STUDY OF THE ROLE OF RETINOIC ACID SIGNALING IN COLON CANCER

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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ABSTRACT

Stem cells in normal and cancer tissues have been studied using different markers developed over the past few years such as ALDH1, CD34, CD133 and many others. Other approaches to identifying stem cells are based on their nuclear morphology and chromosomal organization. Dr. Gostjeva from MIT proposed that stem cells have bell-shaped nuclei (Gostjeva et al. 2009). Cells having this nuclear morphology are called metakaryotes. They undergo amitotic cell division instead of mitosis. These metakaryotic cells are thought to have stem cell properties (stemness) and have been seen more in fetal tissues and in cancers. Fetal mouse intestine and normal and cancer tissue of colon were used in my project to study metakaryotes. A fetal human colon cell line (FHC) was also studied. Different histologic techniques were used to study the presence of this nuclear morphology. While a few cells with bell shaped nucleus could be identified using the Gostjeva protocol, no metakaryotes were detected with any other approach studied. Immunostaining showed that the stem cell marker ALDH1 is expressed in fetal mouse tissue and in the FHC cell line, but no cells were found in these tissues having bell shaped nuclear morphology. These results suggest that the identification of cells with a bell shaped nucleus is dependent on the histologic method and that Gostjeva protocol may affect nuclear morphology.

Retinoid receptors are members of a nuclear hormone receptor family of proteins and are regulators of retinoid acid (RA) signaling pathways. Two types of retinoid receptors that regulate RA signaling are retinoid acid receptors (RAR) and retinoid X receptor (RXR). Both these receptor types are important for normal retinoid acid signaling and homeostasis of cells. Retinoic acid signaling plays a vital role during embryonic development by regulating stem cell function and is also crucial for normal functioning of different types of adult cells. Dysregulation of retinoic acid signaling has been reported in different types of cancer.

Retinoic acid signaling is important for proper proliferation and differentiation of cells because of its role in cell cycle arrest. Retinoic acid derivatives (ATRA and 9-cis RA) are necessary signaling molecules for the activation of this pathway. They act via binding to retinoid receptors. Retinoid receptors are not only important for retinoic acid signaling, but also, for other signaling pathways. They dimerize with other receptor types like pPAR gamma, thyroid hormone receptors and many others. Thus, the involvement of the RA pathway with several other pathways makes understanding normal human RA signaling more complicated. This gets further complicated in cancerous conditions.

Previous studies have shown the involvement of retinoic acid signaling in the development of different cancers including colorectal cancer. I hypothesize that specific retinoid receptors and other members of the RA signaling pathway are crucial

for colorectal cancer development. To study this hypothesis, I investigated expression levels of retinoid receptors (RAR alpha and RXR alpha) and members of the RA signaling pathway (ALDH1, CYP26A1 and CtBP1) in normal and cancer cells of the colon. ALDH1, a marker for normal and cancer stem cells, was used to determine the role of stem cells in colorectal cancer development. First, expression of each protein and protein levels were investigated in colorectal cancer cells and normal and cancer tissue of colon using immunocytochemistry and western blot analysis, respectively. Second, I studied the effects of retinoic acid derivatives on colorectal cancer cells. Colorectal adenocarcinoma cell lines, SW480 and HT29 cells, were used as models to study the effects of RA derivatives on cell survival and growth. To study RA signaling in fetuses, a fetal human colon cell line (FHC) was investigated.

Increased expression of RAR alpha, ALDH1, CYP26A1 and CtBP1 was seen in cancer tissue from colon compared to normal colon, whereas the expression of RXR alpha did not substantially change. Expression of RAR alpha, RXR alpha, and ALDH1 was detected in SW480 and HT29 cells by immunocytochemistry and western blot analysis and by immunocytochemistry in FHC cells.

Treatment of colorectal cancer cell lines with ATRA and 9-cis RA derivatives had different effects on each cell line. ATRA inhibited growth in both SW480 and HT29 cells, but 9-cis RA significantly inhibited proliferation only in SW480 cells but not in HT29 cells. 9-cis RA induced a proliferative response in HT29 cells. Thus different

colorectal cancer cell lines respond differently to these two naturally occurring derivatives of retinoic acid. Taken together, my experimental results suggest that changes in various components of the retinoid signaling pathway in colonocytes can affect cellular response to retinoid derivatives. The study of stem cells in association with retinoic acid signaling in colorectal cancer development can prove crucial for use of retinoic acid derivatives as therapeutic agents for colorectal cancer treatment if they have the potential to kill cancer stem cells.

CHAPTER 1

1. INTRODUCTION

1.1 Background on colon anatomy and colonic stem cells

The large intestine of our body is made up of colon, cecum and rectum. Colon is the last part of our digestive system and its major function is to extract water, salt, potassium and fat soluble vitamins from the solid waste before they are excreted from the body. The colon can be divided into four parts: ascending colon, transverse colon, descending colon and the sigmoid colon, which joins the rectum. The inner surface of the colon has invaginations made of epithelial cells; they form tubular structures called crypts. The size of these crypts and their cell types differ between ascending and descending colon. The crypts are shorter in ascending colon than descending colon. Different cell types are present in descending colon including vacuolated-columnar, goblet, enteroendocrine and vacuolated cells. All these cells are randomly placed throughout the crypt wall of the descending colon, whereas the ascending colon has a smaller number of cells. Stem cells are located at the base of the crypt and daughter cells from stem cell division move up the crypt. Compared to descending colon, cell cycle time in ascending colon is longer and therefore it has fewer proliferating cells. This has been proposed to be the reason why descending colon is at higher risk of cancer than ascending colon (Sell 2004), (www.buzzle.com).

The structural integrity of the GI tract is maintained by GI stem cells that have the potential to proliferate and differentiate into all epithelial cell lineages. They also have regenerative potential and can maintain homeostasis in normal mucosa. For example, the GI tract is a very proliferative organ and replenishes itself in every 5 days. GI stem cells are so multipotent that a single stem cell can regenerate an entire

crypt after the older crypt is radiated. Experimental evidence suggests that when stem cells acquire mutations they may give rise to cancer stem cells. This makes stem cells interesting targets for cancer therapy (Gearhart 2006),(Sell 2004).

Colorectal cancer is the second leading cause of cancer deaths in the US. It is also known as large bowel cancer and includes cancerous growths in colon, rectum as well as appendix. Colon cancer starts in the innermost lining of the colon. If left untreated, it can grow as mushroom shaped small growths called adenomas or polyps, which are premalignant but over the time can develop into cancers. Colon cancer is generally curable if it is restricted to the walls of the colon. The chances of a cure after it metastasizes are much less (www.medicinenet.com).

Colorectal cancer usually starts with the loss of an allele in the *APC* gene followed by a mutation in the RAS family of proteins and in the p53 gene. As many as 85% of human sporadic colorectal cancer cases have a mutated *APC* gene. In FAP (familial adenomatous polyposis) patients have inherited a germline mutation in an *APC* gene on chromosome 5q21, which leads to development of adenomas and carcinomas if the remaining wild type *APC* allele also becomes mutated. This goes along with the two-hit-loss hypothesis of Dr. Knudson for tumor suppressor genes (Sell 2004).

1.2 Wnt signaling in normal colon

In normal colon, the APC complex degrades β-catenin, thus downregulating TCF/LEF activation (Figure 1A)

The *APC* gene is a key player in the Wnt signaling pathway. *APC* forms a complex with glycogen synthase kinase-3β (GSK-3β) and axin, which recruits beta-

catenin to the cytoplasm and leads to phosphorylation, ubiquitination and proteasomal degradation of beta-catenin. When Wnt binds to the Frizzled receptor, it leads to phosphorylation of the Dishevelled protein, which inhibits the APC/GSK-3 β/axin complex formation. Thus, β-catenin cannot be proteosomally degraded, which leads to an increase in the cytoplasmic level of β-catenin, which then translocates to the nucleus and binds to the TCF4/LEF1 (T-cell factor/lymphocyte enhancer factor) DNA binding site and activates genes such as c-myc and cyclin D1, which, in turn, increases cell proliferation (Sell 2004).

1.3 Wnt signaling during colorectal cancer:

During early oncogenesis, Wnt binding to the Frizzled protein degrades the APC complex, thus increasing cytoplasmic β-catenin levels, causing their translocation to the nucleus and, increasing cell proliferation (Figure 1B).

If during sporadic adenoma formation, one allele of the APC gene becomes mutated, cytoplasmic β-catenin translocates to nucleus, activates the transcription factor TCF4 and there is an increase in cell proliferation.

During tumor progression, Wnt ligand binding not only degrades APC, but also it causes APC mutation thus further increasing cytoplasmic levels of β-catenin.

During development of sporadic colorectal cancer development acquired mutations in the APC gene occur in most cases (85%). Even in colorectal adenocarcinoma cases that lack an *APC* gene mutation, mutations of the β-catenin gene are present in many (48%) of tumors. Mutation in β-catenin affects the serine and threonine residue targeted by GSK-3 β, which results in the formation of a phosphorylation resistant protein which cannot be degraded via the Wnt signaling pathway. Other than the Wnt pathway, and before recruitment of β-catenin to the cytoplasm, β-catenin is bound to the actin cytoskeleton and plays a role in cell adhesion via α –catenin (van de Wetering, Sancho et al. 2002), (Baylin and Ohm 2006), (Sell 2004).

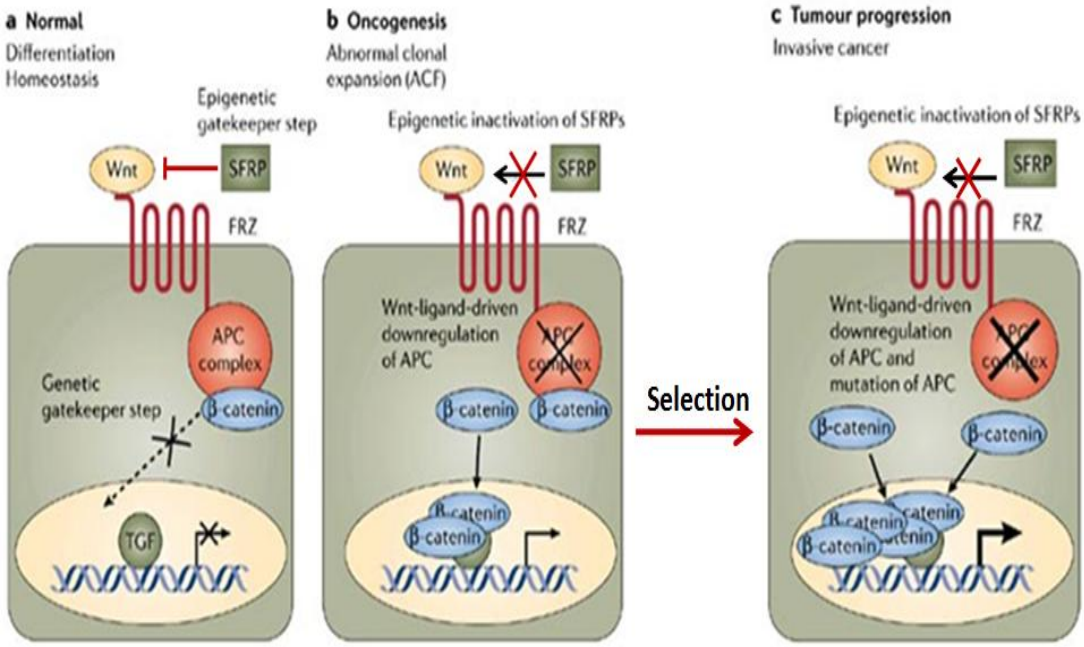


Figure 1: Wnt signaling in a) normal colon, b) during oncogenesis and c) during tumor progression in colon cancer (Baylin and Ohm 2006).

