Goblet Cell Differentiation in Colorectal Cancer

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Thesis submitted for the degree of Master of Science by Research

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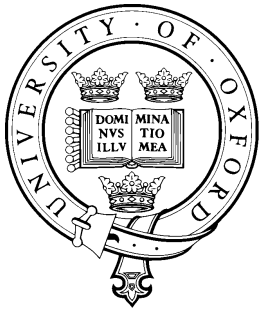

Abstract

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Colorectal stem cells give rise to three cell types in the colorectal crypt: enterocytes, enteroendocrince cells and goblet cells. In colorectal cancer this stem cell differentiation is dysregulated and absence of differentiation usually confers a worse prognosis for the patient. In order to understand goblet cell differentiation in colorectal cancer we first investigated novel goblet cell markers. REG4 was found as a marker through examination of our microarray data on 96 colorectal cell lines and confirmed through flow cytometry and immunohistochemistry. Dll4 was also examined as a potential goblet cell marker but instead stained a separate population of cells that may prove to either influence goblet cell differentiation or be goblet cell progenitors. We then examined the roles of goblet cell differentiation regulators such as Notch and the homeobox genes CDX1 and CDX2 in colorectal cell lines. Treatment of colorectal cell lines with inhibitors of the Notch pathway, including the γ-secretase inhibitor DBZ and anti-Dll4, increased the number of goblet cells. Knockdown of CDX1 and CDX2 decreased the number of goblet cells in goblet cell producing cell lines. Further research will elucidate the mechanisms by which these regulators influence differentiation, which could yield a treatment for colorectal cancer; inducing goblet cell differentiation and abating further tumor growth.

Acknowledgements

I would first like to thank Sir Walter Bodmer for all of his support, guidance and advice. I am indebted to Walter for allowing me to be a part of his group and teaching me what it takes to be a true scientific researcher.

I am extremely thankful of everyone in the Cancer and Immunogenetics Laboratory who have helped me learn essential techniques and who have always been able to find time to discuss and troubleshoot problems and findings from my research. I am especially thankful to Dr. Neil Ashley who has provided not only excellent advice, but who has been involved with most of my experiments and has provided constant support.

I am also appreciative of Dr. Jen Wilding for teaching me how to utilize our colorectal cell line expression data, of Dr. Kamila Koprowska for her help in flow cytometry and FACS, and of Lin-ting Hsai for her western blot expertise. Dr. Djamilla Ouaret, Dr. Trevor Yeung, Jonathan Beckett, Mustak Ayub, and those already mentioned all helped me in my pursuit of this thesis and provided me with excellent advice for completing it. I am also exceptionally grateful to Kevin Clark, who was grateful enough to constantly help me sort goblet cells.

Finally I am especially grateful for the unending and unwavering support of my parents, my sister and Scott.

Author's Declaration

The work in this thesis was performed in the Weatherall Institute of Molecular Medicine in the Department of Oncology. Except where acknowledgement is made, all of the work presented is my own and has not been submitted for another degree in this or any other University.

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List of abbreviations

APC	adenomatous polyposis coli
ATCC	American type culture collection
BCA	bicinchoninic acid
BMP	bone morphogenic protein
BSA	bovine serum albumen
cDNA	complementary DNA
CRC	colorectal cancer
CRISPR	clustered regularly interspaced short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
DBZ	dibenzazepine
DMEM	Dulbecco's modified E4 medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECACC	European collection of cell cultures
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FCGBP	Fc gamma binding protein
g	gram
HRP	horseradish peroxidase
M	molar (moles per liter)
ml	milliliter
µl	microliter
NOD-SCID	non-obese diabetic – sever combined immunodeficiency
PBS	phosphate buffered saline

PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RIPA	radio immuno precipitation assay
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
TBSt	tris buffered saline with tween
TEMED	tetramethylethylenediamine
TFF3	trefoil factor 3

Chapter 1: Introduction

1.1 Colorectal crypts and goblet cells

1.1.1 Colorectal Crypts

Stem cells lie at the base of colorectal crypts. These stem cells give rise to the three cell types that make up the epithelia of the crypts; enterocytes, enteroendocrine cells and goblet cells. A representation of this differentiation and the regulators that will be discussed in this thesis can be seen in Figure 1.1.

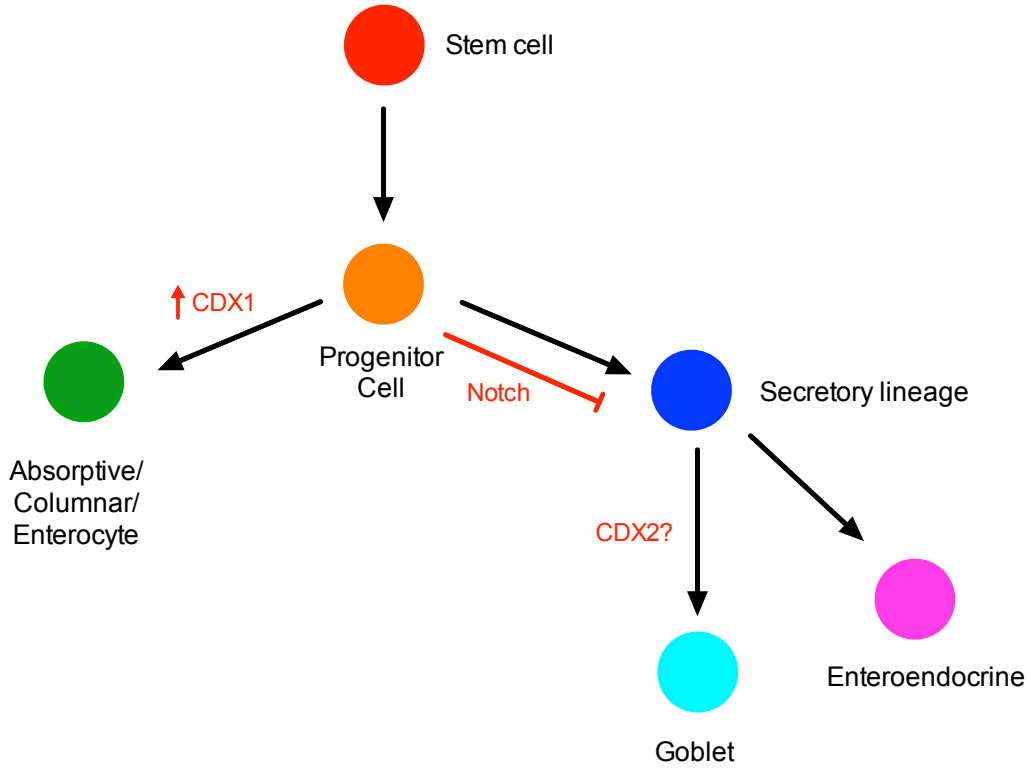


Figure 1.1 Differentiation pathway of colorectal stem cells. Genes of interest from this thesis are shown in red.

Stem cells are self-renewing and multipotent, meaning in this case they can create any epithelial cell type within the colon. In the small intestine Paneth cells send signals to these stem cells, including Wnt, in order to maintain their stemness (Figure 1.2). There are Paneth-

like cells within the colon that also help maintain the stemness of stem cells (Rothenberg et al., 2012). These stem cells give rise to transit-amplifying cells, which as they move away from the base of the crypt stop receiving signaling factors that maintain stemness and start receiving signals that determine their final differentiation state. BMP plays a role at the border of the proliferative zone antagonizing Wnt and thus restricting proliferation (Clevers and Bevins, 2013). Similarly Notch signaling is highly active in the transit-amplifying zone and impacts the differentiation into either absorptive or secretory cells. Those that express Notch that come into contact with another cell expressing one of its ligands, Dll4 for example, will differentiate into an absorptive cell while the ligand presenting cell will differentiate into a secretory cell.

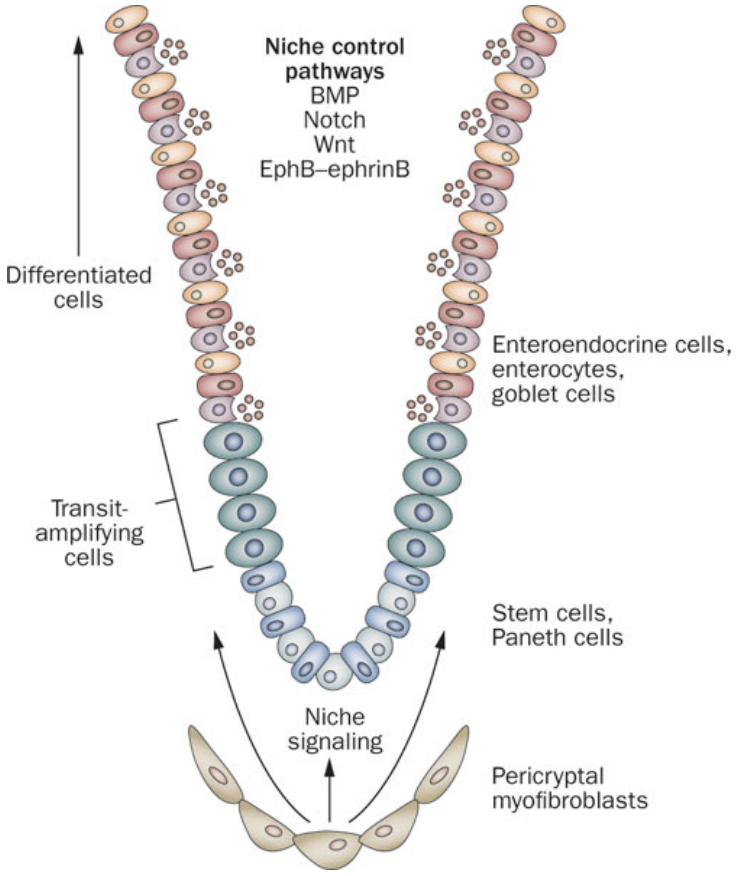


Figure 1.2 Colorectal crypt with zones, cell types and active pathways. Reprinted by permisson from Macmillan Publishers Ltd:Nature Reviews Gastroenterology and Hepatology(Zeki et al., 2011) *Copyright 2011*

1.1.2 Goblet cells

Goblet cells arise from stem cells at the base of the colorectal crypts and are a member of the secretory lineage (Cheng and Leblond, 1974). These cells produce and secrete mucins, mainly MUC2, for maintenance of the mucus blanket, which provides the front line of innate host defense in the colon (Allen et al., 1982). Once they begin differentiation, goblet cells move up the colorectal crypt to eventually be sloughed off into the colon (Specian and Oliver, 1991). Mucin plays a large role in goblet cell morphology and is the identifying feature of a goblet cell (Kim and Ho, 2010). Currently the only means of identifying goblet cells is the presence of MUC2. However, it is possible that there are defective goblet cells in some tumors that do not produce MUC2. Therefore it is necessary to find other markers of goblet cells that are maintained in this dysregulated state or may be present before MUC2 is produced.

The Notch pathway plays a big role in determining the cell fate of a colorectal stem cell into a secretory or an absorptive cell. Active Notch inhibits the secretory cell lineage and pushes cells towards the absorptive lineage (van der Flier and Clevers, 2009). Several other pathways also influence goblet cell differentiation as well as the maintenance of stem cells including Wnt, bone morphogenic protein (BMP), and PI3-kinas/AKT signaling (van der Flier and Clevers, 2009). However, in colorectal cancer abnormal goblet cell differentiation or lack of goblet cell differentiation is not well understood.

There are some tumors such as mucinous adenocarcinomas, which are defined as tumors with more than 50% of their tumor mass represented by mucin and others that have a high number of high mucin producing goblet cells such as signet-ring cell carcinomas (Kim and Ho, 2010). Signet-ring cell carcinomas are very aggressive while it is still debated whether

mucinous carcinomas are also generally more aggressive (Consorti et al., 2000; Goldstein et al., 2000). Other than mucinous tumors, goblet cells are rare and in both CRC tumors and CRC cell lines appear much fewer in number when they are expressed. These goblet cells that do appear are also skewed in their own expression, such as expressing MUC5AC, a gastric mucin that is not normally present in the intestine. MUC6 usually found in the stomach (Bolos et al., 1995) is also present in some colorectal carcinomas (Walsh et al., 2013). Finally, MUC1 is more highly expressed in non-mucinous carcinomas than mucinous carcinomas (Kim et al., 2005). Understanding these altered expression patterns is essential to elucidating goblet cell differentiation in CRC.

1.2 Colorectal cancer

As of 2012 CRC is the third most common cancer in men and second most common in women worldwide with the highest incidence in Oceania and Europe. While at the moment it is more prevalent in more developed countries this is rapidly changing (Ferlay et al., 2014; Jemal et al., 2011). CRC provides an excellent model for understanding the somatic evolutionary process of cancer as well as genetically heritable disease susceptibilities. These forms of heritable susceptibilities led to the identification of key regulators within colorectal and other cancers, including the adenomatous polyposis coli (APC) gene, the Wnt pathway, and the role of mismatch repair genes (Bodmer, 2006). However, the familial cancers only account for about 5% of all incidence of CRC with about another 30% having some form of inherited susceptibility. Those CRC that do not have a recognized inheritable susceptibility and are the only case in a family are classified as sporadic even though they could still be influenced by genetic factors.

Colorectal crypt stem cells have been suggested as the origin for intestinal and specifically colorectal tumors (Barker et al., 2009). Tumorigenic colorectal stem cells were first identified in the colon in two separate laboratories. The two groups described a subset of CD133+ cells in a human colon cancer xenograft in NOD-SCID mice that had cancer-initiating properties. They could maintain themselves in culture in an undifferentiated state, could initiate tumorigenesis once transplanted into a new mouse and could then differentiate into a new tumor (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). One pathological classification of CRC tumors is based on the degree of differentiation of stem cells within the tumors: differentiated or undifferentiated.

Differentiated tumors are recognized by their ability to form crypt-like structures with similar but altered morphology and signaling profile as normal colonic crypts. (Ashley et al., 2013). This differentiation is often times skewed and some cell types, such as goblet cells, are infrequent and morphologically changed (Ashley et al., 2013). Some cancers, such as mucinous carcinomas and signet-cell carcinomas have an unusually high number of goblet cells and in there cases are usually more aggressive and worse for the patient. Signaling factors and pathways that are present in the normal colon also occur in CRC, but the profile and expression is altered based on the cell types that exist within the tumor and the mutations that have occurred during tumorigenesis.

1.3 Notch

Notch and its signaling pathway play an integral role in development and cell fate (Artavanis-Tsakonas et al., 1999). Notch is activated by first binding to one of its four ligands, including Dll4 or Jagged-1, which initiate two proteolytic cleavages. ADAM proteinase cleaves Notch extracellularly first and then the γ-secretase complex cleaves Notch

intracellularly. The cleaved fragment can then move into the nucleus and act as a transcription factor (Grabher et al., 2006)(Figure 1.2). This Notch cell-signaling pathway occurs in many different cell types either to induce cell differentiation or maintain cell stemness.

