Student thesis

NTNU
Norwegian University of Science and Technology
Faculty of Medicine
Department of Cancer Research and Molecular Medicine (IKM)

Helene Kolstad Skovdahl

Differences between the HT29 and HT29-MTX epithelial cell lines

- an analysis of gene expression data

Student thesis in Medicine

Trondheim, June 2016

NTNU
Norwegian University of Science and Technology

Helene Kolstad Skovdahl

Differences between the HT29 and HT29-MTX epithelial cell lines

- An analysis of gene expression data

Student thesis in Medicine
Trondheim, June 2016

Norwegian University of Science and Technology
Faculty of Medicine
Department of Cancer Research and Molecular Medicine

NTNU

Table of contents

1 Acknowledgements

This thesis is the product of my medical student master project at the Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology (NTNU), Trondheim, spring 2016. From this experience, I have gained knowledge on how to analyse microarray data and genego categories, which is highly relevant in a time of big data in research. The skills will be useful in the continuation of my research project as a medical research student, and in a future PhD. For this, I am grateful. Also, the knowledge gained on the HT29 and HT29-MTX cell lines will hopefully benefit me and my colleges when planning and performing investigations involving epithelial cell lines.

I would like to thank my supervisors, Atle van Beelen Granlund and Arne Kristian Sandvik, for their guidance and encouragement throughout the period. Also, thanks to Torunn Bruland for excellent technical assistance. Thanks to the IBD research group, where I feel at home, and to the people working in the 3rd floor at Gastrosenteret Nord. It has been inspiring and informative for me to work and discuss with you all.

Lastly, thanks to Eivind for support and company.

2 Abstract

Introduction:

Epithelial cancerous cell lines are used in a wide variety of research because of the unique possibility to study isolated cellular mechanisms in detail. This can be used for wider understanding of pathological processes of disease but also physiological traits. When treating the epithelial cell line HT29 with methotrexate, the remaining population of cancer cells constitute the HT29-MTX cells, a proposed goblet cell model. In this thesis, we study the mRNA level in HT29 and HT29-MTX to investigate differences in regulation of gene expression and thereby how these cell lines differ in cellular network activity.

Material and methods:

The gene expression levels in the HT29 and HT29-MTX cells after four days of cultivation were measured by microarray. Statistical analysis in Bioconductor gave 6788 differentially expressed genes. Through data analysis in Metacore, differentially regulated cellular processes between the cell lines were characterized.

Results:

We show significant upregulation of goblet cell differentiation genes in HT29-MTX when compared to HT29, but a lack of increase in expression of the main mucus component MUC2. Also, we find that several processes related to immune function and cancerous traits in cells are altered.

Conclusion:

This study reveals that differentiation into goblet cells is indeed a possibility in the HT29-MTX cell, but that important traits such as MUC2 production is more uncertain. Several additional changes in gene expression are seen compared to the HT29 cell, and this must be kept in mind when utilizing the HT29-MTX for mechanistic studies.

3 Sammendrag

Introduksjon:
Epiteliale kreftcellelinjer gir en unik mulighet til å studere isolerte cellefunksjoner og mekanismer. Dette kan brukes til å øke forståelsen for patologiske sykdomsprosesser, og brukes blant annet i forskning på inflammatorisk tarmsykdom, kreft og medikamentopptak. HT29-MTX cellelinjen skapes ved å behandle den epiteliale kreftcellelinjen HT29 med metotrexat, for så å dyrke videre de cellene som overlever. Disse cellene er foreslått som en modell på begerceller. I denne oppgaven ser vi på mRNA nivået i disse to cellelinjene for å undersøke forskjeller i regulering av genuttrykk, og basert på dette hvilke cellulære nettverk som er skrudd på eller av.

Materiale og metode:
Etter fire dager i kultur ble genuttrykket til HT29 og HT29-MTX celler målt med microarray. Statistiske analyser ble gjort i Bioconductor, og dette resulterte i 6788 differensielt uttrykte gener. Differensielt regulerte celleprosesser ble så karakterisert gjennom dataanalyse i Metacore.

Resultater:
I vårt materiale er det en signifikant oppregulering av gener knyttet til begercelle differensiering i HT29-MTX når sammenlignet med HT29, men samtidig mangler det en økning i uttrykk av den viktige mucuskomponenten MUC2. Utover dette finner vi økt uttrykk av gener i flere prosesser knyttet til immunfunksjon og kreftcelletrekk..

Konklusjon:
Studien viser at det er en mulighet for at HT29-MTX cellene er på vei til å differensiere til en begercellevariant. Allikevel ser det ut til at viktige begercellefunksjoner, som MUC2 produksjon, ikke er økt i HT29-MTX sammenliknet med HT29 cellene. Ytterligere ulikheter i genuttrykk finnes, og dette må tas hensyn til ved bruk av HT29-MTX i mekanistiske studier.

4 Introduction

In medical research, good ethical principles prohibit scientists from utilising humans in studies that cause disease, danger and more than negligible unpleasantness. Similar but not identical ethical guidelines apply in work with in vivo animal models. Because of this, experimental research very often utilise in vitro cancerous cell lines to investigate mechanisms of disease and to develop drugs.

Animal models make it possible to perform investigations in complex organ systems that are similar to those in humans. However, there are many examples that research performed in animal models is not applicable to humans. Thalidomide had no adverse effect in in animal models but was devastatingly dangerous in human use. The use of corticosteroids showed a reversed picture where signs of danger were found in animals but not in humans.

Ex vivo models are what we have today that is closest to in vivo in humans. In these studies, human tissue of varying complexity is extracted, kept viable and used to study mechanisms of disease. The clear advantage of these models is the fact that the tissue is human and can be used in research. The tissue can be very complex or very simple, like single cell models. A limitation for ex vivo models is that the tissue rarely survives for long outside the human body, enforcing major restrictions to study design. Research in ex vivo models becomes strictly time bound, which means that an effect cannot be monitored over time. The time until complete tissue death is not the only issue. The lack of circulation can also substantially alter tissue responses to the processes induced during experiments.

Cancerous cell lines are single cell systems that are extracted from tumour biopsies and cultivated for growth and differentiation. The origin of human cancerous cell lines is human tumours, and thus primarily normal tissue undergoing malignant transformation. Therefore, they often resemble differentiated human cells, but have unlimited proliferative potential when grown in culture. Both advantages and limitations are associated with this property. The foremost limitation is the fact that cancerous cells are not normal cells, which means that they do not always respond as such on stimulus. This gives a huge limitation in the potential for scientific results to conclude on disease mechanisms in humans. Cancerous cells can be cultured and their growth and differentiation controlled, giving huge opportunities for performing tightly controlled investigations. Single cell cultures can be kept devoid of

contamination and inflicting factors, and give the possibility to study focused cellular mechanisms. The survival and perpetual growth of cancer cells permits study designs of longer time frame. However, it is important to emphasise that cancerous cell lines are not normal cells, and might react in a different manner than healthy human cells in the same situation.

Colonic cell cultures maintain several important traits that make them similar to intestinal epithelium [1]. To increase the similarity to human tissue of more complexity, several methods of co culturing cancerous cells have been developed. Combining absorptive and secretive enterocytes could model the mucosa of the intestine in a better way than single cell cultures [2-4].

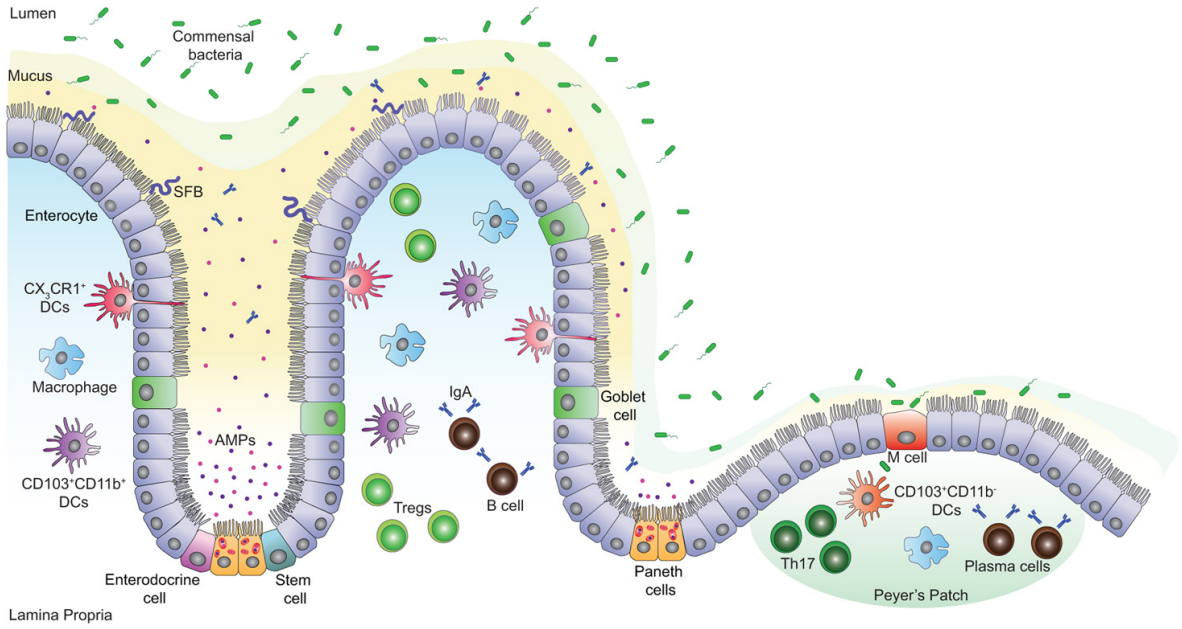


Figure 1. The intestinal mucosal surface at steady state
From "Intestinal antimicrobial peptides during homeostasis, infection and disease" by Muniz L. R. et al [5], reprinted with permission.

A single layer of epithelial cells line the gastrointestinal mucosa, and mainly four cell types are present; the absorptive enterocyte, the mucus-producing goblet cells, the Paneth cells and the enterochromaffin cells. The epithelium is polarized, with an apical side towards the lumen of the gastrointestinal tract and basal side that is attached to the basal membrane and faces the lamina propria. This orientation of the epithelium is highly important for its functions in

secretion, absorption, transepithelial passage of fluid and electrolytes, digestion and also for the immunological properties of the epithelium [6].

There are many different cell lines used in research today that have been derived from cancerous gastrointestinal epithelium. Amongst them is the HT29 colon cancer cell line used as a model of epithelial cells of the intestinal mucosa, and first described in 1975 by Jørgen Fogh [7]. When culturing HT29 cells in absence of glucose, they show signs and functions related to differentiation, and the changes in the cell can be manipulated to create absorptive or secretive cells. In the process of differentiation, the HT29 cells form a monolayer, which is polarized, contains tight junctions between neighbouring cells and has vectorial transportation of molecules [8]. When the growth conditions change, the cell can differentiate in an absorptive enterocyte fashion or become similar to goblet cells [9-11]. The HT29 cell line has been used in investigations of pathophysiological mechanisms of inflammatory bowel disease (IBD) and colon cancer, as well as in studies of bioavailability when developing drugs that have to be absorbed by intestinal enterocytes [12-14].

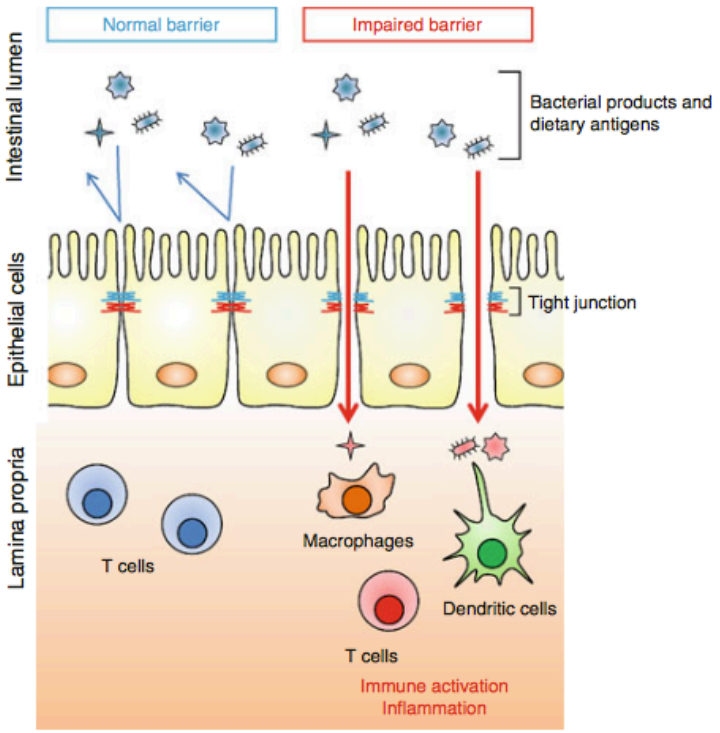


Figure 2. Barrier function of intestinal tight junctions
From "Regulation of intestinal epithelial permeability by tight junctions" by T. Suzuki [15], reprinted with permission.