1.4 Introduction to molecular markers of normal and cancer stem cells:

It has been reported that colorectal cancer formation is due to a small population of cells that have the stem cell phenotype – a subpopulation of mutant cells that increases in size during cancer progression. These stem cells are called cancer stem cells (CSCs) and are important in the development of tumors and in the driving force to tumor growth. There are different markers for identifying normal and cancer stem cells. Some of the markers are cancer and cell type dependent, too. Among a list of stem cell makers some known ones are CD34, CD133 and evidence is mounting for ALDH1 as a marker for both normal and CSCs in colon. Dr. Boman's research team (Huang, Hynes et al. 2009) showed that ALDH1 marks SCs at the base of the normal crypt (Figure 2). Moreover, as tumorigenesis progresses from normal to APC mutant colon, to adenoma, to adenocarcinoma, there is an increase in the number of ALDH1 positive cells per crypt and distribution in crypts.

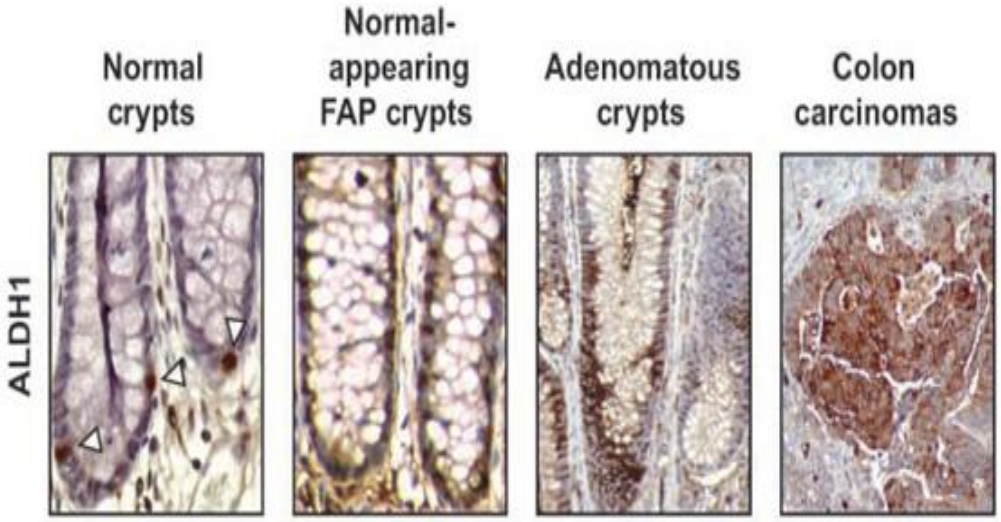


Figure 2: Immunohistochemical staining of ALDH1 in colon: normal, FAP, adenoma and carcinoma. In normal crypt as shown, ALDH1 marks stem cells at the bottom of the crypt. In FAP and adenoma the number of ALDH1 positive cells is increased and these cells have moved up the crypt. In carcinomas, where the crypt structure is completely disrupted, the number of cells positive for ALDH1 progressively increases (Huang, Hynes et al. 2009).

1.5 Background on RA signaling:

Colonic stem cells reside at the base of the crypt and are regulated by specific pathways including the retinoic acid (RA) signaling pathway. RA signaling has been found to regulate stem cells in fetal development and also maintains proper function of adult stem cells. RA signaling is mediated by two nuclear retinoic receptor types: the retinoic acid receptor (RAR) and the retinoic X receptor (RXR). Each receptor type has three isoforms, alpha, beta and gamma. These isoforms are formed by alternative splicing. Retinol, the biological form of vitamin A, is taken up by the cell, and is converted to retinaldehyde by RDH (retinaldehydrogenase). ALDH1, a promising stem cell marker, is a key enzyme of the retinoic acid signaling pathway. It converts retinaldehyde to all-trans retinoic acid (ATRA), a derivative of retinoic acid (RA). ATRA is then transported from the cytoplasm to nucleus which and acts as a ligand and binds to RA receptors (RAR). ATRA only activates RAR receptor types and not RXR receptor types. RXR receptors are activated by another form of RA derivative, 9-cis retinoic acid (9-cis RA). Binding of ATRA leads to replacement of co-repressor complex from the receptor dimer and recruits co-activator, leading to conformational change of RAR receptors, causing their activation. RAR receptors can heterodimerize with PML or RXR alpha, or homodimerize with its own receptor subtypes (Mongan and Gudas 2007).

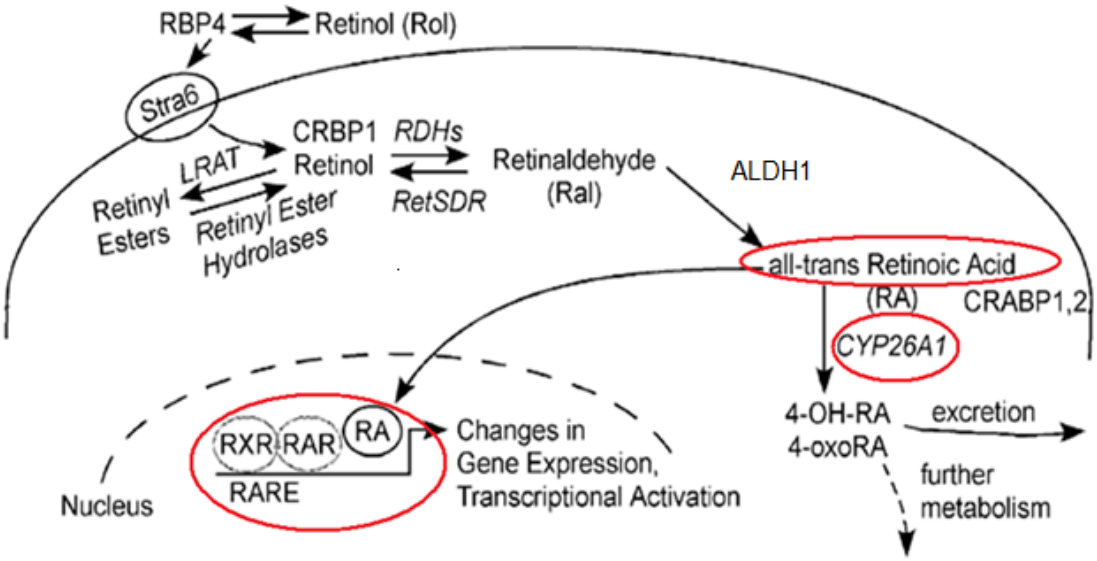


Figure 3: The retinoic acid signaling pathway in normal colon tissue. Retinol is taken up from the circulating blood by cells via Stra 6 receptors, thus leading to conversion of retinol to ATRA in a process involving ALDH1. ATRA is then translocated to the nucleus where it activates the retinoic acid receptor, thus activating the expression of downstream genes (Mongan and Gudas 2007).

Activation of the retinoic acid signaling pathway activates downstream genes like p21, p53 and CDKI (cyclin dependent kinase inhibitor). These are cell cycle arrest genes that maintain proper cell proliferation and differentiation in normal colon. Retinoic acid decreases cyclin D1, D3 and E expression directly and also via activation of CDKI. Retinoids function in intercellular communication as well because retinoids produced by one cell can affect the RA signaling of an adjacent cell. Retinoic acid signaling is compromised in different types of cancer including prostate and breast cancer. Its role in the cell cycle and its dysregulation during cancer makes it an important target for cancer therapeutics. Two of the most common, naturally occurring derivatives of retinoic acid are ATRA and 9-cis RA. ATRA binds only to RAR receptor types and not to RXR receptors. 9-cis RA binds to both RXR and RAR receptors but with higher affinity to RXR. As both RXR and RAR are involved in dimerization with different receptor types, they are the mediators of transcriptional responses to stimuli from multiple kinds of signaling. Both RXR and RAR receptors interact with multiple co-activator and co-repressor proteins. Activation of the RXR/RAR receptor complex recruits different co-activators. One of them is ATP (adenosine triphosphate)-dependent remodeling of chromatin (Mongan and Gudas 2007), (Martin, Lardeux et al. 2005) (Marletaz, Holland et al. 2006). It has been found that binding of estrogen responsive β-box protein to the RAR β_2 binding site on RAR elements (RAREs) in gene promoter regions leads to transcriptional transactivation via the coiled-coil domain (Cheung, Bell et al. 2006). But this transactivation of receptors can be prevented by RXR/RAR signaling repressor, PRAME, when retinoid agonist is present (Epping, Wang et al. 2005). In the absence

of an agonist, transcriptional activation is prevented by co-repressors SMRT and NCoR (Sohn, Kim et al. 2003), (Perissi, Staszewski et al. 1999).

ATRA, a retinoic acid derivative, has been successfully used at pharmacological doses to treat acute promyelocytic leukemia (APL), which responds to the antiproliferative and differentiative actions of ATRA. ATRA binding to RARα receptors that have dimerized with PML, leads to differentiation of leukemic cells. The RARα receptor has also been reported to be mutated many other cancers besides APL. All subtypes of the *RAR* receptor (α, β, γ) all can act as tumor-suppressor genes. RAR β function has been reported to be compromised in different cancers including breast, ovarian and others ((Le, Dawson et al. 2000)). Thus, when mutation occurs in RAR β it cannot induce growth inhibition of cells. This response has also been seen using a breast cancer cell line (MCF7), such that if the cells are treated with retinoic acid derivatives, growth arrest and increased apoptosis are induced by restoration of *RAR* β_2 expression.

From all of the above information, two overall hypotheses were generated. First hypothesis is *"Colonic stem cells have bell shaped nuclei and the number of bell shaped nuclei increases during colon cancer progression."*

Second hypothesis is "Dysregulation of retinoic acid signaling is involved in colon cancer progression and treatment of colorectal cancer cell lines with retinoic acid derivatives induces inhibition of cell growth."

CHAPTER 2

STUDY OF STEM CELLS BASED ON THEIR BELL-SHAPED NUCLEAR MORPHOLOGY DURING COLON CANCER PROGRESSION.

2.1 INTRODUCTION:

The stem cell (SC) population represents a subpopulation of undifferentiated cells found in tissues of most multicellular organisms. This population has the capacity for self renewal and for differentiation into the different cell types and lineages found in each tissue. Normal stem cells are classified into three major types: embryonic SC, fetal SC, and adult SC. Division of SC can give rise to two daughter stem cells by dividing symmetrically or to one stem cell and one progenitor cell by dividing asymmetrically. In colon, these cells reside in the SC niche at the base of the crypt and maintain the crypt structure by dividing both symmetrically and asymmetrically to give rise to SC which remains in the SC niche and progenitor cells which divide further and give rise to transient amplifying cells that migrate out of the SC niche. Transient amplifying cells then undergo several cell divisions while they start to differentiate. After the colonocytes become terminally differentiated, they then undergo apoptosis. There are a number of SC markers that can be used to identify and isolate SC from different tissue types. For example, ALDH1 (aldehyde dehydrogenase1A1) has been used to identify SC in breast, brain and other tissues. More recently, it has been shown that ALDH1 is a new and promising marker for normal and malignant human colonic stem cells (Huang, Hynes et al. 2009**)**. ALDH1 is a key enzyme in retinoic acid metabolism and catalyses the oxidation of aldehydes to carboxylic acid. ALDH1 is expressed in colon, esophagus,

and lung and at very high levels in liver.

A recent study by Dr Boman's research team (Huang, Hynes et al. 2009) (Figure 1) showed that ALDH1 marks cells in the SC niche at the base of the normal human colonic crypt. Moreover as tumorigenesis progresses from normal to APC mutant colon, to adenoma, to adenocarcinoma (Figure1), the number of ALDH1 positive cells per crypt increases and ALDH1+ cells distribute to more upper regions of the crypt.