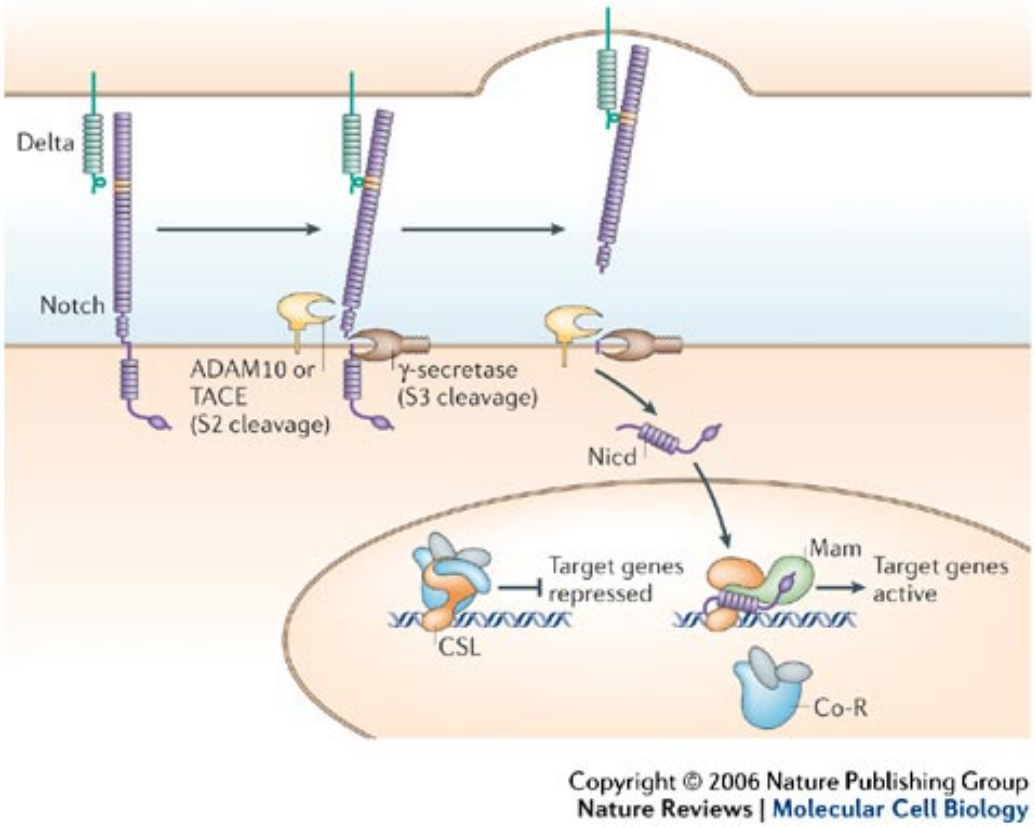


Figure 1.3 Notch signaling pathway. Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: Nature Reviews Molecular Cell Biology (Bray, 2006)

In adult intestinal development constitutively active Notch inhibits goblet cell differentiation *in vivo* (Stanger et al., 2005). It pushes cell differentiation towards the absorptive lineage (van der Flier and Clevers, 2009). A γ-secretase inhibitor, dibenzazepine (DBZ), inhibits Notch activation by preventing its intracellular cleavage, which induces goblet cell differentiation in the colorectal crypt (van Es et al., 2005; Milano et al., 2004). Treatment of CRC cell lines that are goblet cell producing with DBZ has also been shown to increase goblet cell numbers in culture (Yeung et al., 2011). This is the main reason DBZ was used in order to alter the Notch signaling pathway in experimentation.

1.4 Dll4

Dll4 is one of Notch's several ligands; others include Dll1 and Jagged-1. Dll4 was first discovered from a murine white adipose cDNA library where it was determined to be part of the Dll family instead of the Jagged family of Notch ligands (Shutter et al., 2000). Dll4 has previously been suggested as a potential goblet cell marker in colorectal cells (Jubb et al., 2009). Its inhibition through anti-Dll4 treatment has also been shown to inhibit tumor growth (Hoey et al., 2009). Dll4 and Dll1 have been suggested to control cell fate and promote differentiation into goblet cells (Pellegrinet et al., 2011; Akiyama et al., 2010; Noguera-Troise et al., 2006). Dll1 has been seen to directly induce MUC2 expression (Pellegrinet et al., 2011). Dll4 has also been shown to have opposing effects on angiogenesis from Jagged-1, suggesting that Notch signaling through Jagged-1 maintains stemness while signaling through Dll4 stimulates differentiation potentially into goblet cells (Benedito et al., 2009).

1.5 CDX1 and CDX2

CDX1 and CDX2 were first found in Drosophila specifying segment identity and are members of the caudal-related homeobox gene family (Gehring and Hiromi, 1986; Bender et al., 1983). CDX1 and CDX2 are two of four mammalian homologs to the *Drosophila caudal* gene. *Caudal* was first found to be expressed during oogenesis and is involved in determining the posterior end of *Drosophila* (Mlodzik et al., 1985). CDX1 and CDX2 were then found to be involved in embryogenesis and in intestinal development and differentiation. CDX1 was found to be expressed in the murine epithelial cells of the intestine after 14 days gestation and continued to be expressed into adulthood (Duprey et al., 1988). CDX2 was then discovered as a related gene to CDX1 and was only expressed at detectable levels in the intestine (James and Kazenwadel, 1991). These findings along with other results demonstrating their regulatory role in the intestine suggested that CDX1 and CDX2 had a

large role in determining cell fate and differentiation in the mammalian intestine (Suh et al., 1994).

More recently this research into CDX1 and CDX2 intestinal regulation has been further studied. CDX2 has been suggested to specifically regulate MUC2 gene expression in goblet cells, thus suggesting that CDX2 plays a big role in goblet cell differentiation as well (Yamamoto et al., 2003). CDX2 has also shown to have a tumor suppressor like function in the colon where heterozygous knockdown of CDX2 in mice facilitates tumor progression of colorectal cancer (Bonhomme et al., 2003). CDX1 has shown to promote intestinal differentiation in the rat colon (Soubeyran et al., 1999). More specifically regarding goblet cells CDX1 knockdown has not had an effect on goblet cell differentiation in SW1222 (Yeung et al., 2011). CDX1 has also shown to be associated with the triggering of Barrett's metaplasia through promoter demethylation (Wong et al., 2005). The specific effects of CDX1 and CDX2 on goblet cell differentiation are yet unknown. CDX2 must have a broader function than CDX1 since CDX2 mouse knock outs are lethal whereas CDX1 knock outs are not (Chawengsaksophak et al., 1997). The role CDX1 and CDX2 play specifically in goblet cell differentiation was further investigated in this thesis.

1.6 Goblet cell markers PR5D5 and MUC2

PR5D5 is an in-house made antibody most likely to MUC2 (Richman and Bodmer, 1987). PR5D5 is unequivocal in its identification of goblet cells and was chosen over industry made antibodies to MUC2. MUC2 is a colonic mucin, a heavily glycosylated glycoprotein, whose primary function is the protection of the surface of mucosal cells and is the major component in colonic mucus (Allen et al., 1982; Larsson et al., 2009). MUC2 is decreased in most colorectal cancers except in mucinous cancers, which demonstrates its association with

goblet cells (Byrd and Bresalier, 2004). However, in mucinous cancers is not fully glycosylated (Yeung et al., 2011). MUC2 is present in mature goblet cells, either glycosylated or incompletely glycosylated, and is used to identify goblet cells in cultures and tissue samples.

1.7 REG4

REG4 and its family belong to the calcium dependent lectin superfamily. The Reg family is involved in the gastrointestinal response to injury. REG4 was first found through a screening of a cDNA large inflammatory bowel disease library (Hartupee et al., 2001). REG4 is predominantly expressed in the gastrointestinal tract and has been described as a potential differentiation marker in a subset of secretory cells in the colon (Heiskala and Andersson, 2013). REG4 has shown to be overexpressed in mucosal injury and several primary colorectal cancer specimens as compared to their normal colon mucosa counterparts (Violette et al., 2003). The role REG4 plays in normal GI mucosa is not well-understood and further investigation into its role in mucosal injury and metaplasia is necessary.

1.8 Models to be tested and questions asked

I first wanted to examine alternative cell markers of goblet cells, both intracellular and extracellular, with the hopes of being able to isolate goblet cells from a cell line and find another reliable marker that did not rely on the MUC2 protein being expressed. I proposed that I could find other goblet cell markers by categorizing our microarray data on 96 colorectal cell lines into goblet cell producing lines and non goblet cell producing lines and examining their gene expression profiles. Next I wanted to test the Notch model of influence on goblet cell differentiation. I proposed that by inhibiting the Notch pathway either through a gamma-secretase inhibitor, which acts on Notch directly, or by blocking one of its ligands,

Dll4, with anti-Dll4 treatment I would see an increase in goblet cells in goblet cell producing cell lines. Finally I wanted to examine the effects of the homeobox genes CDX1 and CDX2 on goblet cell differentiation. I anticipated that a knockdown of CDX1 or CDX2 would result in a decrease in goblet cell differentiation in goblet cell producing cell lines.

Chapter 2: Materials and Methods

2.1 Cell culture methods

2.1.1 Growth and maintenance

Cells were maintained in 25, 75 or 175 cm^2 flasks (Falcon®, BD Biosciences), at 37°C in a humidified atmosphere of 5% or 10% CO_2 in Dulbecco's modified E4 medium (DMEM) supplemented with 50 ml fetal bovine serum (FBS, Autogen Bioclear) and 5 ml antibiotics (penicillin 200 IU/ml and streptomycin 200 μg/ml, Gibco/Invitrogen).

Cell lines that were adherent were passaged at about 60-80% confluency. Cell lines were passaged by aspirating the medium, washing with about 5 ml of PBSA, and trypsinizing with the appropriate amount of trypsin (see Table 2.1 for specific details on trypsin and cell passage). Cell lines were then allowed to incubate for about 5 minutes inside incubator at 37°C. Once cells had detached culture medium was added to neutralize the trypsin. Cells were normally split in the range of 1:3 to 1:25 at each passage depending on cell line doubling time.

Flask Size	Trypsin	Cell Lines	Doubling Times (hours)	Split
25 cm^2	0.5 ml	C80	45	1:4
75 cm^2	1.0 – 2.0 ml	HCT116	16	1:25
175 cm^2	2.0 – 3.0 ml	HT29	18	1:6
		LS174T	20	1:6
		LS180	20	1:4
		SW1222	44	1:6
		SW480	45	1:6
		T84	54	1:3

Table 2.1 Details of trypsin used and cell passaging

2.1.2 Cryopreservation and Recovery

Cells were passaged then suspended in medium 2x the amount of trypsin used. Cells were transferred to a centrifuge, spun down at 1000 rpm for 5 minutes. The supernatant was drained and the cells were resuspended in the appropriate amount of 10% dimethyl sulfoxide (DMSO) (Sigma) and FBS mixture, aliquoted 500 µl per sterile cryogenic vials (Corning via Fisher Scientific UK), placed in a Mr. Frosty™ (Thermo Scientific) overnight then transferred to liquid nitrogen.

When starting a cell culture from a frozen vial a 25 cm^2 flask was prepared with 10 ml of warm medium. The vial was held for 1 minute in a water bath at 35°C or until it first defrosted. The flask was then removed from the water bath and 2 ml of warm medium were added drop wise. The cells were then mixed gently and transferred to the previously prepared flask. The next day the medium was exchanged for the typical culture volume (5 ml for a 25 cm^2 flask, 10 ml for a 75 cm^2 flask, 20 ml for a 175 cm^2 flask).

2.1.3 Cell line type

The colorectal carcinoma cell lines studied were: C80, HCT116, HT29, LS174T, LS180, SW1222, SW480, and T84. The cell lines were obtained from the American type culture collection (ATCC), the European collection of cell cultures (ECACC), or from the laboratories in which they had originally been raised (Bracht et al 2008).

2.1.4 Cell Counting

The Cellometer® Auto T4 was used when counting cells. 20 µl of cell suspension was taken from approximately 500 µl of the original culture flask and loaded onto the glass slide. The

slide was then inserted into the Cellometer® Auto T4 where the software was used to count cells. The software counts cells based on size and shape on the slide and calculates cell count based on the sample provided.

2.2 Microarray

Microarray data for 96 colorectal cell lines was previously collected and obtained by sending RNA samples to the Molecular Biology Core Facility of the Paterson Institute for Cancer Research in Manchester for gene expression microarray analysis. This research center used the Affymetrix GeneChip system v2.0. The full details of the protocol are on-line (http://www.affymetrix.com/support/technical/manuals.affx). For each sample 10 μg of RNA was used for cDNA synthesis using the T7-T_{24} primer. cDNA was purified with phenol: chloroform: isoamyl alcohol (25:24:1) and was then transcribed *in vitro* to create cRNA. The cRNA was then purified and fragmented before hybridization to the arrays. Finally the chips were washed, stained and then scanned.
https://cbse.soe.ucsc.edu/sites/default/files/affymetrix_protocol050404.pdf

Cell lines were classified into goblet cell producing or non-goblet cell producing based on previous PR5D5 staining. 13 cell lines were classified as goblet cell producing and 33 were classified as non-goblet while the remaining cell lines were kept uncategorized. The cell lines classified can be found in the Table 3.1. Gene expression was measured and compared between the two groups and p-value and fold change were used to identify potential important genes in goblet cell differentiation. Genes of importance were then looked up on the human protein atlas (http://www.proteinatlas.org/) for potential staining ability. Those that showed possible intracellular or surface staining of goblet cells were then purchased and used in several experiments.

2.3 Treatments

2.3.1 DBZ (Dibenzazepine)

Dibenzazepine (DBZ), a γ-secretase inhibitor, was acquired from Herck Millipore (565789). DMSO was used as the solvent and thus also used as the treatment control. DBZ was used at a final concentration of 200 nM during treatment. Preparation of DBZ treatment involved passage of cell lines into new flasks or 96-well plates. The cells were first washed with approximately 5 ml of sterile PBS and incubated for about 10 minutes with the appropriate amount of trypsin. If cells were to be treated in flask the cells were removed and spun down in a 15 ml centrifuge tube for 5 minutes at 1000 rpm. The cells were then resuspended and incubated in the appropriate amount of medium with the addition of DBZ to 200 nM.

If cells were to be treated in a 96 well plate they were measured first on the Cellometer as previously described. The cell concentration was then adjusted in order to dispense about 10,000 cells per 100 µl into each well. The cells were then allowed to adhere for 24 hours and then DBZ was added at a final concentration of 200 nM. The cells were then incubated for an additional 24 hours and then were fixed in preparation for immunostaining.

2.3.2 Dll4

Anti-Dll4 rabbit polyclonal antibody (abcam ab7280) was used at a concentration of 1 µg/µl in order to block Notch from binding one of its ligands, Dll4. Preparation of cells for Dll4 treatment involved passage of cell lines into 96-well plates as previously described in 100 µl of medium (2.3.1). Cells were allowed to adhere overnight to the plate before concentrations of Dll4 were added in 100 µl of medium to each well. The cells were then incubated for another 24 hours after adding the drug and then fixed in preparation for immunostaining.

2.3.2.1 Dll4 and DBZ

When cells were treated in conjunction with DBZ, anti-Dll4 was used at a concentration of 1 μg/μl and DBZ was used at either 200 nM or 100 nM. Concentrations were added in each possible combination as well as with each concentration individually. Cells were treated the same as anti-Dll4 treatment alone.

2.3.3 siRNA treatments

Cells were transfected with 50 pmol of CDX2 siRNA or control siRNA in 96-well plates at a concentration of 10,000 cells in 100 μl of medium per well. Cells were first passaged as previously described before being transfected. Cells were then allowed to incubate after transfection for 24 – 48 hours before fixation and preparation for immunostaining took place.

2.3.3.1 siRNA and DBZ

When cells were treated with both DBZ and siRNA cells were transfected with 50 pmol of CDX2 siRNA or control siRNA and then incubated in 100 μl of 200 nM DBZ for 24 hours. Cells were then fixed in preparation for immunostaining.

2.4 Reverse siRNA transfection

Transfections took place in 96-well plates with cells plated at 30 – 50% confluency using 50 pmol of siRNA. Transfections followed Lipofectamine® RNAiMAX Qiagen kit with a few modifications. 50 pmol of RNAi was diluted in 10 μl Opti-MEM® in each well. 0.3 μl of Lipofectamine RNAiMAX was then diluted in 10 μl Opti-MEM and then added to each well

with RNA. Each well was mixed gently and then allowed to incubate for 15 minutes at room temperature.