Goblet cell models have been sought for research for several decades. Subgroups of cells with diverse morphological attributes have been found within the HT29 cell line, among them cells that are similar to goblet cells because they contain mucus-granules [9]. Treating HT29 cells with methotrexate induce a selection of cells that are resistant to the cytostatic drug, and these are the origin of the HT29-MTX cell line. It has been shown that tight junctions are preserved in this cell line, and that a confluent, adhesive mucus layer is formed on the apical side of the monolayered cell culture [16]. It has similarities to goblet cells, and it has been suggested that a co-culture of HT29 and HT29-MTX cells could serve as an in vitro model of the intestinal mucosa of higher complexity than either of the cells alone, like the co-cultures of HT29 and Caco-2 cells [4]. Whether the mechanism behind the development of the HT29-MTX cell is an initiation of differentiation in response to methotrexate, or simply a selection of a subgroup within the HT29 cell line has been discussed in several articles, and both alternatives are possible [9, 17].

As previously mentioned, cancer cell lines differ from normal human cells in many aspects. When a new cell line is developed from an existing cell line, the difference from human cells may increase further. This study aimed at characterizing the differences between the HT29 and the HT29-MTX cell lines, with the purpose of increased knowledge and critical interpretation of results when using the HT29-MTX cell line in research. Our hypothesis was that more than goblet cell traits distinguish HT29-MTX from HT29. In this thesis, we focus on four topics:

- goblet cell differentiation
- mucus production
- immunological function
- proliferation and cancer development.

5 Material and methods

5.1 Cell lines

Cells from the human cell lines HT29 (colorectal carcinoma, Cat. No. HTB-38, lot no. 59561256) (ATCC, Manassas, VA, USA) and HT29-MTX (methotrexate treated HT29 cells, received as a generous gift from Prof. J. P. Pearson, Newcastle University, U.K.) were cultured at 37°C and 5% CO_2 in RPMI with 10% fetal bovine serum (FBS), 2mM glutamine and 0.05% gentamicin. Detachment of cells from culture flasks was performed using trypsin/EDTA, and after detachment the cells were resuspended in medium and counted in a Countess Automated Cell Counter (Life technologies, Grand Island, NY, USA). The cells were cultured in flat bottom 96-well plates, seeded with 20,000 cells per well, three wells per cell type. After three days, 70% confluence was reached, followed by 24 hours cultivation in serum-free medium. Supernatant was collected and stored at -20, and cells were lysed for RNA extraction.

5.2 Microarray

Isolation of RNA from the cells was performed using Ambion mirVana miRNA isolation kit (Ambion) according to protocol from manufacturer. We used [Bioanalyzer] NanoDropd-1000 Spectrophotometer (Saveen Werner, Malmo, Sweden) to quantify the RNA. Microarray analysis was done on Illumina Beadstation (Illumina, San Diego, CA, USA) in Illumina human HT-12 expression Bead Chips, to analyse the gene expression.

5.3 Statistical analysis

Microarray data analysis was done with Bioconductor [18] in the R software environment. Linear models with least squares regression and empirical Bayes moderated t-statistics was used to determine the differential expression [19]. To adjust the p-values for multiple comparisons, Benjamini Hochberg false discovery rate correction was performed [20]. This resulted in 6788 significantly ($p<0.05$) differentially expressed genes between the cell lines, of which 3312 were downregulated and 3476 upregulated in the HT29-MTX cell line.

5.4 Enrichment in Metacore

The microarray data was assessed by enrichment analysis in the Metacore suite (GeneGo Inc: http://www.genego.com), a pathway analysis tool for functional analysis of omics data. The Metacore database is curated from high quality, peer reviewed experimental evidence, and

incorporates topology in the analysis. Differential expression was considered significant if $p<0.05$.

6 Results

6.1 Goblet cell differentiation genes

Cellular differentiation is the process where a cell changes some of its traits to become a different cell type [21]. This often involves transition from a lower to a higher level of maturation and more specialized cell type. Traits adapted and lost in this process are due to changes in gene expression. When stem cells in the intestinal mucosa differentiate, various gene combinations are expressed for the cell to turn into of the four specialized cell types [6]. Differentiation into goblet cells relies on expression of genes that drive the development towards first, a secretory enterocyte, and second a goblet cell. Final maturation into a specialized cell is the final step.

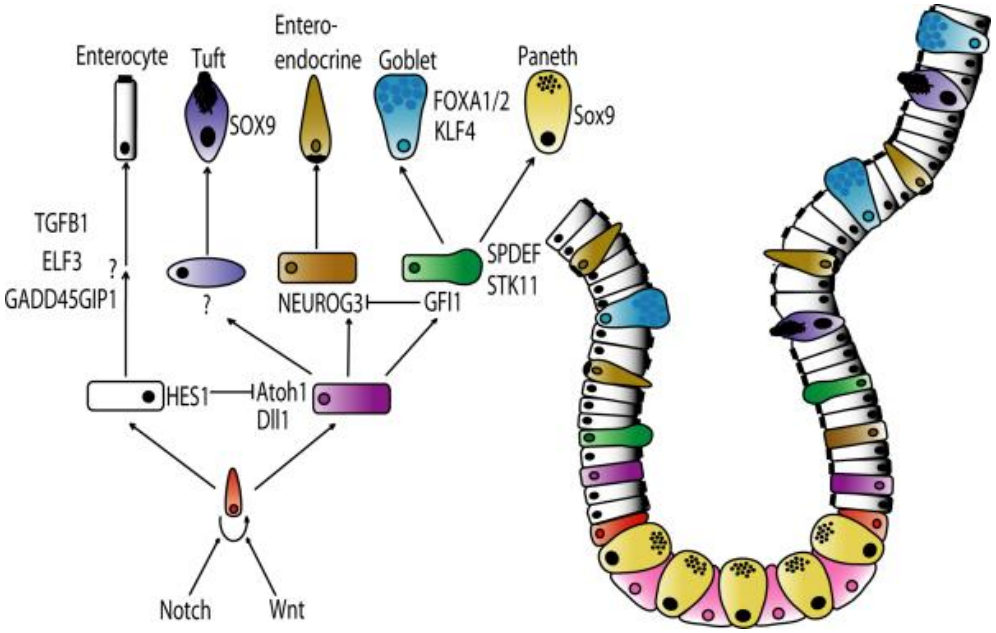


Figure 3. Model of intestinal differentiation
From "Intestinal development and differentiation" by Noah T. K. et al. [6], reprinted with permission.

Several genes involved in early goblet cell differentiation pathways are upregulated in the HT29-MTX cell compared to HT29 (Table 1). Stem cells of embryologic origin are the

source of all the intestinal epithelial cells. Differentiation of cell types from this involves processes that ensure both regeneration of the stem cell, as well as creation of more specialized cells through differentiation.

The Wnt/Beta-catenin pathway is essential for maintenance and proliferation of progenitor and stem cells, while NOTCH signalling drives cells to differentiation. Activity in both pathways is needed for the possibility of perpetual regeneration of the intestinal epithelium, and the transcription factor NOX1 activates both Notch signalling and wnt/b-catenin signalling [22]. NOX1 is downregulated in HT29-MTX, which could be interpreted as the HT29-MTX cell having fewer stem cell traits. Notch1 activates the transcription factor Hes1, which directs the development of epithelial progenitor cells to an absorptive enterocyte [22]. Expression of Atonal Homologue 1 (ATOH1) [23, 24], which encodes a basic-helix-loop-helix transcription factor, regulates differentiation of epithelial progenitor cells to a secretory rather than absorptive linage. The relative levels of ATOH1 and Notch determine whether the epithelial cells differentiate to a secretory or absorptive type [24]. In the HT29-MTX cell, Notch is upregulated while its target Hes1 is not, and lastly, ATOH1 is upregulated, which in concert might drive the differentiation towards the secretory lineage of epithelial cells.

From the decision of becoming a secretory cell to becoming a goblet cell, several steps have to be overcome. There are multiple paths of differentiation, either to tuft cells, enteroendocrine cells or goblet cells and Paneth cells. Atoh1 facilitates production of the transcription factor GFi1, which is important for development of goblet or Paneth cells [25]. The relative activity of GFi1 and Neurog3, a different transcription factor involved in enteroendocrine cells [26], regulates the further development towards a goblet- or Paneth cell phenotype [27]. In the HT29-MTX cells, GFi1 is upregulated, while Neurog3 is not regulated compared to the HT29 cells.

The next step involves expression of STK11 [28], SPDEF[29, 30] and SOX9[31], which are necessary for further maturation of progenitor goblet or Paneth cells. SPDEF is upregulated in HT29-MTX, while STK11 and SOX9 are expressed at the same level as in HT29. For specialization in the goblet cell lineage as opposed to Paneth cells, KLF4 [32, 33], FOXA1 and FOXA2 [34] expression is essential, while Beta-Catenin (CTNNB1) expression, which is involved in maturation of secretory epithelial cells into Paneth cells, has to be blocked [35, 36]. In the HT29-MTX cell, KLF4 and FOXA2 are upregulated, while FOXA1 is downregulated, and lastly, CTNNB1 is downregulated.

Other genes involved in goblet cell differentiation, which are upregulated in the HT29-

MTX cell, are Caudal homebox type 2 (CDX2) and Delta-like ligand 1 (DLL1). CDX2 is involved in differentiation of progenitor cells and regulates homeostasis of the epithelium in the intestine [37]. Downregulation has been associated with lower differentiation of colorectal carcinomas with a poor prognosis for the patient. Upregulation of CDX2 during development of the intestinal mucosa results in increased number of goblet cells and reduced number of Paneth cells [38]. Its upregulation could point to higher differentiation of cells, and a drive towards goblet cell phenotype. DLL1 is a ligand for Notch and is involved in the pathway leading to absorptive enterocytes [39], but it has also been suggested to have effects promoting goblet cell differentiation in NOTCH inactive (more differentiated) cells through cis-acting elements [40].

Some genes involved in terminal differentiation of goblet cells are decreased in HT29MTX compared to HT29. These include E-Cadherin (CHD1) and Protein tyrosine kinase 6 (PTK6). CHD1 is involved in cell sorting, and localization of the different epithelial cells in the crypts, and also in maturation of the goblet cell [41]. PTK6 is responsible for proliferation and growth in breast cancer tumours, but in epithelium of the intestines PTK6 activity causes cell cycle exit and differentiation of epithelial progenitor cells by inactivating Akt [42]. Akt is involved in processes for growth and proliferation, and while PTK6 is decreased, Akt1 is increased in the HT29-MTX cell, which could result in a sum of increased cell division and tumour growth, rather than differentiation into a mature goblet cell.

In conclusion, many steps in the development from a stem cell to a goblet cell are dependent on relative levels of two or more genes. In total, we find that several genes involved in differentiation into a secretory epithelial lineage and goblet cells are upregulated in HT29-MTX and genes that would shift the maturation towards other epithelial cell types, are downregulated. Still, genes involved in final differentiation into a goblet cell are downregulated, and the cells proliferative potential is continued.

Table 1 Goblet cell differentiation genes				
Protein	**Gene**	**Foldchange**	**p-value**	**Sign**
Atonal Homolog 1	ATOH1	+0.66	1.86×e-08	*
Growth Factor independent 1	GFi1	+0.41	1.24×e-08	*
Neurogenin 3	Neurog3	+0.01	0.86	n.s
SRY-Box 9	SOX9	-0.04	0.87	n.s
Kruppel-like factor 4	KLF4	+0.88	5.09×e-07	*
Serine/Threonine Kinase 11	STK11	+0.09	0.11	n.s
SAM Pointed Domain Containing ETS	SPDEF	+0.66	0.0001	*
Catenin Beta 1	CTNNB1	-0.27	0.037	*
Notch1	NOTCH1	+0.75	2.89×e-06	*
NADPH Oxidase 1	NOX1	-0.73	1.88×e-08	*
Caudal type Homeobox 2	CDX2	+0.40	0.00026	*
Delta like 1	DLL1	+0.30	0.00017	*
Forkhead box 2	FOXA2	+1.53	1.2×e-10	*
E-Cadherin	CDH1	-1.31	5.62×e-16	*
Forkhead box 1	FOXA1	-0.49	0.008	*
Protein Tyrosin Kinase 6	PKT6	-0.40	6.46×e-07	*
V-Akt Murine Thymoma Viral	AKT1	+0.49	3.97×e-06	*
Atonal Homolog 1	ATOH1	+0.66	1.86×e-08	*

*Table 1. Goblet cell differentiation genes. * = Differential expression of statistic significance ($p<0.05$). n.s = non significant.*

6.2 Mucus production

Genes involved in differentiation are of interest when characterizing the cells as goblet cells progenitors (table 2). After concluding that the cell expresses genes involved in the development of goblet cells, the next crucial question is whether the cells produce mucus, which is the core phenotypic trait of the goblet cell [43]. The main components of mucus are the glycoprotein mucins, of which 19 types have been characterized in humans. The mucins are divided into two groups; the gel forming mucins and the transmembrane mucins. The function of mucins in the intestine is diverse, from lubrication for passage of luminal contents, to cellular signalling and chemical barrier formation [44, 45]. Retaining the water contained in mucus is also essential. The transmembrane mucins make the inner layer of mucus in the colon adherent to the epithelium, while the gel forming mucins form the non-adherent layer. MUC2 is the core element in organizing the two layers [46]. Co-transportation of water with ions from cells is necessary for water content in mucus, and activity in the ion channels that regulates this cotransportation and thus the balance of water in the mucus is critical for the correct function of mucus.