Cancer stem cells (CSC) are the basis of a very promising evolving concept. They are cells that have many characteristics of a normal SC but they also have a tumorigenic phenotype. In the "cancer stem cell paradigm", CSCs give rise to tumors via increased symmetric SC division. ALDH1 appears as a marker for identifying CSCs in colorectal cancer (Huang, Hynes et al. 2009), lung cancer (Jiang, Qiu et al. 2009), breast cancer (Ginestier, Hur et al. 2007), ovarian cancer (Chang, Liu et al. 2009) and many other cancers.

Different methods have been used to study ALDH1 in normal and cancer stem cells but there are strengths and weaknesses with each technique. Enzyme kinetics are widely used to study the activity of ALDH1, while immunoblotting is commonly used to study the protein level of ALDH1. Both techniques require lysis of the cells and measure ALDH in the cell lysates. Also there is high amount of cross reactivity between ALDH isoforms which makes immunoblotting challenging (Ma and Allan). Thus, it would be helpful to study ALDH1 activity in viable cells and without lysing them.

A potential way to identify SC is based on their nuclear morphology has been suggested by Gostjeva et al (2009), which relays on a method involving Carnoy's

fixation (Gostjeva, Koledova et al. 2009). To detect SC based on a unique nuclear morphology, their study suggests that SC can be characterized by a bell-shaped nucleus and that this distinct nuclear morphologic feature arises due to amitotic SC division (Gostjeva, Koledova et al. 2009). Amitosis has been reported in non-human mammals, humans and plants. In amitotic cell division, cells divide without mitotic spindle formation. During amitosis, the cell undergoes constriction of the nucleus and divides by simple cleavage which can lead to unequal distribution of chromosomes.

In the bell-shaped nucleus, 5% of the total nuclear DNA is concentrated on the mouth of the bell, thus giving it an open mouth structure. The DNA content is diploid. Bell-shaped nuclei were observed in tissues from ecto, endo and mesoderm. Bell-shaped nuclei divide both symmetrically and asymmetrically as do stem cells. Cells having this unique nuclear morphology are called metakaryotes (Gostjeva, Koledova et al. 2009).

Other studies based on SC markers, revealed that SC are few in adult tissues (perhaps 1% of the total number of crypt cells) but the number increases with tumor progression, from normal to adenoma to adenocarcinoma. Similar results have been reported for bell-shaped nuclei during colon tumorigenesis. However, it has not been shown whether bell-shaped nuclei can be identified by histologic methods using different types of tissue processing and fixative (other than Carnoy's fixative) or whether overpopulation of cells with bell-shaped nuclei can be shown to occur during colon tumorigenesis using such different histologic methods.

Based on the above information the following sub-hypothesis is proposed: "*The proportion of bell shaped nuclei increases in colon tumors compared to normal tissue.*" The following aim was designed to test my hypothesis "*To determine if cells*

with bell-shaped nuclear morphology can be identified in colonic tissues using standard histologic procedures."

2.2 MATERIALS AND METHODS:

2.2.1 Bell-shaped nuclei staining

Normal human colon samples and carcinoma samples were received from Christiana Care Hospital after patient consent under ***IRB*** approval and with informed consent. Within 20-25 minutes of surgical removal, tissues were fixed with Carnoy's fixative. The tissue was microdissected on a slide and then stained with nuclear stain Giemsa according to protocol provided by Dr. Gostjeva. Tissues were cut into ~1 mm^2 approximately ~1 mm^2 pieces on a petri dish and then rinsed with de-ionized water. Samples were dried and placed in a box with holes, and the box was placed in a Coplin jar filled with 1N HCl that had been preheated to 60 degrees. The samples were heated for 8 minutes to promote hydrolysis. The dissected pieces rinsed in DI water and placed in a petri dish containing 45% acetic acid for 30 minutes at room temperature. The last step was tissue maceration where solid tissue samples were spread as a single cell layer by placing 0.5mm pieces of tissue on a slide with a drop of acetic acid on top of it and sealing the system with a coverslip was removed with a razor blade. The slide was then dried and stained with Schiff's reagent for 1 hour and rinsed with 2X SSC. Tissue pieces were then stained with 1% Giemsa solution for 5 minutes and washed with Sorenssen's buffer and DI water. The slide was then dried and kept in a Coplin jar Xylene for 3 hours and then dried again and mounted with DePex.

2.2.2 Fetal mouse intestine used as positive control and stained with Hoechst.

A pregnant mouse was sacrificed by carbon-dioxide asphyxiation and 8 fetuses were dissected out at 16.5 days of plague formation. Intestinal tissue was dissected from the embryos. Two embryonic intestines were fixed with 16%paraformaldehyde and the other 6 intestines were fixed with Carnoy's fixative (methanol: acetic acid in a 1:1 ratio). Dissection was done using a Zeiss Stemi 2000C dissection microscope.

The whole fetal mouse intestine was fixed in 16% paraformaldehyde and then stained with Hoechst stain and mounted with 97% TDE (2, 2'-thiodiethanol). Adhesive slides for electron microscopy were purchased for cryosectioning. Slides containing thirty micron thick sections were made to observe the nuclear morphology of the cells residing at the base of the crypt. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with Plan-Apochromat 63X/1.40 oil DIC M27 objective. Z-stacks were done to get a better image of the nuclear morphology at the base of the crypt and also to study the type of cell division going on at the base of the crypt.

2.2.3 Staining with Hoechst and ALDH1A1

Six fetal intestines were fixed with Carnoy's fixative (1:1 ratio of acetic acid and methanol) and then sectioned to obtain slides with16 micron thick tissue sections, which were preserved at -80°C. For staining, fetal tissue was fixed with 4% paraformaldehyde for 40 minutes, washed with PBS, (3 times for 5 minutes), and non-specific sites were blocked overnight at 4°C with 1% BSA, 10% goat serum and 0.2%

triton. After blocking, tissue was stained with anti-ALDH1, a rabbit polyclonal antibody against mouse (Abcam) using with 1:100 dilutions. Secondary antibody was Alexa Fluor 594 goat anti-rabbit IgG (H+L) used at a 1/1000 dilution for 1 hour 30 minutes. Rabbit IgG was used as a negative control. Hoechst stain at a dilution of 1:6,000 was used as a nuclear stain. Nuclei along the crypt were imaged and their size was measured. Some common nuclear morphology like Cigar shaped, Oval shaped, Spherical shaped were found and their size was measured. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with C-Apochromat 40X/1.20 W Korr-VIS-IR objective.

2.2.4 A cell line from Fetal Human colon (FHC) was used as a positive control.

A Fetal Human colon cell line purchased from ATCC was sub-cultured in plated on a chamber slide and then stained with anti-ALDH1A1 and a nuclear stain (DAPI) to identify any Metakaryotic cells. As this is a fetal colon cell line, there is a greater chance of identifying metakaryotic cells. Cells were fixed with 100% ice cold methanol for 10 minutes and then washed twice with ice cold PBS times. Cells were blocked with 1% BSA, 10% serum, 0.3M glycine and PBST overnight at 4°C and then stained with anti-ALDH1, a mouse monoclonal antibody (BD biosciences) with at a1:50 dilution at 4 degrees overnight. The secondary antibody was Alexa Fluor 488 goat anti-mouse IgG (H+L) used at a dilution of 1/1000 for 1hour 30 minutes. Mouse IgG was used as a negative control.

2.3 RESULTS:

2.3.1: Identification of bell-shaped nuclei using the Gostjeva method (Fig: 4)

Bell-shaped nuclei were found in normal colon and in colon cancers. An increase in the number of bell-shaped nuclei was not found in tumor tissues.

A **B**

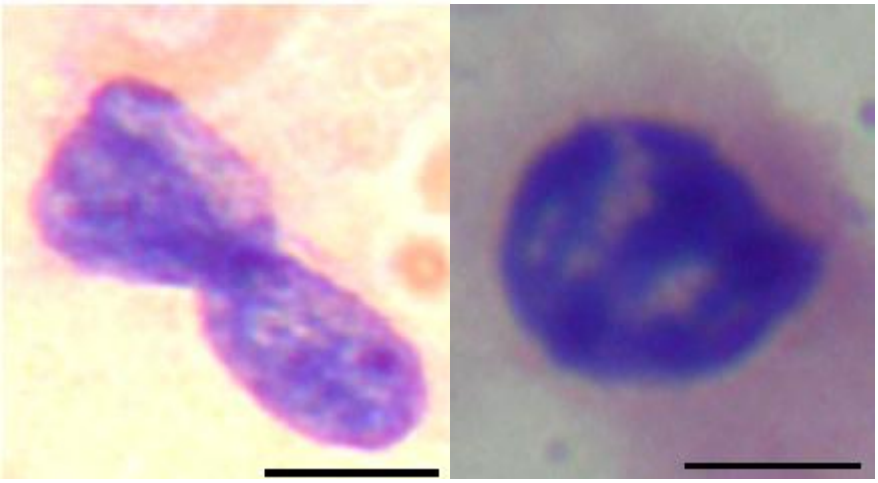


Figure 4: Normal and carcinoma tissue from adult colon stained with Giemsa stain. This image was taken using a 40X objective with a bright field microscope (Olympus Hitech Instrument Inc). A) Shows a bell-shaped nucleus from normal tissue fixed with Carnoy's fixative. B) Shows a bell-shaped nucleus in tumor tissue fixed with Carnoy's fixative. Scale bars represent 15µm.

2.3.2: Hoechst staining of fetal mouse intestine tissue (Fig: 5)

Fetal mouse intestine was used as positive control to identify Metakaryotic cells because in the fetal stage the number of stem cells is higher; hence the chances of finding SC would be higher. Staining fetal mouse intestine tissue with Hoechst stain helped us to understand the shape of the nuclei and the type of cell division going on along the crypt as well as at the base of the crypt. No nuclei with bell-shaped morphology or amitotic cell division were found in any part of the crypt including the base of the crypt. Some cells dividing mitotically were identified at the base of the crypt.

