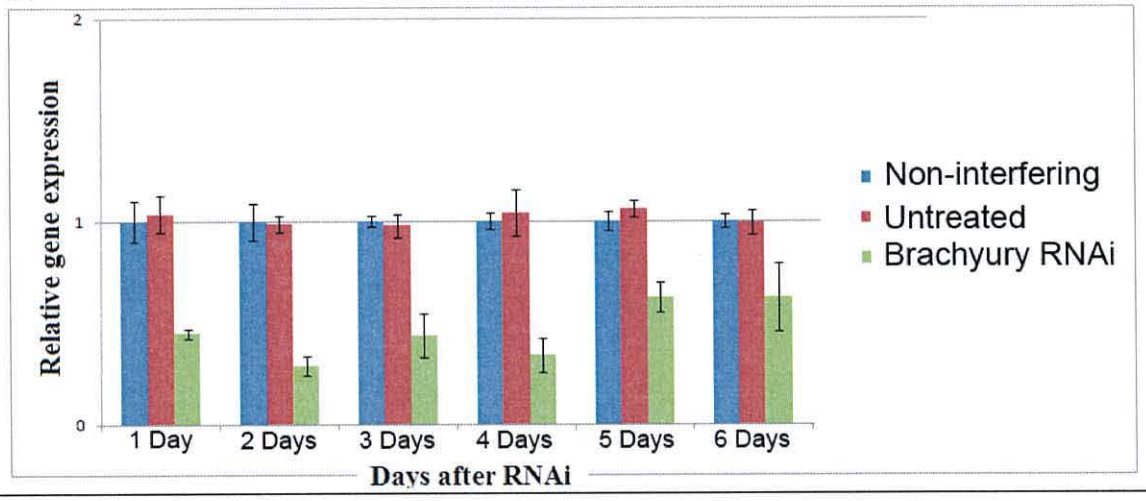


Figure 4.15: *BRACHYURY* knock-down levels after siRNA treatment. A) The relative gene knock-down levels of *BRACHYURY* compared to the SW480 cells treated with non-interfering siRNA. The raw data for this table is presented in **Table 18** of appendix. All data were compared to β-catenin. Experiment was done three times (N=3), each done in triplicate. B) Graphical representation table A showing high knock-down levels on days 1-4 of RNAi, but low RNAi levels on day 5 and 6 after RNAi. The X-axis mentions that BRACHYURY levels are analysed by qRT-PCR under different RNAi conditions on days 1 to 6 after RNAi. Y-axis gives the relative *BRACHYURY* expression levels.

4.4.3 Cell proliferation by CellTiter-Glo Luminescent Cell Viability Assay:

To complement the data provided by the mitotic index count, we also performed a proliferation assay using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Catalogue number G7571, see Materials and Methods section 2.22). This assay is based on quantifying the ATP present, thus can be used for measuring, cell viability and cell proliferation (Crouch *et al.*, 1993). We compared the proliferation of the SW480 cells treated with *BRACHYURY* siRNA to cells treated with non-interfering siRNA and untreated cells using this assay.

The number of proliferating SW480 cells on *BRACHYURY* knock-down was more compared to the untreated control conditions on days 1 to 4 after RNAi. We show that on days 1, 2 and 3 after RNAi, the number of proliferating SW480 cells on *BRACHYURY* knock-down was similar to that of untreated and non-interfering controls (Figure 4.16:A to F). But, 4 days after the RNAi treatment, when the SW480 cells are super-confluent, the number of proliferating SW480 cells on *BRACHYURY* knock-down was more compared to the untreated and non-interfering control conditions (Figure 4.16:G-H), thereby further validating our data obtained from the mitotic index count. [Raw data for Figure 4.16 is in Table 19 of appendix]

Figure 4.17 demonstrates the efficiency of *BRACHYURY* knock-down until 6 days after RNAi. We once again, show that the knock-down levels of *BRACHYURY* on day 5 (27%) and 6 (34%) is not as high compared to the first 4 days after the treatment (day 1=47%, day 2=69%, day 3=58% and day 4=57%). Thus, we obtained the cell proliferation data only for the first 4 days of the RNAi treatment, and not for day 5 and 6 of RNAi. Note: [Raw data for Figure 4.17 is in Table 20 of appendix].

We suggest that *BRACHYURY* might have a role in regulating cell proliferation in super-confluent SW480 cells although the mechanism through which this would work is unclear.

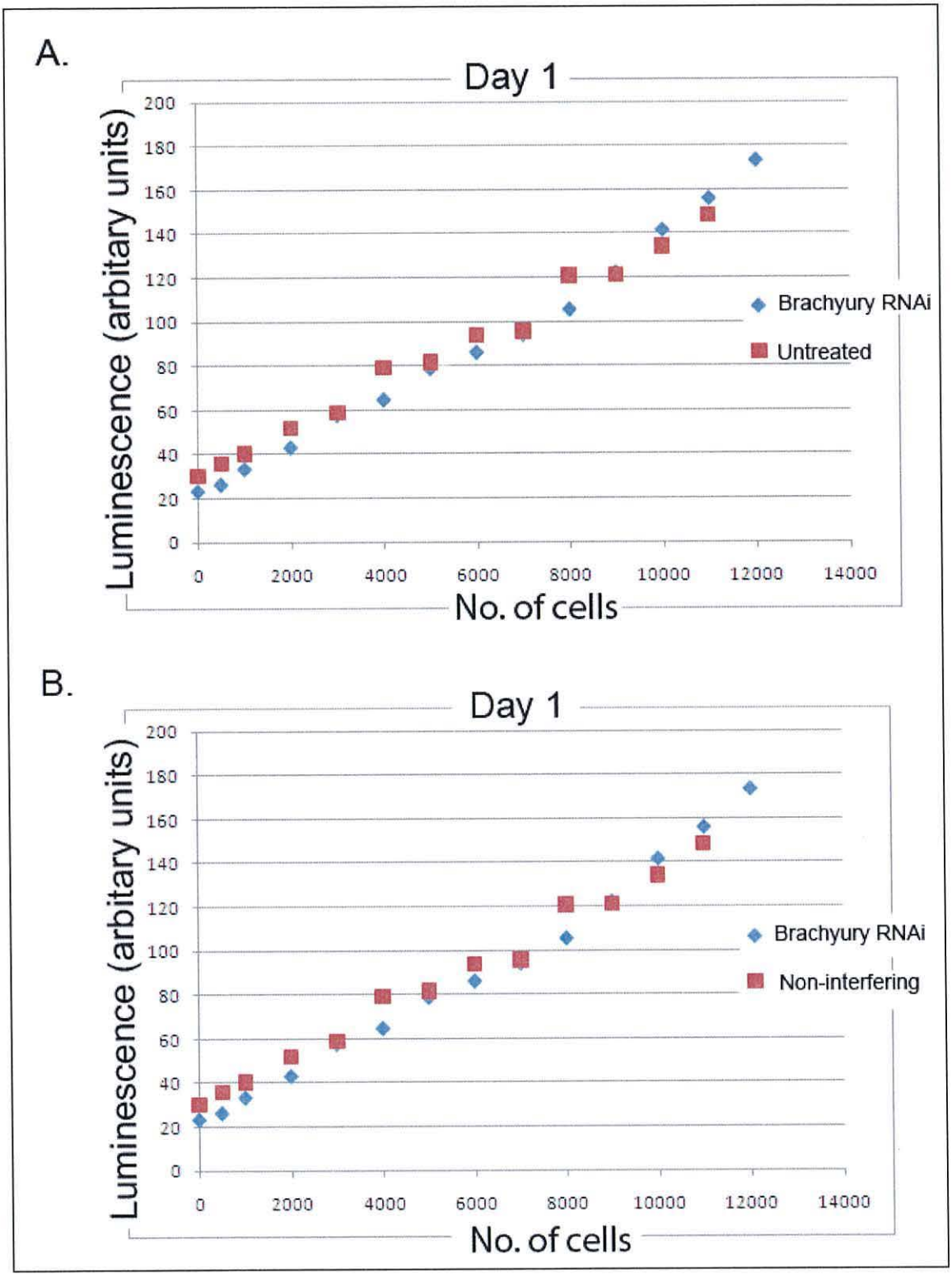


Figure 4.16 A-B: Graphs demonstrating the average luminescence obtained (Y-axis) against the number of viable cells (X-axis). This graph shows that the number of proliferating cells on knocking down *BRACHYURY,* on day 1 of treatment, is similar compared to that of untreated (A) and non-interfering siRNA (B) controls. For each data point, N=1, done in triplicate.

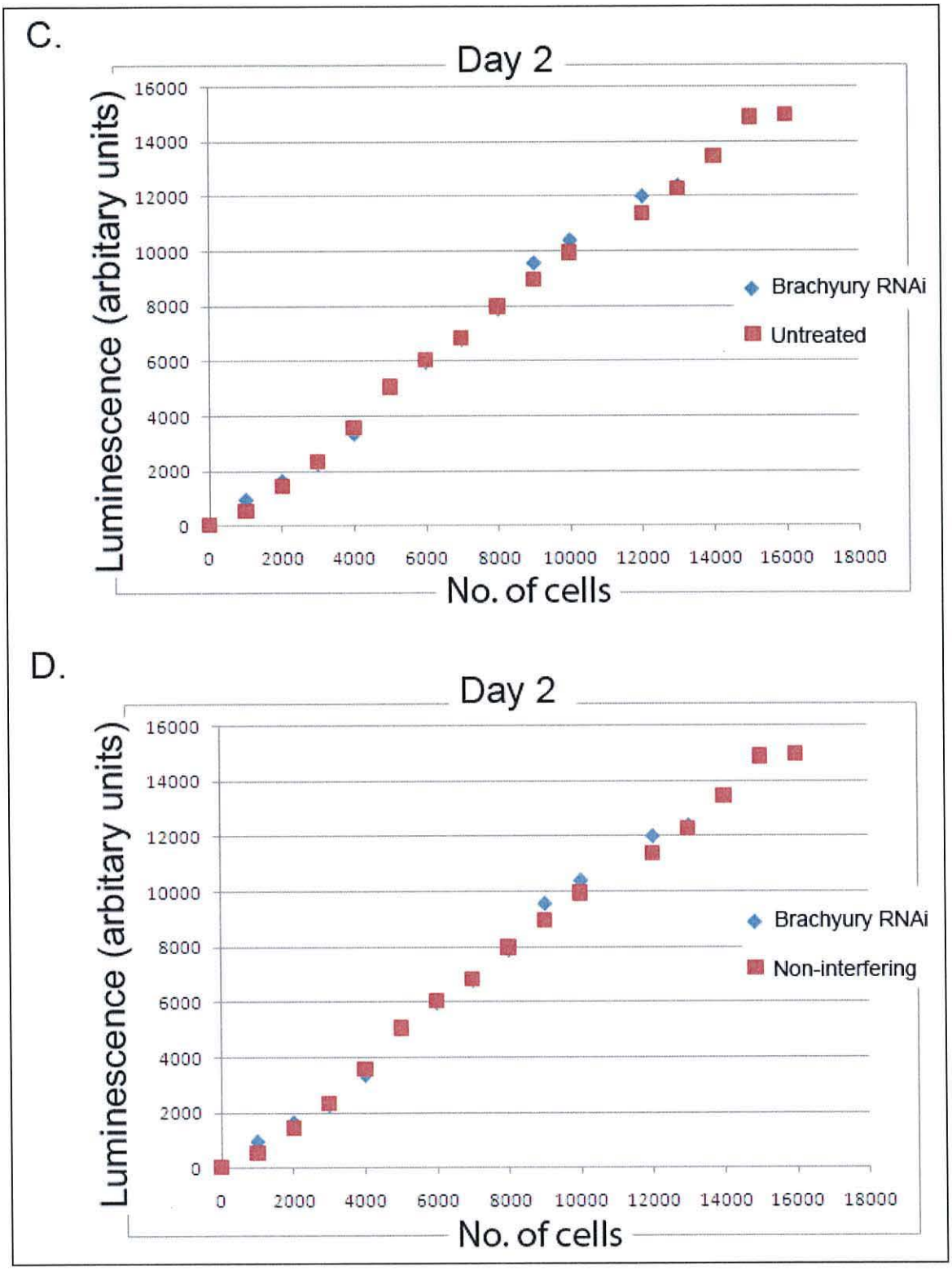


Figure 4.16 C-D: Graphs demonstrating the average luminescence obtained (Y-axis) against the number of viable cells (X-axis). This graph shows that the number of proliferating cells on knocking down *BRACHYURY,* on day 2 of treatment, is similar compared to that of untreated (A) and non-interfering siRNA (B) controls. For each data point, N=1, done in triplicate.

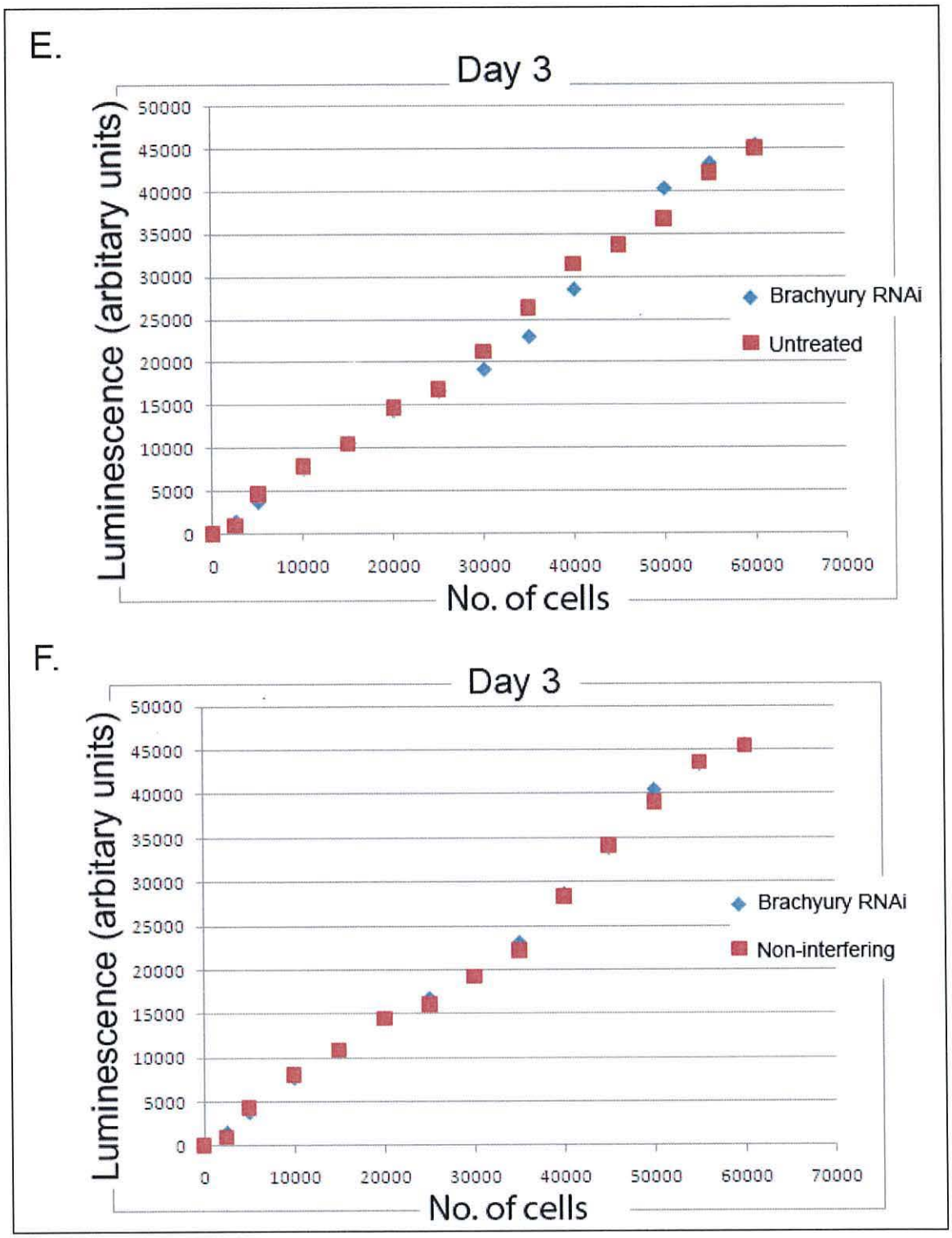


Figure 4.16 E-F: Graphs demonstrating the average luminescence obtained (Y-axis) against the number of viable cells (X-axis). This graph shows that the number of proliferating cells on knocking down *BRACHYURY,* on day 3 of treatment, is similar compared to that of untreated (A) and non-interfering siRNA (B) controls. For each data point, N=1, done in triplicate.