The cells, after being passaged, were diluted in DMEM with 10,000 cells per 100 µl. Cells were then plated 100 µl each well and rotated about 40 times to completely mix the cells and complexes. The cells were allowed to incubate for 24-48 hours and then fixed and prepared for immunostaining.

2.5 Western Blot

LS180 cells were first treated for 24 hours with medium, 200 nM DMSO or 200 nM DBZ. Cells were then lysed with Radio Immuno Precipitation Assay (RIPA) buffer consisting of 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and 50 mM Tris, pH 8.0. The cells were first washed with ice cold PBS and once ice scraped down and transferred into an eppendorf.

24 hours later the sample was allowed to defrost and was spun down at 4°C for 20 minutes at 20,000 rct. Then the supernatant was transferred to a new eppendorf tube and the thermo scientific Pierce BCA protein assay kit was used to measure protein concentration. Briefly, 25 µl of standards and the samples were set up in a 96-well plate in 200 µl of the assay. The samples were then incubated for 30 minutes in 37°C covered in tin foil. The luminescence was then measured and the protein concentrations of the samples were determined. The samples were then diluted in order to load 30 µg of protein into each well with loading buffer with 5x mercaptoethanol and denatured for 10 minutes at 90°C. 30 µl of samples were then loaded into 10% Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) wells. The SDS-PAGE 10% gel consisted of 4.0 ml of water, 3.3 ml 30% Acrylamide mix,

0.1 ml 10% SDS, 2.5 ml 1.5 M Tris (pH 8.8), 0.1 ml 10% Ammonium persulfate and 4 µl TEMED. The SDS-PAGE gel was then submerged in migration buffer containing 25 mM Tris base, 190 mM glycine, 0.1% SDS with a pH of 8.3. The gel was then allowed to run at 90 v for 30 minutes and then 120 v for one hour.

Next the proteins were transferred using a semi-dry transfer for one hour. The membrane was first activated with methanol then placed next to the gel and placed between positive and negative electrodes. The same running buffer was used during the semi-dry transfer. After the one hour transfer the membranes were blocked for 30 minutes in 1% BSA in TBSt. The membranes were then stained overnight with their primary antibody, washed three times and incubated for one hour with their HRP-conjugated secondary antibody. The ECL prime kit was then used in order to detect the proteins, which were then developed using manual film development with an exposure time of 5 minutes for Notch1 and 10 seconds for α-tubulin.

2.6 Immunohistochemistry staining and analysis

Cells were plated at 1 x 10^4 per well in a 96-well plate in order to obtain about 80% confluence in the plate. After cells were treated or other experiments were completed the medium was removed from the wells and rinsed once with PBS. 30 µl of 4 % buffered formaldehyde solution was added to each well in the fume hood and was allowed to incubate for 15 minutes. The wells were then washed twice with PBS to remove the fixative and permeabilized with 0.5% Triton in PBS for 5 minutes. The wells were then washed three times with PBS to remove the permeabilizing agent and incubated for one hour at room temperature in 30 µl of blocking buffer which contained 10% FBS in PBS.

Primary antibodies were diluted appropriately in the blocking reagent (See Table 2.3 for full list of primary and secondary antibodies) ranging from 1:100 – 1:1000, added 100 µl per well and then incubated at 4 °C for 1-2 hours or overnight protected from light. The wells were then washed three times in PBS and the secondary antibody was added, 100 µl per well, and allowed to incubate for either 1-2 hours at room temperature or overnight at 4 °C protected from light. Finally the wells were washed three times with PBS and stained with 300 nM DAPI, 30 µl per well, for 10 minutes then analyzed under the fluorescent microscope (Zeiss Observer).

When cells were prepared on cover slips, they were seeded at 1x 10^8 cells and treated in 24-well plates. They underwent the same staining process but instead of 30 µl of formaldehyde and blocking buffer 100 µl were used. After staining the coverslips were mounted on slides using VECTASHIELD® Hard-Set™ mounting medium with DAPI and allowed to dry for 15 minutes before being incubated at 4°C overnight and then analyzed on the fluorescent microscope.

Target Protein (molecular weight)	Primary Antibody type, clone and supplier	Primary Antibody dilution, dilutant and incubation conditions	Secondary antibody dilution and used for
Dll4	Poly, ab7280, abcam	1:200, 1:400 (Flow Cytometry, Immunostaining)	1:1000 Alexa-fluor 647, 1:1000 Alexa-fluor 555
REG4	Mono, ab89917, abcam	1:200 (Flow cytometry and Immunostaining)	1:1000 Alexa-fluor 488 and Alexa-fluor 488 Goat Anti-mouse IgG_{2b} (Molecular Probes)
CDX2	Mono, CDX2-88, Biogenex	1:100 (Immunostaining)	1:1000 Alexa-fluor 555
PR5D5	Mono, N/A, In-house	1:200 (Flow Cytometry and Immunostaining)	1:1000 Alexa-fluor 488 and 555
Notch1	Mono, Val1744, cell signaling technology	1:200 (Flow Cytometry and Immunostaining)	1:1000 Alexa-fluor 555 and 647 1:10000 Dako polyclonal swine anti-rabbi/HRP (WB)
MUC2	Mono, ab13419, abcam	1:200 (Flow Cytometry)	1:1000 Alexa-fluor 647
TFF3	Mono, ab108599, abcam	1:200 (Flow Cytometry)	1:1000 Alexa-fluor 647
SlC22A11	Poly, sc-134005, Santa Cruz Biotechnology	1:200 (Flow Cytometry)	1:1000 Alexa-fluor 647
α-tubulin	Mono, mouse	1:500 (Western Blot)	1:20000 Dako polyclonal rabbit anti-mouse/HRP

Table 2.2 List of primary and secondary antibodies and dilutions used in various staining methods. I hour incubation for primaries and secondaries for immunostaining, 30 minutes for flow cytometry.

2.7 Flow cytometry

2.7.1 Surface Staining

Cells were first collected by aspirating the medium from the flask and washing the cells with 5 to 10 ml of PBS. The PBS was then aspirated and the appropriate amount of trypsin was

added (see Table 2.1) in order to detach the cells. The flask was then incubated at approximately 37°C for 5 to 10 minutes. The trypsin was then inhibited by adding twice the amount in medium with serum and was mixed well by pipetting. The cells were then transferred to a 15 ml tube with 1 ml being separated in order to count the cells (as discussed in 2.1.4). The cells in the 15 ml tubes were centrifuged for 5 minutes at 1000 rpm. The supernatant was then discarded and the pellet was suspended in an adequate volume of PBS and 2% FBS to obtain 1 x 10^6 per 100 µl. 100 µl of cell suspension was then transferred to each FACS tube.

From this point on the work was done on ice and in the dark. The primary antibodies were added and allowed to incubate for 30 minutes in the dark on ice. After 30 minutes 2 ml of PBS and 2% FBS was added to all the FACS tubes and centrifuged for 5 minutes at 1000 rpm at 4 °C. The supernatant was then discarded and the pellets were resuspended in 100 µl PBS and 2% FBS. The secondary antibodies were then added and were allowed to incubate for an additional 30 minutes. 2 ml of PBS and 2 %FBS was then added to all the FACS tubes and centrifuged for 5 minutes at 1000 rpm at 4°C. The supernatant was then discarded and the pellet was suspended in 500 µl PBS and 2% FBS and then analyzed on the flow cytometer (Beckman Coulter CyAn Analyzer).

2.7.2 Intracellular staining cells

This procedure followed the same method of collecting cells as the surface staining cells protocol up until the cells are centrifuged in 15 ml tubes for 5 minutes at 1000 rpm. The supernatant was then discarded. The cells were resuspended and washed with 5 ml of cold PBS and 2% FBS and centrifuged for 5 minutes at 1000 rpm, RT. The supernatant was discarded again leaving about 50 µl of liquid. The pellet was resuspended and 500 µl of

Fixation Buffer (BD cat#554 655) was added and mixed by vortexing. The cells were then allowed to incubate for 30 minutes on ice and were vortexed on a low setting, 2 or 3, once after 15 minutes.

After incubation the cells were washed twice with 5 ml of PBS and 2% FBS and centrifuged for 7 minutes at 1000 rpm, 4°C. The supernatant was discarded, the pellet was loosened, and 1 ml of cold Permeabilization Buffer III (BD cat. #558 050) was added slowly while vortexing and allowed to incubate on ice for 30 minutes. The cells were then washed twice as previously described and centrifuged for 7 minutes at 1000 rpm, 4°C. The supernatant was discarded and the pellet was suspended and stained exactly as described in the surface staining cell procedure. The samples then were analyzed on the flow cytometer. (Beckman Coulter CyAn Analyzer)

2.7.3 Fluorescent activated cell sorting

Four flasks of LS180 were used to sort goblet cells. Each flask was treated with 200 nM DBZ 24 hours before passage and fixation. This procedure followed the same method of collecting cells as the surface staining cells protocol up until the cells are centrifuged in 15 ml tubes for 5 minutes at 1000 rpm, RT. The supernatant was discarded and the cells were washed with 5 ml of cold PBS and 1% BSA and centrifuged for 5 minutes at 1000 rpm, RT. The supernatant was discarded, the pellet was suspended and 500 µl of 95% ethanol was added and mixed by vortexing. The cells were allowed to incubate for 15 minutes on ice and then the cells were washed twice with 5 ml of PBS and 1% BSA and centrifuged for 7 minutes at 1000 rpm, 4°C. The supernatant was then discarded and the pellet was suspended in 4 ml and transferred to FACs tubes for staining.

100 µl of PR5D5 primary antibody at a concentration of 1:200 were added to the cells, which were then incubated for 30 minutes on ice. After 30 minutes 2 ml of PBS and 1% BSA was added to all FACS tubes, which were then centrifuged for 7 minutes at 1000 rpm, 4°C. The supernatant was discarded and the pellet was suspended in 100 µl PBS and 1% BSA. 100 µl of Alexa-fluor 488 mouse secondary antibody was then added to each tube and allowed to incubate in the dark on ice. After 30 minutes the cells were washed in 2 ml PBS and 1% BSA, centrifuged for 7 minutes at 1000 rpm, 4°C and resuspended in 500 µl of PBS and 1% BSA.

Cells were then sorted into PR5D5 positive and PR5D5 negative tubes containing 1 ml of RNAlater on the Becton Dickinson LSR Fortessa sorter. After being sorted tubes were spun down and prepared for RNA extraction.

2.8 RNA Methods

2.8.1 RNA extraction

PureLink® RNA Mini Kit was used for RNA extraction. In brief after centrifugation all supernatant was removed from the samples by pipetting. Cells were then lysed with 0.3 mL of lysis buffer, manually homogenized by pipetting vigorously for 1 minute and centrifuged at 12,000 g for 2 minutes. The RNA was then purified through a series of binding, washing and elution steps and collected for RNA quantification.

2.8.2 RNA quantification

RNA quality and amount was analyzed on the NanoDrop 1000 spectrophotometer. The NanoDrop was first blanked with water and then the samples were run. Samples were given

in ng/μl and then the concentration of RNA in each sample was calculated. Samples that were above 40 ng were sent off for microarray analysis.

2.8.3 qRT-PCR

The High capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to reverse transcribe RNA into cDNA for analysis. In short, RNA was first extracted using the previously described technique. The RNA was then measured on the NanoDrop and diluted with RNAse free water to 10 ng/μl for a total volume of 50 μl. A master mix containing 2.0 μl 10X RT Buffer, 0.8 μl 25X dNTP Mix (100 mM), 2.0 μl 10X RT Random Primers, 1.0 μl MultiScribe Reverse Transcriptase, 4.2 μl Nuclease-free water per reaction was prepared on ice. 10 μl of RNA was then mixed with 10 μl of master mix and placed into a PCR tube, which then underwent amplification cycling for cDNA synthesis for 3 hours.

For qPCR the TaqMan Fast Universal PCR Master Mix (Applied Biosystems) was used. In brief, after cDNA synthesis was complete, cDNA was first measured on the NanoDrop. cDNA was then diluted to 50 ng/μl and 4.0 μl of cDNA template was added to each well. 1.0 μ of 20x TaqMan Gene expression assay, 10.0 μl of 2X TaqMan Gene Expression master mix and 5.0 μl of RNase-free water was then added to each well that contained cDNA templates. Triplicates of each reaction were made, centrifuged and then loaded into the PCR instrument. After amplification was complete data was obtained and the ΔΔCT of each sample was determined in order to observe expression level.

2.9 Image analysis and Statistical Methods

2.9.1 Image analysis

Fluorescent microscope data was analyzed on FIJI using cell counter plugins and fluorescence analysis. Flow cytometry data was analyzed using Summit v2.0.

2.9.2 Statistical methods

Categorical data were analyzed with either χ^2, paired T-test or where appropriate a two-tailed Fisher's exact test. Treatment data was analyzed using paired t-tests and multiple ANOVA tests.

Chapter 3: Goblet Cell Markers and Isolation

3.1 Introduction

Being able to identify specific marker genes that are unique to a certain cell type is essential in order to genetically dissect the differentiation pathway of that cell type. While functionally we understand what a goblet cell is, the biological markers for what determines a colorectal goblet cell are not as well known. PR5D5, an in-house developed antibody, is an intracellular marker for goblet cells that attaches to MUC2 (Richman and Bodmer, 1987). This antibody posed several problems; firstly one genetic marker is necessary but not sufficient to identify a single cell type, usually a corroboration of multiple markers is more reliable in identification. Secondly, PR5D5 and thus MUC2 is a marker of a differentiated goblet cell, which begs the question as to how many new gene expressions distinguish the mature goblet cell from its progenitor precursor and whether there is a goblet cell that does not make MUC2. Finally an intracellular marker does not allow for the cell in question to be sorted live and undergo experimentation.

The following chapter represents an attempt to identify novel goblet cell markers in order to isolate and characterize them better. Current presumed normal goblet cell markers, for example TFF3 (Wiede et al., 1999) and FCGBP, and our lab's microarray data for 96 colorectal cell lines provided a place to start.

3.2 Aims

In this study I set out to identify novel goblet cell markers from our laboratory's microarray data on 96 colorectal cancer cell lines and other putative markers. Optimization of RNA

extraction from fixed and permeabilized cells was also carried out in the hopes of isolating goblet cells from LS180.

3.3 Results

3.3.1 Microarray Data

Our substantial array of microarray data for 96 CRC lines was the first place to look in order to identify novel goblet cell markers. Data were collected by Dr. Neil Ashley of PR5D5 staining in a sample of the colorectal cell lines and were also analyzed. These staining patterns were used to classify the cell lines into goblet cell and non-goblet cell producing cell lines (Table 3.1) by examining a substantial number of goblet cells per sample (over 1.5%).

PR5D5 positive cell lines (Goblet cell producing)	**PR5D5 negative cell lines (Non-goblet cell producing)**
C125PM	C106
C80	C99
C84	CACO2
HCA46	CC20
HDC114	COLO201
HDC73	COLO320dm
HT29	COLO678
LIM1863	DLD1
LOVO	GP2d
LS174T	HCA7
LS180	HCT116
RW7213	HCT15
T84	HDC142
	HRA19
	HT55
	LS123
	LS1634
	NC1747
	OXCO1
	PMPKO14
	RCM1
	RW2982
	SKO1
	SW1417
	SW403
	SW405
	SW417
	SW48
	SW480
	SW837
	SW948
	VACO70MS

Table 3.1 Sampling of cell lines stained for PR5D5 separated into goblet cell and non-goblet cell producing cell lines.