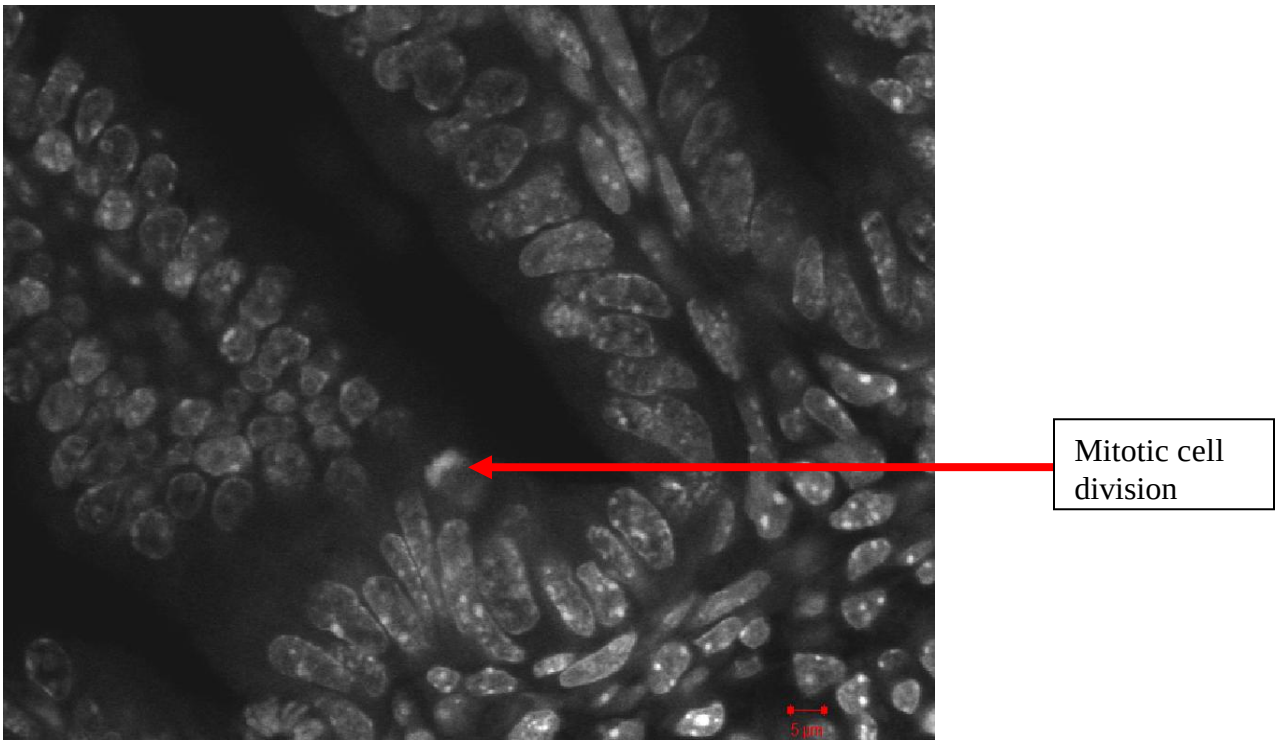
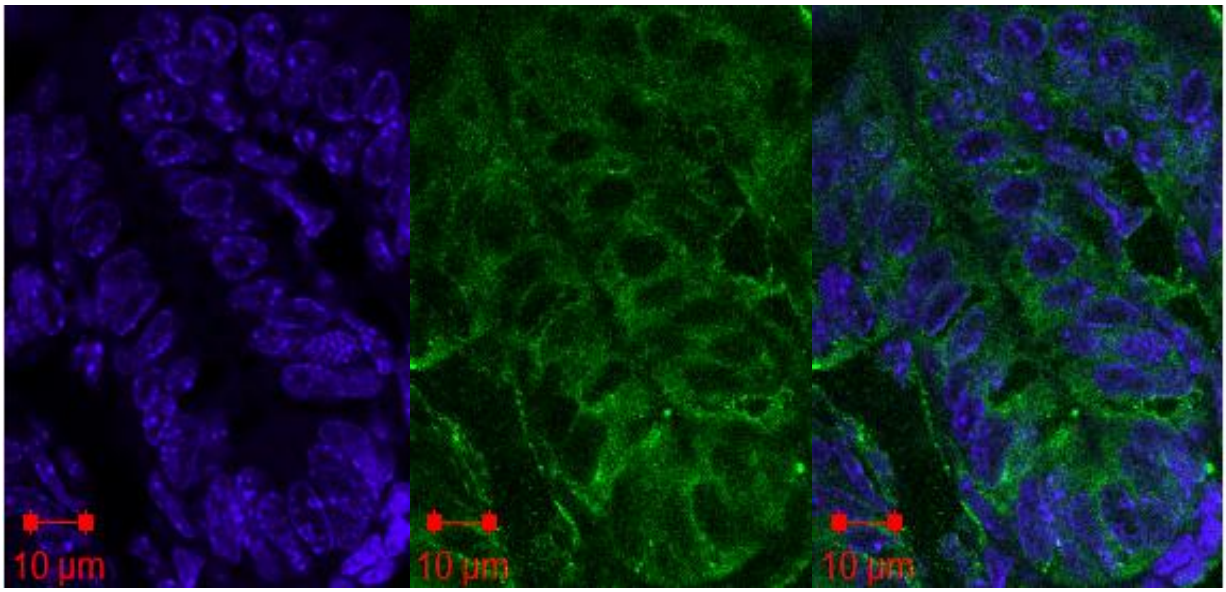


Figure 5: Fetal mouse intestine stained with Hoechst stain. Images of the bottom part of the crypt were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a Plan-Apochromat 63X/1.40 oil DIC M27 objective. Tissue was mounted with 97% TDE.

2.3.3: ALDH1 staining of fetal mouse intestine tissue (Fig: 6)

Fetal mouse intestine tissue was stained with anti-ALDH1 and Hoechst. The crypt shows intense cytoplasmic staining of ALDH1 at the bottom of the crypt, but it also stained the crypt top region. Cells staining positively for ALDH1 at the base of the crypt or along the crypt did not have bell-shaped nuclei. Cigar-shaped, oval shaped and spherical shaped nuclei are the three different types of nuclear morphologies identified in the crypt. Nuclei in 3 crypts per tissue were counted and then averaged to determine how many of the above mentioned nuclear morphologies



are present per crypt with respect to the total number of nuclei present in the crypt.

Figure 6: Fetal mouse crypt stained with ALDH1 and Hoechst stain. First panel (blue) shows nuclear staining using Hoechst stain; second panel (green) shows ALDH1 staining and third panel is the merged image. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a C-Apochromat 40X/1.20 W Korr-VIS-IR objective.

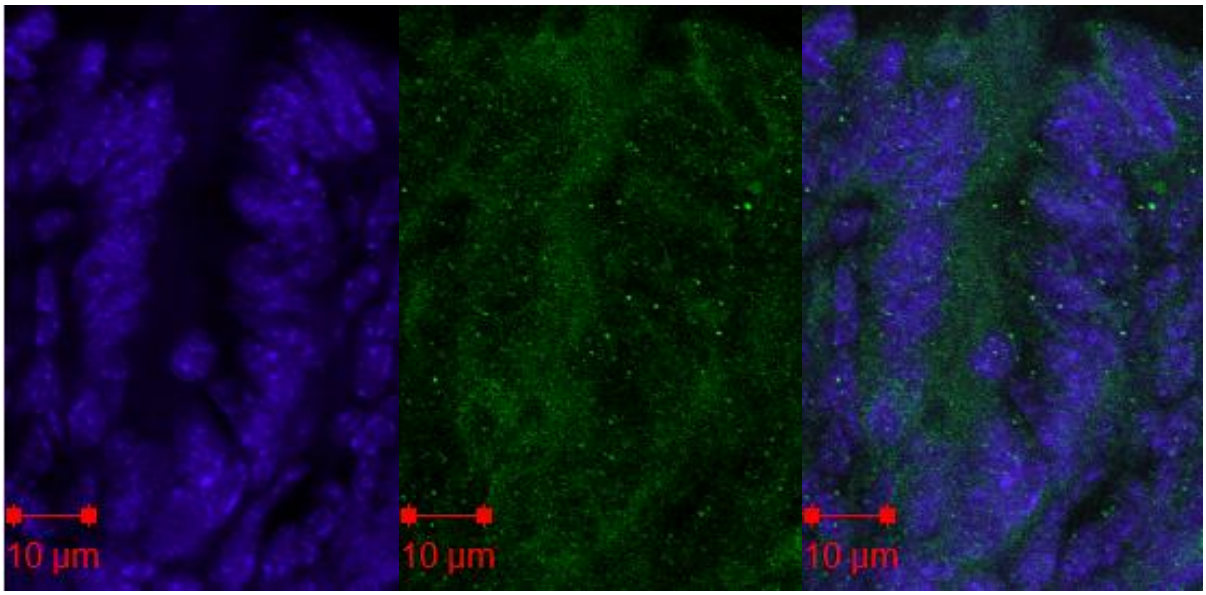
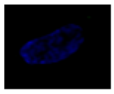
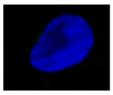
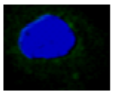


Figure 7: Fetal mouse crypts stained with an IgG control and with a nuclear Hoechst stain. First panel (blue) shows nuclear Hoechst stain, second panel (green) shows IgG staining, and third panel is the merged image. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a C-Apochromat 40X/1.20 W Korr-VIS-IR objective.

Table 1: Different nuclear morphologies per crypt in three different fetal mouse intestinal samples.

Fetal mouse intestine	Cigar -shaped Size- 10-15µ N=3 	Oval- shaped Size-5-7µ N=3 	Spherical-shaped Size-6-8µ N=3 	Average of Total number of cells counted
Sample 1	13	7	3	23
Sample 2	12	5	6	23
Sample 3	16	3	5	24

Average of the total number of nuclei counted and the number of each type of nuclei present along the crypt. Three nuclear morphologies cigar shaped, oval shaped and spherical shaped nuclei were identified in 3 fetal samples fixed with Carnoy's fixative. (µ=micron).

2.3.4: ALDH1 staining of FHC cell lines:

No bell shaped nuclei were found in the FHC cell line and no amitotic cell divisions were seen. Three experiments were done. Ten microscope fields in each chamber were counted and then averaged to determine the total number of cells. Consistent with fetal tissue staining, three nuclear types, cigar, oval and spherical shaped were

identified.

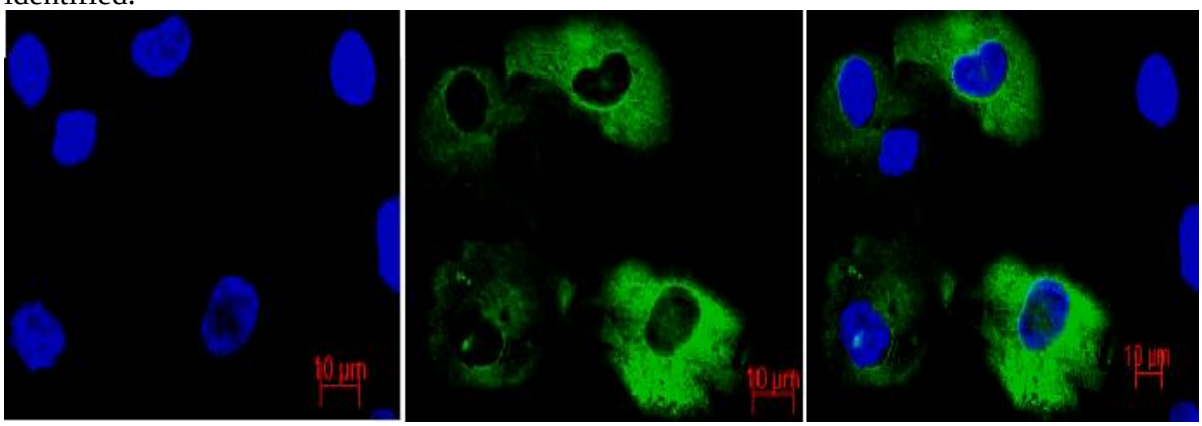


Figure 8: A fetal human colon cell line stained with anti-ALDH1 and DAPI. Blue is the nuclear stain DAPI and green is ALDH. Images were taken with a Zeiss Axio Observer.Z1flourecense microscope with a Plan- Apochromat 20X/0.8 objective.

2.4 DISCUSSION:

It is known that stem cells divide via mitosis and give rise to stem cells and progenitor cells. Dr. Elena V Gostjeva and her colleagues have found metakaryotic cell types in humans and rodents having a bell-shaped nuclear morphology and representing a putative type of stem cell lineage present in fetal organogenesis. Their findings suggested that these cell types can divide both symmetrically and asymmetrically but not via regular mitotic cell division. Consequently, cells dividing non-mitotically with bell-shaped nuclei were denoted as metakaryotes. This nuclear morphology was observed in sarcomeric tubular syncitia of organ anlagen between 5 and 12 weeks of human gestation. These metakaryotic cells with bell shaped nucleus were also found at the base of colonic crypts in juvenile, adults, pre-neoplastic adenomas, as well as in adenocarcinoma and in liver metastasis. The numbers of cells with a bell shaped nucleus were found to increase with tumorigenic progression from normal to carcinoma as was the case for stem cells dividing mitotically (Gostjeva, Koledova et al. 2009)**.**

Our results show that adult human colon (normal and carcinoma) tissues stained with Giemsa have very few bell-shaped nuclei (Figure 1). We had hypothesized that the number of stem cells increases in colon from normal to carcinoma and that the number of bell-shaped nuclei would also increase. But no increase in bell-shaped nuclei was observed.