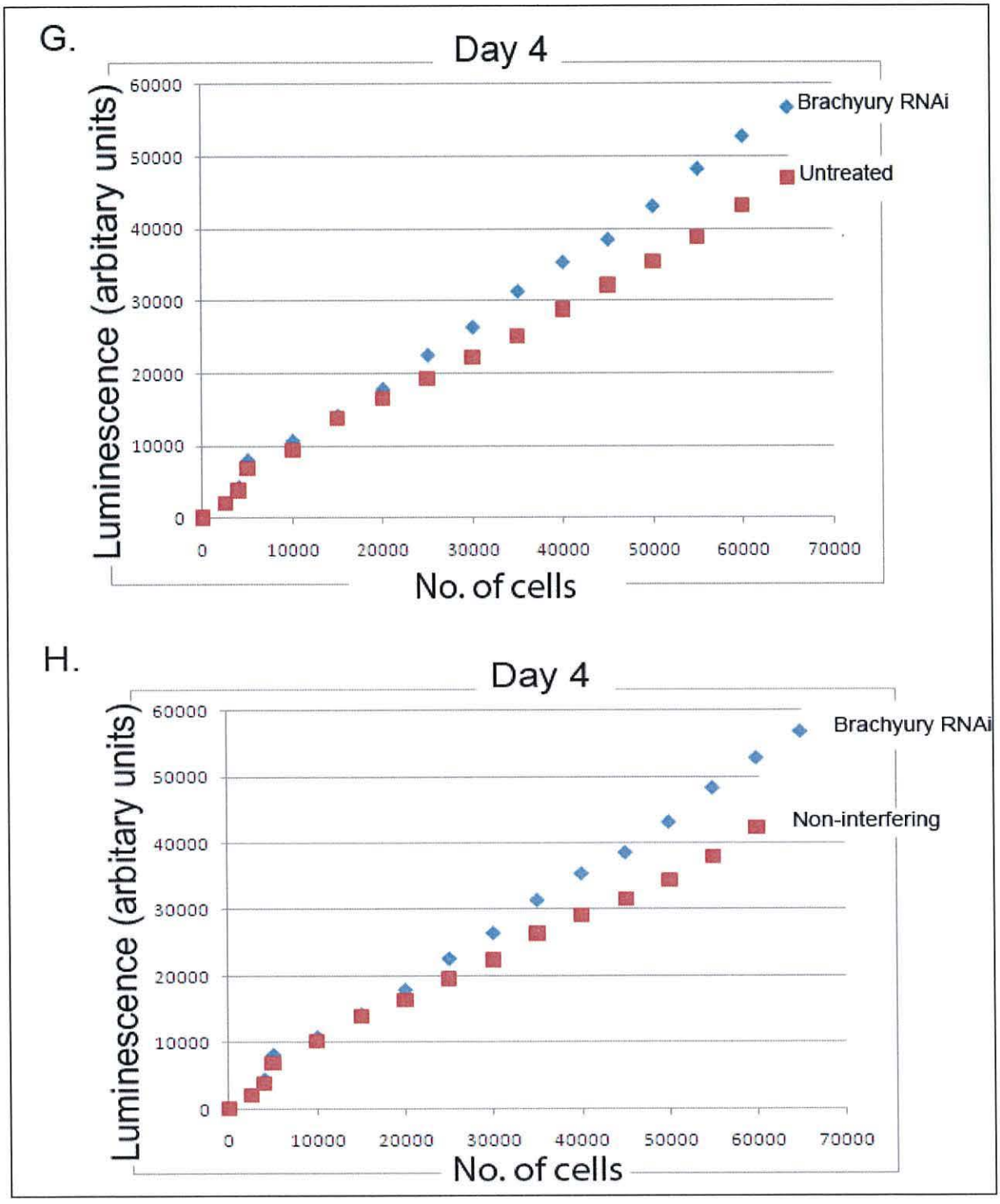


Figure 4.16 G-H: Graphs demonstrating the average luminescence obtained (Y-axis) against the number of viable cells (X-axis). This graph shows that the number of proliferating cells on knocking down *BRACHYURY,* on day 4 of treatment, is similar compared to that of untreated (A) and non-interfering siRNA (B) controls. For each data point, N=1, done in triplicate.

Figure 4.16: Cell proliferation assay by CellTiter-Glo Luminescent Cell Viability Assay kit to demonstrate the proliferation of SW480 cells on *BRACHYURY* RNAi compared to the untreated and non-interfering siRNA treated cells, as the SW480 cells reach from sub- to super-confluent stage.

A.

	1 day after RNAi (Relative expression)	2 days after RNAi (Relative expression)	3 days after RNAi (Relative expression)	4 days after RNAi (Relative expression)	5 days after RNAi (Relative expression)	6 days after RNAi (Relative expression)
Non-interfering	1±0.05	1±0.06	1±0.11	1±0.04	1±0.15	1±0.01
Untreated	0.92±0.05	1.02±0.13	0.96±0.07	0.99±0.08	1.04±0.36	0.97±0.20
Brachyury RNAi	0.53±0.04	0.31±0.02	0.42±0.23	0.43±0.17	0.73±0.26	0.66±0.23

B.

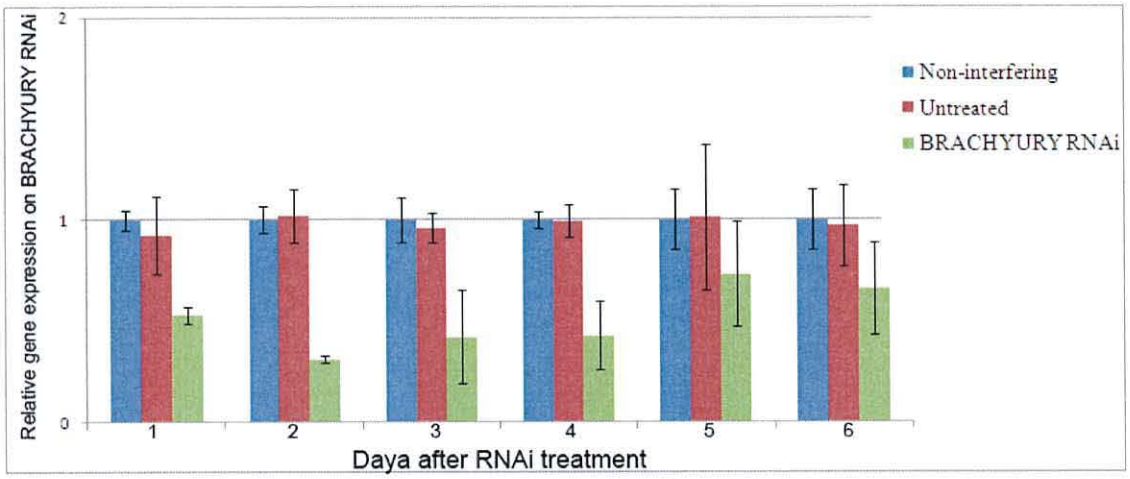


Figure 4.17

Efficiency of *BRACHYURY* RNAi after RNAi in SW480 cells. A) Table showing the levels of BRACHYURY mRNA until 6 days after RNAi. This table shows that the BRACHYURY RNAi levels at day 5 and 6 after RNAi is less than that of days 1-4. B) Graphical representation table A showing high knock-down levels on days 1-4 of RNAi, but low RNAi levels on day 5 and 6 after RNAi. The X-axis mentions BRACHYURY levels are analysed for days 1-6 after RNAi by qRT-PCR under different RNAi conditions. Y-axis gives the relative *BRACHYURY* expression levels. Raw data is in appendix **Table 20**; N=3, in triplicate. All data were compared to β-catenin. p-values obtained for the fold reduction of BRACHYURY RNAi are 0.03 (Day 1), 0.00049 (Day 2), 0.0046 (Day 3), 0.05 (Day 4), 0.173 (Day 5), 0.088 (Day 6).

e) Role of *BRACHYURY* in cell migration

4.5: Role of *BRACHYURY* in cell migration:

Cancer cells become metastatic and move from primary to secondary tumour sites by the process of cell migration. Many signalling molecules are involved in this process. *CDX2* is a homeodomain transcription factor that regulates intestinal differentiation: it is a key intestinal gene down-regulated at the invasive cells of colon cancer (Brabletz *et al.*, 2004) and is also reduced during wound healing. *CDX2* has been shown to prevent cell migration and dissemination in colon cancer, both *in vitro* and *in vivo* (Gross *et al.*, 2008). In human COLO 205 cancer cells, *CDX2* expression is shown to induce E-CADHERIN activity and also increase the cell-cell adhesion (Keller *et al.,* 2004). Over-expression of *CDX2* along with *CDX1* in human colon cancer cell line HT29 (which is low in CDX expressions) showed a reduction of tumourigenic potential and migration; and an increase in sensitivity to apoptosis (Soubeyran *et al.,* 2000). This might show that the loss of expression of *CDX1* and *CDX2* might be related to the development and virulence of colorectal tumours (Soubeyran *et al.,* 2000).

Since, our data shows significant reduction of *CDX2* expression in response to *BRACHYURY* RNAi, we investigated a possible role of *BRACHYURY* in SW480 cell migration.

We used Cytoselect 24 well cell migration assay with 8μm pore size (Cell Biolabs, Cat. Number CBA-101) to analyse this experiment (Materials and Methods 2.23). According to the principle of this assay, the cells which have migrated will pass through a polycarbonate membrane and remain attached to the bottom side of the membrane until detached in cell detachment solution. After adding dye to the cell detachment solution containing the cells, the luminescence is then read at 420/ 530 nm wavelength.

We performed this assay on SW480 cells treated with MitoMYCin-C (to stop cell division and thus to synchronise the cell cycle). We analysed the cells

after *BRACHYURY* knock-down and compared it to the controls at time points 12 hours and 24 hours after RNAi. Our data show (Figure 4.18) that the number of migratory cells decrease over the time span, confirming our data obtained from the scratch wound assay. Also, there was a decrease in migration of SW480 cells treated with *BRACHYURY* RNAi after 12 hours of the treatment, compared to untreated and non-interfering controls. Also, after 24 hours, the number of migratory cells decreased in cells treated with *BRACHYURY* RNAi but this decrease was less compared to 12 hour time-point.

Only two repeats were done for this experiment and *BRACHYURY* knock-down levels were analysed for two sets of data (mentioned as trial 1 and trial 2 in Figure 4.18A). Trial 1 had *BRACHYURY* knock-down 43% for 12 hour time-point (Figure 4.19) and 47% for 24 hour time-point (Figure 4.19). Trial 2 had *BRACHYURY* knock-down to be 66% for 12 hour time point (Figure 4.19). and 62% for 24 hour time point (Figure 4.19) confirming the knock-down being active after 24 hours. Trial 1 (Figure 4.18) did not show any reduction in cell migration after on knocking down *BRACHYURY* at 12 hour time-point whereas trial 2 gave 44% reduction (Figure 4.18) of cell migration in SW480 cells treated with *BRACHYURY* siRNA, at a similar time-point (12 hour). At 24 hour time point, both trial 1 and 2 showed a small decrease in cell migration (~20%) on *BRACHYURY* knocked down SW480 cells.

An average of the two trials was calculated and Figure 4.18B was plotted based on the average values. At 12 hour time point (Figure 4.18B), since the error bar of the RNAi sample overlaps with that of the controls, statistically, this is not a significant difference although biologically, we did not explore its significance. This result was affected by the fact that trial 1 showed no decrease in migration of the cells on treating them with *BRACHYURY* siRNA. This might be due to the lower rate of *BRACHYURY* knock-down (43%) in trial 1.

At 24 hour time point (Figure 4.18B), since the error bar of the RNAi does not overlaps with that of the controls, statistically, this might be of significance, but we need more repeats of the experiment to conclude this result.

A.

RFU (Arbitary Luminescence units)→ RNAI conditions ↓	12 hours (trial 1 RFU)	12 hours (trial 2 RFU)	Average RFU (12 hours)	24 hours (trial 1 RFU)	24 hours (trial 2 RFU)	Average RFU (24 hours)
Untreated	10544	12100	11322	7508	7840	7674
Non-interfering	11442	11985	11713.5	7562	7825	7693.5
***BRACHYURY* RNAi**	11733	7837	9785	6439	6849	6644

B.

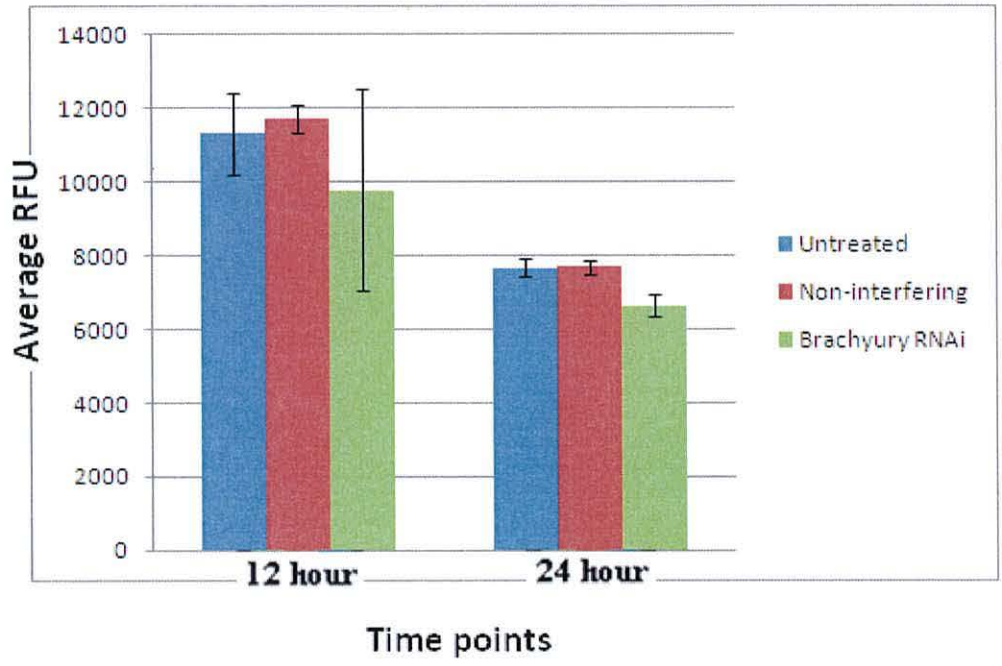
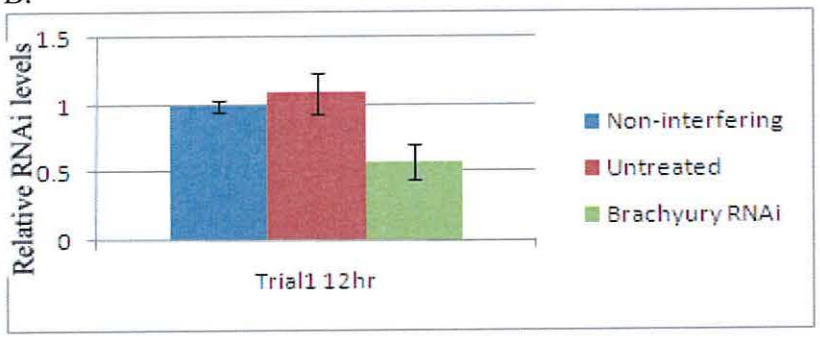


Figure 4.18
Cell migration assay using Cytoselect cell migration assay in SW480 cells. A) Table showing the cell migration rate decreases from 12 hours (blue) to 24 hours (pink) after plating the cells. This table gives the raw RFU values of the migration assay (after BRACHYURY RNAi) for trials 1 and 2, obtained from the luminometer. This table also gives the average of the RFU values obtained for trial 1 and trial 2 for each time point, which is then plotted on the graph B. On knocking down *BRACHYURY*, the rate of cell migration decreases, both 12 and 24 hours after RNAi. The X-axis mentions the time points and the different RNAi conditions, for which the migration assay was analysed. Y-axis gives the average RFU values (migration assay count). RFU = Relative Fluorescence Units. B) Graph depicting data from table A, showing lower cell migration rate on knocking down *BRACHYURY* in SW480 cells at time points 12h and 24h after RNAi. N=2, in triplicate.

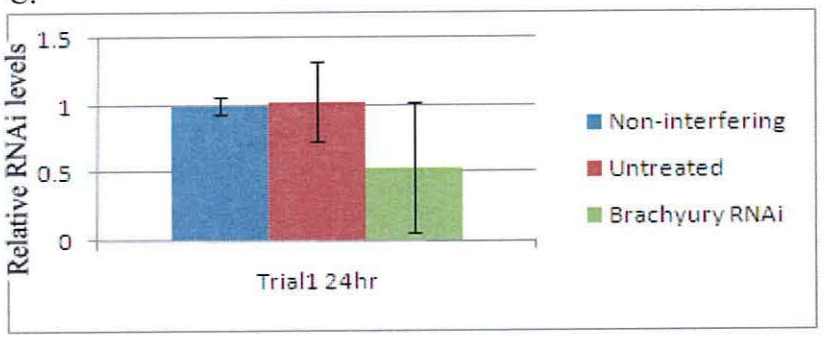
A.