The most important mucin in the intestine, the gel forming MUC2 [43, 46], is not differentially expressed in HT29-MTX compared to HT29 in our material. However, two molecules involved in its function are regulated in HT29-MTX. Anterior gradient homolog 2 (Agr2), which is important for the correct folding of the MUC2 molecule and can be found in the mucus [47], is upregulated in HT29-MTX. Fc-gamma binding protein (Fcgbp) is also upregulated, and is produced by goblet cells in the intestine and responsible for creating covalent bonds within the MUC2 molecule [48].

The transmembrane mucin MUC1 is upregulated in HT29-MTX. It has been shown to be important in upholding the balance between growth and apoptosis of the epithelial layer, and a critical element for the mucosal barrier to infection [46]. Also, MUC1 has been shown to have anti-inflammatory effects, through downregulation of NF-kB activity [49]. This is further describes later.

The rest of the mucins are either not differentially regulated in HT29-MTX, or downregulated. Several other mucus components that are important for the function of mucus are regulated in the HT29-MTX cell, amongst them Intestinal trefoil peptide 3 (TFF3) and Resistin-like molecule beta (RELM β). TFF3 is involved in protecting the epithelium from injury and promoting repair through restitution [50]. It makes the epithelium more mobile to enable it to migrate across nude areas of mucosa without cell division. RELM β is secreted into the mucus, and is important for the susceptibility to inflammation, and can be either proinflammatory or protective, depending on the stimuli. It has been shown to increase MUC2 expression in goblet cells [51-54].

In conclusion, the MUC-genes are not particularly affected in HT29-MTX. If any change is seen, the protective effects of the mucus, including anti-inflammatory effects and epithelium regeneration mediated by MUC1, might be enhanced. However, even if the important mucus component MUC2 is not affected, several regulatory elements that stimulate MUC2 functions are upregulated.

Table 2 Genes related to mucus production				
Protein	**Gene**	**Foldchange**	**p-value**	**Sign.**
Mucin 2	MUC2	-0.07	0.025	*
Anterior gradient 2	Agr2	+0.40	0.0001	*
FC-gamma binding protein	FCGBP	+2.89	5.14×e-15	*
Mucin 1	MUC1	+1.88	2.85×e-15	*
Mucin 4	MUC4	-0.007	0.89	n.s
Mucin 5B	MUC5B	+0.001	0.97	n.s
Mucin 6	MUC6	+0.04	0.05	n.s
Mucin 7	MUC7	+0.007	0.84	n.s
Mucin 12	MUC12	-0.28	3.16×e-18	*
Mucin 13	MUC13	-0.68	0.0003	*
Mucin 15	MUC15	-0.03	0.23	n.s
Mucin 16	MUC16	-0.07	0.007	*
Mucin 17	MUC17	-0.13	0.05	n.s
Mucin 20	MUC20	-0.72	1,79E-10	*
Trefoil factor 3	TFF3	+2.22	2.90×e-03	*
Resistin-like beta	RELMB	+0.34	5.91×e-09	*
Cystic fibrosis Transmembrane Conductance Regulator	CFTR	-0.38	0.0097	*
Tweety family member 3	TTYH3	+0.41	0.0006	*

*Table 2. Genes related to mucus production. * = Differential expression of statistic significance (p<0.05). n.s = non significant.*

6.3 The immune system

The intestinal epithelium plays a vital role in the immunity of the gut through its functions in the innate immune system [55, 56], and epithelial cancerous cell lines are used to investigate inflammatory response mediated by the epithelium in colorectal cancer and inflammatory bowel disease [13, 55, 57-60]. It is therefore important that epithelial cell lines are comparable with respect to immune responses if results from research performed in different cell lines are to be compared. There are a vast number of molecules involved in inflammatory responses, and this part focuses on pathways and molecules where the expression is different in HT29-MTX compared to HT29.

A previously mentioned molecule, MUC1, has immunological functions that protect the cancerous cells from eradication by immune cells. MUC1 suppresses T-cell activity, and thus alters the ability of immune cells to detect and eliminate the cancer cell [61]. Also, MUC1 modulates transcription of IL6 and TNFα and other NF-κB regulated genes [62], and has been shown to downregulate NF-κB activity [49]. Increased MUC1 could thus influence the production of mediators of inflammation.

6.3.1 Interferon pathway

Regulation of antiviral, antiproliferative and immunomodulative mechanisms in cells involves the interferon pathway [63] (table 3A, Fig 4). There are two types of traditional interferons, type I and type II, with several members of the type I group and only one member of the type II group (IFN-γ). Binding of interferon type I to its receptors on cells activate the Jak/STAT signalling pathway, which leads to formation of a transcription factor called Interferon stimulated gene factor 3 (ISGF3). This binds to the IFN-stimulated responsive elements in the DNA of the cell, which in turn leads to transcription of genes involved in antiviral, antiproliferative or immunomodulative mechanisms [64, 65]. For type II interferon, pathways leading to binding of IFN-γ-activated site (GAS) elements in the promotor regions in IFN-stimulated genes are essential. In the HT29-MTX cell, the responsive elements and regulatory factors in the interferon pathways are downregulated, which could reduce the effect of interferon on the cells.

Interferon stimulated gene factor 3 (ISGF3) is a complex of interferon regulatory factor 9 (IRF9), Signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2). ISGF3 mediate actions of interferon in the cell through regulation of transcription [64, 65]. All components of this complex are downregulated in the HT29-MTX cell line. One of the targets for ISGF3 is Interferon-induced GTP-binding protein (MxA), which binds and blocks viral replication [66, 67]. In the HT29-MTX cell MxA is downregulated. The group 2'-5'-Oligoadenylate synthetase, induced by ISGF3 [68], is involved in degradation of RNA in the cell, and all the components are downregulated [69]. Several other targets for ISGF3 are also downregulated (Tryptophan t-RNA synthetase, cytoplasmic (WARS), Interferon induced dsRNA activated protein kinase (PKR), DsRNA-specific adenosine deaminase (ADAR1)) [70], which could give a diminished interferon effect in the cell. Interferon regulatory factor 7 (IRF7), which is under transcriptional control of NF-kB and is involved in the activation of IFN type I stimulated genes, is dramatically downregulated [71, 72].

Interferon regulatory factor 3 (IRF3), which is involved in interferon alpha and beta transcription and thus production of the cytokines in the cell, is downregulated. Two molecules that stimulate IRF3 function are also regulated; CREB binding protein (CBP) is downregulated while Histone acetyl transferace p300 (p300) is upregulated [73] in the HT29-MTX cell. Interestingly, the interferon pathways also affect the major histocompatibility complexes I and II. Ligand binding to the interferon receptors activate STAT1, which in turn regulates transcription of MHC class II transactivator protein, which co-regulates transcription of MHC I and II.

The altered interferon responsiveness in the HT29-MTX cells means that the response to viral infections and included in this the response to TLR5, TLR7, TLR8 and TLR8, and to interferons might be different from the HT29 cells. It also suggests that the antiproliferative effect mediated by this pathway might be reduced.

Table 3 Genes related to immune response

	Protein	Gene	Foldchange.	p-value	Sign.
A					
	Interferon regulatory factor 9	IRF9	-2.42	2.15×e-16	*
	Signal Transducer And Activator Of Trans.1	STAT1	-1.75	8.23×e-08	*
	Signal Transducer And Activator Of Trans.2	STAT2	-0.24	0.01	*
	Interferon -induced GTP -binding protein Mx1	MX1	-4.12	4.71×e-11	*
	2'-5'-Oligoadenylate Synthetase 1, 40/46kDa	OAS1	-1.76	1.30×e-07	*
	2'-5'-Oligoadenylate Synthetase 2, 69/71kDa	OAS2	-2.45	3.65×e-07	*
	2'-5'-Oligoadenylate Synthetase 3, 100kDa	OAS3	-1.89	1.83×e-07	*
	Tryptophanyl-TRNA Synthetase	WARS	-0.83	0.003	*
	Interferon indu. dsRNA activated prot. kinase	EIF2AK2	-0.79	0.0002	*
	DsRNA-specific adenosine deaminase	ADAR1	-0.56	0.0003	*
	Interferon regulatory factor 3	IRF3	-0.63	4.3×e-07	*
	CREB binding protein	CBP	-0.25	9.3×e-05	*
	Histone acetyl transferace p300	p300	+0.16	0.03	*
	Interferon regulatory factor 7	IRF7	-2.28	5.8×e-12	*
	Interleukin 12	IL12	+0.21	0.0001	*
B					
	Cyclin D1	CCND1	-0.56	8.06×e-06	*
	p27	CDKN1B	+0.20	0.0028	*
C					
	Major Histocompatibility Complex, Class I, B	HLA-B	-0.61	0.043	*
	Transp 1, ATP-Binding Cassette, Sub-Fam B	TAP1	-1.45	0.018	*
	Transp 2, ATP-Binding Cassette, Sub-Fam B	TAP2	-1.14	5.23×e-06	*
	TAP binding protein	Tapasin	-0.59	0.049	*
	Beta-2 microglobulin	B2M	-0.76	4.59×e-05	*
	Calreticulin	CALR	-0.35	2.4×e-05	*
	Protein Disulfide Isomerase Family A, Member	PDIA3	-0.05	0.03	*
	Major Histocomp Complex, Class II, DM Alpha	HLA-DMA	-0.25	0.0076	*
	Major Histocomp Complex, Class II, DM Beta	HLA-DMB	-0.24	3.7×e-05	*
	Major Histocomp Complex, Class II, DR Alpha	HLA-DRA	-0.42	2.3×e-11	*
	Major Histocomp Complex, Class II, DR Beta 3	HLA-DRB3	-0.12	0.016	*
	MHC II, gamma chain	CD74	-0.78	7.34×e-06	*
	Cathepsin L	CTSL	-0.42	3.82×e-06	*
	Legumain	LGMN	-0.14	0.0074	*
	Master reg. factor of transcrip. of MHC class II	CIITA	+0.04	0.14	n.s

*Table 3. Genes related to immune response. * = Differential expression of statistic significance ($p<0.05$). n.s = non significant.*

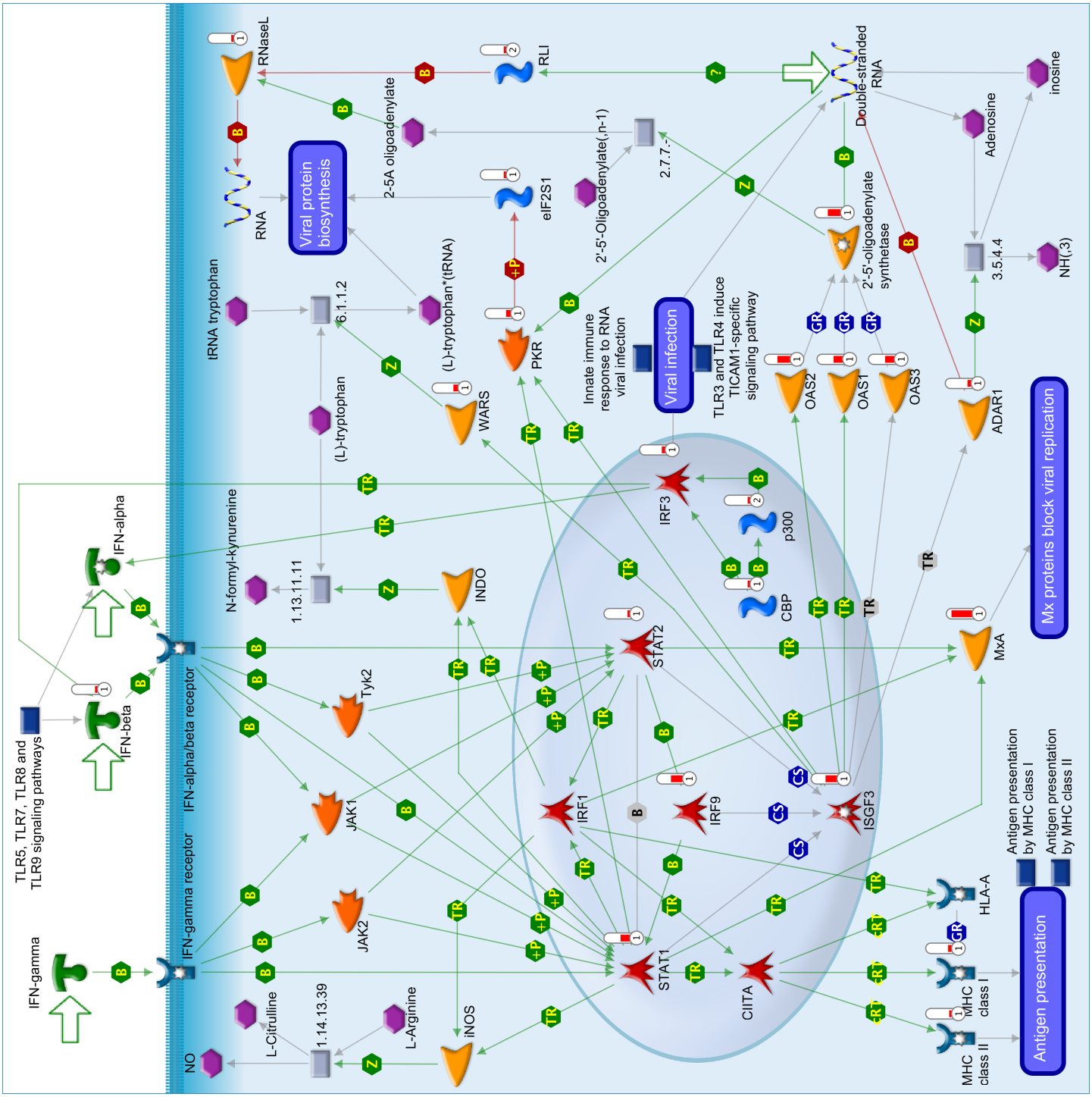


Figure 4. The Regulation of expression in HT-29MTX: Interferon Pathway.