These groups were created as a new labeling criterion in Partek where non-goblet cell producing cell lines were labeled as 1, goblet cell producing cell lines were labeled as 2 and unknown cell lines were 0. From these data mRNA expression levels were compared between the goblet cell producing and non-goblet cell producing cell lines. Potential goblet cell markers were identified by differential expression analysis between goblet cell and non-goblet cell producing cell lines. The results were ordered first by negative high fold change

and then evaluated as potential goblet cell markers by their p-value, specifically looking at $p < 0.05$. A list (Table 3.2) was generated of both genes identified from this process as well as putative normal goblet cell markers. . From this list, staining patterns of antibodies to those specific genes were evaluated through the human protein atlas (http://www.proteinatlas.org/). Some genes were selected based on a literature search relating them to either goblet cells specifically or CRC more generally, such as TFF3, FCGBP, DLL4 and REG4. DLL4 for example was selected because of Notch's role in goblet cell differentiation. Also from microarray data there is a possibility of small highly expressing subsets that would not necessarily show up as overall significant, but would still have high expression in their own subset. ST6GALNAC1 and SLC22A11 were also selected as potential goblet cell markers for their staining patterns on the human protein atlas in both normal and cancerous colon.

Rank	Column #	Gene Symbol	Mean (non)	Mean (goblet)	p-value(non vs. goblet)	Fold-Change (non vs. goblet)
9	12748	FCGBP	3.08853	7.73707	0.000579589	-25.0813
12	37039	ST6GALNAC1	4.49388	9.11846	0.000129241	-24.6682
56	32784	REG4	3.18575	6.30194	0.00783606	-8.67092
81	14180	MUC2	3.45265	6.31377	0.00426542	-7.2658
148	14130	TFF3	5.59689	7.90984	0.0764598	-4.96901
7560	32861	Dll4	4.4566	4.6921	0.238532	-1.17732
41528	29444	SLC22A11	2.23071	2.09189	0.571213	1.101

Table 3.2 List of microarray data expression level of genes upregulated in goblet cell producing cell lines as well as expression levels of supposed goblet cell markers compiled from Partek data as well as literature searches.

MUC2 was highly expressed in goblet cell producing cell lines, which confirmed using PR5D5 staining in order to classify the two groups of cell lines. While REG4 came from the literature search it also came up as highly significant (p-value < 0.01) from the Partek microarray data.

3.3.2 Testing potential and putative goblet cell markers

LS180 was selected as the goblet cell producing cell line to test potential markers on since it has a fairly high percentage of goblet cells, anywhere from 3 – 10%, in its cell line. HCT116 was selected in contrast as a non goblet cell line since it had a similar doubling time as LS180 and could be grown for similar experiments.

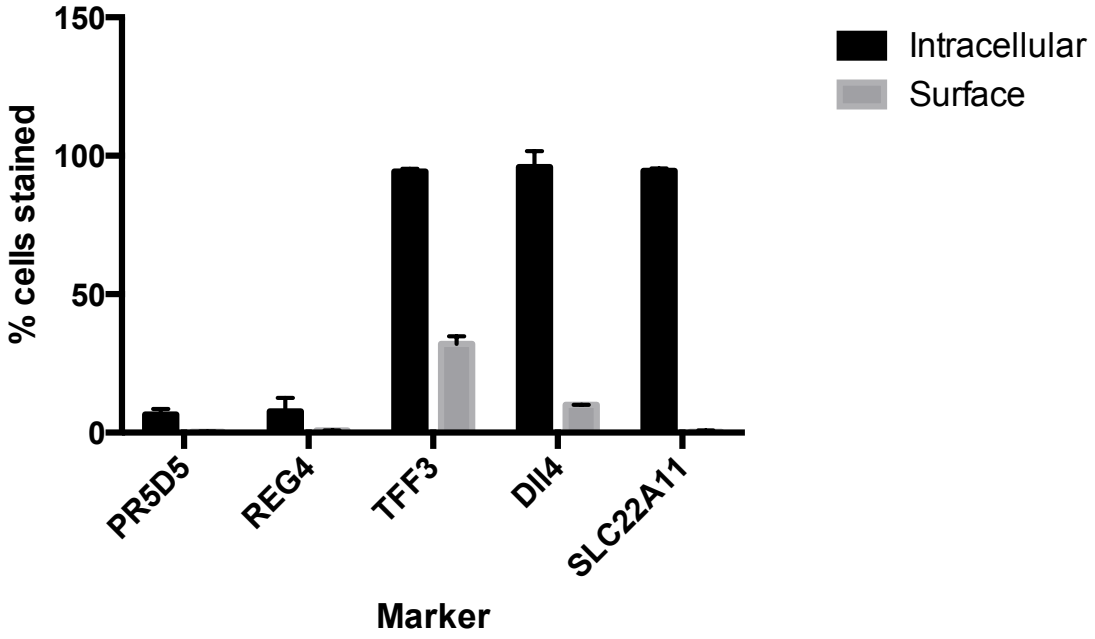


Figure 3.1 Potential goblet cell markers tested against PR5D5 on LS180, staining both intracellularly and on the surface (n=3 staining and measurements were done 3 separate times under the same conditions). No significant difference between PR5D5 and REG4 intracellular staining using one-way ANOVA.

Reg4 showed a similar intracellular staining pattern to PR5D5 (Fig 3.1) and was worth further investigation. TFF3 showed higher fluorescence on those that co-stained with PR5D5, but still stained most of the cells at a low background level intracellularly. TFF3 stained a little less than half of the cells on the surface. SLC22A11 stained almost all of the cells intracellularly and did not work as a cell surface marker. Dll4 stained almost all of the cells intracellularly. However, Dll4 on the surface stained a similar percentage to that of

goblet cells; yet costaining with PR5D5 on LS180 revealed two distinct populations; one staining for Dll4 and the other for PR5D5 with only 1% of the cells staining both (Figure 3.2).

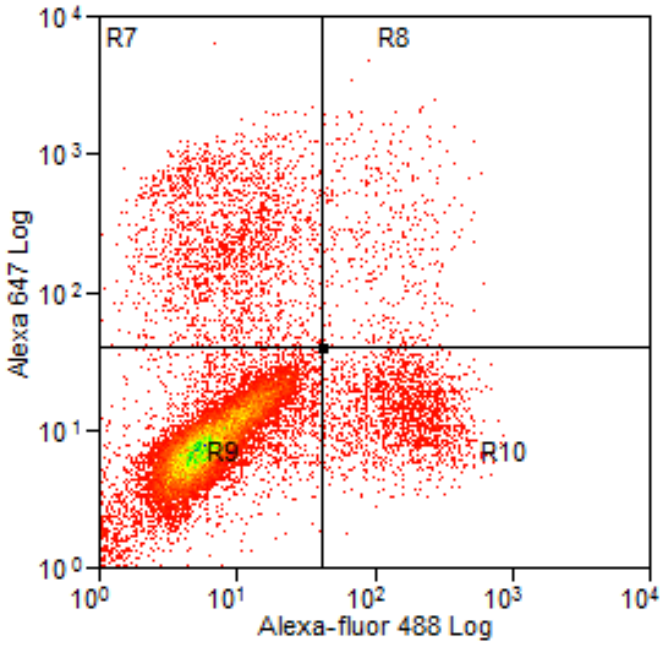


Figure 3.2 LS180 stained with anti-Dll4 and Alexa-fluor 647 on surface then fixed and permeabilized and stained with PR5D5 and Alexa-fluor 488. R7 = 8% of the population, R10 7.03% of the population and R8 = 1.78% of the population, which a significant proportion of the cells that stained only for one or the other ~ 10.6%.

This same staining was tested in an additional two cell lines, HT29 a goblet cell producing cell line and HCT116 a non-goblet cell producing cell line.

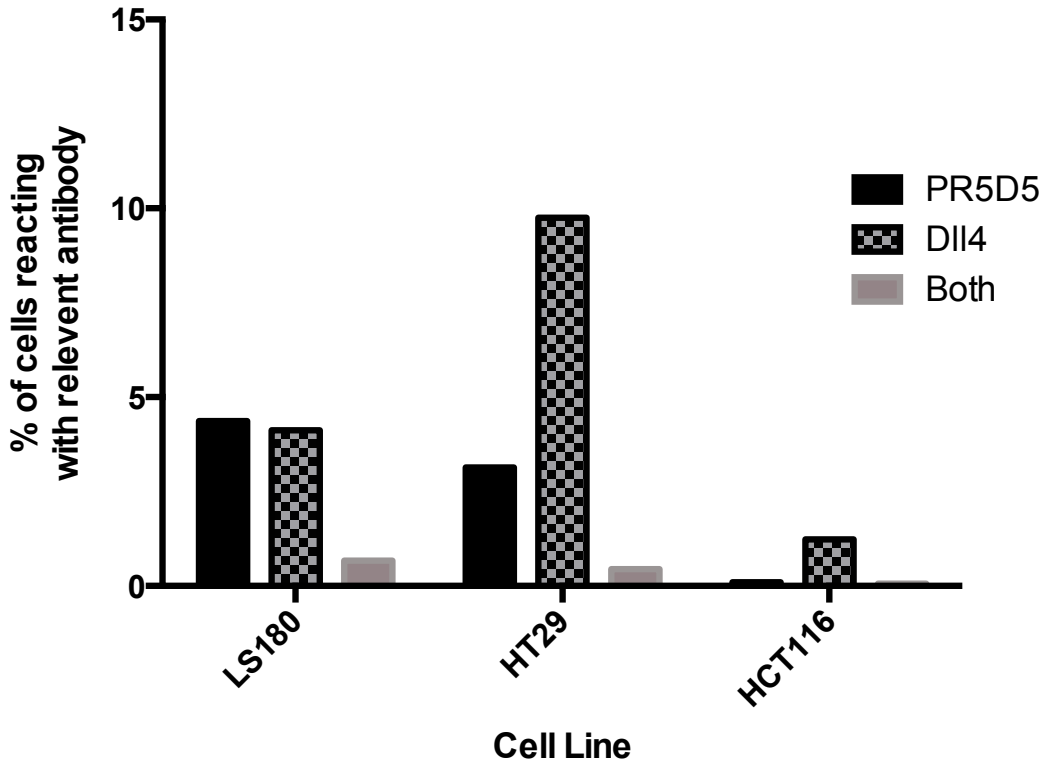


Figure 3.3 Staining for PR5D5 intracellularly and Dll4 extracellularly on three separate cell lines; LS180, HT29 and HCT116.

All three cell lines demonstrated distinct populations of cells staining for Dll4 and only few cells if any staining for both. HT29 had a much higher population of Dll4 staining cells than both LS180 and HCT116. HCT116 has a small population of Dll4 staining cells that demonstrates that Dll4 may be expressed in more colorectal cell lines than we currently believe.

3.3.3 Reg4 as a potential goblet cell marker

Reg4 was then co-stained with PR5D5 in LS180 using immunohistochemistry using a two-day staining process and isotype specific secondary antibodies since both antibodies were anti-mouse. LS180 were first stained with PR5D5 and anti-mouse IgG Alexa-fluor 555 and on the second day they were stained with anti-REG4 and anti-mouse IgG_{2b} Alexa-fluor 488 and examined under oil 100x on the fluorescent microscope.

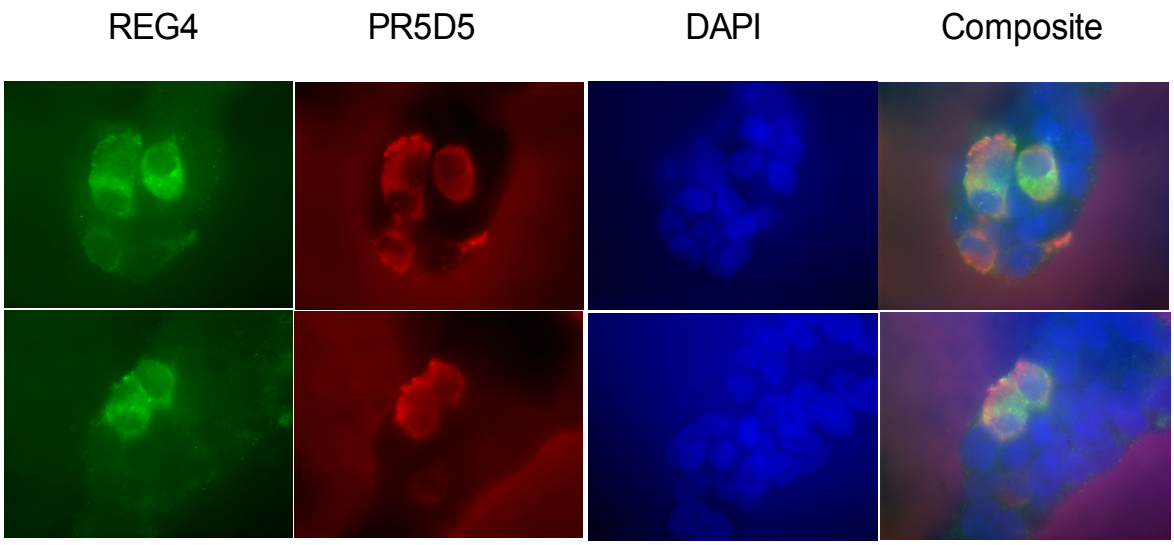


Figure 3.4 LS180 grown over 5 days on coverslips and stained with REG4, PR5D5 and DAPI, to show the nucleus. Staining indicates REG4 as a marker of goblet cells since it stains the same cells as PR5D5 in a colony.

REG4 and PR5D5 stained the same cells within LS180 (Figure 3.4). The staining varied in its intensity between the two antibodies. The antibodies also did not stain the same molecules within the same cell. There were no cells observed from staining that expressed REG4 but not PR5D5 and vice versa. In order to verify the antibody specificity of REG4 staining was done in HCT116, a non-goblet cell producing cell line.

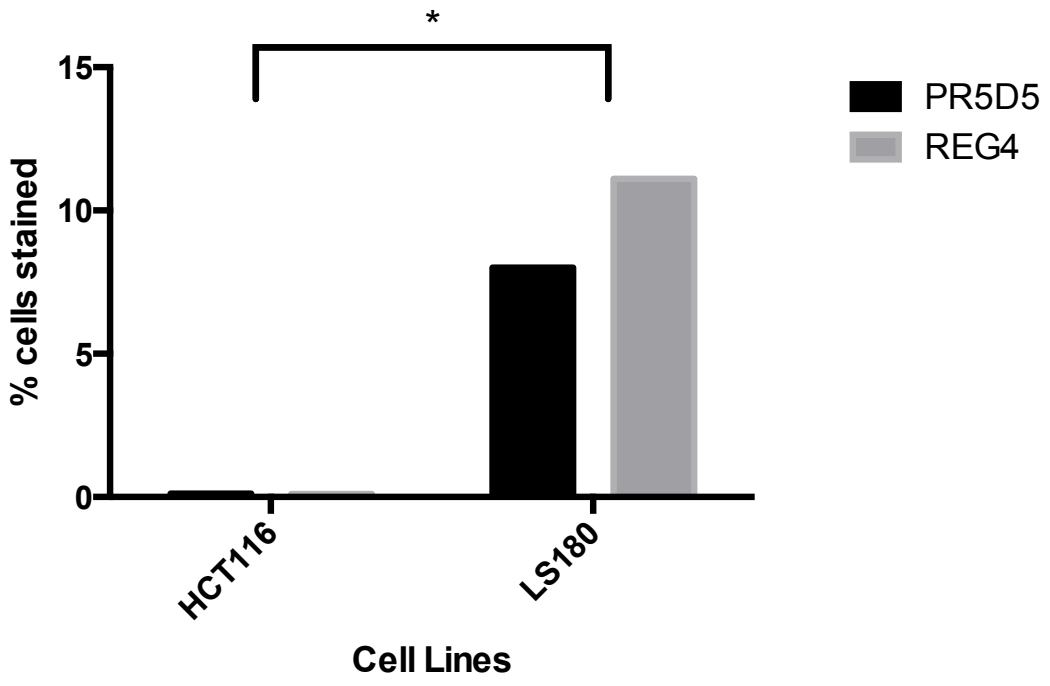


*Figure 3.5 LS180 and HCT116 colorectal cell lines stained intracellularly with PR5D5 and REG4. No significant difference between staining of markers within each cell line using t-test and significant difference between staining between cell lines * indicates p-value < 0.01 using an unpaired t-test. Difference between the number of cells stained for PR5D5 and REG4 may be due to where the boundaries were marked for positive and negative cells.*

REG4 and PR5D5 both did not stain HCT116 intracellularly or extracellularly, while they stained LS180 cells at a similar percentage (Figure 3.5). From our microarray data it was observed that REG4 was substantially less transcribed in HCT116 (Figure 3.6). REG4 again showed a very similar staining pattern to PR5D5 and was not present in HCT116, which positively suggests that the anti-REG4 was specific to REG4 and the results obtained were valid. While visually cells stained for both REG4 and PR5D5 there was a discrepancy from the flow cytometry data that show different staining percentages for PR5D5 and REG4. This experiment was only conducted once and staining intensities differ between both antibodies and could have cut off certain cells that did stain for both, which could have explained this discrepancy. Thus REG4 is another intracellular marker of goblet cells.