	12 hr after RNAi, Trial 1 (Relative expression level)	24 hr after RNAi, Trial 1 Relative expression level)	12 hr after RNAi, Trial 2 (Relative expression level)	24 hr after RNAi, Trial 2 Relative expression level)
Non-interfering	1	1	1	1
Untreated	1.09	1.03	1.13	1.07
Brachyury RNAi	0.57	0.53	0.34	0.38

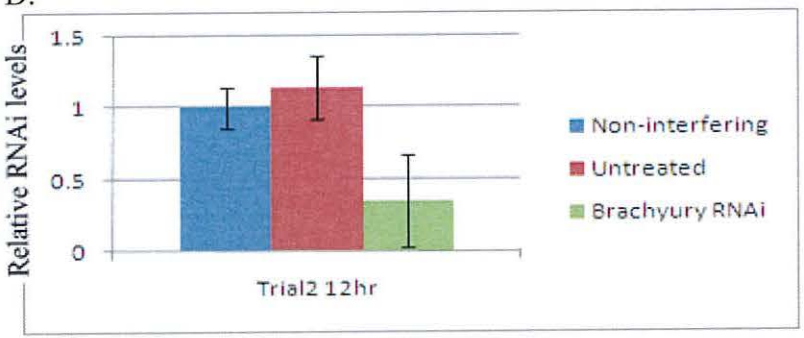
B.



C.



D.



E.

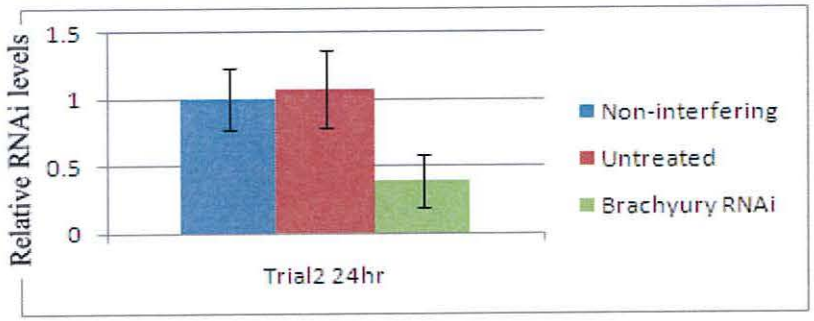


Figure 4.19: BRACHYURY RNAi levels in SW480 cells for migration assay. A) This table shows that relative *BRACHYURY* expression on knocking down *BRACHYURY* at time points 12h and 24h. Graphs B-D gives the graphical representation of table A. X-axis=time points and RNAi conditions; Y axis=relative *BRACHYURY* expression. All data were compared to β-catenin. N=2, done in triplicate. Raw data for this figure can be obtained from Table 21 of appendix.

[Note: We did a cell count of the medium in which the inserts, containing the cells, were dipped in to confirm whether the migratory cells have crossed the membrane and moved to the solution instead of getting attached to the membrane. Since, we did not find any cells in the medium solution; we confirm that the migratory cells were only found at the membrane bottom and not in the medium].

Since, this data is preliminary; more repeats of this experiment should be performed to obtain p-value and the error bars confirming the significance of the data, which will depict a proper scenario of the role of *BRACHYURY* in cell migration.

Discussion:

BRACHYURY was originally shown to have a central role in mouse mesoderm development but its expression has also been shown in chordomas and in the embryonal carcinoma (EC) cell line NTERA2, where it is expressed in the absence of mesodermal differentiation. However, a role of *BRACHYURY* in cancer has not been determined.

In this chapter, we have explored possible roles of *BRACHYURY* in SW480 cells. We have shown that the expression of *BRACHYURY* influences expression of the pluripotency gene *NANOG*, and also the levels of markers of CSCs in low density SW480 cells. We also show that the oncogene *β-catenin* affects the levels of *BRACHYURY* expression, suggesting a possible role of BRACHYURY in the transducing the *β-catenin* signaling pathway and in the maintenance of cells with a CSC-like phenotype in SW480 cells.

β-catenin is a modulator of signaling pathways that regulate 'stemness' and also been shown to be required to maintain a population of CSCs in epidermal tumours . We show a possible mechanism whereby *β-catenin* signals

through *BRACHYURY* to maintain a population of cells potentially having stem-like character is determined by expression of *NANOG*.

NANOG is not absolutely required for stem cell self-renewal, but it increases the efficiency at which the process of self-renewal can be carried out. High levels of NANOG can translate to an efficient self-renewal and low levels of NANOG lead to an increased chance of differentiation. From our data, we show that SW480 cells at a high density, epithelial-like state, display BRACHYURY and NANOG positive cells very rarely. From the limited published studies to date, it is possible that not all tumours/cell lines express *BRACHYURY*. Those tumours that do not express *BRACHYURY* might have evolved an alternative route to activate *NANOG* which is then important in establishing cells bearing characteristics of CSCs. Also, *BRACHYURY* negative tumours might also be less aggressive. It would be interesting to determine the localisation pattern of NANOG and BRACHYURY within a larger panel of colorectal and other cancer tumours and to correlate such data with the presence of CSCs and patient outcomes.

Chapter 5 - The binding of BRACHYURY to the *NANOG* promoter region is dependent on β-catenin in SW480 cells

5.1 Introduction

The T box gene family has been identified in *Drosophila*, mouse, human, *Xenopus*, sea urchin, chick, zebrafish *C. elegans* and the ascidian genomes (reviewed by Papaioannou *et al.,* 1998). These genes have functional roles in early development during organogenesis (such as development of heart and limb, specification of mesoderm, formation of the vertebrae (Papaioannou and Silver, 1998; Smith, 1999; Kavka and Green, 1997) and are also involved in many developmental syndromes in humans (Li Q.Y. *et al.* 1997, Basson *et al.,* 1997; Bamshad *et al.,* 1997). *Brachyury* or *T*, is the founding member of the family of T-box transcription factors and has been the most intensively studied. This gene encodes a sequence-specific DNA-binding protein which acts as a transcriptional activator (Conlon *et al.*, 1996; Herrmann *et al.*, 1990; Kispert and Herrmann, 1993; Kispert *et al.*, 1995a). *Brachyury* is expressed throughout development of the mesoderm of mouse, chick, *Xenopus* and zebrafish embryo and then becomes restricted to the notochord development. (Kispert *et al.*, 1995b; Schulte-Merker *et al.,* 1992; Smith *et al.*, 1991; Wilkinson *et al.,* 1990). In the mouse, Brachyury has been shown to function in mesoderm development where genetic mutation leads to loss of posterior mesoderm formation and aberrant notochord differentiation (Chesley, 1935; Gluecksohn-Schoenheimer, 1938; Herrmann *et al.*, 1990; Halpern *et al.*, 1993; Schulte-Merker *et al.*, 1994; Conlon *et al.*, 1996). The structure, function and expression of Brachyury are highly conserved in mouse, zebrafish, Drosophila and human (reviewed in Papaioannou *et al.,* 1998).

In 1993, Kispert and Herrmann isolated DNA fragments that bind Brachyury protein based on the binding site selection method. The core sequence required for Brachyury binding was found to be an 8 nucleotide sequence, TCACACCT. However, this can be extended either side to 10 nucleotides sequence by adding nucleotides to either sides of it. This can either form a palindrome or an inverted repeat (Kispert and Hermann, 1993; Papaioannau and Silver 1998; Conlon *et al.*, 2001).

The DNA binding ability of Brachyury is confined to the large N-terminal domain of 229 amino acid residues (Busch and Sassone-Corsi, 1990). This domain does not have similarity to any other DNA binding motifs (Harrison, 1991) and is highly conserved between the mouse T protein, and its *Xenopus* (Smith *et al.,* 1991) and zebrafish (Schulte-Merker *et al.*, 1991) homologues (Figure 5.1).

Putative Brachyury (GGGACACACCTAGGGTTCCC) and STAT-3 (TTCCTAGAA) binding sites were reported in the mouse *Nanog* promoter region, 4.91 kb upstream of the translation start site (Suzuki *et al.,* 2006). The Brachyury binding site was identified as a 20-bp sequence which formed an imperfect palindrome and has homology with the proposed Brachyury binding site for *Xenopus*. Further, a STAT3 binding site was also reported, 44 bp upstream of the T-binding site. Reporter assays suggested that the up-regulation of *Nanog* is governed by functional T and STAT3 sites upstream of the mouse *Nanog* gene which act as enhancer elements. When one or both STAT3 and T sites were mutated, the enhancer activity for *Nanog* transcription was lost. T was also found to associate with STAT3 only when the STAT3 translocation was stimulated by LIF (Kispert and Hermann, 1993; Papaioannau and Silver 1998; Conlon *et al.*, 2001).

As discussed above, *Nanog* has been shown to be a direct target of Brachyury binding in the mouse ES cells, and our data (shown in results chapter 4) suggests that *NANOG* might be a target of *BRACHYURY* in human CRC cells (SW480). To determine whether the regulation of *NANOG* by BRACHYURY might involve direct binding of BRACHYURY to the *NANOG* promoter, we carried out ChIP in the upstream regulatory region of the human *NANOG* gene in SW480 cells. In order to carry this out, it was necessary to search for the human *NANOG* upstream regulatory region (chromosome 12) in the *NANOG* contig region (gene sequence NM_024865) on chromosome 12p13.31 for potential BRACHYURY binding sites. There are also 10-11 pseudogenes for *NANOG* in the human genome, some of which are also situated on chromosome 12. It was therefore important to carefully select the functional *NANOG* gene in human in place of any of the *NANOG* pseudogenes.

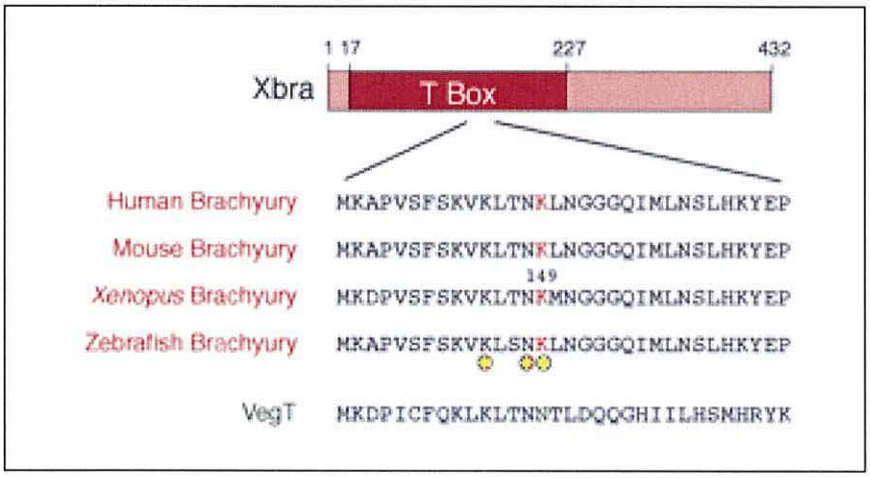


Figure 5.1

Brachyury (T) is conserved in human, mouse, *Xenopus* and zebrafish. This figure highlights the amino acids in the DNA binding region of Brachyury; *Xenopus Brachyury* (Xbra) amino acid sequence, aligned with the T box in human, mouse, *Xenopus* and zebrafish Brachyury. Yellow circles denote amino acids that contact DNA according to Muller and Herrmann, 1997. Lysine 149 is the amino acid which is associated with T box specificity. (Image taken from Conlon *et al.,* 2001).

Results:

Our experimental approach using ChIP to identify binding sites for Brachyury in the human *NANOG* promoter is described in the following sections.

5.2 Identification of BRACHYURY binding sites in the human *NANOG* promoter region:

BRACHYURY has been shown to bind to enhancer elements in the *Nanog* promoter region and to up-regulate the expression of *Nanog* in mouse ES cells in association with STAT3 (Suzuki *et al.*, 2006). We wanted to investigate whether *NANOG* might similarly be regulated by BRACHYURY in SW480 cells. We therefore searched for putative T-binding sites in the sequences upstream of the human *NANOG* gene. The functional human *NANOG* gene is located on chromosome 12 (position 12p13.31) and contained in the contig NT_009714.17. We used this sequence to carry out our search for putative BRACHYURY binding sites. This search was based on the DNA consensus sequence of 8 nucleotides representing $^{5'}$TCACACCT^{3} the core T-box consensus sequence; and its possible palindromes or inverted repeats (Kispert & Hermann, 1993). We found 8 possible sites for both $^{5'}$AGGTGTGAA$^{3'}$ and $^{5'}$TCACACCT$^{3'}$ sequences representing core binding sequences for BRACHYURY (refer to table 5.1 A and B). Out of these sites, we selected two putative T-box binding sequences, since these were arranged as inverted repeats; site 1, a 19 bp inverted repeat (-708 bp relative to the start codon of *NANOG*) and site 2, a 15 bp inverted repeat sequence (-2335 bp relative to the start codon of *NANOG*) (Figure 5.3A). These two sites were chosen based on the length of inverted repeat and proximity to NANOG translation start. [Note: The size of promoter region used was 2331 bp, starting from the BRACHYURY binding site upto the NANOG translation start site. This region of the *NANOG* promoter contained the two putative BRACHYURY binding sites. Refer section 5.3B].

5.3A: BRACHYURY binds to the *NANOG* promoter in human SW480 cells:

Following our identification of two putative BRACHYURY binding sites in

A.

BRACHYURY binding sequence 1: $^{5'}$AGGTGTGAA$^{3'}$

Positions/base site in the *NANOG* promoter region	BRACHYURY binding sequence	Inverted repeats	Palindrome sequence	No. of bases for palindrome
*705591	AGGTGTGAA	705604	CACACC	6
673181	AGGTGTGA	673151 673191	CACCT CACC	5 4
667152	AGGTGTGA	667162	CACCT	5
652812	AGGTGTGA	652782	CACCT	5
616613	AGGTGTGA	616579	CACCT	5
608761	AGGTGTGA	608731	CACCT	5
581831	AGGTGTGA	581846 581846	CACCT CACCT	5 5

B.

BRACHYURY binding sequence 2: $^{5'}$TCACACCT^{3}

Positions/base site in the *NANOG* promoter region	Sequence	Inverted repeats	Palindrome sequence	No. of bases for palindrome
*703971	TCACACCT	703964	AGGTG	5
667771	TCACACCT			
662722	TCACACCT	662761	AGGTG	5
648293	TCACACCT			
598483	TCACACCT	598471	AGGTG	5
595553	TCACACCT			
575713	TCACACCT	575701	AGGTGTGA	8
572012	TCACACCT	575207	AGGTG	5

Table 5.1

Potential BRACHYURY binding sites in the promoter region of the human *NANOG* gene. Locations of possible BRACHYURY binding sites for the sequence A) $^{5'}$AGGTGTGAA$^{3'}$ and B) $^{5'}$TCACACCT^{3} identified in the upstream region of *NANOG* promoter region in the contig; and potential inverted repeat sequences in close proximity to these sites. Two sites at positions 705591 and 703971 were chosen due to their proximity to the *NANOG* gene and their having potential inverted repeat sequences. These two sites are marked with *. Numbers refer to the positions on the *NANOG* contig region (Contig reference: NM_024865, NCBI). described in section 5.3B.

relative close proximity to the *NANOG* promoter, we carried out ChIP (Chromatin Immunoprecipitation) using antibody to BRACHYURY (T), in low density and high density SW480 cells to determine whether these putative binding sites could act as binding sites for endogenous BRACHYURY. The ChIP protocol was followed as We optimized conditions for this as follows.

- Sonication conditions were optimized to 5 repeats of 10 seconds each. These conditions were chosen in order to obtain genomic DNA products ranging in size between 200-1000bp (Figure 5.2A).
- We also carried out IP for BRACHYURY with BRACHYURY antibody to optimize antibody concentration (IP protocol described in M & M section 2.16). This IP gave a very strong product for cell lysate derived from SW480 subconfluent cells, slightly weaker binding with SW480 confluent lysates and almost no binding with supeconfluent cells (Figure 5.2B) confirming our qRT-PCR data (chapter 3) that Brachyury is not abundant in superconfluent SW480 cells.

The ChIP assay was carried out on cell lysates from sub- to superconfluent SW480 cells. Specific binding was observed at both sites 1 and 2, although binding at site 1 may be slightly more efficient as indicated by slightly stronger PCR bands (Figure 5.3B); BRACHYURY binding at the two sites was observed only with lysates from day 1 (low density SW480 cells) and day 2, weaker binding (mid-density cells), and no binding was observed at day 4 (high density cells). The PCR products for the input DNA and ChIP, at sites 1 and 2, were confirmed as being the human *NANOG* promoter region by sequencing. We also included control primers in a ChIP reaction to a region in the *APC* gene (exon 15) [See M & M table 2.9 for APC primer sequence]. Sequencing confirmed this PCR product as *APC* and it did not yield any product following ChIP with BRACHYURY antibody, confirming that no non-specific product was pulled down in the BRACHYURY IP reaction.