Bars marked 1 shows where the expression th HT29-MTX is downregulated, and 2 where it is upregulated. TR= transcription regulation, cRT= co-reguation of transcription, B= binding, Z= catalysis.

6.3.2 Toll-like receptor 3

Toll-like receptors are as stated affected by the alterations in interferon pathways, and they have been shown to be of importance for cancer development, both in antitumour and tumourogenic processes [74-93]. None of the TLRs known in humans are differentially expressed in HT29 TX compared to HT29, but all are expressed. Whether at a level comparable to normal cells is uncertain.

TLR3 is expressed on many types of tumor cells, for example in colon cancer cells. Even if TLR3 is not regulated in HT29 MTX, processes involving TLR3 are affected. Normally, activation of TLR3 decreases cyclin D1, increases p27, which reduces proliferation, and in total the effect is inhibiting tumor survival [94, 95]. Cyclin D1 (CCND1) is downregulated in HT29MTX and p27 (CDKN1B) is increased, while TLR3 is at the same level in HT29-MTX as in HT29 (table 3B). This points to increased TLR3 activity in the HT29-MTX cell cultures with the result of antitumour activity. Also, TLR3 has been shown to activate immune cells in the microenvironment, which leads to activity against the tumor [94, 96], and this would be relevant if the TLR3 activity is indeed increased.

6.3.3 Major Histocompatibility complex

An important mechanism for cells to be recognized by immune cells, either as normal or irregular, is by binding antigens to the surface molecule Major Histocompatibility complex (MHC) I or II. If a cell does not have MHC on its surface or if the MHC molecules bind antigens that are unfamiliar, immune cells will attack and destroy the cell. This mechanism is highly important for protection from cancer, infected cells or altered cells that can be a hazard to the human body. Antigens presented on MHC class I is detected by CD8+ T cells, while MHC class II bound antigens are detected by CD4+ T cells. It has been shown that if the presence of CD8+ cells is high in a tumour, the patient has a higher probability of survival [97].

MHC class I, HLA-B, is downregulated in HT29-MTX (table 3C). Proteins involved in antigen transportation and processing before binding to MHC I, are downregulated as well [98, 99]. Beta-2 microglobulin, calreticulin and PDIA3 are involved in folding and conformation of the MHC class I molecule before it can bind the antigen peptides, and these are also downregulated [98, 100]. Downregulation of PDIA3 is actually associated with chemoresistance in colon tumors [101]. Thus, the HT29-MTX might be less capable of

activating CD8+ T-cells and the immune response towards the cell, which is a way of evading the immune system.

A similar picture is seen for MHC class II (Table 3C). Several of the MHC class II molecules are downregulated in HT29-MTX. Also, associated molecules to MHC class II are regulated in the HT29-MTX cell. The gamma chain of MHC II (CD74), which is crucial for a functional molecule, is downregulated [102]. CD74 is implicated in cancer progression through its function as a binding site for macrophage migration inhibitory factor (MIF) [103, 104]. MIF increases cell survival and proliferation [105]. As CD74 is downregulated in HT29-MTX, this function could be decreased. Cathepsin L (CTSL), which is involved in lysosomal degradation of peptides and the preparation of antigens to be bound by MHC [106], is downregulated, and Legumain (LGMN), which hydrolyses peptides, is also downregulated [107].

Master regulator factor of transcription of MHC class II, CIITA, which positively regulates the expression of MHC class II, is not differentially regulated, and is found at the same level in HT29 and HT29-MTX cell cultures. This is somewhat in accordance rather than conflicting with the downregulation of MHC class II molecules, though it seems impossible that it is the single causative. However, other factors are involved, such as the reduced interferon responsiveness explained in the previous section. Interferon is a stimulus that can alter the epithelial cells from their normal state of no expression of MHC class II into production of the molecule, and this can occur in response to a infection, trauma or inflammation [108].

We conclude that the HT29-MTX cell might, through the downregulation of MHC class I, MHC class II and associated molecules to both molecules, evade the CD4+ and CD8+ T-cells. Both ways of portraying the irregularity of the cell to the adaptive immune system seem less functional, which could mean that the HT29-MTX cell can evade immune cells to a more efficiently than the HT29 cell.

Thus, the HT29-MTX has several traits that that results in an altered immune response compared to the HT29 cell, and which increases the proliferative potential of the cell. These are direct inhibition of immune cells, altered mechanisms for immune cells to detect the cancer cell, regulation of NF-kB and altered response to cytokines.

6.4 Cancer

As HT29-MTX is derived from a colorectal cancer cell line (HT29), it is not surprising that it has several traits characteristic for cancer cells. However, insights into whether the process of designing the HT29-MTX cell line has altered the cancer genotype is interesting, as it might provide information relevant to the question of whether the methotrexate treatment induces a cellular change, or if the mechanism for HT29-MTX development is selection of already existing cells in the heterogenic HT29-cell line. Also, increased cancer traits would make the cell less appropriate as a model system in which to do research.

6.4.1 MUC1 and cancer

MUC1 is important for cancer development for several reasons. First, it has been shown to supress T-cells, and through this it alters the immunosurveillance to cancer cells, which increases the survival of the cells [61]. Second, upregulation of MUC1 decreases E-cadherin expression, and through this it inhibits cell-cell and cell-matrix adhesions. Reduced adhesion of cells is associated to progression of a tumour [109]. Also, it has been shown that downregulation of E-cadherin is an important mechanism for resistance to metothrexate in HT29-MTX [110]. E-Cadherin is downregulated in our HT29-MTX, while MUC1 is upregulated, as previously mentioned (table 4A). Furthermore, MUC1 interacts with selectin and ICAM-1 in heterogenic adhesions, and this has been linked to intravascular cancer metastasis [111, 112]. Neither selectins nor ICAM-1 is differentially expressed between HT29 and HT29-MTX.

Lastly, MUC1 has been shown to increase and activate MAPK, the latter through a MUC1/EGFR action. Activation of MAPK negatively regulates tight junctions possibly affecting the ability of cancer cells to migrate from the primary tumour [113]. MUC1 could therefore be involved in metastasis of cancer, and this could be an increased trait in HT29-MTX.

In essence, MUC1 is involved in cancer development through the inhibition of immune cells, which mediates cell survival, and through increased mobility of the cancer cells by reduced adhesions to neighbouring cells and extracellular matrix. It could mean that the HT29-MTX has an increased metastatic potential when compared to HT29 cell cultures.

Table 4 Genes related to cancer evolvement					
	Protein	**Gene**	**Foldchange**	**p-value**	**Sign.**
A					
	Mucin 1	MUC1	+1.88	2.85×e-15	*
	E-Cadherin	CDH1	-1.31	5.62×*e-16*	*
	Intercellular Adhesion Molecule 1	I-CAM1	-0.22	0.23	n.s
	Selectin E	SELE	-0.016	0.69	n.s
	Selectin P	SELP	-0.015	0.66	n.s
B					
	Cyclin B1	CCNB1	+0.25	0.011	*
	Forkhead Box M1	FOXM1	+0.35	4.59×e-05	*
	Transcription Factor AP-2 Alpha	TFAP2A	+0.22	0.0008	*
	M-phase inducer phosphatase B	CDC25B	+1-27	5.23×e-08	*
	M-phase inducer phosphatase C	CDC25C	+0.22	0.0011	*
	Cyclin H	CCNH	-0.33	6.61×e-07	*
	CDK7&CDK-activation kinase assem. factor MNAT1	MNAT1	+0.12	0.00073	*
	Membr. ass. tyr. threo. spec. CDC2 inhib. kinase	MYT1	-0.26	6.12×e-06	*
	V-Akt Murine Thymoma Viral Oncogene Homolog 1	Akt1	+0.49	3.97×e-06	*
	Ribosomal Protein S6 Kinase, 90kDa, Polypeptide 1	RPS6KA1	+0.22	0.00026	*
	Wee1-like protein kinase	Wee1	+0.18	0.0034	*
	Polo-Like Kinase 1	PLK1	-0.11	0.04	*
	Ubiquitin B	UBB	-0.19	0.01	*
	Ubiquitin C	UBC	-0.1	0.04	*
	Kinesin-like protein KIF11	KNSL1	+0.29	0.002	*
	Histone 1	H1F0	+0.22	0.01	*
	Histone 1	HIST1H1C	-0.18	0.01	*
	Histone 3	H3F3B	+0.3	6.68×e-05	*
C					
	Carcinoembryonic antig. cellul. adhesion mol. 1	CEACAM	-3.18	3.86xe-16	*
	Carcinoembryonic antig. cellul. adhesion mol. 6	CEACAM	+3.48	1.20xe-20	*
	Carcinoembryonic antig. cellul. adhesion mol. 5	CEACAM	+0.07	0.07	n.s

*Table 4. Genes related to immune response. * = Differential expression of statistic significance (p<0.05). n.s = non significant.*

6.4.2 Cell cycle regulation

Proliferation of cancer cells involves increased mitosis rate. The cell cycle includes several phases; Interphase consists of Gap1, Synthesis phase and Gap2, and this is where the growth and DNA synthesis occurs. Mitosis phase is where the cell nuclear division is completed, and is directly followed by the cytokinesis, where the cell divides. Activation of the processes than result in cell division are stimulated by growth factors and cytokines in the environment in or around the cell. Regulation of the cell cycle progression is important because if irregularities are allowed to happen, this can give aberrant cells that may threaten the life of the organism.

Transition from Gap2 to M-phase (Fig 5) is driven by formation of the Cyclin-dependent kinase 1 (CDK1) / Cyclin B1 complex, which activates enzymes that catalyse the processes in the cascade of cell division [114]. The regulation of this complex is quite intricate, with inhibitory and activating factors. In the HT29-MTX cell, Cyclin B1 is upregulated, and also, the transcription factors FOXM1 and AP-2alpha, which controls the Cyclin B1 amount in the cell, are upregulated [115, 116] (table 4B). M-phase inducer phosphatase 2 (CDC25B) and 3 (CDC25C) activate the binding of CDK1 (previously called CDC2) to Cyclin B1, forming the CDK1/Cyclin B1 complex [117], and both are upregulated in HT29-MTX.

The Cyclin-activating kinase complex (CAK) also promotes assembly of CDK1 and Cyclin B1, and consists of Cyclin H (CCNH), CDK7 and CDK-activation kinase assembly factor MNAT1 [114]. In the HT29-MTX, CCNH is downregulated, while MNAT1 is upregulated.

Membrane associated tyrosine and threonine specific CDC2 inhibitory kinase (MYT1) inhibits CDK1 function [114]. This molecule is in turn regulated by the inhibitory molecules Akt(PKB) and p90RSK1 [118, 119], which are upregulated in the HT29-MTX cell, and this may result in a reduction of MYT1 activity and thus less inhibition of CDK1.

Wee1-like protein kinase (Wee1) is an inhibitor of CDK1 [114], and this is actually also upregulated in HT29-MTX, contributing to the complex picture of cell cycle control. Molecules that inhibit Wee1, PLK1 and Ubiquitin (UBB, UBC), are both downregulated in HT29-MTX, increasing the probability of inhibitory function of Wee1 in CDK1 activity. However, CDK1 itself is an inhibitor of Wee1. Serin/threonine protein kinase (PLK1) has complex functions, activating Cyclin B1 directly, but also activating Cyclin B1 promoting factors like CDC25C and APC, while at the same time inhibiting the CDK1 inhibitor MYT1 [120]. PLK1 is downregulated in HT29-MTX.

The regulation of CDK1/Cyclin B1 is complex and it is highly regulated in the HT29-MTX cells (table 4B). In sum, the activating factors are mostly upregulated and the inhibitory factors inhibited, leading to an increased promoting surge on the CDK1/Cyclin B1 complex.