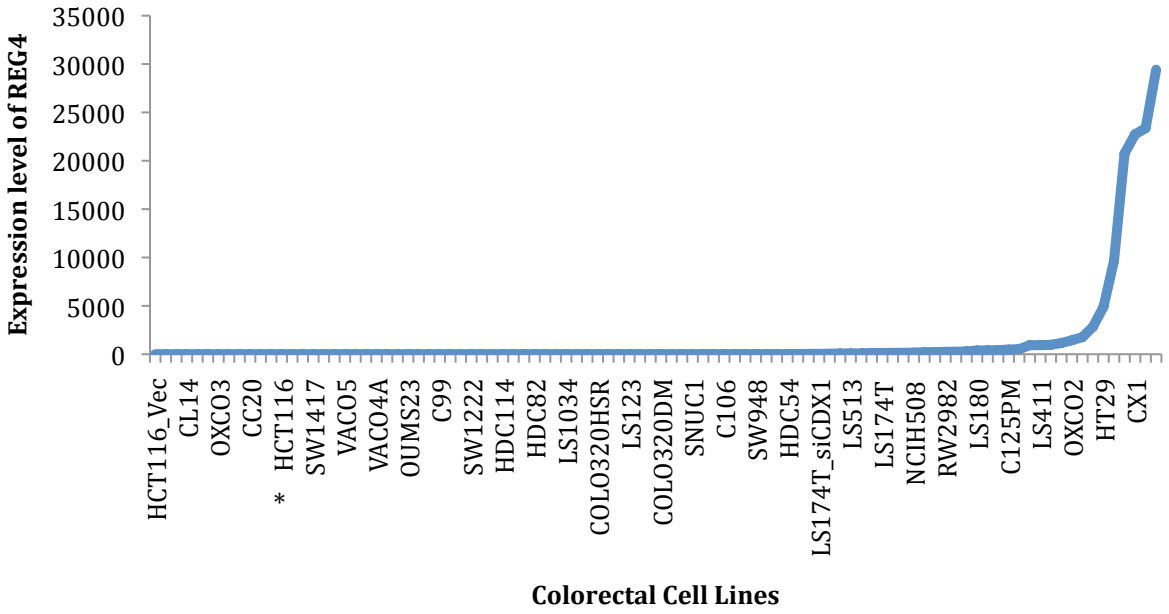


*Figure 3.6 Abbreviated data of expression level of REG4 in 96 colorectal cell lines. * Indicates HCT116 and low expression level of REG4.*

Those cell lines that are goblet cell producing appear on the right of this graph, having a high expression of REG4, while those such as HCT116 that are non-goblet cell producing appear on the left. It must be remembered that in microarray expression data the majority of cells that have low expression levels can overshadow data from small subsets with high expression levels. However, since staining was conducted in HCT116 for REG4 and REG4 was not found we can accept that these microarray data are useful for identifying low to no expression of REG4 in HCT116 and adding to the evidence that REG4 is indeed a goblet cell marker.

3.3.4 Optimization of RNA extraction from fixed and permeabilized cells

PR5D5 is an in-house made monoclonal antibody to MUC2 and is unequivocal in its identification of goblet cells. Thus PR5D5 was used in order to sort goblet cells out of the CRC line LS180. PR5D5 is an intracellular marker, which means that in order to stain the cells they first needed to be fixed and permeabilized. This posed a threat to both the quality

and quantity of extractable RNA since these processes take time and involve using preservatives such as formaldehyde that may make chemical modifications to the RNA. In order to extract a high quantity of high quality RNA from sorted goblet cells of LS180 several modifications were made to the normal staining procedure. Four large flasks of LS180 were grown to 70% confluence and incubated for 24 hours with 200 nM DBZ. Then techniques taken from several texts were used for the permeabilization and staining process. Cells were fixed and permeabilized in 95% ethanol for 15 minutes and washed with PBS and 1% BSA (Esser et al., 1995). Cells were then sorted into 500 µl RNAlater© and extracted using previously described methods. These techniques doubled the number of cells able to be obtained (Figure 3.7) as well as the amount of extracted RNA (Figure 3.8). Cells were sorted into PR5D5 positive and PR5D5 negative samples and RNA was extracted accordingly. This RNA was then sent off for microarray analysis in the hope that the return data will provide a clue to novel surface markers of goblet cells.

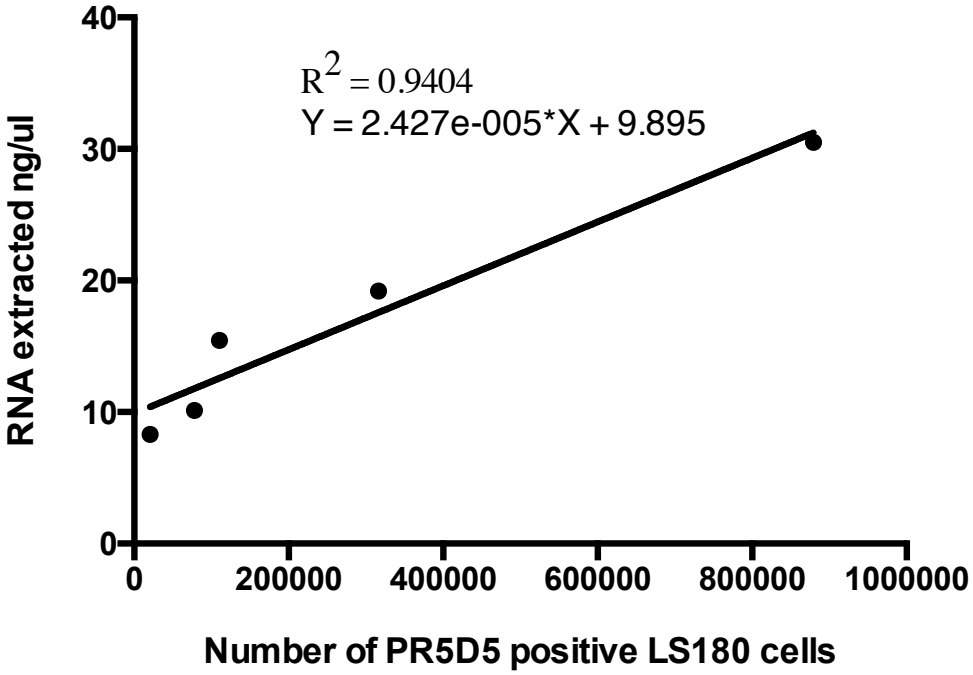


Figure 3.7 Number of PR5D5 positive cells in LS180 versus the amount of RNA extracted. The final extraction after optimizing the protocol is represented as the final point. This is linearly significant with an R^2 of 0.94. The highest population of positive PR5D5 sorted cells were incubated for 24 hours in 200 nM DBZ while all other points were not.

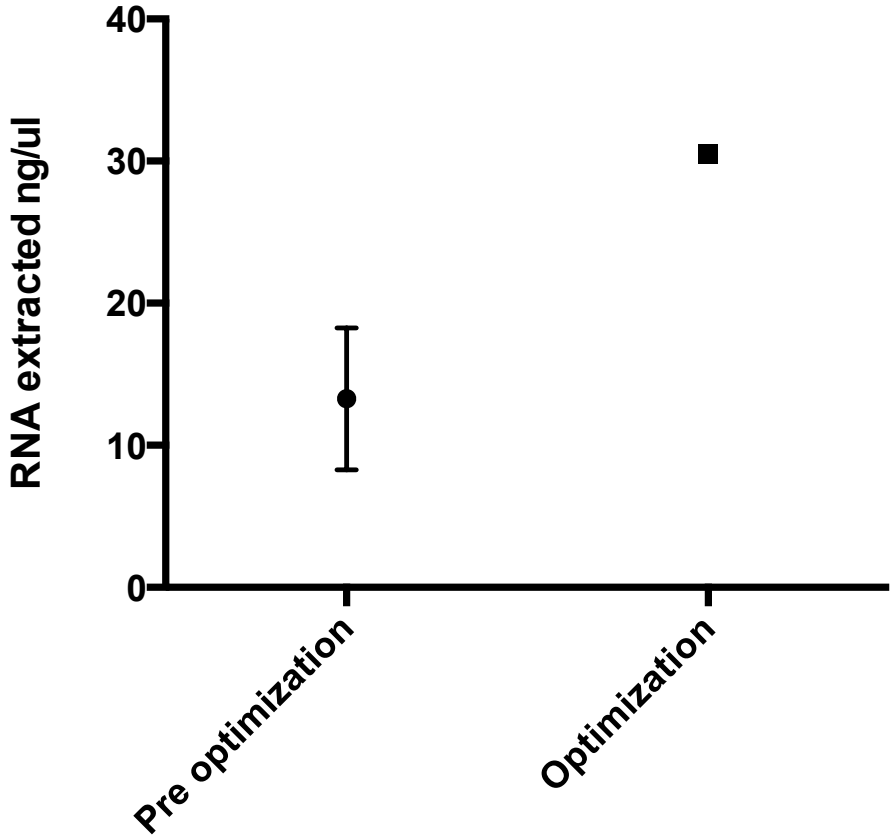


Figure 3.8 Amount of RNA extracted from positive PR5D5 sorted LS180 cells before the protocol was optimized and after the protocol was optimized (n=5).

Unfortunately the RNA was not of high enough quality for microarray analysis, most likely due to degradation by RNAses and the length of time from fixation to sorting. In order to preserve the RNA, RNAlater© was then used in experiments. First it was tested if it would interfere with the staining of PR5D5 and its secondary antibody, Alexa-Fluor 488, to goblet cells. LS180 was separated into three treatment groups, the first was treated with RNAlater© before fixation, the second was treated with RNAlater© after fixation and the third was not treated with RNAlater©. The addition of RNAlater© at each point before analysis on the flow cytometer disrupted the staining pattern and also changed the morphology of the cells. Therefore RNAlater© could not be used prior to sorting in order to maintain the quality of

the RNA. Further work is needed to devise protocols that would yield better quality RNA using inhibitors of RNases.

3.4 Discussion

Using our sizeable information of microarray data as well as literature searches, several genes came up as potential goblet cell markers, including REG4, TFF3, and Dll4. However, microarray data can only give so much information. Especially since goblet cells represent a small proportion of the cell line, anywhere from as few as 1% to 10%, microarray data has a major drawback. Looking at the over all expression of mRNA in a cell line may not enable the detection of a small subset of cells that are highly expressing the mRNA in question. Thus it was important to first verify the new potential markers as well as develop a way to isolate and examine goblet cells through flow cytometry, which allows you to see such sub-populations.

TFF3 has been described as a goblet cell marker (Kim and Ho, 2010) and is a secreted product of mucin producing cells in the intestine (Durual et al., 2005). In this study TFF3 proved to stain the majority of LS180 cells intracellularly and a little less than half extracellularly. There was a correlation between higher fluorescence TFF3 staining with PR5D5 stained cells. However, this correlation was not substantial enough to mark a discrete population since there were cells highly fluorescent for TFF3 but not fluorescent at all for PR5D5. Thus TFF3 could not be used to exclusively mark goblet cells (Figure 3.1).

Another reputed goblet cell marker that was described in the literature was Dll4. Dll4 is a ligand of Notch and has been implicated in tumor angiogenesis(Jubb et al., 2009). Dll4 stained a similar percentage extracellularly as did PR5D5 intracellularly in LS180, suggesting

that Dll4 could be a surface goblet cell marker. However, when Dll4 was co-stained extracellularly with PR5D5 intracellularly it revealed distinct populations of cells staining separately for both Dll4 and PR5D5 (Figure 3.2). This is interesting because previously it was believed that LS180 was not a Dll4 producing cell line from the microarray data collected in our laboratory. These Dll4 expressing cells could be the same previously described Paneth-like cells of the colon that have been described as cKit positive and influencing the fate of colonic stem cells (Rothenberg et al., 2012). They could also be goblet cell precursors that are closer to the colorectal stem cells. Further experiments would be necessary in order to positively identify these cells and to understand their function in goblet cell differentiation.

REG4 was first identified through differential expression analysis in our 96 cell lines by comparison of goblet to non-goblet cell producing cell lines and was confirmed as a goblet cell marker through staining. REG4 stained within the same cells as PR5D5 in LS180 goblet cells and was determined to be another intracellular marker for goblet cells (Figure 3.4). REG4 is highly expressed in most of the goblet cell producing cell lines (Figure 3.6) and is not present in HCT116, a non-goblet cell producing cell line, which once again confirms it as a goblet cell marker (Figure 3.5). Its high expression has also been noted in neoplastic goblet cells of appendiceal mucinous cystadenomas (Heiskala et al., 2006). REG4 is not a well understood protein and was first discovered by isolating cDNA from an inflammatory bowel disease library (Hartupee et al., 2001). REG4 is a secreted C-lectin protein thought to be involved in intestinal differentiation and proliferation (Heiskala and Andersson, 2013). The Reg family was first discovered in pancreatic islet β cells for their regulation of regeneration of the islet β cells. They have also been implicated in mucosal injury regeneration in the stomach and are a calcium dependent lectin superfamily (Asahara et al.,

1996; Okamoto, 1999). Little is known about REG4 and it has been implicated in being overexpressed in 71% of colorectal cancers, but they are most likely just more expressed in mucinous CRC or ones that produce goblet cells. REG4 could thus be involved in the regulation of goblet cell differentiation and provides another marker from which to identify goblet cells.

Finally the isolation of goblet cells is a necessity if we are to discover the full goblet cell differentiation pathway. Goblet cells were extracted from LS180 through PR5D5 intracellular staining and FACs. Using previously described techniques for extracting RNA from fixed and permeabilized sorted cells this protocol was optimized (Esser et al., 1995). This optimization was necessary to both sort more goblet cells as well as obtain a higher quantity of RNA from them (Figure 3.7, 3.8). The optimization worked for sorting a large number of goblet cells and could be successfully repeated with similar quantity of RNA. RNA was then extracted from the sorted PR5D5 positive goblet cells and the PR5D5 negative non-goblet cells. These data were sent off for microarray analysis with the hopes of gaining new insight on goblet cell expression level compared to non-goblet cell expression level in LS180. The RNA proved to be of too poor quality to be analyzed so RNAlater© was tested in an effort to prevent RNA degradation. However, RNAlater© proved to interfere with staining and thus would not be useful for sorting of goblet cells with PR5D5. RNAlater© has elsewhere been seen to interfere with GFP staining, which may describe its interference with Alexa-fluor 488, which is on a similar wavelength (Zaitoun et al., 2010). Using a different secondary antibody may solve the interference problem, but the morphology may be altered too much to use RNAlater©. RNAlater© has previously been used to maintain RNA integrity, but only in live sorted cells and only after the sort instead of directly after fixation (Nishimoto et al., 2007). Therefore other measures may need to be taken in order to prevent

RNA degradation and remove RNases from the procedure. Further investigation is necessary into a surface cell goblet marker to circumvent this problem as well as to continuing to optimize this procedure.