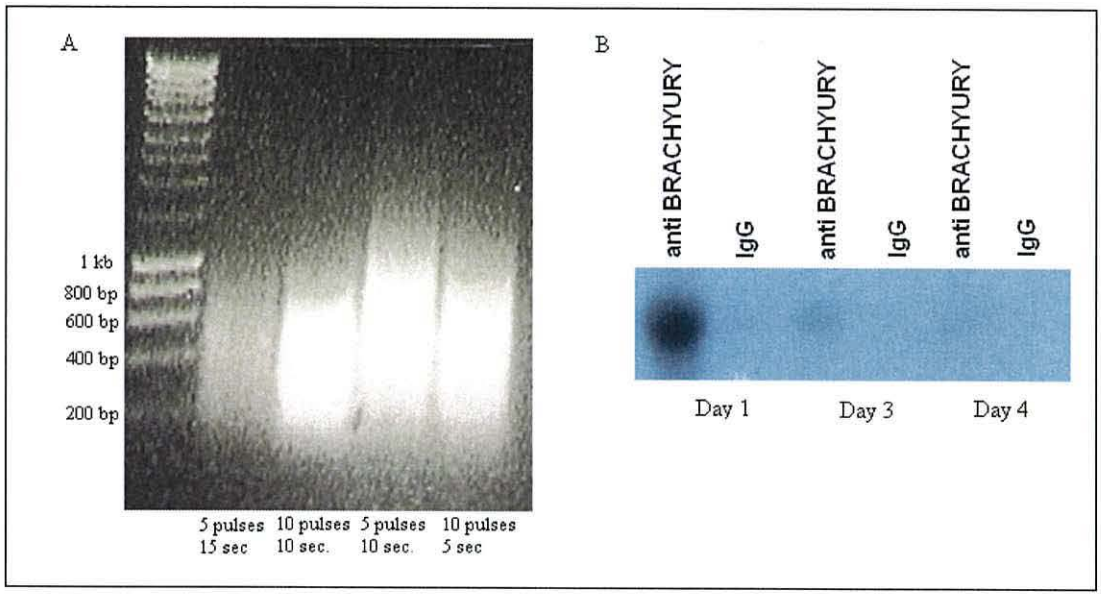


Figure 5.2

Optimization of ChIP conditions. A) Optimization of sonication conditions with four different pulses and duration of sonication. The number of pulses and the time of sonication are mentioned below each lane in the image. B) The amount of BRACHYURY antibody was optimized using SW480 cell lysates from Day1 (sub-confluent), Day 3 (confluent) and Day 4 (super-confluent) confirming strongest binding on day 1 SW480 lysates and the weakest on day 4. The IgG was used as control without BRACHYURY antibody to confirm specific antibody binding.

5.3B: *NANOG* promoter sequence for BRACHYURY binding (human)

caatacgtga aaattggtga atctccattc tgacctatta acacccagtg tgggagcttt
tgagcatcag tttaaagcaa aaggtggtag cgccggcctg atgaggtggc tcacacctgt
aatctcagta cttcgggagg ccgaggcagg cggatcacga ggtcaggagt tcgagaccag
cctcaccaac attgagaaac cctgtctcta ctaaaaatac aaaattagcc aggcgtgatg
gtgcctgcct gtaatcccag ctactcagga ggctgaggca ggagaatcgc ttgaacccag
gagatggagg ttgcagtgag ccgagattgc accatcgcac ttcagcctgg gcaacaagag
tgaacctcca tcctaatttt aaaaaaaagg ggtagcgccg ctcagcaatg cattggccac
cattatagat ctctcccaac gcagtctatt atgtacaaaa tggagatact gataagactt
cttggagtaa tactatataa agcttgccaa agtgccaggg ctgcttaaga aattgcttct
tattacttag atctggggtt ctgggaatta tcaaagtact ttgaaaacaa ttttttaaa
ggatatttta atatttgaaa aattttagac aaaagtgtcc ttttatttgt tcccaacagt
ctctcctctt ccttcctcca tggatctgct tattcaggac agccctgatt cttccaccag
tcccaaaggc aaacaaccca cttctgcaga gaagagtgtc gcaaaaaagg aagacaaggt
cccggtcaag aaacagaaga ccagaactgt gttctcttcc acccagctgt gtgtactcaa
tgatagattt cagagacaga aatacctcag cctccagcag atgcaagaac tctccaacat
cctgaacctc agctacaaac aggtaggctt gttttgtcct tggaataagg tgaacaaaaa
ttggactaat ttgcatggct aagacctctg tggatgcatg tagatgtgtg tactatgtgt
ccgtacatcg cctcttgcaa ataatttatg aagatgaaat gcttttgtaa cttccttcac
ctctttcttt ccttaaatat tctattatgt gaataattat gtcataattt aaccactttc
ttgcacagac caatattgtg aaaatctttc caacgtttcc ttaataaaaa agaagtatct
aactccactt accagggtag gagaaaccct aactcacact ggttctcatt aaaataaaaa
ctttttttt ccttttttc tttctgccag caactccagc ttgttttgtt ttttatctgt
gtgcattcaa taaggccaat cattgtttat tacttgttgg ccattttcct tcattaattg
gcttatcctc ttctaggctt tatttccttc ttttcttttg tttatttttg agacggagtc
tcactctgtc tcccaggctg gagtgcagtg gcctcataat gagacatcat aatgacatta
gcattacttg gtttaagtac aaatggatga aacctccacc tcctgggttc aagggattct
cctgcctcag cctcccaagt agctgggatt acaggcgccc gccactacac ctggctaatt
ttactgtatt ttttagtaga gatggggctt caccatgttg gccaggctgg ttttcaactc
ctgacctcaa gtgatccacg tgcctctgcc tcctaaagtg ctaggattac aggtgtgaac
taccacaccc ggcccttgcc tttatttttct atccaaaatg ttcatttctc tctttttttc
tgagacagtc tggttctgtt gtcacgcagc tggagtgcag tgatgcaatc ttggctcact
gcagccttca cttcccaggt gcaagcgatc ctcccgtctc agcctcctga gaagctggga
ccacagacgt gcagcattac acccggctaa ttttttgtat ttttgtagac acggtgtttc
ctcatgttgg ctagcctgta gcgaactcct gggctcaagc aatctgcccg ccttggcctt
ccaaagtgtt gggattacag gcatgagata tggcacctgg ccaggaataa aagttagcaa
tatactttga ttcaaagtac ctctgtatta tgaatatttt acaatttcta tcatttttc
ctgcaggtga agacctggtt ccagaaccag agaatgaaat ctaagaggtg gcagaaaaac
aactggccga agaatagcaa tggtgtgacg caggtaacag gaaacttcat tctgttcttt
cctttcagtg atctttcaat cttgtccatc cctgaaacac acaactccag tcacagacag
ttctggttgt ccttgtaccc tttctgttaa tccctccttc tctttcagaa ggcctcagca
cctacctacc ccagccttta ctcttcctac caccagggat gcctggtgaa cccgactggg
aaccttccaa tgtggagcaa ccagacctgg aacaattcaa cctggagcaa ccagacccag

Nanog translational site: atg
Two putative Brachyury binding sites: tcacacct and aggtgtgaa

L2a: attcca ggtac **gtgtgggagcttttgagcatc**
L2C: attcca ggtac gtcaggagt tcgagaccag cctc
L0F: attcca ggtac **gagacagtctggttctgttgtcacgc**
Reverse primer, LR1: attcca gagctc ctggtggtaggaagagtaaagg
Construct 1(no sites) = L0F + LR1 = 635 bp
Construct 2(1 sites) = L2C + LR1 = 2275 bp
Construct 3(both sites) = L2a + LR1 = 2389 bp
Size of the NANOG promoter = 2331 bp (from the BRACHYURY binding site, tcacacct to NANOG start site, atg)

5.4 BRACHYURY binding to the *NANOG* promoter depends on β-catenin:

BRACHYURY has been shown to be a downstream target of Wnt 3a during mesoderm specification in the mouse embryo (Terry *et al.,* 1999). Since, the Wnt signalling pathway is critically altered in CRC and *β-catenin* is the key oncogene of this pathway, we wanted to know if the binding of BRACHYURY to sequences in the *NANOG* promoter region was dependent on signaling through β-catenin. We therefore repeated the ChIP, but in the presence of interfering RNA to β-catenin; to knockdown *β-catenin* in the SW480 cells. Again, specific binding of BRACHYURY at both sites 1 and 2 was observed for day 1 and 2 in untreated and non-interfering control samples, but no binding was observed for samples treated with siRNA to knockdown *β-catenin* in day1 and 2 samples. None of the samples at day 4 (super-confluent SW480 cells) displayed binding of BRACHYURY to either binding site under any of the conditions used to knockdown *β-catenin* (control or experimental) (Figure 5.3C). These results are consistent with the expression patterns for BRACHYURY and NANOG observed previously, i.e. strong expression at day 1 (sub-confluent cells) and rare expression at day 4 (super-confluent cells); and demonstrate that binding sites for BRACHYURY exist within the *NANOG* promoter in human CRC cells and that BRACHYURY binding to these sites is dependent on β-catenin.

In summary, we demonstrated the presence of two putative T binding sites (19bp and 15bp long) at 708bp and 2335bp upstream of the *NANOG* promoter in human CRC cells. We were not able to find a putative STAT binding site near these

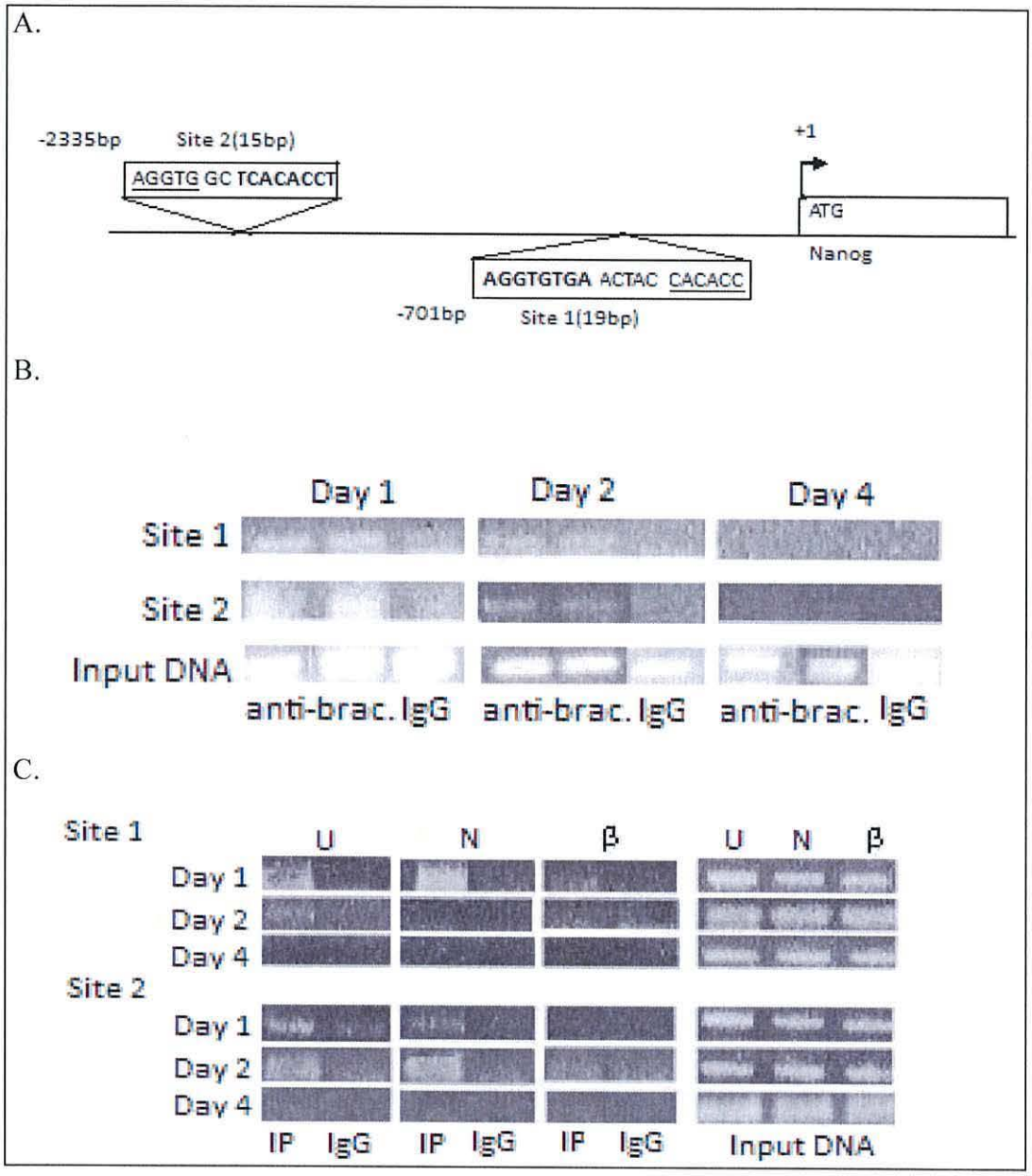


Figure 5.3
ChIP assay results for BRACHYURY. A) The putative two BRACHYURY binding sites in the upstream of human *NANOG* promoter with nucleotide base pair positions in relation to the promoter (+1). B) ChIP assay showing BRACHYURY binding to *NANOG* promoter in sub-confluent (Day 1), confluent (Day 2) and super-confluent (Day 4) SW480 cell lysates. C) ChIP with knocking down β-catenin indicating that β-catenin is essential for this binding of BRACHYURY to *NANOG* enhancer site in human. Site 1 and Site 2 are the two putative BRACHYURY binding sites in the *NANOG* promoter region. Abbreviations: U=untreated SW480 cells, N=non-interfering RNAi, β= *β-catenin* RNAi. Input DNA is the qRT-PCR product without IP. IgG=immunoglobulin G as a control for the IP.

sequences. The ChIP assay revealed that BRACHYURY can bind to these sites in the human *NANOG* enhancer region with one site apparently being slightly weaker for binding than the other. The binding was shown to be the strongest in sub-confluent SW480 cell lysates at day 1 and this affinity becomes weaker and non-detectable as the cells reached higher density (input DNA was used as a control with no antisera directed to BRACHYURY). This correlates to our data presented in chapter 3 where the amount of BRACHYURY was shown to be high in SW480 sub-confluent cells and lowest in the super-confluent cells. Our ChIP assay with *β-catenin* knockdown confirmed that β-catenin is essential for this binding of BRACHYURY to the *NANOG* enhancer region in human and acts upstream of BRACHYURY.

5.5 BRACHYURY binding sites act as BRACHYURY -dependent enhancers of expression from the *NANOG* promoter:

Cloning, transfection and luciferase assay

So far, in this chapter, we have shown that BRACHYURY binds to sites within *NANOG* promoter and this is dependent on β-catenin. We next wanted to determine if these sites were functional i.e. whether binding of BRACHYURY to these sites could influence transcription. We made three constructs with and without these sites along with a construct with none of the sites for control. Our constructs (Figure 5.5) were as follows:

- Construct 1 = no BRACHYURY binding site
- Construct 2 = containing site 1
- Construct 3 = containing both sites 1 and 2

The PGL3-Basic vector used to make the constructs (1-3) is illustrated on Figure 5.4 with KpnI and SacI restriction sites in the multiple cloning site of the vector. The constructs were made by cloning the DNA into PGL3-Basic vector (4818bp) (Figure 5.4). Restriction enzymes KpnI and SacI were used for restriction digest since these two sites are present in the multiple cloning site of the PGL3 basic vector and do not within cut the *NANOG* regulatory sequence being studied. [Refer section

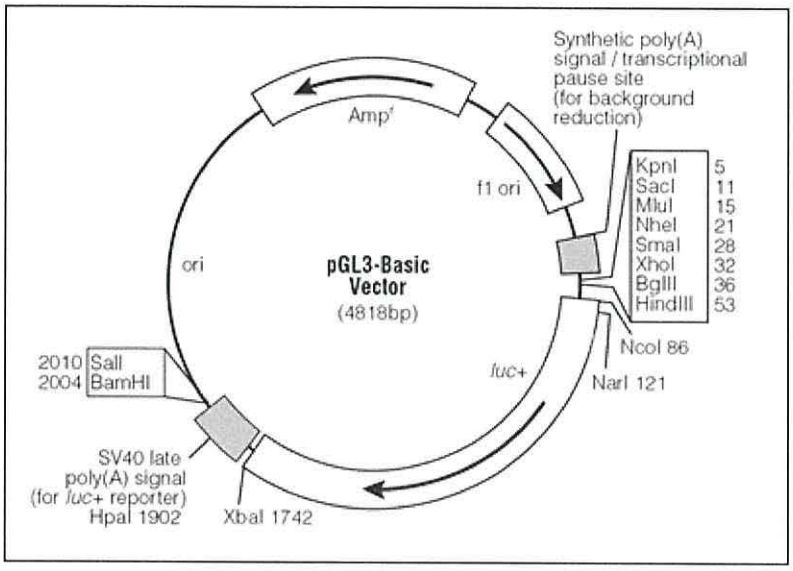


Figure 5.4

PGL3 basic vector map used for cloning. The multiple cloning site contains the KpnI and SacI sites. The size of the vector is 4818bp.