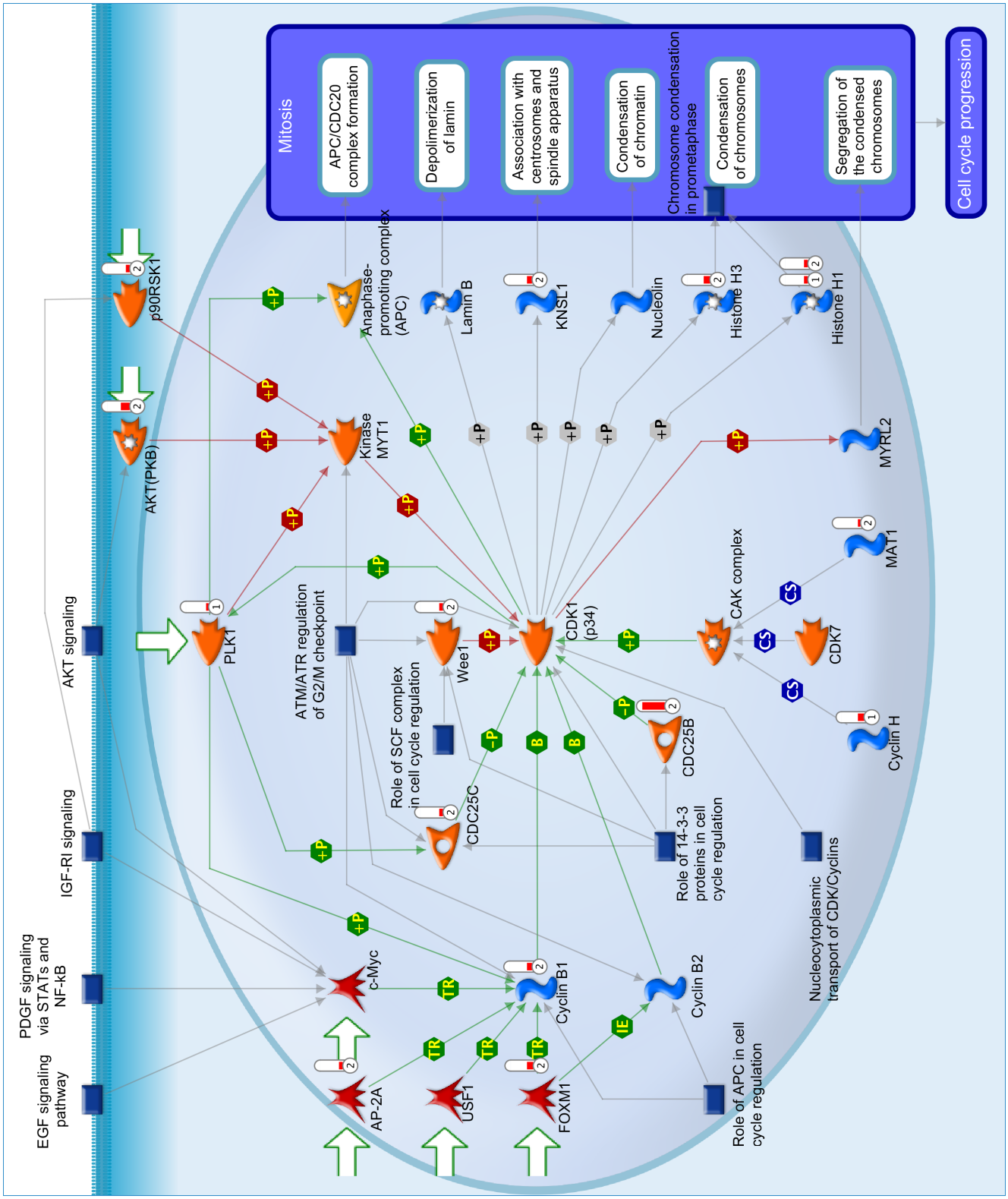


Figure 5. Regulation of cell cycle: Transition from Gap 2 to M-Phase

Bars marked 1 shows where the expression in HT29-MTX is downregulated, and 2 where it is upregulated. TR= transcription regulation, B= binding, -P= dephosphorylation, +P = phosphorylation, CS= complex subunit, IE = influence of expression. Green arrow = activation, red arrow = deactivation, grey arrow = unspecified effect.

Prophase is the first step of mitosis. It involves condensation of the chromatin, the two identical chromosomes after DNA synthesis remain connected, and the spindle apparatus consisting of microtubuli and centrosomes is assembled for the separation of identical chromosomes in anaphase. The spindle is organised by the centrosome, and there are two centrosomes on separate sides of the cell, polarizing the cell. CDK1/Cyclin B1 initiate activity in several molecules involved in this process; Nucleolin, Kinesin-like protein KIF11 (KNSL1) and Histone 1and Histone 3. Nucleolin regulates the condensation of chromatin during mitosis [121]. Histone H3 (H3F3B) and Histone H1 (H1F0 and HIST1H1C) regulate the chromosome condensation in prophase [122], and in the HT29-MTX cells H3F3B is upregulated along with H1F0, while HIST1H1C is downregulated. KNSL1, which associated with the centrosome and spindle apparatus, is essential for the formation of a spindle within the cell [123], and is upregulated in HT29-MTX.

Throughout M-phase, CDK1/Cyclin B1 are important for several steps. This complex initiate activity in Lamin B, which drives the depolarisation of the nuclear membrane [124], a hallmark of prometaphase. It inactivates myosin regulatory light polypeptide 9 (MYRL2), which allows for separation of chromosomes during anaphase [125]. Also, Anaphase Promoting Complex (APC) is activated by CDK1/Cyclin B1, and this complex is essential for the degradation of enzymes, which allows transition to anaphase [126].

Several molecules either activate or inhibit the complex CDK1/Cyclin B1 function, and several processes involved in driving the cell through the M-phase are upregulated in the HT29-MTX cell (table 4B). This is in turn could be important for the proliferative potential of the cell.

6.4.3 Carcinoembryonic antigen cellular adhesion molecule (CEACAM)

CEACAMs are a group of molecules that are well characterized, and several of them are highly sensitive and specific biomarkers of colon cancer development. Especially CEACAM5 (CEA) [127]. CEACAM1 [128] and CEACAM6 [129] can be found in epithelium of the gastrointestinal tract.

Expression of CEACAM6 has been shown to correlate with reduced degree of differentiation in tumours, and is increased in early adenomas compared to healthy colon tissue [129, 130]. CEACAM6 has traits that increase tumour invasiveness and metastasis

potential, as well as inhibit differentiation and anoikis [130-132]. CEACAM6 downregulates E-Cadherin [131], an adhesion molecule shown to suppress invasion and metastasis of tumours [133], often suppressed in cancer [134]. E-Cadherin inhibits activation of the Wnt pathway, which leads to transcription through binding to β-cathenin. Loss of E-cadherin increases the activity in this oncogenic pathway [135-137]. In the HT29-MTX cell, CEACAM6 is upregulated (table 4C), E-cadherin is downregulated, which would increase the Wnt-pathway, but however, β-cathenin is downregulated as well.

CEACAM1 in cancer is more complicated, as it has been shown to act tumour suppressive and to be downregulated in early stages of tumour development [138-141], but to promote growth, invasiveness and metastasis in later stages where it is overexpressed [142, 143]. CECAM1 overexpression is correlated to more aggressive cancer. In the HT29-MTX cell, CEACAM1 is upregulated (table 4C), which could indicate that the HT29-MTX cell is a more aggressive cancerous cell type, in a later stage of tumour development.

CEACAM5, expressed at the same level in both cell lines, is the most sensitive and specific marker of colorectal cancer [144], and when bound to CEACAM1 acts as an inhibitor of NK-cell attack [145, 146]. It has properties to inhibit anoikis and apoptosis as well [147]. To conclude, the CEACAMs 1 and 6 are upregulated in HT29 MTX, which could result in a more invasive and aggressive tumour type. Levels of the sensitive and specific marker of colorectal cancer CEACAM5 cannot differentiate between the two cell lines (table 4C).

7 Discussion

This study aimed at characterizing differences between the HT29 cell and its derivate, the HT29-MTX cell, with the hypothesis that more traits than those included in goblet functions are distinctive. Indeed, through our microarray data analysis in the Metacore platform, we found many differentially regulated pathways. In the biological areas we chose to study (goblet cell differentiation, mucus production, immunology and cancer development), there were several interesting findings.

The first issue is whether the HT29-MTX is suitable to be used as a goblet cell model. We found several essential goblet cell differentiation genes to be upregulated, combined with downregulation of genes associated with differentiation into other epithelial cells like absorptive or Paneth cells. The genes involved in final differentiation, however, were not differentially regulated and thus transition into a mature goblet cell has not occurred in our experimental setup. As the HT29-MTX is a cancerous cell line with self-renewal potential and the ability of tumourogenicity in nude mice [148], this is not surprising, as final maturation would prevent the survival of the in vitro model.

One of the major final differentiation genes which is downregulated is E-cadherin, and this has been associate with resistance to methotrexate and increased metastasis potential in cancer cells [110, 149, 150]. Also, in the epithelial cell line of origin, HT29, final differentiation into enterocytes with microvilli, polarization and tight junctions necessitates growth for weeks under specific conditions such as glucose-deprived media or sodium butyrate [11, 151]. Our cells were cultivated for three days in medium with FBS followed by 24 hours of cultivation in serum-free medium, and it is very unlikely for final differentiation to occur within this time under the given conditions.

Mucus production is an essential trait of goblet cells, distinguishing it from other enterocytes. We found an upregulation of the MUC1 gene; the membrane bound mucin, which has several immunogenic traits. Surprisingly, we find no particular change in expression of the core mucus component MUC2 in the HT29-MTX cell. An explanation could lie in the time of culture. Goutyer et al. studied the expression of mucin genes from four to 21 days in culture, and found expression of MUC1, MUC3, MUC4 and MUC5AC at day four, expression of MUC2 and MUC5B at day five and expression of MUC11 at day seven. All the mucin genes reached maximum expression levels at the point of induction of cell differentiation [152]. Lesuffleur et al. found that cultures of HT29-MTX cells form mucus droplet after confluency, but that the expression of MUC1 begins earlier and is detectable in preconfluent cells [153]. It

has been described that at late confluence, a mucus layer forms on the apical side of the cells, where the main component is MUC2 [148]. Our cells were cultivated for four days, which could explain why MUC2 was found to not be differentially expressed in our data.

Because epithelial model systems are used in research on immune mechanisms on the gut, it is interesting to study how immunological function of the epithelial cancerous cell lines are and how they differ. This is highly relevant if HT29-MTX is to be used in co-cultures with cells differentiated into an enterocytic phenotype. Previous studies by Lesuffleur et al. and others have concluded that HT29 cells treated with methotrexate differentiate in culture and form homogenous monolayers of polarized cells with apical brush borders containing intestinal hydrolases, which secrete mucins of gastric immunoreactivity [148, 154]. These traits resemble the differentiation of human foetal colon epithelium [155]. It has been stated that HT29-MTX can be a relevant model for studying mucin biosynthesis in vitro [153], and it has largely been used to this exact cause. However, it has also been used in co-cultures as a goblet cell model to investigate digestion, cellular transportation, intestinal permeability and absorption of drugs [2-4]. If the next avenue of use for the HT29-MTX cell is in co-cultures to study immunological mechanisms, a wider understanding of the cell is paramount.

Recently, the goblet cell is emerging as a possible major immune cell of the intestinal mucosa [156]. Goblet cells have for a long time been known to be essential for the mechanical, chemical an immunological barrier of the mucosa through the production of mucus which covers the epithelium. During the last couple of years it has however become evident that the immunological mechanisms are much more intricate. Mucus secretion is partly controlled extrinsic to the goblet cell. Firstly, secretion of mucus has been linked to the active inflammasome, where a deficiency in inflammasome activity results in defective MUC2 and mucus secretion from goblet cells [157]. Second, during exposure to possible dangers to the mucosa, great exocytosis from the goblet cells is initiated as a response, and this is triggered by autophagy protein Atg5 [158]. The goblet cell has also been designated sensory functions, as it has been shown to internalize through endocytosis luminal material for display to the lamina propria dendritic cells [159]. In this way, the goblet cells may play a vastly more complex role in the innate immune system than previously expected. In this context, it is more relevant than ever to have a goblet cell model in which to study immunologic and inflammatory mechanisms of this cell. Our study reveals that the HT29-MTX cell has several traits of importance to the immune system that are altered compared to the HT29 cell, which could be of importance if the cell line is to be introduced as a model in which to study goblet cell immunological functions. There are alterations in core pathways

important for the innate immune system (interferon) and for the adaptive immune system (MHC class I and II). Because alterations in these pathways have the potential of being substantial due to the effect on a wide variety of connected pathways, it will be of great importance to investigate how the expression patterns and successive protein function in the HT29-MTX correspond to a primary culture of goblet cells.

Resistance to anticancer drugs is a well-studied field as it is highly important clinically in treatment of patients with cancer. The HT29-MTX cell is proven to be methotrexate resistant through its ability to survive in culture under influence of this anticancer drug. In addition to this, we show that there is an upregulation of mitosis and thus a positive effect on proliferation in the HT29-MTX cell, which is an important mechanism for development of resistance to anticancer drugs [160]. Specific mechanisms behind the HT29-MTX resistance to Methotrexate have been investigated, and downregulation of E-Cadherin in concert with upregulation of Enolase-2 and Caveolin is suggested as important [110].
Resistance to methotrexate in it self may prove the HT29-MTX cell is a more successful cancer cell than the HT29 cell, but several other traits typical of cancer distinguish the cell line. Escaping the immune system is important for a cancerous tumour to survive and grow [161]. In our material, important elements for discovering to destroy abnormal cells, namely MHC class I and II, are downregulated. Also, MUC1 has a T-cell inhibitory effect which would serve to the same purpose. Increased ability to exist separate from neighbouring cells and migrate from a tissue, in short the possibility to metastasize, is also increased in the HT29-MTX cell. Furthermore, downregulation of tight junction molecules and upregulation of molecules give migratory traits. Combined, the HT29-MTX cell is in the naïve state a cell with more advanced cancer cell traits than the HT29 cell. However, the HT29-MTX retains its ability to differentiate when grown in culture, which might result in downregulation of proliferative potential and the migratory abilities.
It is important to emphasise that microarray analysis only gives information on the gene expression status of the cells at a specific time of culture. We can know what is expressed, but come no closer to knowing why and to what effect. Posttranscriptional and translator regulation will affect the protein levels in the cell. We still believe a thorough study of the expression pattern on a global gene expression level presents an excellent vantage point for further mechanistic studies on a protein level.