Chapter 4: Regulators of Goblet Cell Differentiation

4.1 Introduction

While differentiation is highly deranged in CRC, tumors usually retain some form of differentiation (Ashley et al., 2013). Those tumors that exhibit less differentiation are mostly made up of stem cells that continue to divide. Differentiated cells have limited division and thus do not greatly add to tumor growth. In cancer there is a strong selection for blocking differentiation and maintaining a highly proliferative and undifferentiated phenotype. If the majority of the tumor is made up of constantly dividing stem cells that cannot differentiate then the tumor can grow at a faster rate and is much more aggressive.

While the differentiated forms of tumors are not uncommon in CRC, their possession of differentiated goblet cells is. Since any differentiation proves to slow down tumor growth it is essential to understand this process of differentiation in those tumors that do possess goblet cells. Great insight into this area would be derived from examining the role of normal goblet cell differentiation regulators.

One of the major known regulators of goblet cell differentiation is Notch. Notch and its pathway act as regulators in many embryonic tissues by inducing or inhibiting certain cell fates (Artavanis-Tsakonas et al., 1999) In adult intestinal development, Notch regulates the balance between the absorptive and secretory cell type differentiation (Stanger et al., 2005) by inhibiting the secretory lineage and thus goblet cell differentiation. In order to examine this regulation in CRC, CRC cell lines were treated with the γ-secretase inhibitor DBZ.

DBZ has been shown to induce goblet cell differentiation in both intestinal crypts and adenocarcinomas of mice (van Es et al., 2005) through inhibition of the Notch pathway. Another form of Notch inhibition is through one of its many ligands. Previous studies using anti-Dll4 in order to block activation of Notch through its Dll4 ligand showed an inhibition of tumor growth (Hoey et al., 2009), thus suggesting increased differentiation of cells within the tumor.

Two additional regulators that have come up within our own laboratory's research and elsewhere are the homeobox genes CDX1 and CDX2. CDX1 and CDX2 are members of the caudal-related homeobox gene family first found in Drosophila and are known to regulate intestinal differentiation and intestine-specific gene transcription (Suh et al., 1994; Fang et al., 2000). Specifically, CDX2 has been shown to activate MUC2 transcription and potentially play a role in goblet cell differentiation (Yamamoto et al., 2003). Furthermore, CDX1 has been associated with inducing crypt progenitor cells into mature epithelial cells (Fujii et al., 2012; Yeung et al., 2010). The balance between these two homeobox genes may determine cell lineage fate of intestinal crypt stem cells.

4.2 Aims

In this study I focused on identifying the degree of regulation by Notch, CDX1 and CDX2 of goblet cell differentiation through the inhibition of their pathways in CRC cell lines that were goblet cell and non-goblet cell producing.

4.3 Results

4.3.1 Gamma-secretase inhibitor dibenzazepine

LS180 was selected in order to test the effects of DBZ since it already produces a relatively high number of goblet cells, anywhere between 3 - 10% of the culture. 200 nM of DBZ, a dosage that has been used previously in our laboratory (Yeung et al., 2011), was administered to LS180 over a period of 5 days, changing medium every two days.

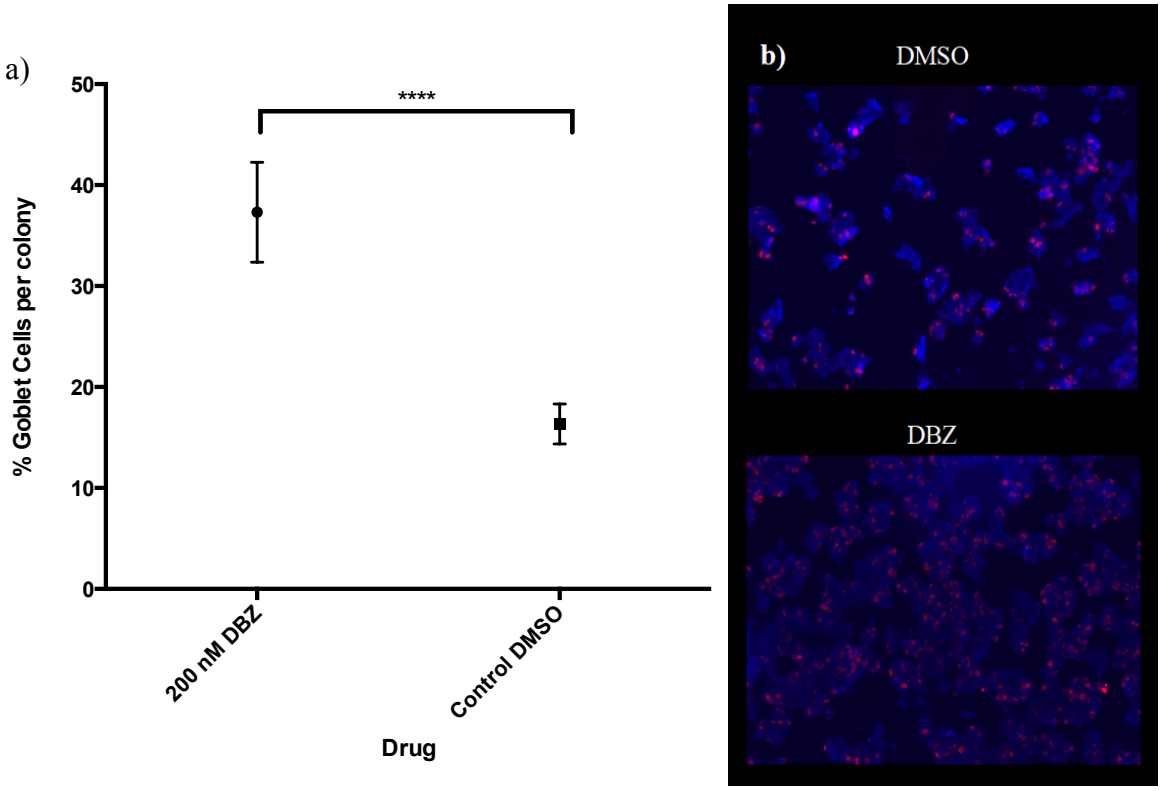
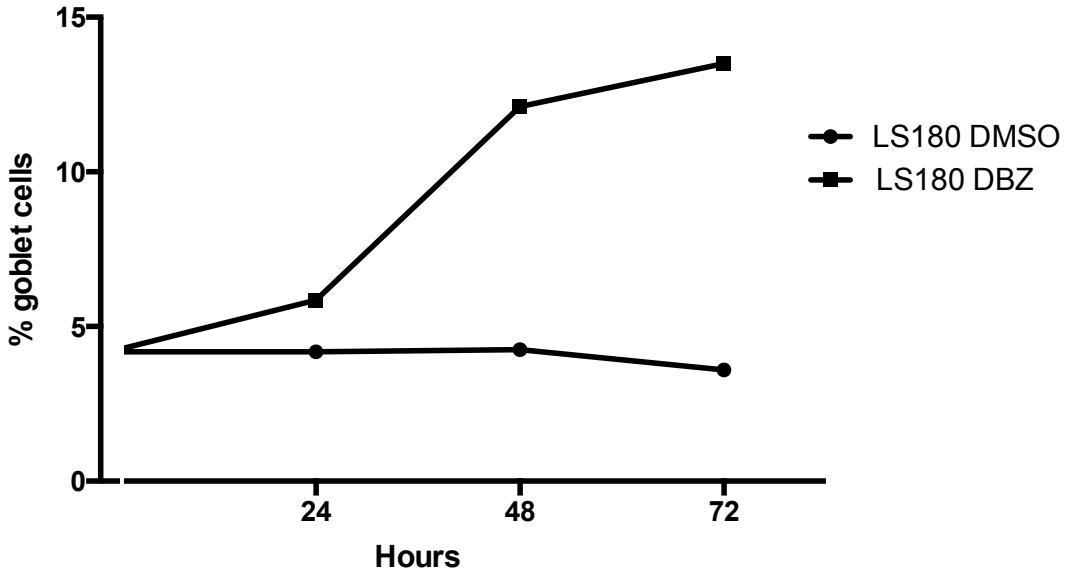


Figure 4.1 LS180 incubated for 5 days with or without 200 nM DBZ (γ-secretase inhibitor) changing medium and DBZ every 2 days a) n = 10 colonies randomly selected based on area and counted percent of goblet cells present b) representative colonies of wells measured. Significant with p-value < 0.0001

Goblet cells were measured through PR5D5 staining at the end of the five-day period. There was a significant difference in the number of goblet cells per colony treated with DBZ rather than DMSO (Figure 4.1). The percentage of goblet cells in this experiment was much higher than otherwise found because of how the goblet cells were measured. This data was obtained by counting number of goblet cells per colony while further measurements were conducted

by flow cytometry, a much more objective approach. From here the question became at what time did DBZ cause an increase in goblet cell differentiation. A time course experiment was then set up to test the effects of 200 nM DBZ on LS180 and HCT116, a non-goblet cell producing cell line, over 48-72 hours.

a)



b)

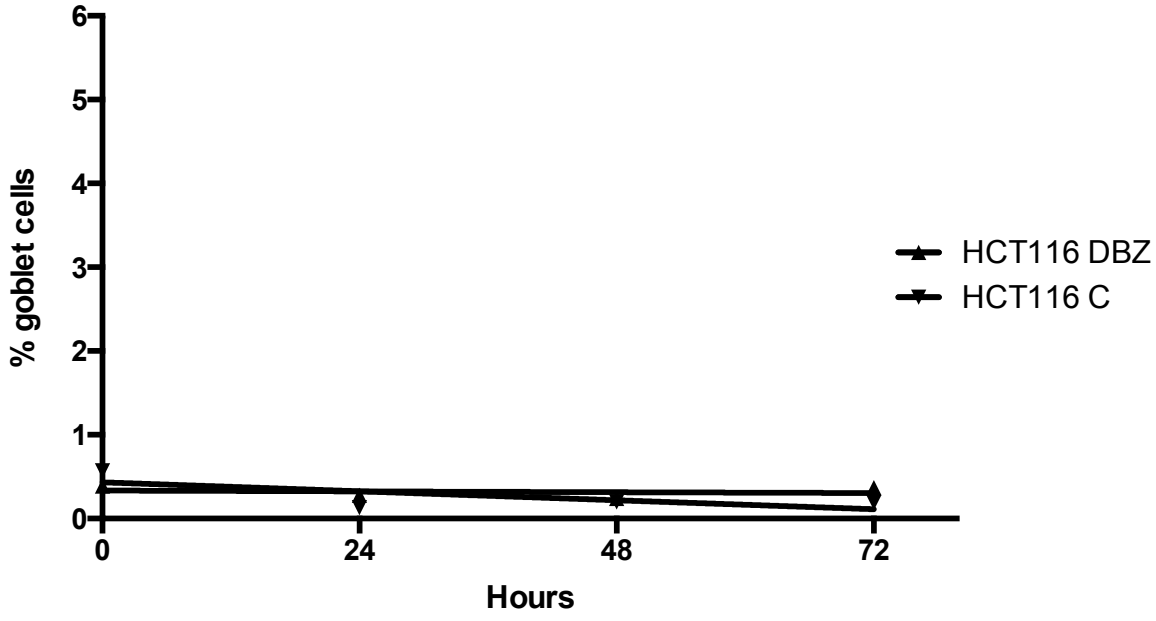


Figure 4.2 a) LS180 and b) HCT116 incubated for 72 hours with 200 nM DBZ or 200 nM DMSO. Medium was not changed for the full 72 hours. Regression test of slopes significant with p-value = 0.0091 between LS180 and LS180 DMSO and of elevation < 0.0001 between LS180 and HCT116.

There was no change in goblet cell number in HCT116 over 72 hours, but there was a significant increase in goblet cell number after 24 hours and a greater than two-fold increase after 48 hours in LS180 (Figure 4.2). 24 hours was determined to be enough to result in an increase in goblet cell number and therefore optimum dosage for the greatest number of goblet cells was determined next. While there was still further increase after 72 hours and percent of goblet cells looked as if it may just be starting to plateau, 24 hours was enough to make a significant change and was chosen in order to maximize number of experiments conducted. Many more experiments could be conducted and analyzed with an incubation time of 24 hours than of more than 72. Also past 72 hours without changing cell medium, cells would start to die and become confluent, changing their morphology and potentially expression. Thus, LS180 was incubated for 24 hours with doses ranging from 0 nM to 1600 nM DBZ.

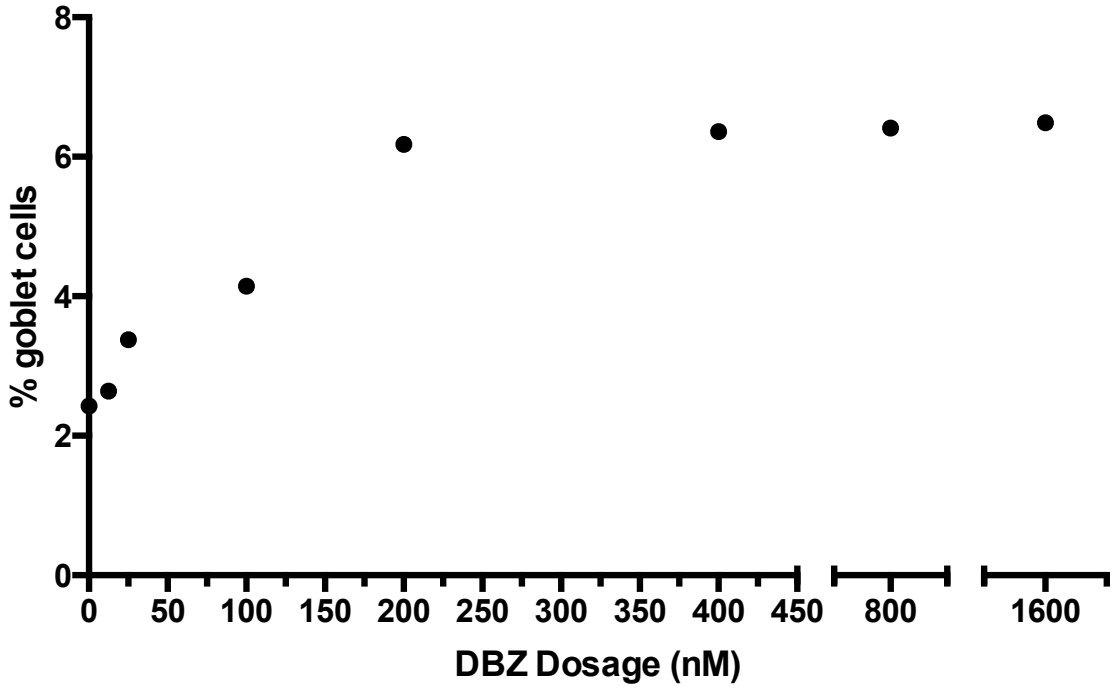


Figure 4.3 LS180 incubated for 24 hours with varying dosage of DBZ (0, 12.5, 25, 50, 100, 200, 400, 800, 160)) measuring goblet cells through % goblet cells in population (n=2).