2.19 of Materials and Methods for the cloning procedure]. The three constructs were co-transfected into SW480 cells in turn (transfection protocol- Qiagen, refer to section 2.19 of Materials and Methods) alongside a renilla contruct that contains renilla luciferin (pGL4.73 [*hRluc*/SV40]) under the control of the SV40 promoter. SW480 cells were grown for 24 hrs at 37°C. After 24 hrs, a luciferase assay (according to Promega protocol listed in M & M section 2.19) was performed and the intensities were recorded from the luminometer. The luminescence was then stopped by adding Stop & Glo reagent (E2920, Promega Dual-Glo Luciferase Assay kit) and a separate assay was carried out on the same cells to determine the luminescence intensity of the Renilla using the Promega Dual-Glo Luciferase Assay System protocol. The data was normalized to luminescence obtained from the Renilla vector to account for variation in transfection efficiencies. The experiment was controlled by including untreated cells, mock-treated cells (only transfection reagent and no DNA) and cells with transfection reagent and PGL3 basic vector only. Construct 1 contained 645 bp of sequence upstream of *NANOG* (relative to translation start site) with no BRACHYURY binding sites; construct 2 contained 2285 bp upstream of the start site and 1 BRACHYURY binding site (site 1); construct 3 contained 2399 bp of sequence upstream of the start site, and 2 BRACHYURY binding sites (site 1 and 2). Transient transfection of these constructs in low density SW480 cell showed an increase in the luciferase activity of construct 2 by 1.2 fold relative to control (promoterless luciferase construct, PGL3-Basic), and a 2.3 fold increase in luciferase activity (relative to a promoterless control, PGL3-Basic) for construct 3 that contained both the BRACHYURY binding sites (Figure 5.5A). The p-values showed that this increase in the luciferase activities of constructs 2 (p-value = 0.0008) and 3 (p-value = 0.0269) are statistically significant. The experiment was controlled by using cells with no transfection treated (untreated) and cells with only Superfect transfection reagent (Mock-treated) which gave similar luciferase activities to that of PGL3-Basic (which contains only PGL3-Basic vector and no DNA) (Figure 5.5A). The luminescence obtained from PGL3-Basic was adjusted to 1 and the luciferase activities of contructs (1-3) were compared to PGL3-

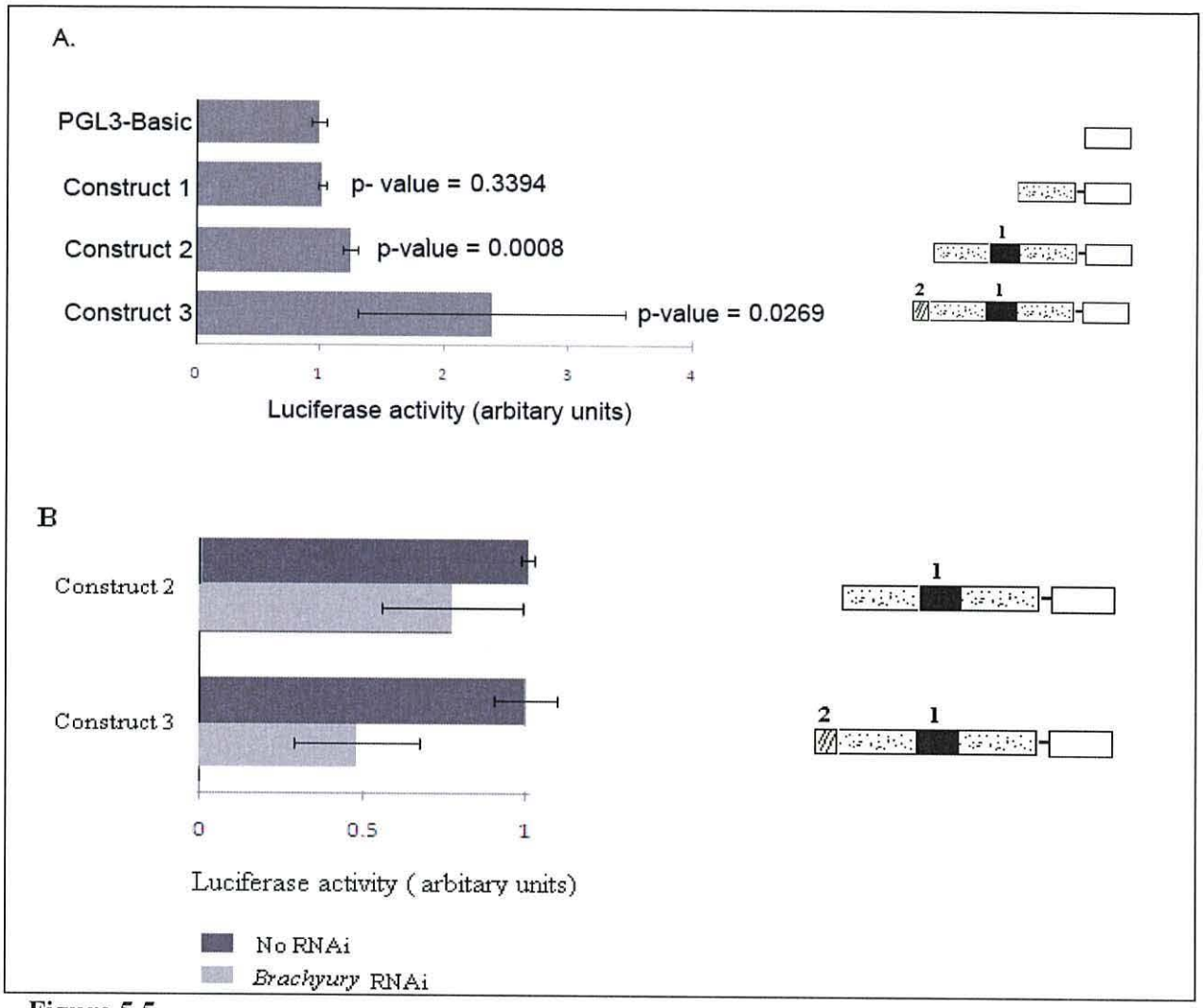


Figure 5.5

Luciferase assay. A) Image showing luciferase activity of construct 2 and 3 increased compared to cells with PGL3-Basic vector as a control. N=5 (for Pgl3-Basic and Construct 3); N= 4 (for constructs 1 and 2). P-values for final F/R value of **Construct 1** compared to that of PGL3-Basic construct = **0.3394 (>0.05)**, p-value for **Construct 2** compared to PGL3-Basic construct = **0.0008 (<0.05)**, p-value for **Construct 3** compared to PGL3-Basic construct = **0.0269 (<0.05).** B) Luciferase activity of constructs 2 and 3 in response to *BRACHYURY* RNAi decreases. The final F/R values for constructs 2 and 3 without RNAi were plotted on the graph against those of constructs 2 and 3 with RNAi . N=3. SDs gave the error bars. Knocking down the *BRACHYURY* reduces the luciferase activity of the **Construct 2** by almost **19%** (comparing 1.79 and 1.19) and that of the **Construct 3** by **60%** (comparing 0.961 and 0.77). The p-value obtained by comparing the final F/R values from the three trials of **Construct 2** with RNAi to that of Construct 2 without RNAi was found to be **0.1362 (>0.05).** The p-value obtained by comparing the final F/R values from the three trials of **Construct 3** with RNAi to that of Construct 3 without RNAi was found to be **0.0012 (<0.05)**. '1' and '2' on the contructs mention the site 1 and 2 for BRACHYURY binding on Nanog promoter.

Basic luminescence. Further, we analyzed whether knocking down *BRACHYURY* in cells transfected with constructs 2 and 3 would show changes in the luciferase activities of the constructs (Figure 5.5B). This would tell us how efficient is the binding of BRACHYURY to these putative sites in *NANOG* promoter region.

Our knock-down data was plotted along with non-knockdown data to analyze the luciferase activities of the constructs 2 and 3. Our data show that knocking down the gene expression of *BRACHYURY* reduces the luciferase activity of the construct 2 by almost 19% and that of the construct 3 by 60%. The p-values for these data show that reduction in luciferase activity of construct 2 were statistically not significant (p-value = 0.1362), but that of construct 3 is significant (p-value = 0.0012). This shows that BRACHYURY can bind to the two putative BRACHYURY binding sites in the *NANOG* promoter, but binding to site 1 is less efficient than binding to site 2. [Raw data for Figure 5.5A in appendix Table 22A-D and for Figure 5.5B in Table 23A -D].

5.6 Discussion:

The ChIP assay in this chapter shows a direct binding of BRACHYURY to putative specific regulatory elements in the *NANOG* promoter region. The ability of BRACHYURY to bind to these sites was removed on knocking down *β-catenin* demonstrating a dependence of binding upon *β-catenin*. In mouse ES cells, the binding of Brachyury to sites in the *Nanog* promoter was co-ordinate with the binding of STAT3 (Suzuki *et al.*, 2006). However, we did not find any STAT3 binding sites close to either of the T-binding sites that we located in the promoter region of the human *NANOG* gene. It has been shown that signaling through STAT3 does not promote self-renewal in human ES cells in culture (Daheron *et al.*, 2004). β-catenin signaling increases *Nanog* promoter activity in a Tcf3-independent manner in mouse ES cells suggesting that a complex control mechanism exists in mouse ES cells (Pereira *et al.*, 2006). Again, this is consistent with our data (chapter 4) where

β-catenin acts to positively regulate the binding of BRACHYURY to the promoter region of *NANOG* in SW480 cells.

The luciferase assay data presented in this chapter indicates that expression levels from the luciferase construct are low when a 701bp *NANOG* upstream element is inserted (construct 2): these levels appear to be close to the control levels, suggesting that the promoter elements contained in the sequence are not efficient under the conditions used in this experiment. Construct 3 (with both BRACHYURY binding sites) appears to give an increase in the transcription activity that is 2.3 fold compared to controls, suggesting that enhancer activity may be contained in a 114bp sequence element (2399bp to 2285bp) that contains the second BRACHYURY binding site. Whilst ChIP data showed that BRACHYURY binds to both sites, it is likely that one site alone is insufficient to enhance transcription.

Removing the sites was a proper experiment preliminary. In future, replacing with random sequence and then mutating specific sequences with real sites will be performed. Future studies would involve more detailed analysis of these binding sites and their composition. Mutation analysis of the sites and further studies on the sequence elements upstream of the *NANOG* promoter will also be beneficial. Also, preparing constructs with site 2 only, and not site 1 will further confirm our results. Other constructs for this analysis would involve deletion or mutation of bases in-between the two BRACHYURY binding sites.

Chapter 6 - The role of *BRACHYURY* in NTERA-2, SW626 and T84 cells

In chapter 4, we have demonstrated a role for the mesoderm inducing gene, *BRACHYURY* in regulating the pluripotency gene *NANOG* and levels of the CSC markers CD166 and CD133 in the human colon cancer cell line, SW480. We next wanted to determine whether these results were specific to the CRC cell line SW480, or whether they could be applied more generally to other cell lines. From our results obtained, we hypothesized that this pathway may be initiated/ active in cell lines that were competent for differentiation along a mesodermal lineage. Therefore, we looked at this pathway in the germ cell tumour cell line, NTERA2.

6.1 Role of *BRACHYURY* in NTERA-2 cells

NTERA-2 is an embryonal carcinoma (EC) cell line obtained from the lung metastasis of a testicular teratocarcinoma (Fogh & Trempe, 1975; Andrews *et al.*, 1984). It is a sub-clone obtained from TERA-2 which was derived by culturing embryonal carcinoma (EC) cells from the lung metastasis of testicular cancer (Andrews *et al.*, 1984).

The EC cells behave as pluripotent stem cells and give rise to all other cell types in the tumour. These cells may originate from a displaced embryonic stem (ES) cell which might have retained pluripotency into postnatal life. Alternatively, they may originate from germ cells which may proliferate without entering into meiosis.

We grew NTERA-2 cells to 70-80% confluency, collected cell lysates and assessed the levels of BRACHYURY by Western blot analysis. Once we confirmed the existence of BRACHYURY in these cells, we then knocked down *BRACHYURY* through RNAi in these cells (as described in materials and methods, section 2.17), collected the cell lysates and assessed the levels of NANOG, Oct-4 and CD166 by Western blot analysis. Our experiment shows that BRACHYURY is present in NTERA-2 cells, the knockdown of which reduces the levels of NANOG,

BRACHYURY and Oct-4. We could not detect CD166 in these cells as expected since this is a specific marker for CRC-SC. Lamin B was used as a loading control confirming equal amount of protein loaded for experimental and control lanes (Figure 6.1B). Like NANOG, Oct-4 is another determinant of pluripotency (Pesce *et al.,* 2001; Nicholas *et al.,* 1998) and is known to be expressed in undifferentiated NTera2 cells (Rosner *et al.,* 1990). We were intrigued to see that Oct-4 was also regulated by BRACHYURY. Oct-4 has been shown to be expressed in some cancers such as bladder cancer (Xu *et al.*, 2007), human non-small cell lung cancer (Karnoubi *et al.,* 2009) and many other cancer (Tai *et al.,* 2005; Wang *et al.,* 2003; Monk *et al.*, 2001; Gidekel *et al.,* 2003; Looijenga *et al.,* 2003).

Thus, we have shown that *BRACHYURY* knockdown in NTERA2 cells decreased the levels of NANOG in similar manner to that observed in SW480 cells (Figure 6.1A). Based on our results from colon cancer cell line, SW480 and EC cell line NT2, we then checked the role of BRACHYURY in the colon cancer cell line T84 and the ovarian cancer cell line, SW626 which is a metastasis of a primary colon carcinoma. The cell lines SW626 and T84 were previously reported to express *BRACHYURY* (qRT-PCR) (Palena *et al.*, 2007).

6.2 Role of *BRACHYURY* in T84 cells

T84 is a human colon cancer cell line derived from a lung metastasis of colon carcinoma in a 72 year old male. The tumour tissue was innoculated sub-cutaneously and transplanted into nude mice. T84 are epithelial cells which grow in monolayer and have receptors for many peptide hormones and neurotransmitters.

Earlier we have shown that the expression of *BRACHYURY* is very low or almost negligible in T84 cells (assessed by qRT-PCR). To check the protein levels,

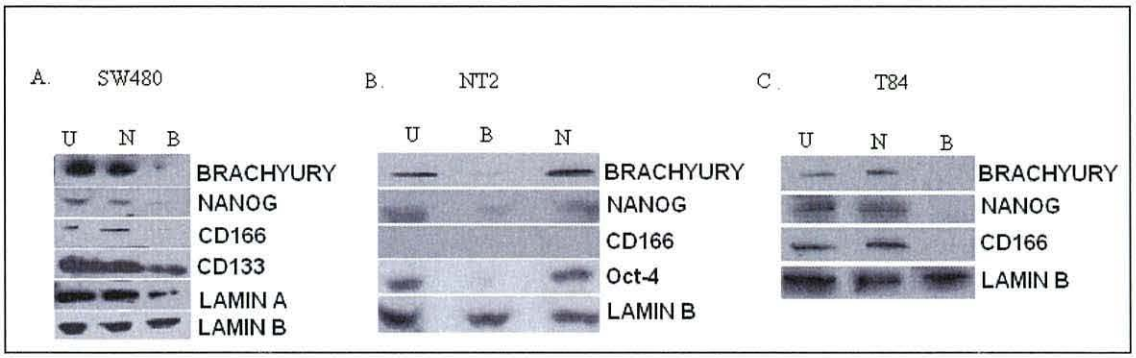


Figure 6.1

Expression of BRACHYURY and other proteins in SW480, NT2 and T84 cells with respect to *BRACHYURY* RNAi. A) *BRACHYURY* knockdown in SW480 sub-confluent cells regulates the expression of NANOG and colon cancer SC-markers CD133, CD166 and Lamin A. B) *BRACHYURY* RNAi in NTERA-2 cells also shows decrease in levels of NANOG, Oct-4 and colon cancer SC-marker CD166. C) Levels of BRACHYURY, NANOG and CD166 in sub-confluent T84 cells were shown here to be lower than that observed in SW480 sub-confluent cells. *BRACHYURY* knockdown by RNAi regulates the levels of NANOG and CD166 in sub-confluent T84 cells. The loading was controlled by LAMIN B whose levels do not change on knockdown of *BRACHYURY*. U=untreated cells, N= cells treated with non-interfering siRNA, B=*BRACHYURY* knockdown.

we performed Western blot analysis to study the levels of BRACHYURY in low and high density T84 cells. We show that BRACHYURY is present inT84 cells but not differentially expressed in low and high density cells (data not shown). We also performed *BRACHYURY* RNAi experiment in low density T84 cells to investigate the effect of BRACHYURY on the levels of NANOG and CD166 (Figure 6.1C). We show that level of NANOG and the CRC stem cell marker CD166 are decreased by knocking down the expression of *BRACHYURY*. Therefore, this regulation of NANOG and the CRC stem cell marker CD166 on *BRACHYURY* knockdown is similar to that observed in SW480 cells (Figure 6.1A). This might suggest that a population of stem cells is maintained by BRACHYURY in the T84 sub-confluent cells by regulating NANOG and CD166 in these cells.