8 Conclusion

The present study demonstrates that HT29-MTX differs from the HT29 cell in several aspects of gene expression beside goblet cell-associated expression patterns. This is becoming more important to take into account, as the goblet cell is emerging as possibly an important immune cell of the mucosa and mechanistic studies need to be conducted. One of the venues of cell biology where there is differential expression between the HT29-MTX and the HT29 cell is indeed the immune system. Also, more pronounced cancerous traits could affect how close the cancer cell line will come to a normal cell, even if differentiation can be controlled in culture. However, drawing specific conclusion in regard to HT29-MTX features would require additional mechanistic studies on the protein level, and comparison to a primary goblet cell culture.

9 References

1. Zweibaum A, Laburthe M, Grasset E, Louvard D. Use of cultured cell lines in studies of intestinal cell differentiation and function. Comprehensive Physiology. Oxford: Wiley Blackwell; 2011. p. 22.
2. Hilgendorf C, Spahn-Langguth H, Regardh CG, Lipka E, Amidon GL, Langguth P. Caco-2 versus Caco-2/HT29-MTX co-cultured cell lines: permeabilities via diffusion, inside- and outside-directed carrier-mediated transport. Journal of pharmaceutical sciences. 2000;89(1):63-75.
3. Mahler GJ, Shuler ML, Glahn RP. Characterization of Caco-2 and HT29-MTX cocultures in an in vitro digestion/cell culture model used to predict iron bioavailability. The Journal of nutritional biochemistry. 2009;20(7):494-502.
4. Walter E, Janich S, Roessler BJ, Hilfinger JM, Amidon GL. HT29-MTX/Caco-2 cocultures as an in vitro model for the intestinal epithelium: in vitro-in vivo correlation with permeability data from rats and humans. Journal of pharmaceutical sciences. 1996;85(10):1070-6.
5. Muniz LR, Knosp C, Yeretssian G. Intestinal antimicrobial peptides during homeostasis, infection and disease. Frontiers in immunology. 2012;3.
6. Noah TK, Donahue B, Shroyer NF. Intestinal development and differentiation. Experimental cell research. 2011;317(19):2702-10.
7. Fogh J, Trempe G. New human tumor cell lines. In: Fogh J, editor. Human tumor cells in vitro. New York: Plenum; 1975. p. 115-41.
8. Pinto M, Appay MD, Simon-Assmann P, Chevalier G, Dracopoli N, Fogh J, et al. Enterocytic differentiation of cultured human colon cancer cells by replacement of glucose by galactose in the medium Biol Cell. 1982;47:4.
9. Huet C, Sahuquillo-Merino C, Coudrier E, Louvard D. Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation. The Journal of cell biology. 1987;105(1):345-57.
10. Wice B, Trugnan G, Pinto M, Rousset M, Chevalier G, Dussaulx E, et al. The intracellular accumulation of UDP-N-acetylhexosamines is concominant with the inability of human colon cancer cells to differentiate. J Biol Chem. 1985(260):7.

11. Zweibaum A, Pinto M, Chevalier G, Dussaulx E, Triadou N, Lacroix B, et al. Enterocytic differentiation of a subpopulation of the human colon tumor cell line HT-29 selected for growth in sugar-free medium and its inhibition by glucose. Journal of cellular physiology. 1985;122(1):21-9.
12. Brand S, Olszak T, Beigel F, Diebold J, Otte JM, Eichhorst ST, et al. Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK, and Akt signaling resulting in proliferation and migration of colorectal cancer cells. Journal of cellular biochemistry. 2006;97(4):709-23.
13. Ostvik AE, Granlund AV, Bugge M, Nilsen NJ, Torp SH, Waldum HL, et al. Enhanced expression of CXCL10 in inflammatory bowel disease: potential role of mucosal Toll-like receptor 3 stimulation. Inflammatory bowel diseases. 2013;19(2):265-74.
14. Collett A, Sims E, Walker D, He YL, Ayrton J, Rowland M, et al. Comparison of HT29-18-C1 and Caco-2 cell lines as models for studying intestinal paracellular drug absorption. Pharmaceutical research. 1996;13(2):216-21.
15. Suzuki T. Regulation of intestinal epithelial permeability by tight junctions. Cellular and molecular life sciences : CMLS. 2013;70(4):631-59.
16. Navabi N, McGuckin MA, Linden SK. Gastrointestinal cell lines form polarized epithelia with an adherent mucus layer when cultured in semi-wet interfaces with mechanical stimulation. PloS one. 2013;8(7):e68761.
17. Lesuffleur T, Violette S, Vasile-Pandrea I, Dussaulx E, Barbat A, Muleris M, et al. Resistance to high concentrations of methotrexate and 5-fluorouracil of differentiated HT-29 colon-cancer cells is restricted to cells of enterocytic phenotype. International journal of cancer Journal international du cancer. 1998;76(3):10.
18. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome biology. 2004;5(10):R80.
19. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Statistical applications in genetics and molecular biology. 2004;3:Article3.
20. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. . J R Statist Soc B. 1995;57(1):11.
21. Slack JM. Metaplasia and transdifferentiation: from pure biology to the clinic. Nat Rev Mol Cell Biol. 2007;8(5):369-78.

22. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature. 2005;435(7044):959-63.
23. VanDussen KL, Samuelson LC. Mouse atonal homolog 1 directs intestinal progenitors to secretory cell rather than absorptive cell fate. Dev Biol. 2010;346(2):215-23.
24. Kazanjian A, Noah T, Brown D, Burkart J, Shroyer NF. Atonal homolog 1 is required for growth and differentiation effects of notch/gamma-secretase inhibitors on normal and cancerous intestinal epithelial cells. Gastroenterology. 2010;139(3):918-28, 28 e1-6.
25. Bjerknes M, Cheng H. Cell Lineage metastability in Gfi1-deficient mouse intestinal epithelium. Dev Biol. 2010;345(1):49-63.
26. Lee CS, Perreault N, Brestelli JE, Kaestner KH. Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity. Genes Dev. 2002;16(12):1488-97.
27. Jenny M, Uhl C, Roche C, Duluc I, Guillermin V, Guillemot F, et al. Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. The EMBO journal. 2002;21(23):6338-47.
28. Shorning BY, Zabkiewicz J, McCarthy A, Pearson HB, Winton DJ, Sansom OJ, et al. Lkb1 deficiency alters goblet and paneth cell differentiation in the small intestine. PloS one. 2009;4(1):e4264.
29. Gregorieff A, Stange DE, Kujala P, Begthel H, van den Born M, Korving J, et al. The ets-domain transcription factor Spdef promotes maturation of goblet and paneth cells in the intestinal epithelium. Gastroenterology. 2009;137(4):1333-45 e1-3.
30. Noah TK, Kazanjian A, Whitsett J, Shroyer NF. SAM pointed domain ETS factor (SPDEF) regulates terminal differentiation and maturation of intestinal goblet cells. Experimental cell research. 2010;316(3):452-65.
31. Bastide P, Darido C, Pannequin J, Kist R, Robine S, Marty-Double C, et al. Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. The Journal of cell biology. 2007;178(4):635-48.
32. Ghaleb AM, McConnell BB, Kaestner KH, Yang VW. Altered intestinal epithelial homeostasis in mice with intestine-specific deletion of the Kruppel-like factor 4 gene. Dev Biol. 2011;349(2):310-20.
33. Katz JP, Perreault N, Goldstein BG, Lee CS, Labosky PA, Yang VW, et al. The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. Development. 2002;129(11):2619-28.

34. Ye DZ, Kaestner KH. Foxa1 and Foxa2 control the differentiation of goblet and enteroendocrine L- and D-cells in mice. Gastroenterology. 2009;137(6):2052-62.
35. Wang Y, Giel-Moloney M, Rindi G, Leiter AB. Enteroendocrine precursors differentiate independently of Wnt and form serotonin expressing adenomas in response to active beta-catenin. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(27):11328-33.
36. Andreu P, Peignon G, Slomianny C, Taketo MM, Colnot S, Robine S, et al. A genetic study of the role of the Wnt/beta-catenin signalling in Paneth cell differentiation. Dev Biol. 2008;324(2):288-96.
37. Ann Crissey M, Guo RJ, Funakoshi S, Kong J, Liu J, Lynch JP. Cdx2 levels modulate intestinal epithelium maturity and Paneth cell development. Gastroenterology. 2011;140(2):517-28 e8.
38. Crissey MA, Guo RJ, Funakoshi S, Kong J, Liu J, Lynch JP. Cdx2 levels modulate intestinal epithelium maturity and Paneth cell development. Gastroenterology. 2011;140(2):517-28 e8.
39. Pellegrinet L, Rodilla V, Liu Z, Chen S, Koch U, Espinosa L, et al. Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells. Gastroenterology. 2011;140(4):1230-40 e1-7.
40. Akiyama J, Okamoto R, Iwasaki M, Zheng X, Yui S, Tsuchiya K, et al. Delta-like 1 expression promotes goblet cell differentiation in Notch-inactivated human colonic epithelial cells. Biochemical and biophysical research communications. 2010;393(4):662-7.
41. Schneider MR, Dahlhoff M, Horst D, Hirschi B, Trulzsch K, Muller-Hocker J, et al. A key role for E-cadherin in intestinal homeostasis and Paneth cell maturation. PloS one. 2010;5(12):e14325.
42. Haegebarth A, Bie W, Yang R, Crawford SE, Vasioukhin V, Fuchs E, et al. Protein tyrosine kinase 6 negatively regulates growth and promotes enterocyte differentiation in the small intestine. Molecular and cellular biology. 2006;26(13):4949-57.
43. Johansson MEV, Hansson GC. Mucus and the Goblet Cell. Dig Dis. 2013;31(0):305-9.
44. Johansson ME, Ambort D, Pelaseyed T, Schutte A, Gustafsson JK, Ermund A, et al. Composition and functional role of the mucus layers in the intestine. Cellular and molecular life sciences : CMLS. 2011;68(22):3635-41.
45. Johansson ME, Sjovall H, Hansson GC. The gastrointestinal mucus system in health and disease. Nat Rev Gastroenterol Hepatol. 2013;10(6):352-61.

46. Johansson MEV, Larsson JMH, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host–microbial interactions. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(Suppl 1):7.
47. Park SW, Zhen G, Verhaeghe C, Nakagami Y, Nguyenvu LT, Barczak AJ, et al. The protein disulfide isomerase AGR2 is essential for production of intestinal mucus. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(17):6950-5.
48. Johansson ME, Thomsson KA, Hansson GC. Proteomic analyses of the two mucus layers of the colon barrier reveal that their main component, the Muc2 mucin, is strongly bound to the Fcgbp protein. J Proteome Res. 2009;8(7):3549-57.
49. Sheng YH, Triyana S, Wang R, Das I, Gerloff K, Florin TH, et al. MUC1 and MUC13 differentially regulate epithelial inflammation in response to inflammatory and infectious stimuli. Mucosal immunology. 2013;6:11.
50. Wright NA, Hoffmann W, Otto WR, Rio M-C, Thim L. Rolling in the clover: trefoil factor family (TFF)-domain peptides, cell migration and cancer. The FEBS journal. 1997;408(2):3.
51. Hogan SP, Seidu L, Blanchard C, Groschwitz K, Mishra A, Karow ML, et al. Resistin-like molecule beta regulates innate colonic function: barrier integrity and inflammation susceptibility. The Journal of allergy and clinical immunology. 2006;118(1):257-68.
52. Krimi RB, Kotelevets L, Dubuquoy L, Plaisancie P, Walker F, Lehy T, et al. Resistin-like molecule beta regulates intestinal mucous secretion and curtails TNBS-induced colitis in mice. Inflammatory bowel diseases. 2008;14(7):931-41.
53. Nair MG, Guild KJ, Du Y, Zaph C, Yancopoulos GD, Valenzuela DM, et al. Goblet cell-derived resistin-like molecule beta augments CD4+ T cell production of IFN-gamma and infection-induced intestinal inflammation. Journal of immunology. 2008;181(7):4709-15.
54. Kim YS, Ho SB. Intestinal Goblet Cells and Mucins in Health and Disease: Recent Insights and Progress. Curr Gastroenterol Rep. 2010;12(5):12.
55. Davies JM, Abreu MT. The innate immune system and inflammatory bowel disease. Scandinavian journal of gastroenterology. 2015;50(1):24-33.
56. Kraus S, Arber N. Inflammation and colorectal cancer. Current opinion in pharmacology. 2009;9(4):405-10.
57. Ostvik AE, Granlund AV, Torp SH, Flatberg A, Beisvag V, Waldum HL, et al. Expression of Toll-like receptor-3 is enhanced in active inflammatory bowel disease and