The fetal colon cell line and fetal tissue served as a positive control for the presence of bell shaped nuclei in early stage of organogenesis. If cells with bell-shaped nuclei were detected, this finding would have supported the possibility that this nuclear morphology serves as a stem cell marker. Strong cytoplasmic staining for the stem cell marker ALDH1 was observed in the fetal colon cell line and at the

bottom of the crypt in fetal tissue. This finding indicated that these fetal tissues contain stem cells. But no nuclei resembling bell shaped morphology or amitotically dividing cells were detected in any of the fetal tissues in our experiments using standard histologic methods. However, mitotically dividing cells – ones with a mitotic spindle- were detected in fetal tissues. Most mitotic cell divisions were located at the base of the crypt in slides containing 30 micron thick section and a few mitotic cells were found in 16 micron thick. Sixteen micron sections are optimal for the ALDH1 staining to work in mouse tissue but have less depth than 30 micron section; probably that's why fewer mitotic cell divisions were seen at the base of the crypt. No amitotic cell divisions were detected at the base of the normal crypt or in carcinoma were observed using standard histologic methods.

In our experiments on adult human colon using Dr. Gostjeva's protocol, a few bell shaped nuclei were observed but no amitotic cell divisions were found. The general absence of bell shaped nuclei and amitotic cell divisions in our experiments (Figure 2, 3 and 5) make the presence of the nuclear morphology questionable. Two different techniques were used to confirm the data obtained using Carnoy's fixative and Giemsa staining. An immunofluorescence technique, as mentioned in Materials and Methods (1.2.2 and 1.2.3), was used for ALDH1 staining, which does not involve any harsh treatment with chemicals, and should have been ideal for detecting metakaryotic cells.

The question arises why bell shaped nuclei are seen using one procedure and not with the other approaches used. The differences in experimental findings might be explained by different effects of tissue preparation on nuclear shape and size

and chromosome organization. Eukaryotic cells are mostly uni-nuclear surrounded by nuclear envelop (NE) that separates the chromatin in the nucleus from the cytoplasm. The nuclear membrane is made of a meshwork of proteins called lamins. These lamins are intermediate filaments and are of two major types-A and B. These lamins are farnesylated thereby making them able to attach to the inner nuclear membrane. Thus they may play a role in organization of the nucleus. Experimental evidence indicates that the nuclear lamina maintains the spatial organization during interphase of chromosomes which have discrete location and are not randomly placed. The non random placement of chromosomes is linked with gene density. Chromosomes that are gene rich are preferentially located on the interior side of the nucleus, whereas chromosomes with a lower gene density are located to the outer side and locate to the nuclear periphery (Nathan, Baker et al.), (Dahl, Ribeiro et al. 2008).

It has been seen in many different cell types that changes in nuclear shape are dependent on changes in nuclear lamina. Nuclear shape can also be changed due to changes in force from the cytoplasm. While the nucleus is the stiffest organelle in a cell, its shape can be altered by mechanical stress from the extracellular environment through the cytoskeleton. Mechanical forces change nuclear structure, cell signaling and gene expression. Cells can withstand these mechanical stresses up to a certain extent, but prolonged mechanical stress can lead to severe stress on the cell and thus lead to changes in nuclear shape (Nathan, Baker et al.), (Dahl, Ribeiro et al. 2008).

Cells with an abnormal nucleus are also observed in disease states like in cancer where lamina proteins are mutated or even in normal aging where the lamina protein level decreases. Alterations in nuclear shape have also been seen in cancerous cells. The exact mechanism of how these changes occur is unknown, but it has been

proposed that cancerous cells lose nuclear stiffness and experience changes in their nuclear morphology which increases their metastatic potential (Dahl, Ribeiro et al. 2008).

So finding these bell-shaped nuclei structures using the Gostjeva protocol may be explained by the fact that her protocol involves the tissue maceration step, which must cause mechanical stress on the cells. This mechanical stress puts pressure on the plasma membrane and cytoplasm, which then affects the cytoskeleton and nuclear lamina. Changes in nuclear lamina can lead to rearrangement of interphase chromosomal organization and cause change in nuclear shape. When using the Gostjeva protocol, the frequency of bell shaped nuclei observed may depend on the state of rigidity of the nucleus. As mentioned above, some conditions such as aging and cancer conditions lead to a decrease in nuclear lamina; this may be the reason why we see few bell shaped nuclei in adult tissues. In situations, such as cancer, where cells lose nuclear rigidity, number of these bell-shaped nuclei would increase. A decrease in nuclear rigidity would help these cells squeeze through tight spaces and invade tissues as in the case of metastasis. It has been reported that stem cells are more sensitive to mechanical tension than terminally differentiated fibrochondrocytes. A study on scaffold architecture (random vs non random organized fiber) and deformation of nuclear structures by tension showed that tension increased alteration of nuclear morphology of stem cells. This explanation may be the answer to why Gostjeva et al. (2009) found bell shaped nuclear structure in stem cells that are exposed to mechanical tension and sensitive to mechanical stress (Nathan, Baker et al.).

Further study needs to be conducted to understand the nuclear structure of stem cells and organization and its effect on the properties of stem cells. It would be a very interesting finding if these bell shaped nuclei that are thought to have stem cell properties can be detected via other techniques and also if their properties can be studied widely.

Chapter 3

STUDY OF THE EXPRESSION LEVEL OF RETNOID RECEPTORS AND OTHER MEMBERS OF RETINOID SIGNALING PATHWAY IN COLON CANCER LINES AND NORMAL AND CANCER TISSUES

3.1 INTRODUCTION:

Retinoic acid (RA) is a metabolite of vitamin A. It modulates a wide range of processes such as cell proliferation, differentiation, development and apoptosis. Retinoic acid signaling is dependent on how much RA is transported from blood to each cell and the amount taken up by the cell. Along with the presence of RA in the cell, the receptors that mediate all the RA signaling are the retinoic acid receptor (RAR) and retinoic X receptor (RXR). Both the receptor types belong to the nuclear receptor family and are primarily activated when retinoic acid derivatives are present as ligand. Both RXR and RAR have three isoforms-alpha, beta and gamma- and all of them play important roles in RA signaling. These receptors need to heterodimerize or homodimerize to be active. RXR can heterodimerize with thyroid, vitamin D and other receptors and RAR can heterodimerize with RXR, PML, PPARɣ and some other receptors (Mongan and Gudas 2007). Two biologically active derivatives of retinoic acid that can act as ligand are all-trans retinoic acid (the carboxylic form) and 9-cis retinoic acid. RAR's become activated by both all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cis RA) and RXR's becomes activated only by 9-cis retinoic acid (Egea, Mitschler et al. 2000).

RXR receptor family can dimerize in three ways to produce three different type of signaling mechanism. RXR can homodimerize with other RXR subtypes, ligand binding leads to activation of receptor to recruit co-activators like histone

acetyltransferase and induces transcription of downstream genes. When RXR heterodimerizes with RAR receptor subtype or with some other receptor types, it is called non-permissive heterodimerization. This is because even if the ligand is bound to RXR, there is transcriptional activation of downstream genes only when the ligand for RAR is present and binds to it. On the other hand, in permissive heterodimerization RXR dimerizes with PPAR (Peroxisome proliferator-activated receptor), LXR (Liver X Receptor) and some other receptor types, and ligand binding to any of the receptor types activates the transcription of downstream genes. Both receptors can also get activated together, leading to a synergistic effect ((Martin, Lardeux et al. 2005), (Cheung, Bell et al. 2006)). But in case of RAR subtype, ligand binding is crucial for replacement of co-repressor with co-activator and activation of downstream targets (Marletaz, Holland et al. 2006).

The systemic level of ATRA is higher than that of 9-cis RA, so ATRA can be responsible for a lot of the retinoic acid signaling in our body. RA signaling also plays role in cell cycle arrest by increasing p21, p27 and CDKI expression (Suzui, Masuda et al. 2002; Suzui, Shimizu et al. 2004),(Wang, Barsky et al. 2002). It also increases cyclin D1 proteosomal degradation increasing cell turnover without altering the mRNA level of cyclin D1. With a decrease in activity of cyclin D1- CDK activity, the retinoblastoma (Rb) tumor suppressor protein cannot be hyperphosphorylated. Thus E2F transcription factor cannot be released, which then restricts cells from transitioning from G1 phase to S-phase. In this way retinoic acid maintains a check on cell cycling and maintains cell growth and signaling (Mongan and Gudas 2007), (Wang, Barsky et al. 2002).

Retinol is the form of vitamin A that circulates in the blood and is taken up by the cells via STRA 6 receptors. Inside the cell, retinol can be esterified by LRAT (lecithin:retinol acyl trasferase) and converted to retinyl ester or can be converted to retinaldehyde by RDH (retinaldehydrogenase) by a reversible process. Retinaldehyde is then converted to ATRA (all-trans retinoic acid) by RALDH1 (Retinaldehyde dehydrogenase type 1) by an irreversible process. ATRA is then transported from cytoplasm to the nucleus where it acts as a ligand and binds to the retinoid receptors, which leads to activation of downstream genes. CYP26A1, a cytochrome P450 enzyme metabolizes RA in the cytoplasm and breaks it down into 4-OH-RA or 4-OXO-RA. CtBP1 (C-terminal binding protein 1), a transcriptional repressor, has been found to play an important role by binding with APC to degrade nuclear beta-catenin and thus decrease TCF/beta-catenin binding (Hamada and Bienz 2004). CtBP1 is high in colon adenocarcinoma. It also inhibits the transcription of RDH. Loss of functional APC protein due to mutation not only leads to increased nuclear beta-catenin and cell proliferation; it also affects other signaling molecules: dysregulation of CtBP1 and increase in K-ras expression. Increase in intestinal cell proliferation is due to accumulation of beta-catenin in the nucleus and activation of K-ras/RAF1 signaling, which leads to adenoma formation. APC degrades CtBP1 and also degrades beta-catenin. Beta-catenin on the other hand degrades CYP26A1. Thus, in the case of colorectal cancers in which APC is mutated, CtBP1 and CYP26A1 expression are high. Indeed, retinoic acid signaling has been found to be aberrant in colorectal cancer. In colorectal cancer, colonocytes are not able to take up retinol and they become RA deficient. Due to decreased RA, the retinoic acid receptors cannot be

activated, and, thus, retinoic acid signaling is compromised as well (Mongan and Gudas 2007).

Retinoic acid signaling is aberrant in many other types of cancer. It was first reported by Hu et al (9) for oral and epidermal human squamous cell carcinoma cell lines versus normal human epidermal keratinocytes in culture, and by Gebert et al (Yamazaki, Shimizu et al. 2007) for human lung cancer. The later found that retinoic acid signaling is aberrant due to hypermethylation of the RAR beta 2 promoter. Subsequent hypermethylation of the RAR beta 2 promoter was found in head and neck cancer and in breast cancer etc as well (Youssef, Lotan et al. 2004). In APL (Acute Promyelocytic Leukemia) the RAR alpha receptor is mutated via PML-RAR alpha fusion proteins.

A study by Yamazaki et al. (2007) suggested that the phosphorylated form of RXR alpha (p-RXR alpha) is associated with colorectal cancer. It was found that p-RXR alpha accumulates in colorectal cancer and loses its ability to cause cell cycle arrest. When the colorectal cancer cell line Caco 2 was treated with 9-cis RA and a pPAR gamma agonist in the presence of MEK inhibitor, there was a decrease in expression of p-RXRalpha followed by inhibition of cell growth and increased apoptosis (Yamazaki, Shimizu et al. 2007). A study of hepatocellular carcinoma showed similar malfunctions of RXR alpha. It was observed that p-RXR alpha loses its ability to dimerize with RXR or other retinoid receptors, which results in loss of control over cell growth and thus accelerates tumor development (Yoshimura, Muto et al. 2007). Thus both RXR alpha and RAR alpha play major roles in maintaining proper retinoic acid signaling in human colon.