6.3 Role of *BRACHYURY* in SW626 cells

SW626 cells are epithelial-like cells derived from a human ovarian tumour which was a metastasis of primary adenocarcinoma of the colon (Furlong *et al.*, 1999). This cell line was first initiated in January 1974 by A.Leibovitz at the Scotland White Clinic, Texas from a surgical specimen from a cystadenocarcinoma of the ovary of a 46 year old Caucasian female (Anti Cancer Research 1998; 18:1153).

In the first instance, we wanted to determine whether SW626 cells undergo an EMT-like transition similar to that observed in SW480 cells. Therefore, we grew SW626 cells to low and high cell density and performed immunofluorescence to study the localization of β-catenin in these cells. [Note: β-catenin localization shifts from membrane to nucleus in cells undergoing EMT.] But, here, we were able to show that there is no such transition in these cells and since the localization of β-catenin is to the membrane in low density SW626 cells and to the cytoplasm in high density SW626 cells (Figure 6.2).

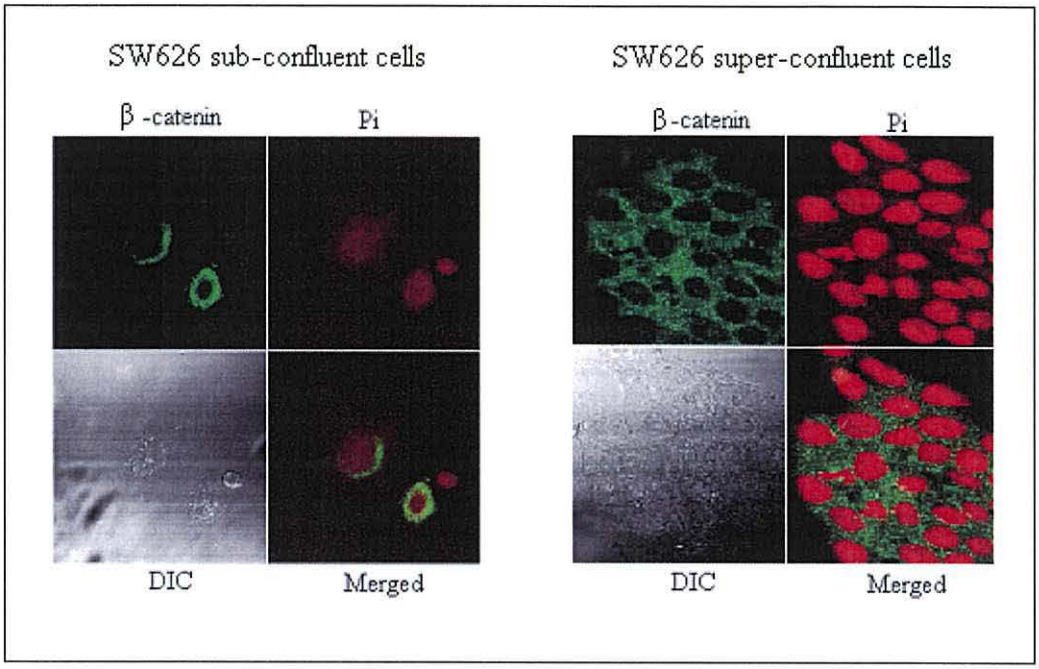


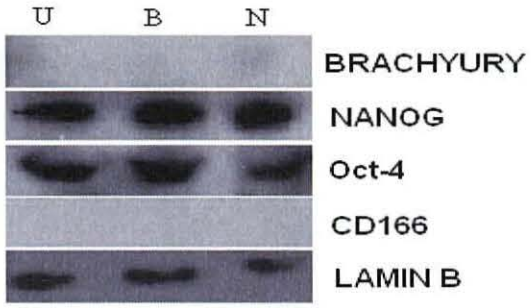
Figure 6.2

Confocal image showing localization of β-catenin in low and high density SW480 cells using β-catenin antibody. β-catenin (green) is shown to be localized to the cell membrane in low density SW626 cells and to the cytoplasm in high density SW626 cells. Pi staining (red) stains the nucleus of the cells. DIC image shows the cells without any staining and merged image shows the localization of β-catenin in the cells with respect to the Pi staining that stains the nucleus of the cells.

We did Western blot analysis in low density SW626 cells to study the levels of BRACHYURY in these cells. BRACHYURY had previously been reported to be expressed in SW626 cells (Palena *et al.,* 2007) as detected by qRT-PCR. Our data show that BRACHYURY is not expressed in SW626 sub-confluent cells, but the cells did stain positive for NANOG and Oct-4 (Figure 6.3A). The CRC stem cell marker, CD166 could not be detected in these cells by Western blot (Figure 6.3A). Since, we could not detect BRACHYURY in SW626 sub-confluent cells by Western blot analysis; we examined its expression by qRT-PCR and we did not observe a great change in the expression of *BRACHYURY* in low density SW626 cells compared to high density SW626 cells (Figure 6.3B). But the absolute levels for the Ct (thermal cycle) of BRACHYURY was found to be 37 which was similar to water control used in this experiment, which confirms negligible expression of *BRACHYURY* in sub and super-confluent SW626 cells (Figure 6.3C).

This confirms our Western blot data that SW626 cells do not express BRACHYURY (Figure 6.3A). We also checked the expressions of *E-CADHERIN*, *SNAIL*, *NANOG*, *FGF8* and mesoderm forming gene *NODAL* in SW626 cells also showed no significant changes in low density SW626 cells compared to high density SW626 cells (Figure 6.3B). But, the cell polarity markers such as *ZEB1* and *ZEB2* showed a significant decrease in expression (by 50%) in high density SW626 cells compared to low density SW626 cells (Figure 6.3B). Intestinal cell-differentiation marker *CDX2* also showed decrease in expression in high density SW626 cells (by 78%) compared to low density SW626 cells. The absolute levels for each of these genes mentioned above are depicted in Figure 6.3C which shows that there is negligible expression of *FGF8* and *NODAL* in SW626 cells whereas, *SNAIL*, *CDX2*, *NANOG* and *E-CADHERIN* are highly expressed and *ZEB1* and *ZEB 2* are moderately expressed in these cells. The absolute levels of these expressions were compared to water control which gave the CT value of 39.

A.



B.

Genes	SW626 Sub-confluent (Relative expression)	S.D.	SW626 Super-confluent (Relative expression)	S.D.
SNAIL	1	0.12	0.97	0.22
E-CADHERIN	1	0.09	1.02	0.20
BRACHYURY	1	0.24	0.99	0.38
ZEB1	1	0.22	0.54	0.21
ZEB2	1	0.08	0.55	0.20
NANOG	1	0.08	1.14	0.17
CDX2	1	0.16	0.50	0.17
FGF8	1	0.11	0.29	0.24
NODAL	1	0.10	0.97	0.14

C.

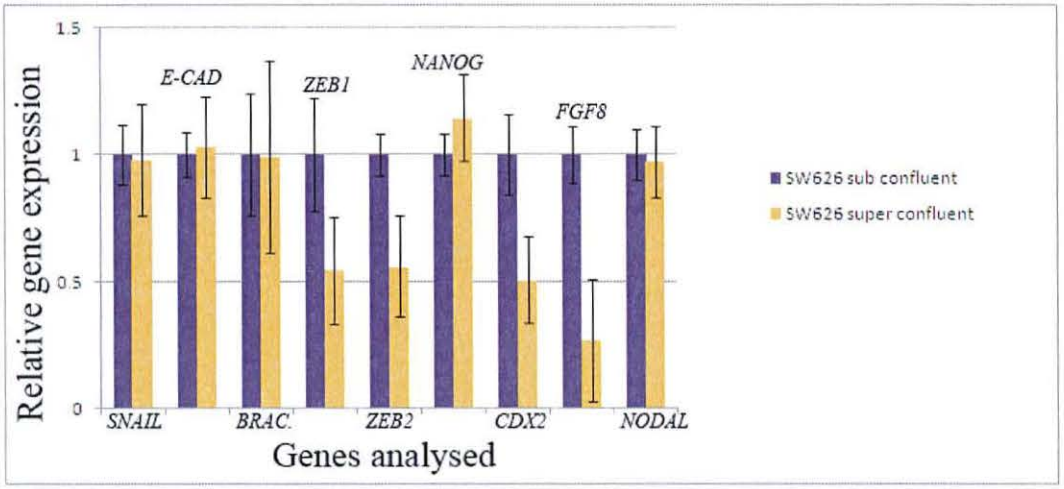


Figure 6.3: Protein levels and gene expressions in SW626 cells. A) Western blot analysis depicting the levels of BRACHYURY, NANOG, Oct-4 and CD166 in low density SW626 cells. U=untreated cells, B=*BRACHYURY* RNAi, N=non-interfering cells. LAMIN B was used as loading control. B) Gene expressions in low and high density SW626 determined by qRT-PCR. Here, low density SW626 cells were adjusted to 1 and the high density expression was compared relative to normalized value of 1 in low density SW626 cells. Data was compared to β-catenin. N=3, each done in triplicate. C) Graph demonstrating the relative gene expression levels in low and high density SW626 cells.

[Note: Though *BRACHYURY*, *NODAL* and *FGF8* are plotted on the graph, their absolute Ct values (**appendix table 24)** show that there are negligible levels of these genes in SW626 cells. (Ct value for *BRACHYURY* was 39-40; *FGF8* = 34-36; *NODAL* =37). Water control (Ct value for water control=39). Part B of this figure is obtained from **appendix table 24**]. Refer appendix Table 26 for the statistical significance data of the fold differences obtained for each gene in low and high density cells.

6.4 Discussion:

In this chapter, we studied the role of BRACHYURY in various carcinoma cells lines. In human embryonal carcinoma cell line, NTERA-2 and human colonic adenocarcinoma cell line T84, NANOG is shown to be regulated by BRACHYURY in a manner to that seen in SW480 cells. Thus, it shows that the mechanism of BRACHYURY regulating NANOG is not only shown in SW480 cells but also in other forms of cancer cells. It is possible that this regulatory pathway of BRACHYURY regulating NANOG is detected in cell lines that are competent for mesodermal differentiation, such as NTERA2, whilst T84 did not really undergo EMT-like transitions which were observed in SW480 cells. We note that the levels of BRACHYURY in these T84 cells are considerably reduced compared to SW480 cells. It will be beneficial and necessary to determine the status of other pathways in these cells to try elucidating the mechanism by which this pathway operates or is important in.

Chapter 7 - Preliminary characterization of an extracellular factor with *BRACHYURY* regulatory activity

7.1 Introduction:

In chapter 3, we showed that the expression of *BRACHYURY* in low density SW480 cells is twice as higher compared to high density SW480 cells. Based on this result, we proceeded to show that the density dependent expression of *BRACHYURY* is able to bind and regulate expression from the *NANOG* promoter control region, in low density SW480 cells (chapter 4). We were interested to explore the possibility that factors controlling the levels of BRACHYURY might be secreted into the medium and as such they might be isolated and identified. To approach this, we grew the SW480 cells to a high density, collected the medium from the cells (conditioned medium, materials and methods section 2.24) and removed dead cell debris with a centrifugation step. Conditioned medium is often used for studying growth factor and cytokines secreted into the medium. This conditioned medium was then used to plate out and grow low density SW480 cells, under various conditions as described below.

7.2 Results:

SW480 cells were plated out at low density ($2X10^6$ cells/ T75) under the following four conditions:

- 100% (v/v) conditioned medium collected from SW480 cells grown at a high density (grown for 24 hours after confluency) with no additional FBS. [Note: cell count for high density cells are $2X10^9$ cells/ T75].
- 50% (v/v) conditioned medium collected from SW480 cells grown at a high density (grown for 24 hours after confluency) with 50% (v/v) fresh DMEM supplemented with 10% (v/v) FBS. Therefore, conditioned medium:fresh medium:FBS = 1:1:0.1
- 100% (v/v) conditioned medium with fresh 10% (v/v) FBS. Therefore, conditioned medium:fresh medium = 1:1

- 100% (v/v) fresh DMEM with 10% (v/v) FBS (control). Therefore, fresh medium:FBS = 1:0.1

Figure 7.1A demonstrates that the levels of BRACHYURY is reduced in sub-confluent SW480 cells grown in 100% (v/v) conditioned medium irrespective of adding fresh FBS demonstrating that this effect is not brought about by depletion of components in FBS. The sub-confluent cells grown in fresh DMEM (control) and the sub-confluent cells grown in 50% (v/v) conditioned medium show levels of BRACHYURY similar to control levels. This suggests that there is a dose dependent effect and only 100% (v/v) conditioned medium decreases the level of BRACHYURY in low density SW480 cells. This result was indicative of a factor that may be present in the conditioned medium (secreted or otherwise) which is responsible for the reduction in levels of BRACHYURY.

To explore this further, we next grew sub-confluent SW480 cells in 100% (v/v) and 50% (v/v) conditioned medium which had been boiled at 100°C for 5 minutes (cooled to room temperature and centrifuged to remove debris), before adding to the SW480 cells. It was expected that this would denature the majority of proteins. This experiment was controlled by growing the cells in 100% (v/v) and 50% (v/v) conditioned medium which was not boiled. Figure 7.1B shows that the expression of *BRACHYURY* is reduced even after boiling the 100% (v/v) conditioned medium. The boiled and non-boiled 50% (v/v) conditioned medium still has no effect on this activity. This suggested that the 'factor' in the conditioned medium responsible for regulating BRACHYURY is highly stable and does not get deactivated on boiling.

We have shown (chapter 5) that BRACHYURY regulates the expression of *NANOG* in low density SW480 cells and also that β-catenin act upstream of BRACHYURY in these cells. Thus, we explored the expression of NANOG and β-catenin in the low density SW480 cells grown in boiled and non-boiled 100% (v/v) and 50% (v/v) conditioned medium. Figure 7.1 B shows that β-catenin levels remain high in the cells grown in 100% (v/v) conditioned medium irrespective of boiling the medium. NANOG levels have were still high on growing SW480 cells in 100% (v/v)

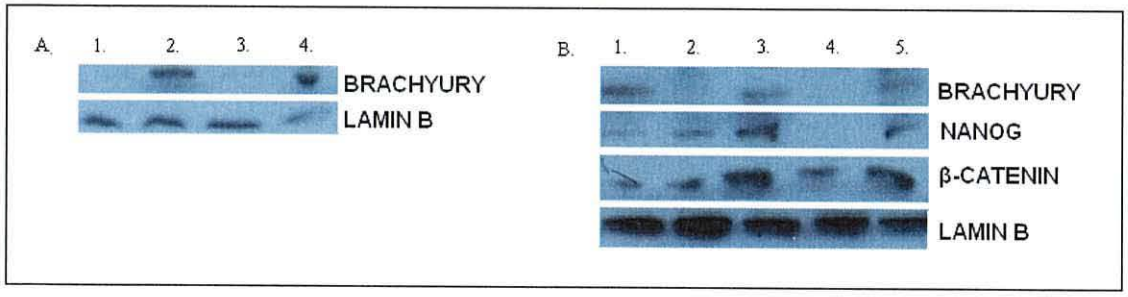


Figure 7.1

Western blot analysis showing the effects of growing sub-confluent SW480 cells in conditioned medium. A) Levels of BRACHYURY in low density SW480 cells grown under different conditions. Lane 1 = 100% (v/v) conditioned medium collected from SW480 cells grown at a high density with no additional FBS; Lane 2 = 50% (v/v) conditioned medium collected from SW480 cells grown at a high density with 50% (v/v) fresh DMEM with 10% (v/v) FBS; Lane 3 = 100% (v/v) conditioned medium with fresh 10% (v/v) FBS; Lane 4 = 100% (v/v) fresh DMEM with 10% (v/v) FBS. B) Western blot analysis demonstrating the levels of NANOG and β-catenin in SW480 sub-confluent cells grown under various conditions. Lane 1=SW480 cells grown in fresh medium; Lane 2 = cells grown in 100% (v/v) conditioned medium; Lane 3 = cells grown in 50% (v/v) conditioned medium; Lane 4 = cells grown in 100% (v/v) conditioned medium boiled for 5 minutes; Lane 5 = cells grown in 50% (v/v) conditioned medium boiled for 5 minutes.

conditioned medium but its levels went down on boiling the 100% (v/v) conditioned medium. Again, 50% (v/v) conditioned medium has no effect on these levels though NANOG levels are decreased in SW480 cells grown in conditioned medium boiled for 5 minutes. Thus, it suggests that mechanisms regulating BRACHYURY and NANOG levels in SW480 cells are complex and involve multiple components. Whilst most proteins would be denatured by boiling, peptides may still remain fully active. We therefore, next carried out a series of purification steps aimed at identifying the nature of the 'factor' in the conditioned medium. These purification steps will now be described.