mediates the excessive release of lipocalin 2. Clinical and experimental immunology. 2013;173(3):502-11.
58. Sugiura Y, Kamdar K, Khakpour S, Young G, Karpus WJ, William DePaolo R. TLR1-induced chemokine production is critical for mucosal immunity against Yersinia enterocolitica. Mucosal immunology. 2013;2013 Nov; 6(6):9.
59. Dieu-Nosjean MC, Massacrier C, Homey B, Vanbervliet B, Pin JJ, Vicari A, et al. Macrophage inflammatory protein 3alpha is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. The Journal of experimental medicine. 2000;192(5):705-18.
60. Izadpanah A, Dwinell MB, Eckmann L, Varki NM, Kagnoff MF. Regulated MIP-3alpha/CCL20 production by human intestinal epithelium: mechanism for modulating mucosal immunity. American journal of physiology Gastrointestinal and liver physiology. 2001;280(4):G710-9.
61. Gimmi CD, Morrison BW, Mainprice BA, Gribben JG, Boussiotis VA, Freeman GJ, et al. Breast cancer-associated antigen, DF3/MUC1, induces apoptosis of activated human T cells. Nature medicine. 1996;2(12):1367-70.
62. Cascio S, Sciurba J, Finn O. MUC1 modulates NF-kB-dependent gene expression of pro-inflammatory cytokines by altering their epigenetic regulation (TUM7P.944). The Journal of Immunology. 2014;192(1 Supplement):203.26.
63. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nature reviews Immunology. 2005;5(5):375-86.
64. Fu XY, Kessler DS, Veals SA, Levy DE, Darnell JE, Jr. ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains. Proceedings of the National Academy of Sciences of the United States of America. 1990;87(21):8555-9.
65. Darnell JE, Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264(5164):1415-21.
66. Horisberger MA. Interferon-induced human protein MxA is a GTPase which binds transiently to cellular proteins. Journal of virology. 1992;66(8):4705-9.
67. Holzinger D, Jorns C, Stertz S, Boisson-Dupuis S, Thimme R, Weidmann M, et al. Induction of MxA gene expression by influenza A virus requires type I or type III interferon signaling. Journal of virology. 2007;81(14):7776-85.

68. Samuel CE. Antiviral actions of interferons. Clinical microbiology reviews. 2001;14(4):778-809, table of contents.
69. Rebouillat D, Hovanessian AG. The human 2',5'-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 1999;19(4):295-308.
70. Morrow AN, Schmeisser H, Tsuno T, Zoon KC. A novel role for IFN-stimulated gene factor 3II in IFN-gamma signaling and induction of antiviral activity in human cells. Journal of immunology. 2011;186(3):1685-93.
71. Moschonas A, Ioannou M, Eliopoulos AG. CD40 stimulates a "feed-forward" NF-kappaB-driven molecular pathway that regulates IFN-beta expression in carcinoma cells. Journal of immunology. 2012;188(11):5521-7.
72. Ning S, Pagano JS, Barber GN. IRF7: activation, regulation, modification and function. Genes and immunity. 2011;12(6):399-414.
73. Yang H, Lin CH, Ma G, Orr M, Baffi MO, Wathelet MG. Transcriptional activity of interferon regulatory factor (IRF)-3 depends on multiple protein-protein interactions. European journal of biochemistry / FEBS. 2002;269(24):6142-51.
74. Fukata M, Shang L, Santaolalla R, Sotolongo J, Pastorini C, Espana C, et al. Constitutive activation of epithelial TLR4 augments inflammatory responses to mucosal injury and drives colitis-associated tumorigenesis. Inflammatory bowel diseases. 2011;17(7):1464-73.
75. Szczepanski MJ, Czystowska M, Szajnik M, Harasymczuk M, Boyiadzis M, Kruk-Zagajewska A, et al. Triggering of Toll-like receptor 4 expressed on human head and neck squamous cell carcinoma promotes tumor development and protects the tumor from immune attack. Cancer research. 2009;69(7):3105-13.
76. Huang B, Zhao J, Li H, He KL, Chen Y, Chen SH, et al. Toll-like receptors on tumor cells facilitate evasion of immune surveillance. Cancer research. 2005;65(12):5009-14.
77. Kelly MG, Alvero AB, Chen R, Silasi DA, Abrahams VM, Chan S, et al. TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. Cancer research. 2006;66(7):3859-68.
78. Waldner MJ, Foersch S, Neurath MF. Interleukin-6--a key regulator of colorectal cancer development. Int J Biol Sci. 2012;8(9):1248-53.
79. Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology. 2007;132(4):1359-74.

80. Podolsky DK, Gerken G, Eyking A, Cario E. Colitis-associated variant of TLR2 causes impaired mucosal repair because of TFF3 deficiency. Gastroenterology. 2009;137(1):209-20.
81. Huang B, Zhao J, Shen S, Li H, He KL, Shen GX, et al. Listeria monocytogenes promotes tumor growth via tumor cell toll-like receptor 2 signaling. Cancer research. 2007;67(9):4346-52.
82. Cai Z, Sanchez A, Shi Z, Zhang T, Liu M, Zhang D. Activation of Toll-like receptor 5 on breast cancer cells by flagellin suppresses cell proliferation and tumor growth. Cancer research. 2011;71(7):2466-75.
83. Klimosch SN, Forsti A, Eckert J, Knezevic J, Bevier M, von Schonfels W, et al. Functional TLR5 genetic variants affect human colorectal cancer survival. Cancer research. 2013;73(24):7232-42.
84. Cherfils-Vicini J, Platonova S, Gillard M, Laurans L, Validire P, Caliandro R, et al. Triggering of TLR7 and TLR8 expressed by human lung cancer cells induces cell survival and chemoresistance. The Journal of clinical investigation. 2010;120(4):1285-97.
85. Vaisanen MR, Vaisanen T, Jukkola-Vuorinen A, Vuopala KS, Desmond R, Selander KS, et al. Expression of toll-like receptor-9 is increased in poorly differentiated prostate tumors. The Prostate. 2010;70(8):817-24.
86. Gonzalez-Reyes S, Marin L, Gonzalez L, Gonzalez LO, del Casar JM, Lamelas ML, et al. Study of TLR3, TLR4 and TLR9 in breast carcinomas and their association with metastasis. BMC Cancer. 2010;10:665.
87. Hasan UA, Bates E, Takeshita F, Biliato A, Accardi R, Bouvard V, et al. TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. Journal of immunology. 2007;178(5):3186-97.
88. Brody JD, Ai WZ, Czerwinski DK, Torchia JA, Levy M, Advani RH, et al. In situ vaccination with a TLR9 agonist induces systemic lymphoma regression: a phase I/II study. J Clin Oncol. 2010;28(28):4324-32.
89. Brignole C, Marimpietri D, Di Paolo D, Perri P, Morandi F, Pastorino F, et al. Therapeutic targeting of TLR9 inhibits cell growth and induces apoptosis in neuroblastoma. Cancer research. 2010;70(23):9816-26.
90. Gekeler V, Gimmnich P, Hofmann HP, Grebe C, Rommele M, Leja A, et al. G3139 and other CpG-containing immunostimulatory phosphorothioate oligodeoxynucleotides are potent suppressors of the growth of human tumor xenografts in nude mice. Oligonucleotides. 2006;16(1):83-93.

91. Wang H, Rayburn ER, Wang W, Kandimalla ER, Agrawal S, Zhang R. Chemotherapy and chemosensitization of non-small cell lung cancer with a novel immunomodulatory oligonucleotide targeting Toll-like receptor 9. Molecular cancer therapeutics. 2006;5(6):1585-92.
92. Merrell MA, Ilvesaro JM, Lehtonen N, Sorsa T, Gehrs B, Rosenthal E, et al. Toll-like receptor 9 agonists promote cellular invasion by increasing matrix metalloproteinase activity. Molecular cancer research : MCR. 2006;4(7):437-47.
93. Berger R, Fiegl H, Goebel G, Obexer P, Ausserlechner M, Doppler W, et al. Toll-like receptor 9 expression in breast and ovarian cancer is associated with poorly differentiated tumors. Cancer science. 2010;101(4):1059-66.
94. Levy HB, Law LW, Rabson AS. Inhibition of tumor growth by polyinosinic-polycytidylic acid. Proceedings of the National Academy of Sciences of the United States of America. 1969;62(2):357-61.
95. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. Journal of immunology. 2006;176(8):4894-901.
96. Sarma PS, Shiu G, Neubauer RH, Baron S, Huebner RJ. Virus-induced sarcoma of mice: inhibition by a synthetic polyribonucleotide complex. Proceedings of the National Academy of Sciences of the United States of America. 1969;62(4):1046-51.
97. Hadrup S, Donia M, Thor Straten P. Effector CD4 and CD8 T cells and their role in the tumor microenvironment. Cancer Microenviron. 2013;6(2):123-33.
98. Androlewicz MJ, Ortmann B, van Endert PM, Spies T, Cresswell P. Characteristics of peptide and major histocompatibility complex class I/beta 2-microglobulin binding to the transporters associated with antigen processing (TAP1 and TAP2). Proceedings of the National Academy of Sciences of the United States of America. 1994;91(26):12716-20.
99. Grandea AG, 3rd, Van Kaer L. Tapasin: an ER chaperone that controls MHC class I assembly with peptide. Trends in immunology. 2001;22(4):194-9.
100. Michalak M, Corbett EF, Mesaeli N, Nakamura K, Opas M. Calreticulin: one protein, one gene, many functions. The Biochemical journal. 1999;344 Pt 2:281-92.
101. Menoret A, Drew DA, Miyamoto S, Nakanishi M, Vella AT, Rosenberg DW. Differential proteomics identifies PDIA3 as a novel chemoprevention target in human colon cancer cells. Mol Carcinog. 2014;53 Suppl 1:E11-22.
102. Claesson L, Larhammar D, Rask L, Peterson PA. cDNA clone for the human invariant gamma chain of class II histocompatibility antigens and its implications for the protein

structure. Proceedings of the National Academy of Sciences of the United States of America. 1983;80(24):7395-9.
103. Gold DV, Stein R, Burton J, Goldenberg DM. Enhanced expression of CD74 in gastrointestinal cancers and benign tissues. International journal of clinical and experimental pathology. 2010;4(1):1-12.
104. Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, et al. MIF signal transduction initiated by binding to CD74. The Journal of experimental medicine. 2003;197(11):1467-76.
105. Oliveira CS, de Bock CE, Molloy TJ, Sadeqzadeh E, Geng XY, Hersey P, et al. Macrophage migration inhibitory factor engages PI3K/Akt signalling and is a prognostic factor in metastatic melanoma. BMC Cancer. 2014;14:630.
106. Nakagawa T, Roth W, Wong P, Nelson A, Farr A, Deussing J, et al. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. Science. 1998;280(5362):450-3.
107. Dall E, Brandstetter H. Structure and function of legumain in health and disease. Biochimie. 2015.
108. Reith W, LeibundGut-Landmann S, Waldburger J-M. Regulation of MHC class II gene expression by the class II transactivator. Nature reviews Immunology. 2005;5(10):793-806.
109. Kondo K, Kohno N, Yokoyama A, Hiwada K. Decreased MUC1 expression induces E-cadherin-mediated cell adhesion of breast cancer cell lines. Cancer research. 1998;58(9):2014-9.
110. Selga E, Morales C, Noe V, Peinado MA, Ciudad CJ. Role of caveolin 1, E-cadherin, Enolase 2 and PKCalpha on resistance to methotrexate in human HT29 colon cancer cells. BMC medical genomics. 2008;1:35.
111. Mannori G, Crottet P, Cecconi O, Hanasaki K, Aruffo A, Nelson RM, et al. Differential colon cancer cell adhesion to E-, P-, and L-selectin: role of mucin-type glycoproteins. Cancer research. 1995;55(19):4425-31.
112. Kam JL, Regimbald LH, Hilgers JH, Hoffman P, Krantz MJ, Longenecker BM, et al. MUC1 synthetic peptide inhibition of intercellular adhesion molecule-1 and MUC1 binding requires six tandem repeats. Cancer research. 1998;58(23):5577-81.
113. Schroeder JA, Thompson MC, Gardner MM, Gendler SJ. Transgenic MUC1 interacts with epidermal growth factor receptor and correlates with mitogen-activated protein kinase activation in the mouse mammary gland. The Journal of biological chemistry. 2001;276(16):13057-64.