Cytosolic ALDH1, which converts retinaldehyde to ATRA, has three major isoforms in humans: ALDH1A1, ALDH1A2 and ALDH1A3. ALDH1 is not just a stem cell marker in several tissues, but also, it has capability of self protection and differentiation. Studies conducted in mouse by Elizondo et al. in 2000 and 2009 have shown that ALDH1 transcription is dependent on the presence or absence of retinoic acid in the cell (Elizondo, Corchero et al. 2000), (Elizondo, Medina-Diaz et al. 2009). ALDH 1, which converts retinaldehyde to ATRA, is an important regulator of retinoic acid pathway. A decrease in cellular retinoic acid, leads to transcription of ALDH 1 due to binding of the RAR alpha and the CCAAT/ Enhancer binding protein beta, which increases the oxidation of retinaldehyde to ATRA, thus increasing retinoic acid signaling. High levels of ATRA also have an inhibitory effect in ALDH1. A high cellular level of retinoic acid has a negative feedback effect on ALDH1 transcription, and thus decreases its expression (Ma and Allan). As mentioned in the introduction and Chapter 1, ALDH1 in colorectal cancer is a promising cancer stem cell marker.

"Dysregulation of retinoic acid signaling is involved in colon cancer progression and treatment of colorectal cancer cell lines with retinoic acid derivatives induces inhibition of cell growth."

To study the above hypothesis, I propose the following aim: "*To determine the expression level of retinoid receptors and other members of retinoid signaling pathways in colon cancer cell lines and in normal and cancer tissues."*

3.2 MATERIALS AND METHODS:

3.2.1 Cell line maintenance:

SW480 cells are grown in L-15 media and HT29 cells are grown in MacCoy's media (purchased from Cellgro) with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics. SW480 cells cultured without 5% carbon dioxide and HT29 cells grown with 5% carbon dioxide. FHC cells are grown in DMEM F: 12 media, both cell line and media purchased from ATCC. Additives added to DMEM F: 12 media are 0.005mg/ml insulin, 0.005mg/ml transferring, 100ng/ml hydrocortisone, 10ng/ml cholera toxin, extra 10nM HEPES and 10% fetal bovine serum with 5% carbon dioxide. With 10% fetal bovine serum and 1% penicillin/ streptomycin antibiotics with 5% carbon dioxide.

3.2.2 Immunocytochemistry (ICC)

Two adult human colon adenocarcinoma cell lines SW480 and HT29 cells, and one fetal human colon cell line (FHC) were used. The SW480 and HT29 cells were between passages 6-10, the FHC cells between passages 4-10. ICC was done in 8-well chamber slides bought from Lab tek II. SW480 and HT29 cells were fixed with 4% paraformaldehyde for 40 minutes and then, based on the antibody used the protocol changed. For ALDH1, the cells were then blocked with 1% BSA, 10% serum, 0.2% triton and PBS. For RXR alpha, RAR alpha and CtBP1, 0.2% triton extraction for 20 minutes was done first and the cells were then blocked with 1% BSA, 10% BSA and PBS. Blocking was done overnight at 4 degrees, followed by overnight primary antibody incubation at 4 degrees. Primary antibody was washed off using blocking buffer three times for 10 minutes each time. Secondary antibody,

Alexa flour goat anti-rabbit, was used at a dilution of 1:1000 and incubated at room temperature for 1.5 hours. After incubation with secondary antibodies, three washes were done with blocking buffer for 10 minutes. Cells were dried and later stained with SlowFade® Gold antifade DAPI and sealed with coverslips.

Table 2: Anitbodies used for immunocytochemistry

Antibody	Company	Dilution	Positive control cell line
ALDH	Abcam and BD	1:50	HepG2
RXR alpha	Santa Cruz	1:50	Hela
RAR alpha	Santa Cruz	1:50	MCF7

The table shows all the antibodies for ICC, the company from which they were purchased, the dilution used and also the respective positive controls.

3.2.3 Western blot:

Western blot analysis was performed with HT29, SW480, FHC cells and three matched normal and tumor pairs. SW480 and HT29 cells were between passages 6-10; FHC cells were between passage number 4-10. Normal human colon samples and carcinoma samples were received from Christiana Care Hospital after patient consent under ***IRB*** approval. Polyaccralamide gels (10%) bought from Lonza, Bio-rad gel electrophoresis apparatus and 1X SDS gel running buffer were used for running

the proteins. Proteins first ran at ninety seven volts until they came out of the wells and were saturated. Then the voltage was increased to 130 volts and ran the gel for one hour. The transfer was done using a wet transfer apparatus from Invitrogen, PVDF membranes, and wet transfer buffer (for 1L, 14.4g of glycine, 5.4 g of Tris base and 200 ml of methanol were dissolved in DI water). Transfer was done at 23 volts, 253mA for one hour at room temperature. To keep the water cool, transfer was done on a stir plate. For ALDH1, RXR alpha and CtBP1, membranes were blocked with 3% BSA overnight at 4 degrees on a shaker. For CYP26A1 and RAR alpha membranes were blocked with 5% milk overnight at 4 degrees on a shaker. Both primary and secondary antibodies were prepared in 3% BSA for ALDH1, RXR alpha and CtBP1 and in 5% milk for CYP26A1 and RAR alpha. Overnight primary antibody incubation was done at 4 degrees on a shaker. The primary antibody was washed three times with 0.1% TBST for 10 minutes each time. Secondary antibody was also made in 3% BSA for ALDH1, RXR alpha and CtBP1 and in 5% milk for RAR alpha and CYP26A1. Secondary incubation was done for 1 hour at room temperature on a shaker. Again we washed three times with 0.1% TBST for10 minutes. SuperSignal West Dura Chemiluminescent Substrate was used for horseradish peroxidase (HRP) activity from antibodies.

3.2.4 Protein extraction:

Cells were trypsinized to obtain a pellet and RIPA buffer was added. RIPA buffer was made with 0.1% triton, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl and 150 mM NaCl. Protease inhibitor was added to the RIPA just before

protein extraction. Cells were then vortexed to break up the pellet and incubated on ice for 0.5 h. Proteins were stored at -20° C. Repeated freeze thaw was avoided.

Table 3: Antibodies used for immunobloting

Antibody	Company	Primary antibody Dilution	Secondary antibody Dilution	Positive control cell line
ALDH	Abcam	1:300	1:20,000	HepG2
RXR alpha	Abcam	1:500	1:20,000	MCF7
RAR alpha	Santa Cruz	1:800	1:20,000	MCF7
CYP26A1	Abcam	1:500	1:20,000	Hela
CtBP1	Abcam	1:500	1:20,000	Hela

Listed are the antibodies for western blots, the company from which they were purchased, the dilutions used and the respective positive controls.

3.2.5 Table 4: Patient Samples

Matched tissue	**Patient 1**	**Patient 2**	**Patient 3**	**Patient 4**
Normal	**10224**	**10332**	**10431**	**10554**
Tumor	**10223**	**10331**	**10430**	**10553**
Age	**66**	**62**	**52**	**82**
Part	**Right colon**	**Splenic flexure**	**Transverse colon**	**Right colon**

Four matched normal and CRC tissue samples were collected from Christiana Hospital after patient consent under the IRB approval. Both male and female patients were included. Their ages ranged between 50 and 80 years. The protein samples were made in RIPA buffer with protease cocktail and stored at -20°C.

3.3 RESULTS:

3.3.1 Immunocytochemistry results:

A

B

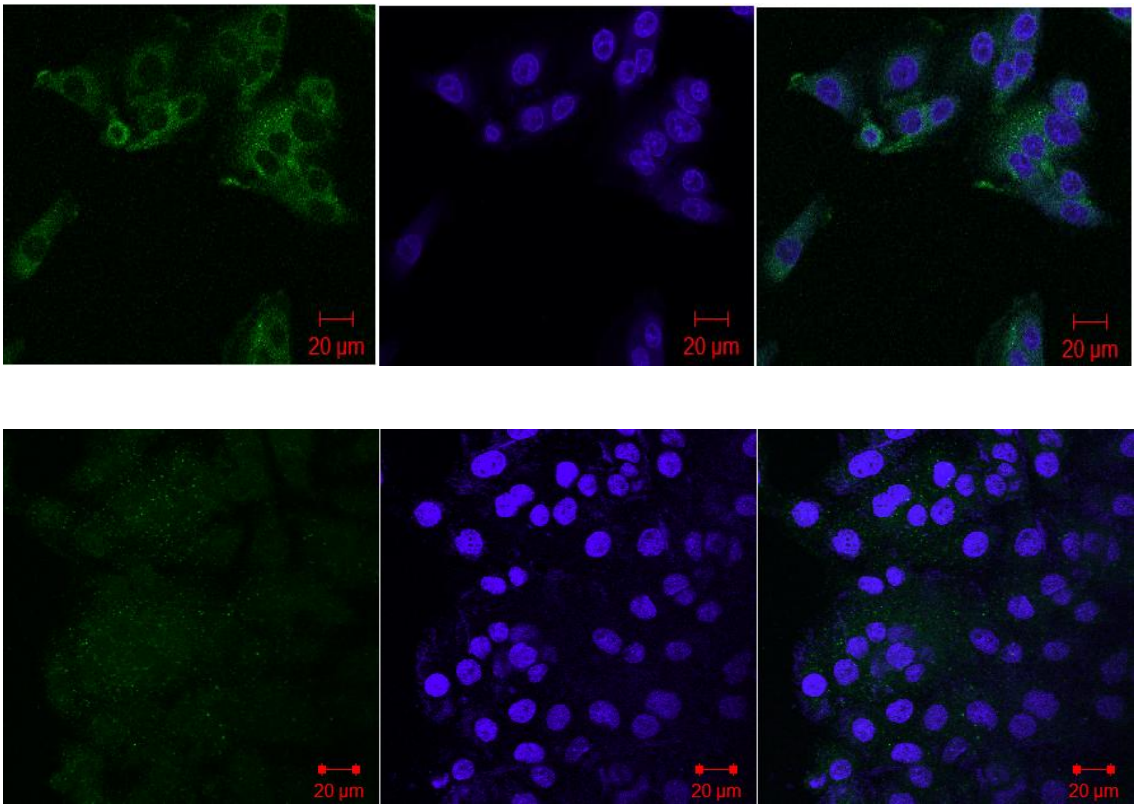


Figure 9: SW480 cells stained with anti- ALDH1 and DAPI. A) The first panel (green) shows ALDH1 staining and second panel (blue) shows nuclear DAPI staining and third panel shows the merged images. B) Shows rabbit IgG in first panel and DAPI staining in second panel followed by merged image in third panel. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a C-Apochromat 40X/1.20 W Korr-VIS-IR objective. For better contrast, false color green was chosen in the confocal software for antibody and IgG labeling.