We performed a range of fractionation procedures (materials and methods section 2.24) to help elucidate the conditions best suited to isolate the peptide/ factor of interest from the conditioned medium (Figure 7.2). The following columns were used:

- Amicon ultra-15 centrifugal units (Millipore) with membrane of 10 kDa: The conditioned medium was centrifuged in this unit at 4000 g for 30 minutes at room temperature, which gave an upper fraction with molecules bigger than 10kDa and a lower fraction of molecules smaller than 10kDa.
- HiTrap™ Blue HP Column (GE healthcare): This affinity column has Blue Sepharose to purify albumin, enzymes (including NAD^+ and $NADP^+$), coagulation factors, interferons, and related proteins. This purification yields a flow through fraction, not bound to the column, and elution fractions which have affinity for the column. The binding buffer used was 20 mM sodium phosphate buffer pH 7.0 and the elution buffer was sodium phosphate buffer pH 7.0 + 2 M NaCl. The pH of the sample (boiled and filtered conditioned medium) was adjusted to 7 using 1M HCl.
- MonoS column (GE healthcare): This is a cationic exchange column which binds anionic molecules to the column. We used this column to separate biomolecules are that negatively charged (elution fractions). We obtained a flow through fraction which contains molecules with a positive and neutral

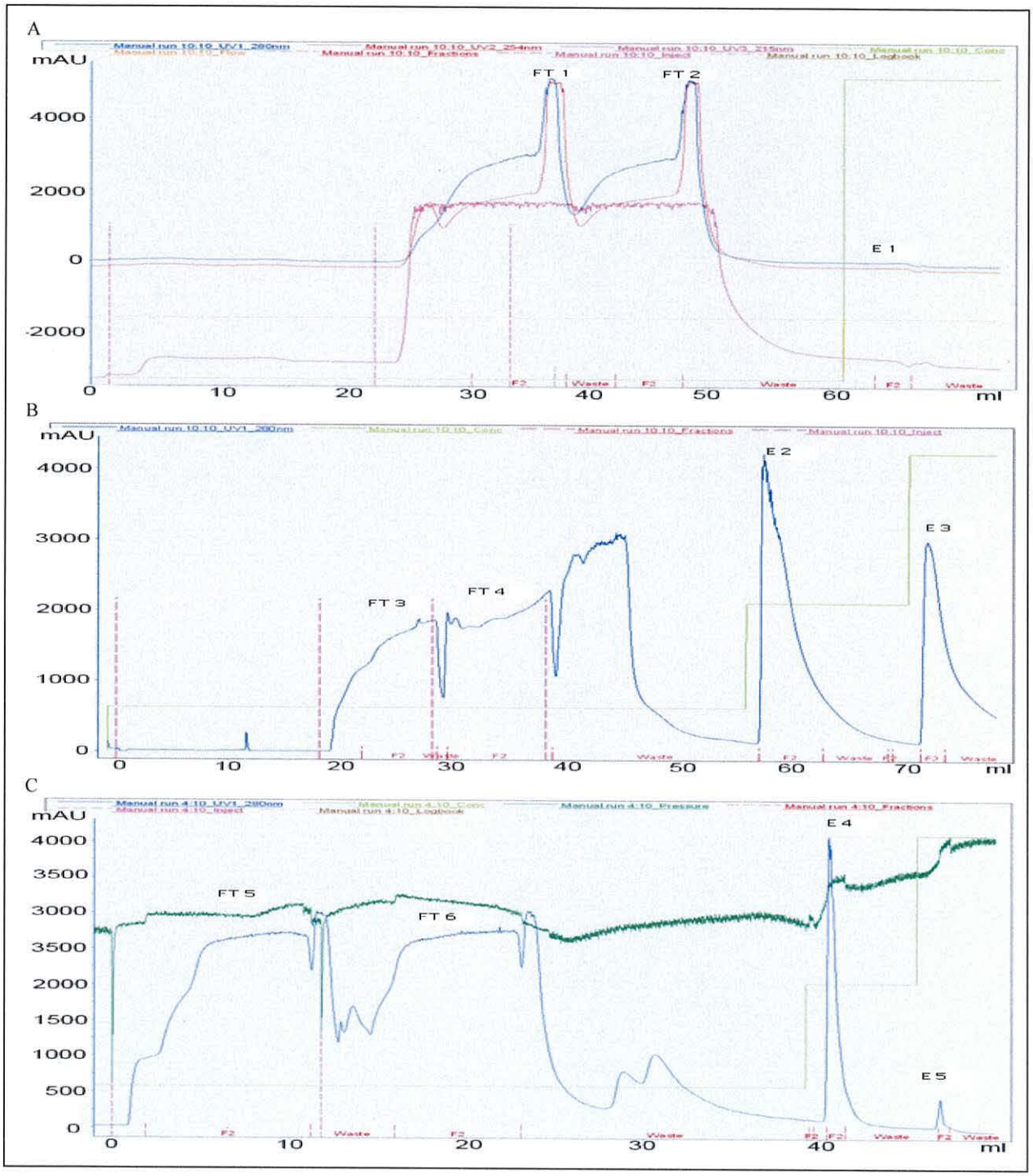


Figure 7.2: Fractions obtained from the protein fractionation. A) Flow through (FT1, 2) and elution (E1) fractions obtained from HiTrap Blue HP. B) Flow through (FT3, 4) and elution (E2, 3) fractions obtained from cationic exchange column. C) Flow through (FT5, 6) and elution (E4, 5) fractions obtained from anionic exchange column. The X-axis gives the volume (ml) of sample flown through. The Y-axis gives the OD (blue) and the pressure (green). The flow through are the fractions that are not bound to the column and elution fractions are bound to the column. FT= flow through, E= elution. [Note: Total volume loaded for each of these columns was 25ul].

charge. The binding buffer for this column was 20 mM MES (2-(*N*-morpholino) ethane sulfonic acid) pH 6.0 and elution buffer was 20 mM MES pH 6.0 + 1 M NaCl. The pH of the sample (boiled and filtered conditioned medium) was adjusted to 6 using 1M HCl.

- MonoQ column (GE healthcare): This is an anionic exchange column to isolate the cationic components from the sample (elution fraction) whilst the negative and neutral charged molecules are released as flow through fractions. The binding buffer for this was 20 mM Tris-HCl pH 8.0 and the elution buffer was 20 mM Tris-HCl pH 8.0 + 1M NaCl. The sample was adjusted to pH 8.0 1M NaOH.

Separation of putative 'peptides' was carried out using column fractionation and each of these fractions was then examined for BRACHYURY levels. Figure 7.3A shows the details of initial volume of boiled and filtered conditioned medium run through each column and the final volume of each of the fractions. We grew SW480 sub-confluent cells in each of these fractions diluted (as appropriate, according to relative concentration following fractionation) in fresh medium, adjusted to a total volume of 2ml. Our results (Figure 7.3B) confirm that the levels of BARCHYURY remain high when SW480 cells are grown at low density in fresh medium with FBS, whereas BARCHYURY is not detected when cells are grown in conditioned medium and filtered conditioned medium. The level of BARCHYURY was found to be low in the anion exchange column but the elution fractions of the anionic exchange column shows high levels of BARCHYURY. In contrast, there is no BRACHYURY detected in SW480 cells grown in medium isolated from the 'eluted' fraction from the cationic exchange column. This suggests that the elution fractions from the cationic exchange column, containing molecules with an anionic charge have biological activity associated with reducing the levels of BARCHYURY.

From Figure 7.3B, we also confirm that level of BARCHYURY remains high in the upper fraction of the ultra 15 spin column and is reduced in the lower

A.

Column	Initial volume run through the column (ml)	Fractions collected	Volume of fractions collected (ml)
Ultra-15 spin column	15 ml	Upper fraction	1 ml
		Lower fraction	14 ml
Hitrap-blue- affinity column	25 ml	Flow through FT1	6 ml
		Flow through FT2	6 ml
		Elution E1	2-3 ml
Mono S- cationic exchange column	25 ml	Flow through FT3	5-6 ml
		Flow through FT4	6 ml
		Elution E2 (500 mM)	4 ml
		Elution E3 (1M)	.5 ml
Mono Q-anionic exchange column	25 ml	Flow through FT5	6 ml
		Flow through FT6	6 ml
		Elution E4 (500 mM)	1 ml
		Elution E5 (1M)	1 ml

B.

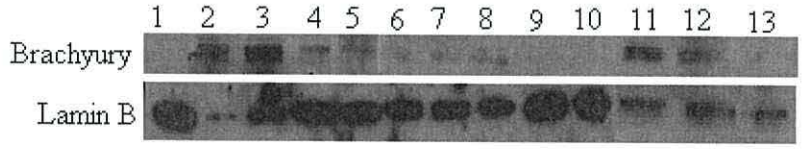


Figure 7.3

Result of the peptide/ protein fractionation. A) Table demonstrating the details of fractions obtained from the protein fractionation. B) Western blot analysis of BRACHYURY levels in low density SW480 cells grown in different fractions of conditioned medium; Lane 1 = Conditioned medium, lane 2 = fresh medium, lane 3 = upper fraction of the ultra 15 spin column (>10 kD), lane 4 = lower fraction of the ultra 15 spin column (<10 kD), lane 5 = Flow through of affinity column, lane 6 = Flow through of exchange column, lane 7 = Flow through of anionic exchange column, lane 8 = Elution affinity column, lane 9 = Elution of cationic column (peak 1), lane 10 = elution of cationic column (peak 2), lane 11 = elution of anionic column (peak 1), lane 12 = elution of anionic column (peak 2), lane 13 = filtered medium. [Note: All boiled, conditioned medium was filtered using 115-ml filter unit, (Cat. Number 121-0020, Nalgene) prior to loading on the columns. Lane 13 was included to confirm that the biological activity was not lost following filtration].

fraction of the same column. This suggests that the 'factor' is of less than a 10kDa size.

7.3 Discussion:

We have shown that *BRACHYURY* is differentially expressed in SW480 cells, in a density dependent manner. Specifically, BRACHYURY is present in relatively high amounts and in relatively low levels in SW480 super-confluent cells. Our aim was to identify whether a possible 'factor' carrying biological activity to regulate levels of BRACHYURY could be secreted in the medium and therefore, isolated when SW480 cells are grown at high density. Thus, we collected this medium (conditioned medium) and carried out preliminary fractionation to try to identify factors present in the conditioned medium that might be responsible for reducing the levels of BRACHYURY. Our results from protein fractionation from the conditioned medium suggest that BRACHYURY is not detected in the elution fraction of a cationic exchange column. This elution contains anions since it is not bound to cationic column. This suggests that there might be a possible anionic factor responsible for the low level of BRACHYURY in super-confluent SW480 cells. It would be important to examine this anionic fraction in greater detail, isolate its components and determine whether it is a protein, peptide or any other biological compound. Also, our result confirms that the possible 'factor' is likely to be of less than 10kDa size which guides us to look for components less than 10kDa size.

Most proteins are not heat resistant, although few of them are. Our experiment shows that on boiling the 100% (v/v) conditioned medium, the BRACHYURY activity remain high suggesting that the 'factor' possibly is not a protein, unless it is heat resistant protein.

Chapter 8 - Summary

8.1 SW480 cell line: validation as a model to study molecular events involved in cells undergoing EMT and MET

Changes in cell density of the CRC cell line, SW480 lead to changes in gene expression and protein localization, resulting in a phenotype that resembles cells transitioning between mesenchymal-like and epithelial-like states (Brabletz *et al.*, 2001; Davies *et al.*, 2004). In low density SW480 cells (mesenchymal-like), the cells have low levels of E-CADHERIN located in the cytoplasm and nuclear β-catenin. In high density SW480 cells (epithelial-like), the cells exhibit higher levels of E-CADHERIN, mainly membrane associated, and membrane bound β-catenin. Such changes in levels of expression of E-CADHERIN and localization of E-cad and β-catenin are hallmarks of cells transitioning between epithelial and mesenchymal states. In accordance with previous literature, we provided further evidence that SW480 cells grown at different densities are a good *in vitro* model to study the molecular events involved in EMT. Nuclear NFκB has previously been shown to be associated with induction and maintenance of EMT (Huber *et al.*, 2004). One of the NFκB family members, Rel A (Nuclear factor, NFκB p65 subunit), which is the most abundant forms of NFκB, translocates from the nucleus to the cytoplasm as the SW480 cells progress from low to high density. The change in localization of Rel A is in accordance with the changes in localization of β-catenin and E-CADHERIN and further validates the use of SW480 cells grown to different densities as a system to study EMT.

We have also shown that in low density, mesenchymal-like SW480 cells, *BRACHYURY* is expressed at levels that are double those observed in the epithelial-like high density SW480 cells. We also found a number of other developmental genes which were also differentially expressed in the SW480 cell line and that were indicative of cells undergoing EMT/MET. These genes include *TBX2, TBX3, CDX2, ZEB1, SNAIL, FGF8, BRCAHYURY, E-CADHERIN* and *NANOG.*

8.2 Why was *BRACHYURY* studied?

Although we found a range of developmental genes differentially expressed in low and high density SW480 cells (such as *TBX2, TBX3, CDX2, ZEB1, SNAIL, FGF8*), we focused our studies on *BRACHYURY* because it plays a critical role in embryogenesis and at the time of staring this thesis, had not had a published association with colorectal cancer. Recently, Fernando and co-workers (2010) have published that *BRACHYURY* is expressed in many cancer cell lines including SW480 and also in few lung cancer cell line, such as H460. This now gives a broader chance of studying BRACHYURY in cancer.

BRACHYURY is the founding member of the T-box transcription factor family of genes and is involved in early mesoderm development (Wilson and Conlon, 1990; Vujovic *et al.*, 2006). *BRACHYURY* has recently been identified as a possible target for T-cell mediated cancer immunotherapy and was associated with many tumour cell types, including colorectal tumour cell lines (Palena *et al.*, 2007). This further validates our results. *BRACHYURY* is also expressed in chordomas and in the embryonal carcinoma (EC) cell line NTERA2, where it was found to be expressed in the absence of mesodermal differentiation (Vujovic *et al.*, 2006; Gokhale *et al.*, 2000). However, no role of *BRACHYURY* in cancer has been described as yet. Human *BRACHYURY (T)* is located on chromosome 6, which makes it important to study because chromosome 6 is associated with colorectal cancers (Ezaki 2003).

Another critical observation was that *BRACHYURY* was not expressed in other human colorectal cancer cell lines examined such HCT116, LoVo and HT29. This makes the study of BRACHYURY in SW480 even more interesting because SW480 is obtained from a very aggressive tumour. Therefore, we wanted to investigate the role of BRACHYURY specifically in SW480 cells.

8.3 Possible roles of *BRACHYURY* in SW480 cells

We have investigated possible roles of *BRACHYURY* in SW480 cells using a range of techniques. We wanted to examine whether *BRACHYURY* is directly involved in EMT. To address this, we knocked down *BRACHYURY* in SW480 cells, and checked the translocation and accumulation of β-catenin and the loss of E-CADHERIN expression (the two key hallmarks of EMT). (Hirohashi *et al.*, 2003). Since, we did not see any change in the localization and expression of β-catenin and E-CADHERIN in response to *BRACHYURY* RNAi in these cells; we concluded that BRACHYURY might not be sufficient on its own to induce EMT. Further, we concluded this observation by knocking down *BRACHYURY* in low density SW480 cells, and observing no change in the expression of cell polarity marker genes such as *ZEB1, ZEB2, PALS1, EVA1* and *CRB.* Fernando and colleagues (2010) stated that BRACHYURY is involved in the induction of EMT. But, their experiment was done by over-expressing *BRACHYURY* in their cells which induced EMT; whereas, our experiments were done without over-expressing *BRACHYURY.* This explains the fact that we did not see any change in EMT markers on *BRACHYURY* RNAi. If we over-express a gene, there is always a possibility that we change balance of other factors in the cell. Therefore, in our system, BRACHYURY is released but other factors might inhibit its EMT inducing properties, which might be active on over-expressing *BRACHYURY.*

Further, we investigated the role of *BRACHYURY* in migration of SW480 sub-confluent cells. From our screen of differentially expressed developmental genes in low density SW480 cells, we found that *CDX2* (a marker of intestinal epithelium) expression was reduced by 78% in high density SW480 cells compared to the low-density cells. *CDX2* is a biomarker of gastrointestinal differentiation, especially colorectal cancer (Liu *et al.*, 2007). Therefore, we studied *CDX2* expression in low density SW480 cells in response to *BRAHCYURY* knockdown, which reduced the expression of *CDX2* by 74%. This may suggest that *BRACHYURY* might play a role in regulating *CDX2* at the invasive fronts but not at

the central mass of the tumour because we do not expect BRACHYURY to be present in the central mass of the tumour. Gross and co-workers (2008) showed that expression of *CDX2* is high at the invasive front and its expression opposes cell migration in colorectal tumours. Therefore, we investigated a possible role of *BRACHYURY* in influencing colon cancer cell migration. (This study is currently in progress). Fernando and co-workers (2010) found that *BRACHYURY* induces cell migration in the pancreatic cell line PANC-1. This validates our preliminary migration assay data whereby, migration of SW480 cells was reduced by knocking down *BRACHYURY* expression.