At 200 nM DBZ the maximum number of goblet cells able to be achieved after a 24-hour period leveled off (Figure 4.3). 200 nM DBZ greater than doubled the number of goblet cells within the population and was thus used as the normal dosage from there on. At the higher dosages, 800 and 1600 nM, DBZ had a negative effect on cell survival. If this experiment were to be repeated dosages between 200 nM and 400 nM would have been examined over a period of 72 hours in order to observe maximum proportion of goblet cells. Next, examination of inhibition of Notch was conducted through a western blot for active Notch, Notch-1, in LS180.

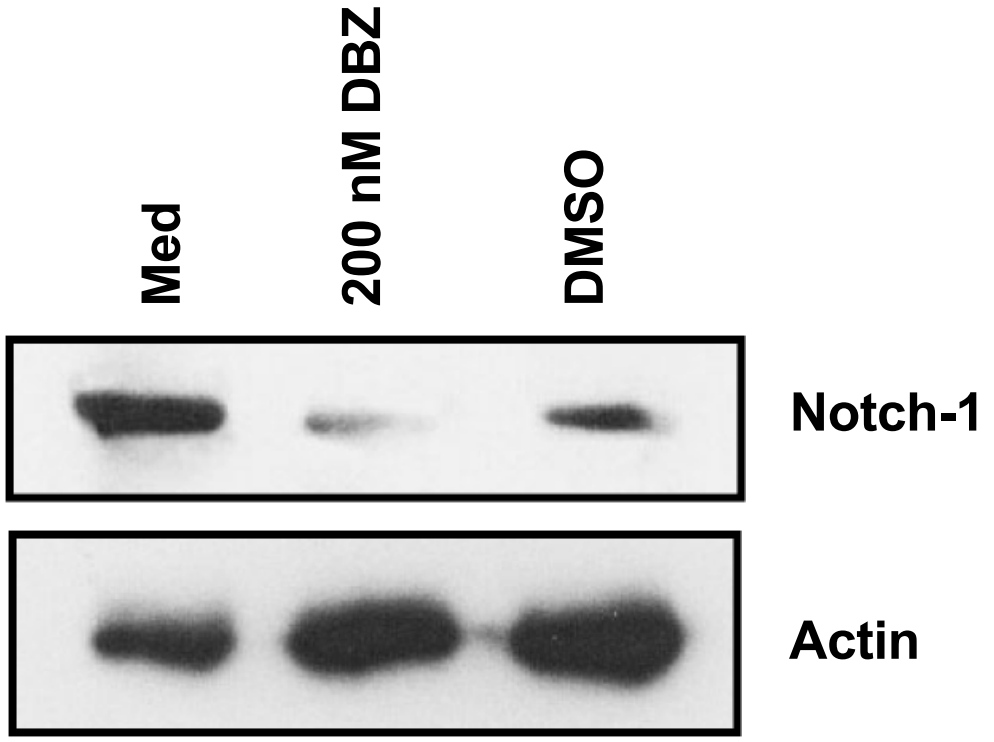


Figure 4.4 Western blot of active Notch-1 in LS180 after 24-hour treatment with either DMEM, 200 nM DBZ or DMSO.

200 nM DBZ sufficiently inhibited Notch activation in LS180 after 24 hours as compared to treatment with either 200 nM DMSO or plain medium. While Notch-1 was inhibited there is still significant residual active Notch. For this reason a slightly higher concentration of DBZ and more importantly a longer time of incubation should have been tried for better Notch inhibition. DMSO caused a slight decrease in Notch-1, which could have been caused by human error and with more time this western would have been repeated for a more accurate comparison between DBZ and DMSO.

4.3.2 DBZ and Dll4

Effects on goblet cell number were compared between DBZ and anti-Dll4 treatment. A concentration of 1 μg/μl of anti-Dll4 was used to inhibit the Dll4 ligand. LS180 cells were treated 24 hours with 200 nM DBZ, 1 μg/μl anti-Dll4 or both.

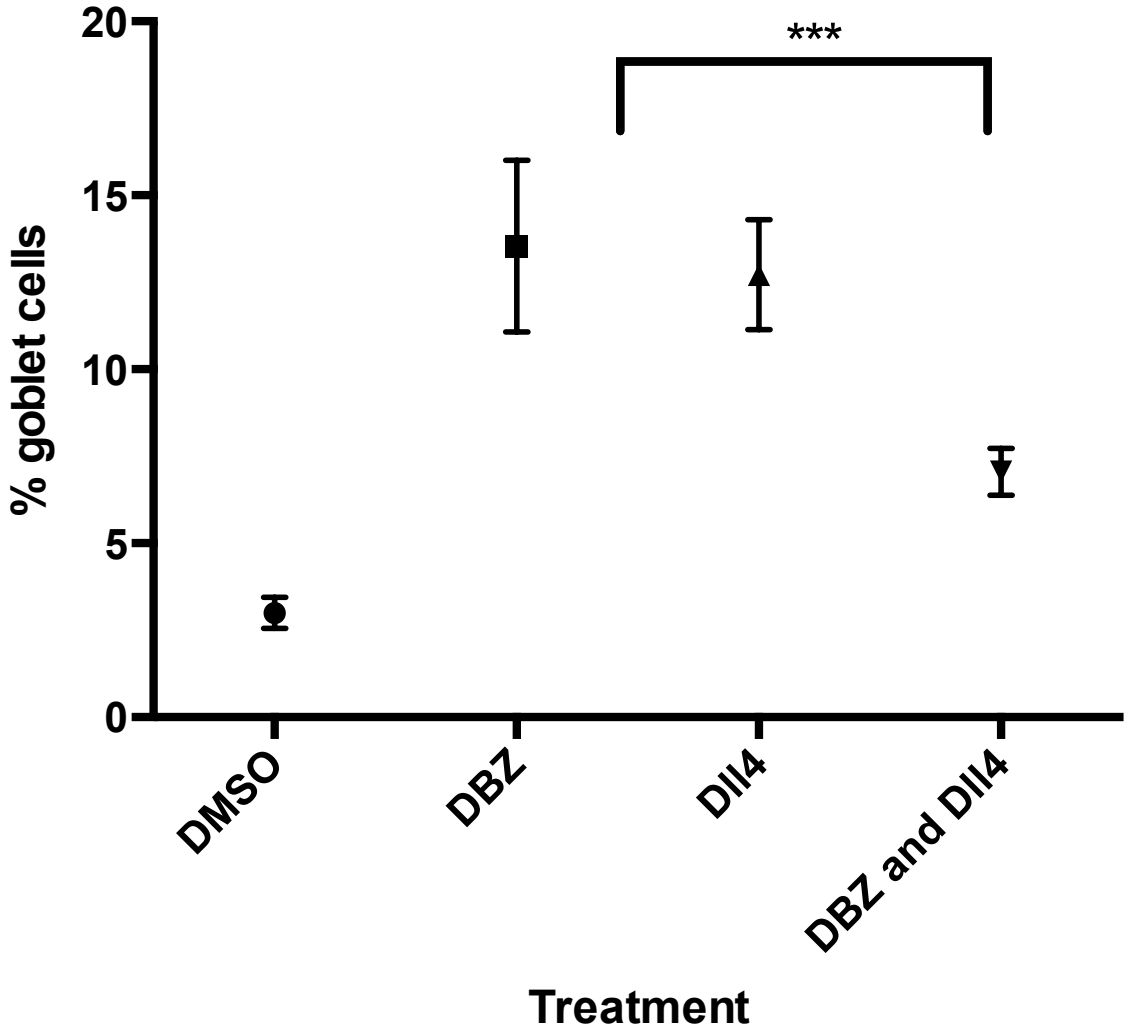


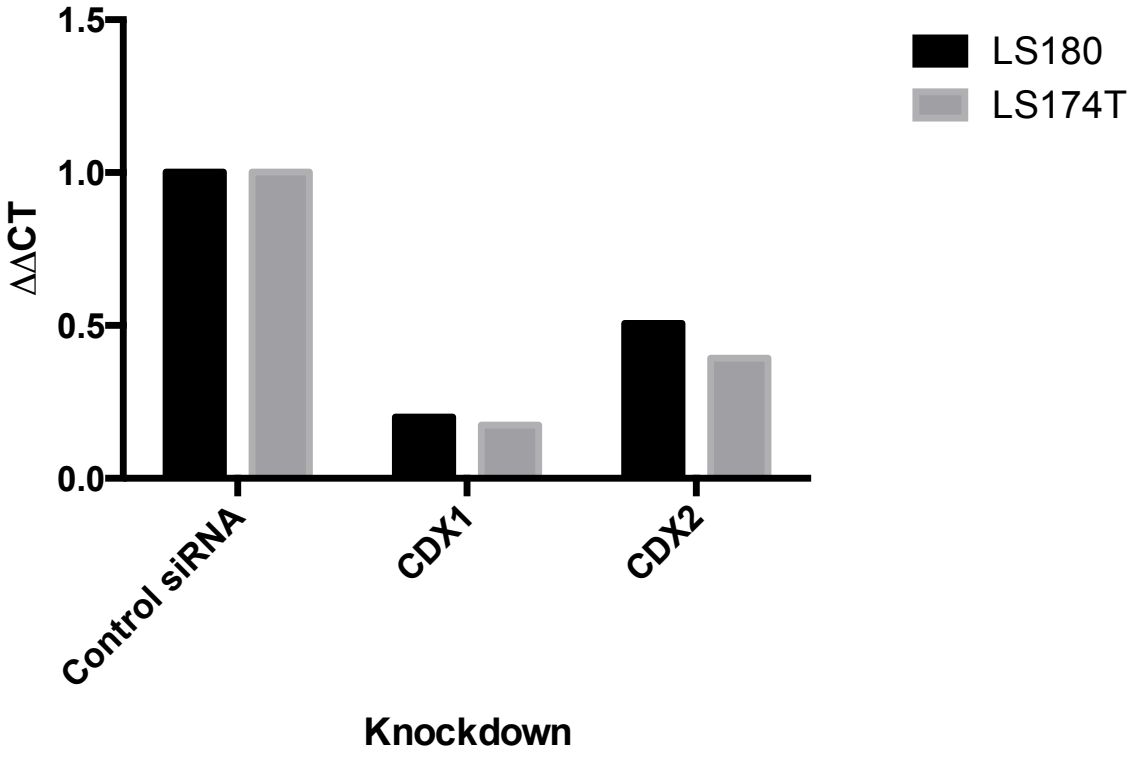
*Figure 4.5 Percent goblet cells of four treatment groups of LS180 Colonies treated for 24 hours. *** Indicates p < 0.001 statistically significant differences in number of goblet cells per culture. P-value < 0.05 between DMSO and treatment of both. Vertical bars represent standard error of the mean (n=2).*

Both DBZ and anti-Dll4 increased the number of goblet cells two-fold with no significant difference between them. However, when LS180 cells were treated with both there was an antagonistic effect between the two treatments (Figure 4.5). There was still a significant difference between those cells treated with both and the control, however, there was also a significant difference between each individual treatment compared to the combined treatment with a p-value < 0.01 (Figure 4.5).

4.3.3 CDX2 and CDX1

LS180 and LS174T, which are both goblet cell producing cell lines that produce similar numbers of goblet cells, were used to examine the effects of CDX1 and CDX2 knockdown on goblet cell number. Cell lines were knocked down for 72 hours with siRNA against CDX1 and CDX2 and then number of goblet cells per population was assessed using PR5D5.

a)



b)

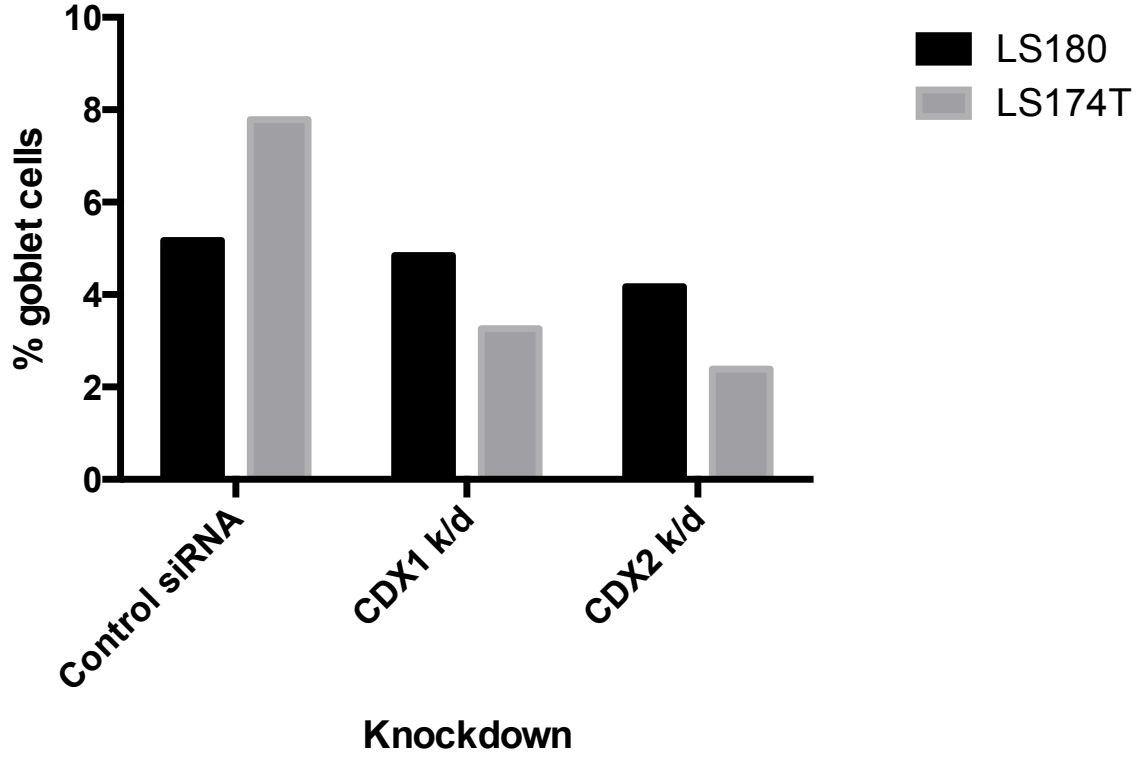


Figure 4.6 CDX1 and CDX2 knockdown in LS180 for 24 hours on goblet cell number . a) RT-PCR verification of knockdown through ΔΔCT with ANOVA significant between both CDX1 and CDX2 knockdowns and the control with a p-value < 0.0001 for both LS180 and LS174T. b)In LS180 t-test significant with a p-value < 0.01 between CDX1 and control and p-value < 0.001 between CDX2 and control, both CDX1 and CDX2 knockdowns were ANOVA significant in LS174T with a p-value of < 0.0001. Difference in number of goblet cells in LS174T and LS180 due to inter experimental variation.

Significant knockdown was achieved in both LS180 and LS174T for CDX1 and CDX2 (Figure 4.6b). There was also a significant decrease of goblet cells in both LS180 and LS174T for knockdown of either CDX1 or CDX2 as compared to their controls. In LS180 there was a larger significant decrease in number of goblet cells for CDX2 knockdown (p-value < 0.001) than for CDX1 knockdown (p-value < 0.01). However, this decrease isn't as convincing as that in LS174T. LS174T had a more significant decrease of goblet cells for knockdown of CDX1 and CDX2 (p-value < 0.0001) than did LS180. Knockdown in LS174T was more sufficient than in LS180 and may result in the larger effects on goblet cell number.

This experiment was only conducted once, however, these data are suggestive of CDX2 and CDX1 of having a large impact on goblet cell differentiation but this should be further verified and tested in several other cell lines.