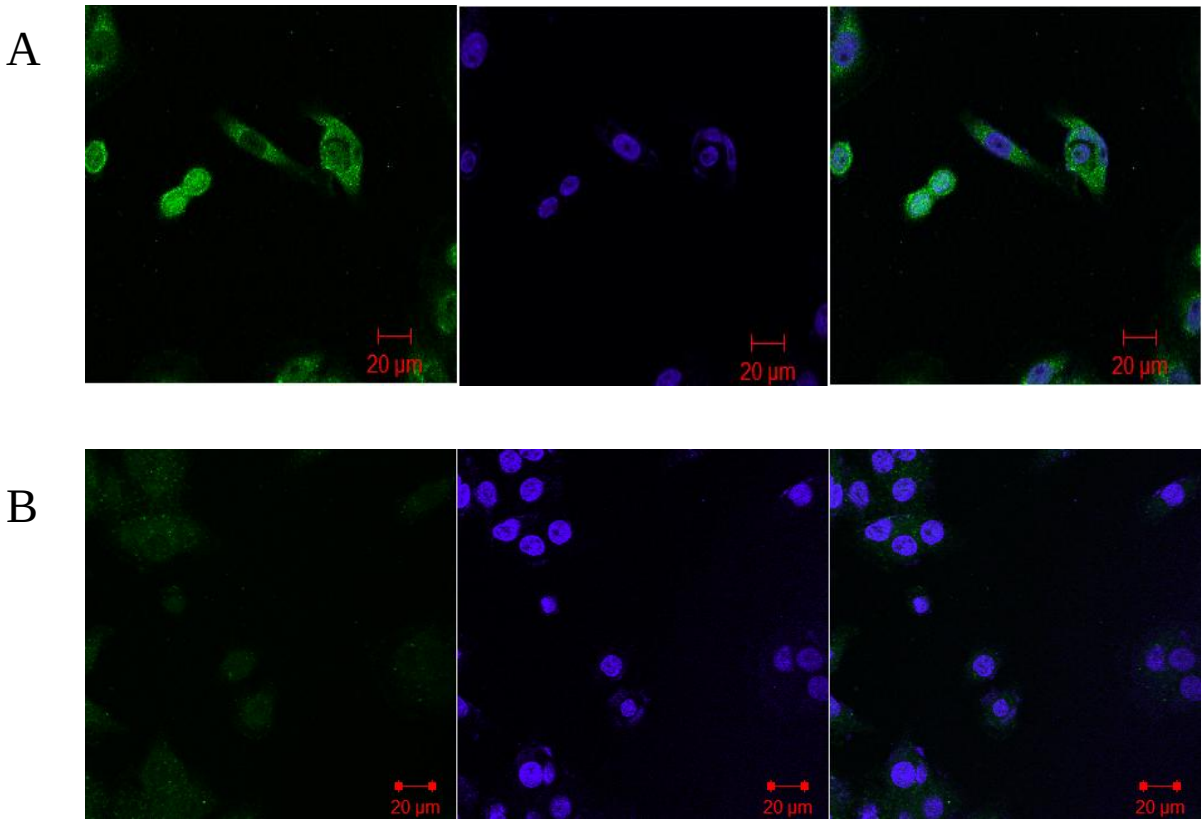


Figure 10: SW480 cells stained with anti-RXR alpha and DAPI. A) The first panel (green) shows RXR alpha staining and second panel (blue) shows nuclear staining DAPI and third panel shows the merged images. B) Shows rabbit IgG in first panel and DAPI staining in second panel followed by merged image in third panel. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a C-Apochromat 40X/1.20 W Korr-VIS-IR objective. For better contrast, false color green was chosen in the confocal software for antibody and IgG labeling.

A

B

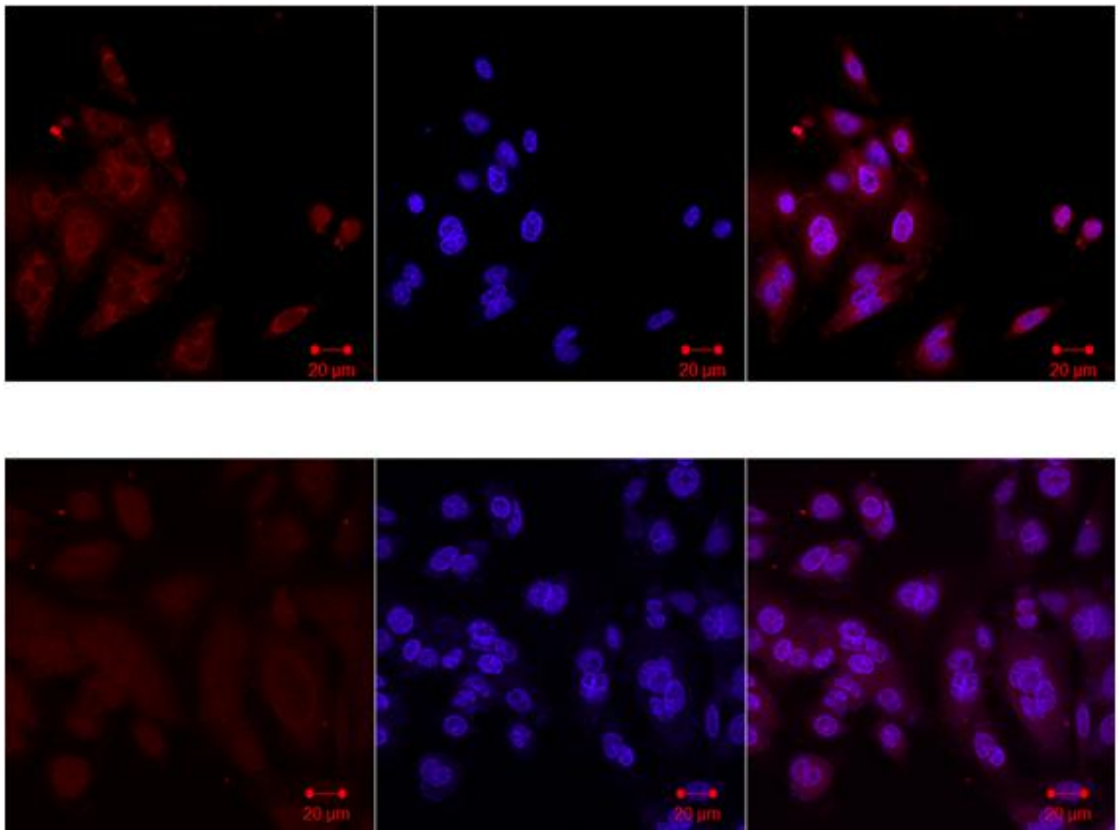


Figure 11: SW480 cells stained with anti-RAR alpha and DAPI. A) The first panel (red) shows RAR alpha staining. The second panel shows nuclear DAPI staining and third panel shows the merged images. B) Shows rabbit IgG in first panel and DAPI staining in second panel followed by merged image in third panel. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a C-Apochromat 40X/1.20 W Korr-VIS-IR objective.

A

B

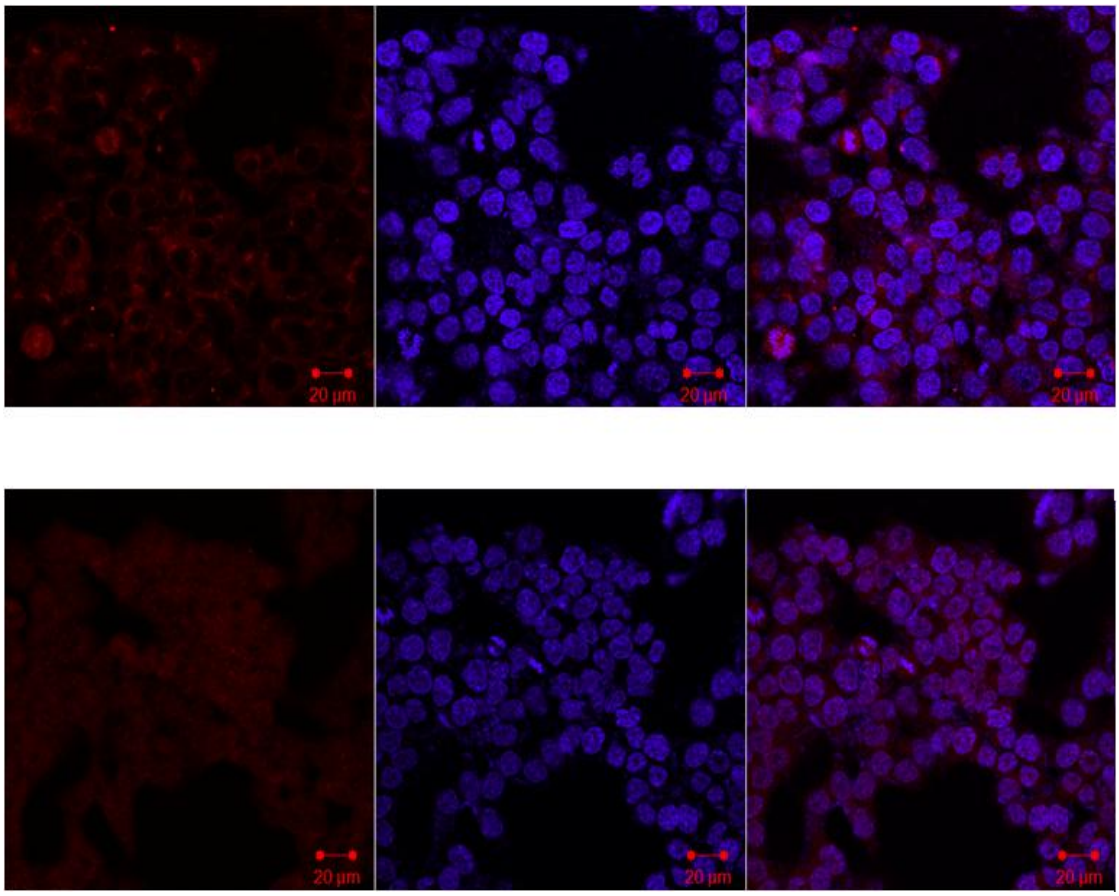


Figure 12: HT29 cells stained with anti-ALDH1 alpha and DAPI. A) The first panel (green) shows ALDH1 staining and second panel (blue) shows nuclear DAPI staining and third panel shows the merged images. B) Shows rabbit IgG in first panel and DAPI staining in second panel followed by merged image in third panel.Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a C-Apochromat 40X/1.20 W Korr-VIS-IR objective.

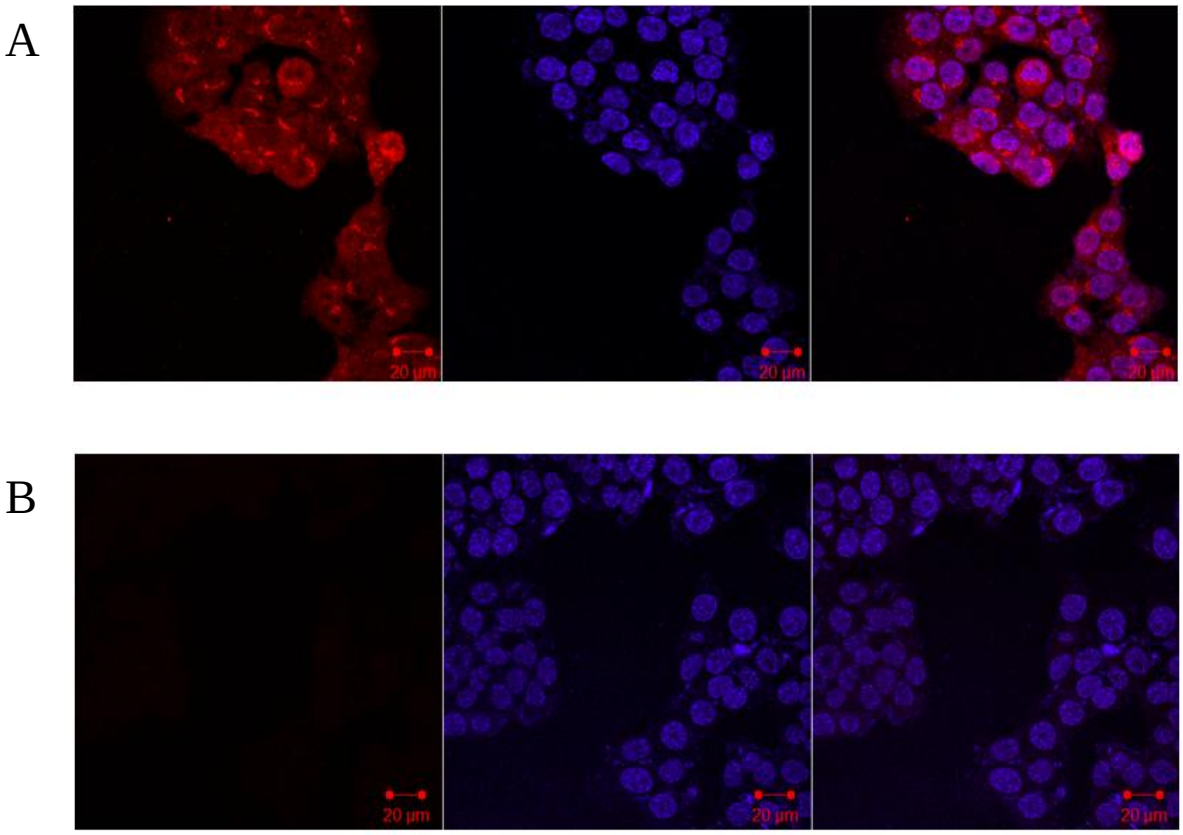


Figure 13: HT29 cells stained with anti-RXR alpha and DAPI. A) The first panel (red) shows RXR alpha staining. The second panel (blue) shows nuclear DAPI staining. The third panel shows the merged images. B) Shows rabbit IgG in first panel and DAPI staining in second panel followed by merged image in third panel. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a C-Apochromat 40X/1.20 W Korr-VIS-IR objective.