The role of *BRACHYURY* was also studied in NFκB pathway. The activation of the NFκB pathway is involved in many cancers including colon cancer (Rayet and Gelinas 1999). It was also shown that *NANOG* inhibits the NFκB signalling pathway in mouse ES cells, thereby maintaining pluripotency (Watt, F. and Torres, J., 2008). The NFκB family consists of 5 member proteins, out of which *REL A and REL B* were extensively expressed in ES cells. *NANOG* was shown to bind to REL A in ES cells where it acts to inhibit NFκB regulated genes (Watt, F. and Torres, J., 2008). Therefore, we wanted to determine whether *NANOG* was regulating targets of NFκB genes similarly in SW480 cells, for which, we examined the expression of NFκB regulated genes (such as *VIEMNTIN, PLAUR, BCL2L1* and *IL10*) in response to *BRACHYURY* knockdown in low density SW480 cells by qRT-PCR. Our observations show that on *BRACHYURY* RNAi in low density SW480 cells, there is an upregulation of NFκB regulated genes. Also, our Western blot analysis shows that phosphorylated REL A (p-REL A) and VIMENTIN are up-regulated on knocking down *BRACHYURY* in low density SW480 cells. This suggests a possible activation of NFκB signalling in low density SW480 cells in response to knockdown of *BRACHYURY*. Therefore, *BRACHYURY* may be involved in inhibition of NFκB signalling in low density SW480 cells, which may occur through activation of *NANOG*. Since, NFκB signaling is activated in many form of tumours, this observation was interesting, which would suggest that at low density SW480, cells

are invasive, where BRACHYURY is in high amounts, and it can inhibit the NFκB signaling which can make the cells less aggressive.

8.4 *BRACHYURY* regulates *NANOG*

Nanog is one of the genes which were found to be differentially expressed in low and high density SW480 cells. Its expression was reduced by 70% in high density SW480 cells compared to low density SW480 cells. *Nanog* is shown to be expressed in a number of cancers such as primordial germ cell tumours (where it is involved in malignant cell transformation of gonads), osteosarcoma and lung cancer (Nirasawa *et al.,* 2009). *Nanog* is also a key gene involved in the maintenance of pluripotency of stem cells.

In mouse, NANOG has *Brachyury* and *Stat3* binding sites *in vivo* (Suzuki *et al*., 2006) which regulate the expression of *Nanog* in mouse ES cells. Knocking down expression of *BRACHYURY* in low density SW480 cells, results in 85% reduction in the expression of *NANOG* in these cells. Therefore, we present data that link the expression of *BRACHYURY* with the regulation of the pluripotency gene *NANOG* and the maintenance of markers of CSCs in low density SW480 cells. Therefore, we further studied the relation between *BRACHYURY* and *NANOG* in the low density SW480 cells. We also confirmed the influence of BRACHYURY on the regulation of Nanog for the germ cell tumour cell line, NTERA2, and the CRC cell line T84, similar to SW480 cells.

Therefore, it might be possible that in invasive cells (such as low density SW480), *BRACHYURY* expression is high which regulates the expression of pluripotency gene, NANOG thereby maintaining a population of cancer stem cells. In a tumour , a population of cancer cells can turn nearby cells cancerous. This will make a benign tumour malignant. Therefore, targeting a cancer cell population is very important for drug targets.

8.5 *β-catenin* is upstream of *BRACHYURY*

β-catenin is a modulator of signaling pathways that regulate 'stemness' (Gokhale *et al.*, 2000) and is required to maintain a discrete population of CSCs in epidermal tumours (Malanchi *et al.*, 2008; Braun *et al.*, 2008). BRACHYURY is shown to be a target of β-catenin signaling (Arnold, et al., 2000) in mouse ES and NIH3T3 cells. In low density SW480 cells, β-catenin is localized to the nucleus of the cells confirming active Wnt signaling. In these cells, BRACHYURY is highly expressed. Therefore, to study whether β-catenin is upstream of BRACHYURY in low density SW480, we knocked down β-catenin in low density SW480 cells. Our western blot data shows that knock-down of β-catenin in low density SW480 cells reduced the BRACHYURY levels in these cells. This demonstrates a possible mechanism whereby β-catenin signals through BRACHYURY to maintain a population of cells potentially having stem-like character, as determined by expression of *NANOG*.

8.6 BRACHYURY binds to *NANOG* promoter region in SW480 cells

In mouse ES cells, the binding of BRACHYURY to sites in the *Nanog* promoter was co-ordinated with the binding of STAT3 (Suzuki *et al.*, 2006). In addition to this, our ChIP assay showed direct binding of BRACHYURY to specific regulatory elements in the *Nanog* promoter region, and this binding was diminished following knockdown of β-catenin. However, we did not find any STAT3 binding sites close to either of the T-binding sites that we located in the promoter region of the human *Nanog* gene. This observation agrees with the fact that signalling through STAT3 does not promote self-renewal in human ES cells in culture (Le *et al.*, 2008).

In cancer cells, *NANOG* mRNA is derived mostly from the retrogene *NANOGP8* (*NANOG* pseudogene) (Jeter et al., 2008); and *NANOGP8* is expressed in many forms of cancer (Zhang et al., 2006) but not all. Therefore, we are currently

doing ChIP assay to clarify whether we are detecting binding of BRACHYURY to *NANOGP8* promoter region. Before doing this, we will first make sure whether SW480 cells do express *NANOGP8* or not. If *NANOGP8* is not expressed in SW480 cells, then we will conclude that we are looking at the binding of BRACHYURY to *NANOG* promoter region. If we see any expression of *NANOGP8* in SW480 cells, we will do the ChIP assay for clarification.

Pereira and co-workers (2008) suggested that β-catenin signaling increase *Nanog* promoter activity in a Tcf3-independent manner in mouse ES cells. This suggests a complex control mechanism existing in these cells. This observation is consistent with our data where β-catenin acts to positively regulate the expression of *Nanog* in SW480 cells.

The luciferase assay data presented indicates that the expression levels from the luciferase construct are low when a 701bp *NANOG* upstream element is inserted (Construct 2): whose level is close to the control levels, suggesting that the promoter elements contained in the sequence are not efficient under the conditions used in this experiment. Construct 3 (with both BRACHYURY binding sites) gives an increase in the transcription activity that is 2.3 fold compared to controls, suggesting that enhancer activity may be contained in a 114bp sequence element (2399bp to 2285bp) that contains the second BRACHYURY binding site. Whilst ChIP data showed that BRACHYURY binds to both sites, it is likely that one site alone is insufficient to enhance transcription.

Further, it will be interesting to make a construct with site 2 and not site 1. This will tell us whether the binding affinity is dependent on site 2 or site 1 alone. Also, deletion or mutation of the bases in-between the two sites will also suggest whether the activity is due to the bases in-between the two sites and not site 1 or site 2 alone. Direct mutation of site 1 and 2 will also be done in the laboratory.

High levels of NANOG translate to efficient self-renewal and low levels of Nanog lead to an increased chance of differentiation (Lluis *et al.,* 2008). Although,

Nanog is not absolutely required for self-renewal of ESC, it is found to be essential for the development of primordial germ cells (PGCs), where it is estimated to have a role in re-setting the epigenetic state. It is possible that the presence of NANOG in low density SW480 cells serves to deter differentiation. Changes in the media or growth conditions over time might lead to changes in expression profiles that favour differentiation with a corresponding decrease in levels of NANOG.

Furthermore, it is also possible that NANOG plays a role in low density SW480 cells, in altering the epigenetic state of the cells to either protect an 'immature cell state' (through high expression) or alternatively to allow differentiation (through low expression).

The initial tumour-generating events in CRC are of defined nature, but the phenotype of the cells alters with progression of the tumour, irrespective of genetic mutations. This observation may imply that epigenetic changes are involved, or alternatively, that the cells respond to extrinsic cues (Brabletz *et al.*, 2005). In accordance with this, our work shows that the SW480 cancer cells can transition reversibly between two states (depending on cell density), one state bearing characteristics/markers of CSCs, and one state bearing epithelial-like character. The expression of *NANOG* in SW480 cells might therefore permit a cell to express stem cell markers (such as CD166) and also to have stem cell characteristics. We also detected expression of other genes associated with the undifferentiated state such as *OCT4*, *SOX2* and *FOXD3* in SW480 cells at low density.

8.7 *BRACHYURY* expression in other tumour types

From the limited published studies, it is likely that not all tumours/cell lines express *BRACHYURY*. It may therefore be that those tumours that do not express *BRACHYURY* have evolved an alternative route to activate NANOG which might be important in establishing cells bearing characteristics of CSCs. Alternatively,

tumours not expressing *BRACHYURY* and *NANOG* may be less aggressive or may not have the ability to transition between different differentiation states. It would be interesting to determine the generality of the presence and localisation pattern of NANOG and BRACHYURY in a larger panel of colorectal tumours and tissue and to correlate such data with the presence of CSCs and patient outcomes.

We have also examined expression of BRACHYURY in the human ovarian tumour cell line SW626 cells by Western blot analysis and qRT-PCR. In these cells, BRACHYURY is not present but the expression of NANOG is very high. We suggest that in tumour types where BRACHYURY is not present, they develop an alternative route to activate NANOG which is centrally important for progression of the tumour by establishment of tumour initiating stem cells.

We have shown that in human embryonal carcinoma cell line, Ntera-2 and human colonic adenocarcinoma cell line T84, NANOG is shown to be regulated by BRACHYURY in a similar manner to that seen in SW480 sub-confluent cells. Therefore, it shows that the mechanism of BRACHYURY regulating NANOG is not restricted to SW480 cells. It is possible that this regulatory pathway of BRACHYURY regulating NANOG is detected in cell lines that are competent for mesodermal differentiation, such as NTERA2, whereas T84 did not really undergo EMT-like transitions which were observed in SW480 cells. The levels of BRACHYURY in these T84 cells are considerably reduced compared to SW480 cells. It will be necessary to determine the status of other pathways in these cells to try examining the mechanism by which this pathway operates.

8.8 Conditioned media 'factor' might be responsible for differential expression of *BRACHYURY* in SW480 cells

We have already mentioned that the expression of *BRACHYURY* in low density SW480 cells is twice as higher compared to high density SW480 cells. We

wanted to explore the possibility that factors controlling the levels of BRACHYURY might be secreted into the media and as such they might be isolated and identified.

The results we observed in the protein purification experiment, suggest that the possible 'factor' influencing *BRACHYURY* expression is an anionic factor. It would be important to examine this anionic fraction in greater detail, isolate its components and determine whether it is a protein, peptide or any other biological compound. Our result also suggests that the 'factor' is likely to be of less than 10kDa size and thus, we should look for components less than 10kDa size. Further study of this 'factor' will be beneficial in determining an element that is involved in regulation of BRACHYURY in SW480 colon cancer cell.

Also, when we repeated our experiment on boiled lysates, the 'factor' was still sustained. This might suggest that the 'factor' is unlikely to be a protein, since most proteins would degrade by heat. The possibility lies that the 'factor' could be a peptide or even peptidase.

8.9 A model pathway derived:

Based on our study so far, we have derived a model pathway (Figure 8.1A) explaining the results we obtained so far. We have shown SW480 transitioning between low (mesenchymal-like) and high density (epithelial-like) and BRACHYURY expression and levels transitions between these two density types of SW480 cells. Since, we have shown that β-catenin signals upstream of BRACHYURY, we predict that β-catenin activates BRACHYURY in low density SW480 cells, which in turn activates the expression of NANOG to maintain a population of colorectal cancer stem cells defined by CSC markers (CD133 and CD166). A tumour needs cancer stem cell to make a benign one malignant. In high density SW480 cells, β-catenin signaling is low and therefore, BRACHYURY level

is also low. This gives very rare NANOG and CRC stem cell marker cells at high density SW480 cells. Based on these observations, we predict a model pathway (Figure 8.1B) whereby, Wnt pathway activates BRACHYURY in SW480 cells; which in turn activates NANOG and the CRC stem cell markers. Nevertheless, this pathway has to be further verified in other tumour cell types.

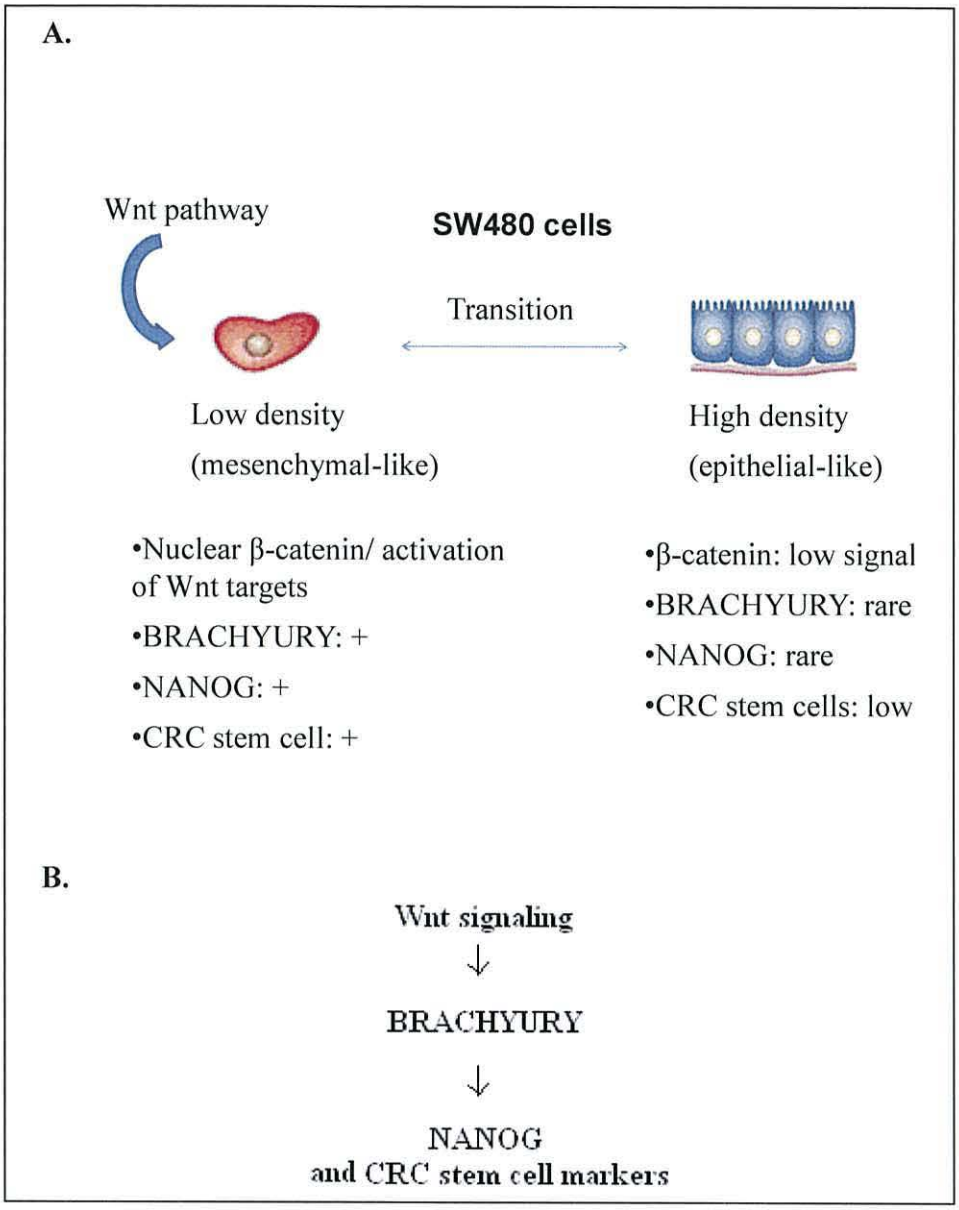


Figure 8.1: Model pathway derived. SW480 cells transition between low and high density. A) At low density, β-catenin, BRACHYURY, NANOG and CRC stem cell markers are highly expressed. At high density SW480 cells, β-catenin signaling is low and expression of BRACHYURY, NANOG and CRC stem cell markers are very rare. CRC = colorectal cancer. B) We predict that Wnt pathway activates BRACHYURY in SW480 cells which in turn activates the pluripotency gene, NANOG and the CRC stem cell markers.

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