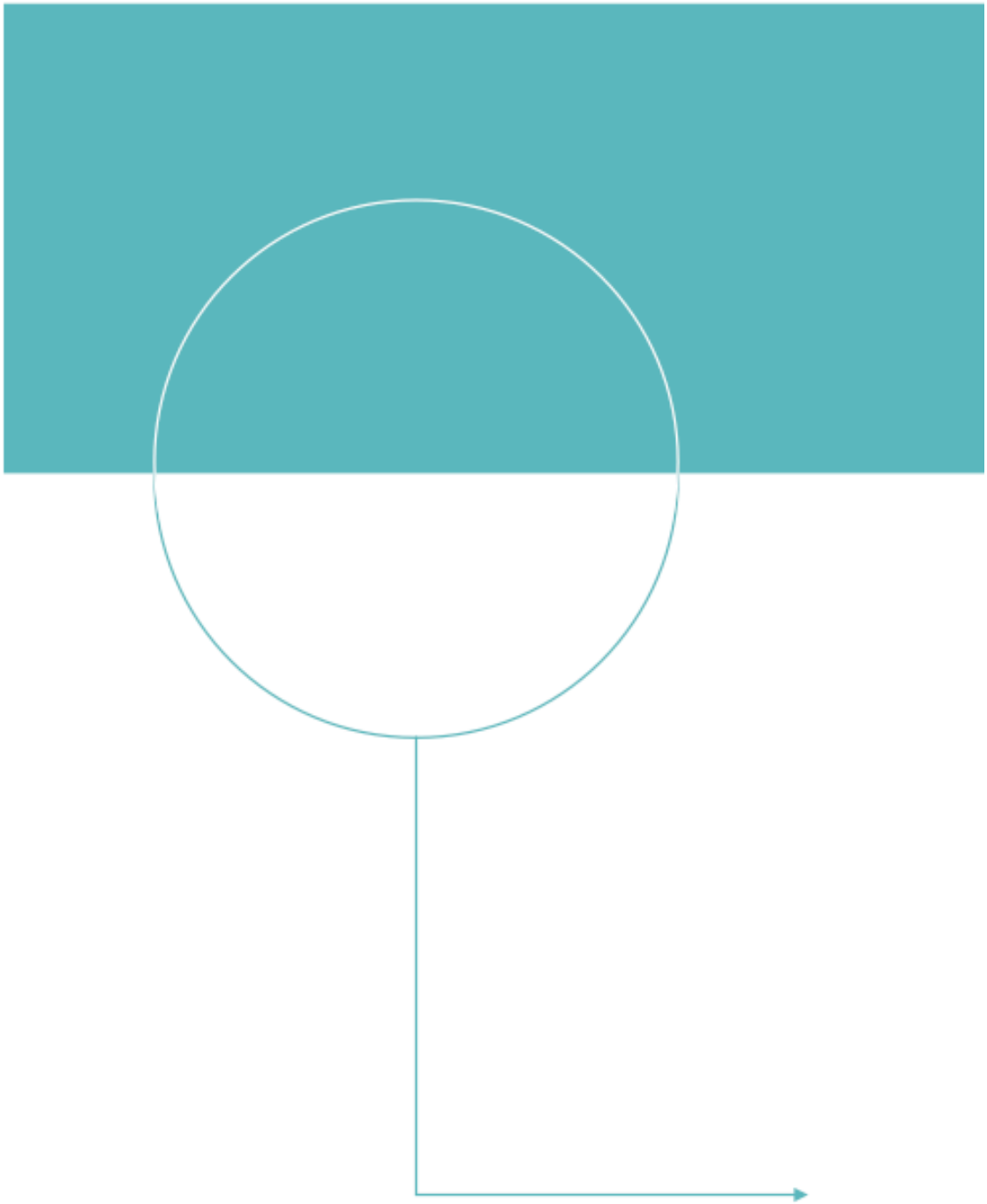
114. Castedo M, Perfettini JL, Roumier T, Kroemer G. Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. Cell death and differentiation. 2002;9(12):1287-93.
115. Cogswell JP, Godlevski MM, Bonham M, Bisi J, Babiss L. Upstream stimulatory factor regulates expression of the cell cycle-dependent cyclin B1 gene promoter. Molecular and cellular biology. 1995;15(5):2782-90.
116. Leung TW, Lin SS, Tsang AC, Tong CS, Ching JC, Leung WY, et al. Over-expression of FoxM1 stimulates cyclin B1 expression. FEBS letters. 2001;507(1):59-66.
117. Taylor WR, Stark GR. Regulation of the G2/M transition by p53. Oncogene. 2001;20(15):1803-15.
118. Palmer A, Gavin AC, Nebreda AR. A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. The EMBO journal. 1998;17(17):5037-47.
119. Okumura E, Fukuhara T, Yoshida H, Hanada Si S, Kozutsumi R, Mori M, et al. Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition. Nat Cell Biol. 2002;4(2):111-6.
120. Nakajima H, Toyoshima-Morimoto F, Taniguchi E, Nishida E. Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. The Journal of biological chemistry. 2003;278(28):25277-80.
121. Srivastava M, Pollard HB. Molecular dissection of nucleolin's role in growth and cell proliferation: new insights. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1999;13(14):1911-22.
122. Ajiro K, Yoda K, Utsumi K, Nishikawa Y. Alteration of cell cycle-dependent histone phosphorylations by okadaic acid. Induction of mitosis-specific H3 phosphorylation and chromatin condensation in mammalian interphase cells. The Journal of biological chemistry. 1996;271(22):13197-201.
123. Blangy A, Lane HA, d'Herin P, Harper M, Kress M, Nigg EA. Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. Cell. 1995;83(7):1159-69.
124. Peter M, Nakagawa J, Doree M, Labbe JC, Nigg EA. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. Cell. 1990;61(4):591-602.

125. Satterwhite LL, Lohka MJ, Wilson KL, Scherson TY, Cisek LJ, Corden JL, et al. Phosphorylation of myosin-II regulatory light chain by cyclin-p34cdc2: a mechanism for the timing of cytokinesis. The Journal of cell biology. 1992;118(3):595-605.
126. Golan A, Yudkovsky Y, Hershko A. The cyclin-ubiquitin ligase activity of cyclosome/APC is jointly activated by protein kinases Cdk1-cyclin B and Plk. The Journal of biological chemistry. 2002;277(18):15552-7.
127. Hammarstrom S. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. Seminars in cancer biology. 1999;9(2):67-81.
128. Frangsmyr L, Baranov V, Prall F, Yeung MM, Wagener C, Hammarstrom S. Cell- and region-specific expression of biliary glycoprotein and its messenger RNA in normal human colonic mucosa. Cancer research. 1995;55(14):2963-7.
129. Scholzel S, Zimmermann W, Schwarzkopf G, Grunert F, Rogaczewski B, Thompson J. Carcinoembryonic antigen family members CEACAM6 and CEACAM7 are differentially expressed in normal tissues and oppositely deregulated in hyperplastic colorectal polyps and early adenomas. The American journal of pathology. 2000;156(2):595-605.
130. Ilantzis C, Jothy S, Alpert LC, Draber P, Stanners CP. Cell-surface levels of human carcinoembryonic antigen are inversely correlated with colonocyte differentiation in colon carcinogenesis. Laboratory investigation; a journal of technical methods and pathology. 1997;76(5):703-16.
131. Kim KS, Kim JT, Lee SJ, Kang MA, Choe IS, Kang YH, et al. Overexpression and clinical significance of carcinoembryonic antigen-related cell adhesion molecule 6 in colorectal cancer. Clin Chim Acta. 2013;415:12-9.
132. Jantscheff P, Terracciano L, Lowy A, Glatz-Krieger K, Grunert F, Micheel B, et al. Expression of CEACAM6 in resectable colorectal cancer: a factor of independent prognostic significance. J Clin Oncol. 2003;21(19):3638-46.
133. Vleminckx K, Vakaet L, Jr., Mareel M, Fiers W, van Roy F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell. 1991;66(1):107-19.
134. Birchmeier W, Behrens J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochimica et biophysica acta. 1994;1198(1):11-26.

135. Fagotto F, Funayama N, Gluck U, Gumbiner BM. Binding to cadherins antagonizes the signaling activity of beta-catenin during axis formation in Xenopus. The Journal of cell biology. 1996;132(6):1105-14.
136. Heasman J, Crawford A, Goldstone K, Garner-Hamrick P, Gumbiner B, McCrea P, et al. Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early Xenopus embryos. Cell. 1994;79(5):791-803.
137. Cox RT, Pai LM, Kirkpatrick C, Stein J, Peifer M. Roles of the C terminus of Armadillo in Wingless signaling in Drosophila. Genetics. 1999;153(1):319-32.
138. Neumaier M, Paululat S, Chan A, Matthaes P, Wagener C. Biliary glycoprotein, a potential human cell adhesion molecule, is down-regulated in colorectal carcinomas. Proceedings of the National Academy of Sciences of the United States of America. 1993;90(22):10744-8.
139. Rosenberg M, Nedellec P, Jothy S, Fleiszer D, Turbide C, Beauchemin N. The expression of mouse biliary glycoprotein, a carcinoembryonic antigen-related gene, is down-regulated in malignant mouse tissues. Cancer research. 1993;53(20):4938-45.
140. Kunath T, Ordonez-Garcia C, Turbide C, Beauchemin N. Inhibition of colonic tumor cell growth by biliary glycoprotein. Oncogene. 1995;11(11):2375-82.
141. Hsieh JT, Luo W, Song W, Wang Y, Kleinerman DI, Van NT, et al. Tumor suppressive role of an androgen-regulated epithelial cell adhesion molecule (C-CAM) in prostate carcinoma cell revealed by sense and antisense approaches. Cancer research. 1995;55(1):190-7.
142. Fiori V, Magnani M, Cianfriglia M. The expression and modulation of CEACAM1 and tumor cell transformation. Ann Ist Super Sanita. 2012;48(2):161-71.
143. Ieda J, Yokoyama S, Tamura K, Takifuji K, Hotta T, Matsuda K, et al. Re-expression of CEACAM1 long cytoplasmic domain isoform is associated with invasion and migration of colorectal cancer. International journal of cancer Journal international du cancer. 2011;129(6):1351-61.
144. Tiernan JP, Perry SL, Verghese ET, West NP, Yeluri S, Jayne DG, et al. Carcinoembryonic antigen is the preferred biomarker for in vivo colorectal cancer targeting. British journal of cancer. 2013;108(3):662-7.
145. Conaghan P, Ashraf S, Tytherleigh M, Wilding J, Tchilian E, Bicknell D, et al. Targeted killing of colorectal cancer cell lines by a humanised IgG1 monoclonal antibody that binds to membrane-bound carcinoembryonic antigen. British journal of cancer. 2008;98(7):1217-25.

146. Zheng C, Feng J, Lu D, Wang P, Xing S, Coll JL, et al. A novel anti-CEACAM5 monoclonal antibody, CC4, suppresses colorectal tumor growth and enhances NK cells-mediated tumor immunity. PloS one. 2011;6(6):e21146.
147. Ordonez C, Screaton RA, Ilantzis C, Stanners CP. Human carcinoembryonic antigen functions as a general inhibitor of anoikis. Cancer research. 2000;60(13):3419-24.
148. Lesuffleur T, Barbat A, Dussaulx E, Zweibaum A. Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells. Cancer research. 1990;50(19):6334-43.
149. Munro SB, Turner IM, Farookhi R, Blaschuk OW, Jothy S. E-cadherin and OB-cadherin mRNA levels in normal human colon and colon carcinoma. Experimental and molecular pathology. 1995;62(2):118-22.
150. Masur K, Lang K, Niggemann B, Zanker KS, Entschladen F. High PKC alpha and low E-cadherin expression contribute to high migratory activity of colon carcinoma cells. Molecular biology of the cell. 2001;12(7):1973-82.
151. Wice BM, Trugnan G, Pinto M, Rousset M, Chevalier G, Dussaulx E, et al. The intracellular accumulation of UDP-N-acetylhexosamines is concomitant with the inability of human colon cancer cells to differentiate. The Journal of biological chemistry. 1985;260(1):139-46.
152. Gouyer V, Wiede A, Buisine MP, Dekeyser S, Moreau O, Lesuffleur T, et al. Specific secretion of gel-forming mucins and TFF peptides in HT-29 cells of mucin-secreting phenotype. Biochimica et biophysica acta. 2001;1539(1-2):71-84.
153. Lesuffleur T, Porchet N, Aubert JP, Swallow D, Gum JR, Kim YS, et al. Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. Journal of cell science. 1993;106 (Pt 3):771-83.
154. Lesuffleur T, Barbat A, Luccioni C, Beaumatin J, Clair M, Kornowski A, et al. Dihydrofolate reductase gene amplification-associated shift of differentiation in methotrexate-adapted HT-29 cells. The Journal of cell biology. 1991;115(5):1409-18.
155. Bara J, Gautier R, Daher N, Zaghouani H, Decaens C. Monoclonal antibodies against oncofetal mucin M1 antigens associated with precancerous colonic mucosae. Cancer research. 1986;46(8):3983-9.
156. Johansson ME, Hansson GC. Is the intestinal goblet cell a major immune cell? Cell Host Microbe. 2014;15(3):251-2.

157. Wlodarska M, Thaiss CA, Nowarski R, Henao-Mejia J, Zhang JP, Brown EM, et al. NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. Cell. 2014;156(5):1045-59.
158. Patel KK, Miyoshi H, Beatty WL, Head RD, Malvin NP, Cadwell K, et al. Autophagy proteins control goblet cell function by potentiating reactive oxygen species production. The EMBO journal. 2013;32(24):3130-44.
159. McDole JR, Wheeler LW, McDonald KG, Wang B, Konjufca V, Knoop KA, et al. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. Nature. 2012;483(7389):345-9.
160. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nature reviews Cancer. 2013;13(10):714-26.
161. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nature immunology. 2002;3(11):999-1005.



NTNU

Norwegian University of
Science and Technology