4.4 Discussion

Addition of 200 nM DBZ over 24 hours proved to significantly increase the number of goblet cells in a culture of LS180. These results has been paralleled in mouse studies as well as in another goblet cell producing cell line, SW1222 (van Es et al., 2005; Yeung et al., 2011). However, addition of 200 nM DBZ to HCT116 had no effect on goblet cell number. HCT116 does not differentiate at all and thus Notch must be downstream of an initial regulator of general stem cell differentiation.

In this study Notch inhibition through blockage of Dll4 with anti-Dll4 proved to increase goblet cell number just as much as 200 nM DBZ treatment (Figure 4.5). Inactivation of Dll4 in mice has also shown to induce goblet cell differentiation in intestinal crypts (Pellegrinet et al., 2011). Further studies have also shown that blocking Dll4 signaling can reduce tumor growth in human colon tumor xenografts in NOD-SCID mice (Hoey et al., 2009), which makes sense since cells differentiate into goblet cells and then die. It was interesting that when used in conjunction 200 nM DBZ and 1 μg/μl anti-Dll4 proved to have an antagonistic effect, while still significantly increasing goblet cell number.

This may be due to the fact that since Dll4 is being blocked, Notch may be preferentially using other ligands instead of using Dll4. This suggests that Dll4 may be the primary ligand of Notch for goblet cell inhibition, but further studies are necessary. Notch may have different signaling fates depending on which ligand binds and activates it. By blocking

Notch signaling more broadly goblet cell differentiation may be stimulated. However, blocking Notch activation by Dll4 may be causing Notch to be seeking other ligands that usually confer the fate of the absorptive lineage of the cell. These two treatments together may be somewhat canceling each other out and thus may explain the antagonistic effect of the two treatments.

Similarly Dll1 has specifically been shown to be involved in goblet cell differentiation (Akiyama et al., 2010), which suggests the two may work together. It has been suggested that Dll1 may activate ATOH1 and then induce MUC2 if it is not bound to its Notch ligand, in which case it would be interesting to treat cells with anti-Dll1 and see if a similar effect occurs (Akiyama et al., 2010). The combination of both DBZ and anti-DLL4 treatment could block both DLL4 as well as the relevant Jagged ligand that Notch may be interacting with. Dll4 could similarly to Dll1 be able to activate goblet cell differentiation when it is not bound to Notch. Thus it would be worth continuing this research by looking at some of Notch's other ligands including Jag1 and Dll1 and their effects on goblet cell differentiation. FACS could be used on LS180 staining with both anti-Dll4 as well as anti-Jagged antibodies in order to determine if Dll4 is only expressed on goblet cell progenitors or separate cells to those expressing Jagged that may influence goblet cell differentiation.

CDX1 and CDX2 were significantly knocked down in both LS180 and LS174T after 72 hours (Figure 4.6b). In LS174T there was a significant decrease in the number of goblet cells in the CDX2 knockdown culture and a smaller yet still significant decrease in LS180. CDX2 has previously shown to activate MUC2 transcription and play an integral role in goblet cell differentiation in COS-7 cells, a monkey kidney cell line (Yamamoto et al., 2003). Thus

implying that the knockout of CDX2 inhibits MUC2 transcription and thus inhibits goblet cell differentiation, which is consistent with these results.

Knockdown of CDX1 also proved to significantly decrease the percent of goblet cells in both LS180 and LS174T, however, not as much as CDX2 (Figure 4.6a). This result is inconsistent with the literature, which has previously described knockdown CDX1 as not inhibiting goblet cell differentiation in SW1222 (Yeung et al., 2011). This discrepancy may be due to an insufficient knockdown of CDX1 and CDX2. This experiment could be repeated using a more substantial knock down such as the CRIPSR-cas9 technology to fully knock out CDX1 and CDX2 in LS180 and LS174T. These results should be verified and repeated again in LS180 as well as other goblet cell and non-goblet cell producing cell lines.

Chapter 5: Conclusions and future directions

Each conclusion derived from this thesis is a subheading. Each statement is followed by a summary and examination of future work that would expand this body of knowledge.

5.1 REG4 is a marker for intestinal goblet cells

Greater insight into the physiological functions of REG4 is needed. Currently it has been shown to stimulate cell growth and promote migration and invasion of tumor cells in colorectal cancer cell lines HT-29 (Rafa et al., 2010) as well as having control of multiple growth factor pathways (Vanderlaag et al., 2012). REG4 has also been shown to be present in goblet cells of other organs including the appendix (Heiskala et al., 2006) and elsewhere in the GI-tract (Heiskala and Andersson, 2013). This research has shown that REG4 is a goblet cell marker that is separate from MUC2. REG4 is expressed within cells of the GI-tract, but the true function of REG4 is much less understood. While REG4 has been linked to tumor growth and growth factors, it may also be involved in the repair process of mucosal injury (Biton et al., 2011). REG4 has also been shown to be a direct target of CDX2 in gastric cancers (Naito et al., 2012). Since CDX2 has elsewhere been associated with goblet cell differentiation this correlation is worth further study. It would be worth examining if this same connection is true in CRC and if this activation could induce goblet cell differentiation and thus be a potential therapeutic target.

5.2 Dll4 stains a discrete population in goblet cell producing cell lines to PR5D5

Dll4 and all other Notch ligands, excluding Dll3, have been shown to be expressed in epithelial cells in human intestinal biopsy specimens and the human CRC cell lines LS174T

and HT29 (Akiyama et al., 2010). However, the data from this thesis contradicts the conclusions of Akiyama and colleagues that Dll4 stains goblet cells in LS174T and HT29 (Akiyama et al., 2010). This may be due to the fact that instead of staining mature goblet cells, Dll4 might be staining a goblet cell progenitor. This discrepancy needs to be resolved and may be due to this lack of knowledge or the fact that this may only be true in normal tissue. It should thus be determined, using the antibodies from this thesis, whether Dll4 is present in goblet cells from normal tissue samples as well as several other colorectal cell lines. What is interesting is that Dll1 and Dll4 expression has been linked to MUC2 expression and goblet cell differentiation, while expression of another ligand of Notch, Jagged-1, has been linked to decreased expression of MUC2 and less goblet cell differentiation in LS174T (Akiyama et al., 2010). Our laboratory's microarray expression data shows that Jagged-1 is widely expressed in both goblet cell and non-goblet cell producing cell lines and thus is in agreement with Akiyama et al. that Jagged-1 is not involved in goblet cell differentiation.

Additionally, distinct population of Dll4 positive cells may prove to influence goblet cell differentiation in a similar way that Paneth cells maintain stem cells in the small intestine. Paneth cells secrete various factors that modulate the colorectal crypt stem cells and progenitor cells in the small intestine (Clevers and Bevins, 2013). Blocking of Dll4 has also been demonstrated to block tumor growth in colon tumor human xenograft tumor models in NOD-SCID mice, which agrees with the data from this thesis that anti-Dll4 increases goblet cell differentiation (Hoey et al., 2009). As previously stated Dll4 cells may indeed be impacting cell fate and influencing goblet cell differentiation much like Paneth cells do in the small intestine. Rothenberg and colleagues have described such potential cells in mice that are Dll4 positive and may influence the fate of stem cells towards goblet cell differentiation

(Rothenberg et al., 2012). In order to understand the function of these Dll4 positive cells within goblet cell producing cell lines they should be sorted and used in further experiments. It would also be worth sorting those cells that expressed other Notch ligands and potentially co-culturing them with stem cells in order to see what differentiation, if any, they undergo. Dll4 expression should be further investigated to determine whether the positively stained cells in CRC cell lines are goblet cell progenitors or if they are helping to determine cell fate by inducing differentiation into goblet cells.

5.3 DBZ and anti-Dll4 both increase goblet cell percentage in LS180 and LS174T, but together demonstrate an antagonistic phenotype

Other studies have previously demonstrated that administration of DBZ increases goblet cell differentiation (van Es et al., 2005; Milano et al., 2004). While studies have shown that inhibition of Dll4 inhibits tumor growth (Hoey et al., 2009; Noguera-Troise et al., 2006), its inhibition has not previously been linked to goblet cell differentiation. In contrast, knockdown of Dll1 expression in LS174T and HT29 CRC cell lines has abrogated goblet cell differentiation (Akiyama et al., 2010). Akiyama and colleagues found a decrease in Dll1, Dll4, ATOH1 and MUC2 expression with forced Notch1 signaling as well as increased expression with Notch signaling inhibition in both HT29 and LS174T. This research did not examine the influence of Dll4 knockdown on goblet cell differentiation due to an insufficient knockdown as compared to Dll1 and therefore would be interesting to investigate further. Similar to these data Dll4 has also shown to have opposing effects to Jagged1 on angiogenesis in mouse retina (Benedito et al., 2009) and may show similar effects in the colon as suggested by Akiyama and colleagues (Akiyama et al., 2010). Since Dll1 has been shown to induce goblet cell differentiation when Notch has been inhibited and binding has

been blocked then the same may be true for Dll4. If Notch binding to Dll1 is not required for goblet cell differentiation then what triggers goblet cell differentiation is still unknown. The goblet cell precursor may be involved in blocking Notch binding and allowing this signaling process to occur. This experiment should be repeated to examine the effects of Dll4 on goblet cell differentiation and the antagonistic relationship between anti-Dll4 and DBZ treatment as well as testing anti-Dll1 on this and several other cell lines. If Dll4 and Dll1 hold the key to goblet cell differentiation that knowledge could be exploited in using a therapy to stop tumor growth in patients.

5.4 CDX2 knockdown significantly decreases percent of goblet cells in LS174T and LS180, while CDX1 does so as well but to a lesser extent

The data from this thesis are in agreement with other literature that CDX2 is involved in goblet cell differentiation. It has been shown that an increase in CDX2 expression in gastric carcinoma cell lines also increases MUC2 expression (Tamagawa et al., 2012) and is in agreement with our own laboratory's expression data. Similarly, CDX2 has been shown to directly activate MUC2 transcription in gastric carcinoma cell lines and in COS-7 cells (Mesquita et al., 2003; Yamamoto et al., 2003). This thesis has shown that the knockdown of CDX2 has led to a decrease in goblet cells in CRC cell lines and thus CDX2 is most likely essential to goblet cell differentiation. This knockdown should be conducted in several other CRC cell lines, including those cell lines that are not goblet cell producing cell lines in order to see if the effects are more widely found. Increased expression of CDX2 should also be conducted in both cell lines to see if its higher expression would increase goblet cell differentiation.

Previous data from our own laboratory and others shows that the primary role for CDX1 is enterocyte differentiation (Soubeyran et al., 1999; Wong et al., 2005). CDX2 may in this case take the primary role in goblet cell differentiation while CDX1 plays a minor role (Figure 4.6a). A better knockdown of both CDX2 and CDX1 would be required to truly examine the effects on goblet cell differentiation such as using CRISPR-cas9 technology. From here it would be interesting to knock out both CDX1 and CDX2 in LS180 and observe the effects it has on goblet cell differentiation. These knockdowns should also be carried out on several other goblet cell lines both non-goblet and goblet cell producing in order to better understand the limits of being able to induce goblet cell differentiation. If CDX2 plays a large enough role in goblet cell differentiation measures can be taken towards finding a way to induce CDX2 expression and thus differentiation.

5.5 Summary of Conclusions

The main conclusions of this thesis on goblet cell differentiation can be presented in an updated figure of the intestinal crypt differentiation pathway.

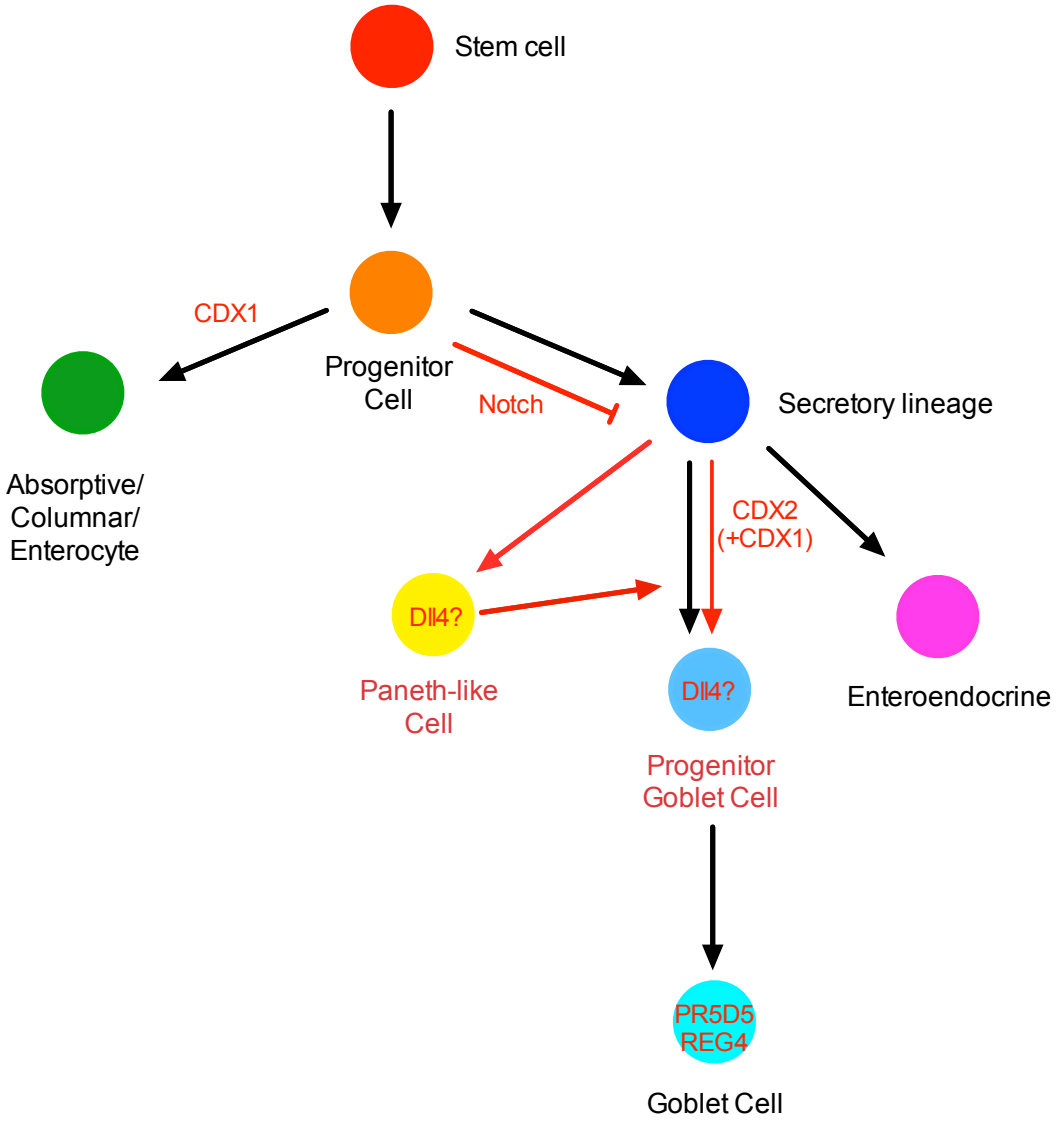


Figure 5.1 Summary of thesis findings of goblet cell differentiation in colorectal cancer. Novel regulators their influences and markers are marked in red. Notch inhibits goblet cell differentiation. CDX2 promotes goblet cell differentiation. CDX1 still regulates absorptive cell differentiation, but may also play a minor role in goblet cell differentiation. REG4 is an additional intracellular marker of goblet cells along with PR5D5. Dll4 is either a marker of progenitor goblet cells or of Paneth-like cells that induce goblet cell differentiation.

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