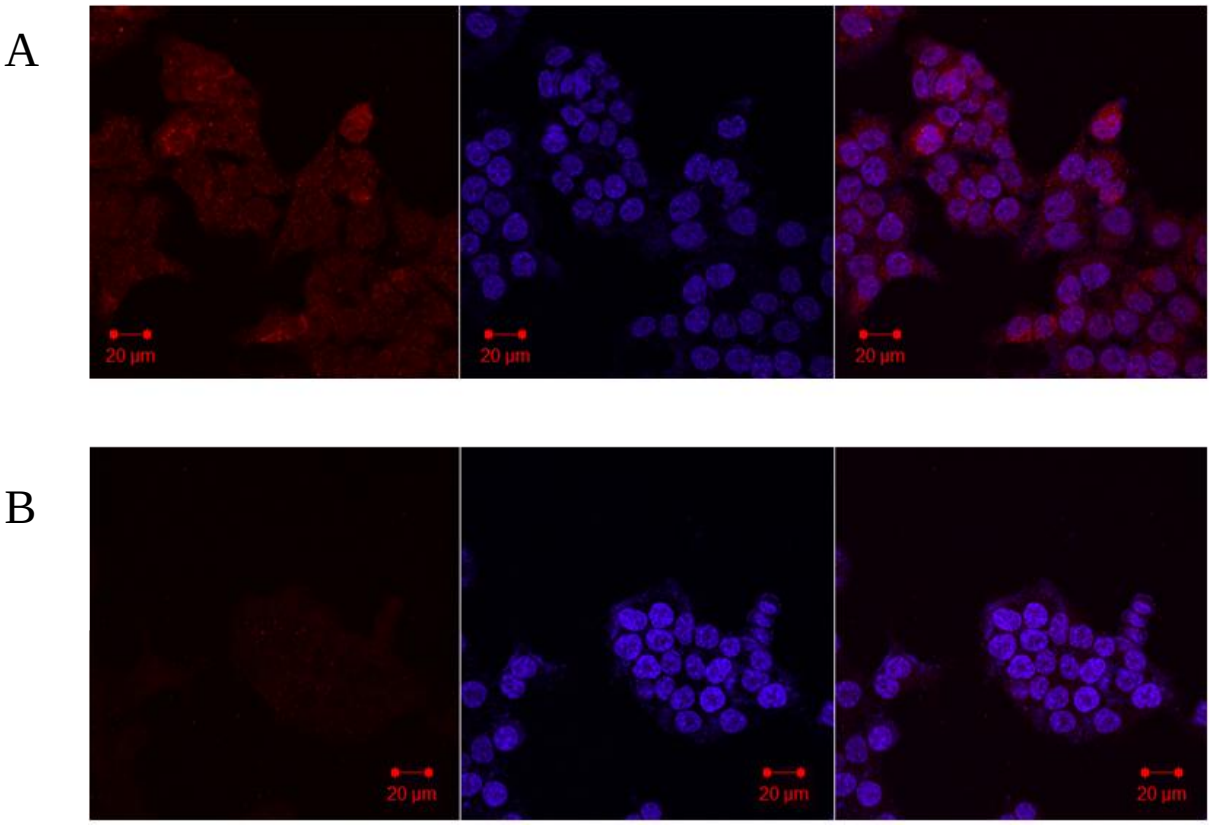


Figure 14: HT29 cells stained with anti-RAR alpha and DAPI. A) The first panel (red) shows RAR alpha staining and second panel (blue) shows nuclear DAPI staining. The third panel shows the merged images. B) Shows rabbit IgG in first panel and DAPI staining in second panel followed by merged image in third panel. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a C-Apochromat 40X/1.20 W Korr-VIS-IR objective.

A

B

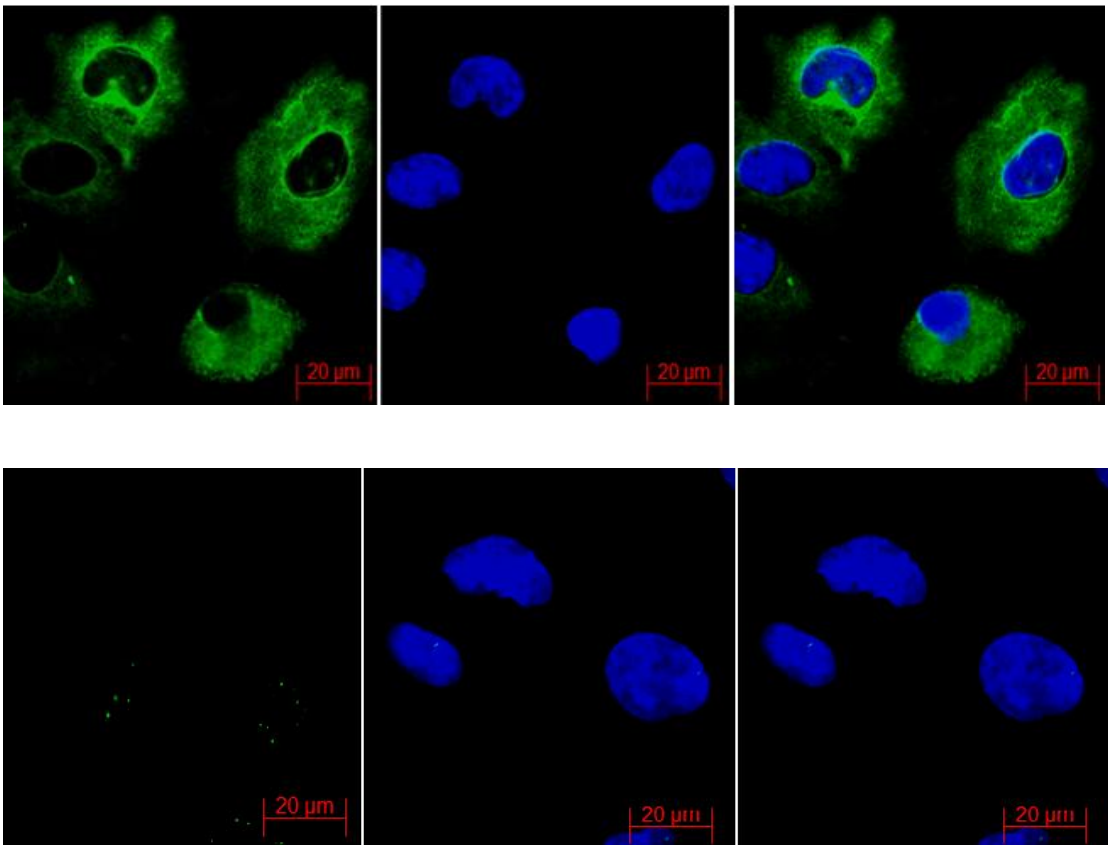


Figure 15: FHC cells stained with anti-ALDH1 alpha and DAPI. A) The first panel (green) shows ALDH1 staining and second panel shows nuclear DAPI staining. The third panel shows the merged images. B) Shows rabbit IgG in first panel and DAPI staining in second panel followed by merged image in third panel. Images were taken with a Zeiss Axio Plan-Apochromat 20X/0.8 objective.

A

B

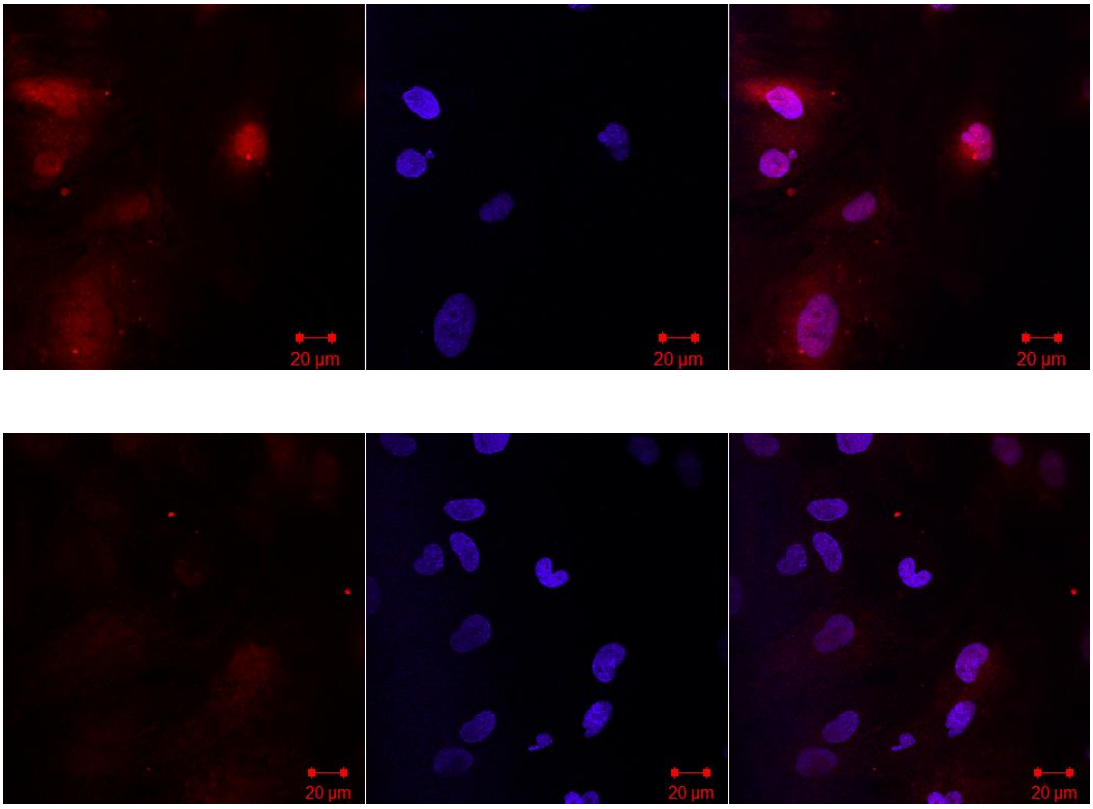


Figure 16: FHC cells stained with anti-RXR alpha and DAPI. A) The first panel (red) shows RXR alpha stain and second panel shows nuclear DAPI staining. The third panel shows the merged images. B) Shows rabbit IgG in first panel and DAPI staining in second panel followed by merged image in third panel. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a C-Apochromat 40X/1.20 W Korr-VIS-IR objective.

A

B

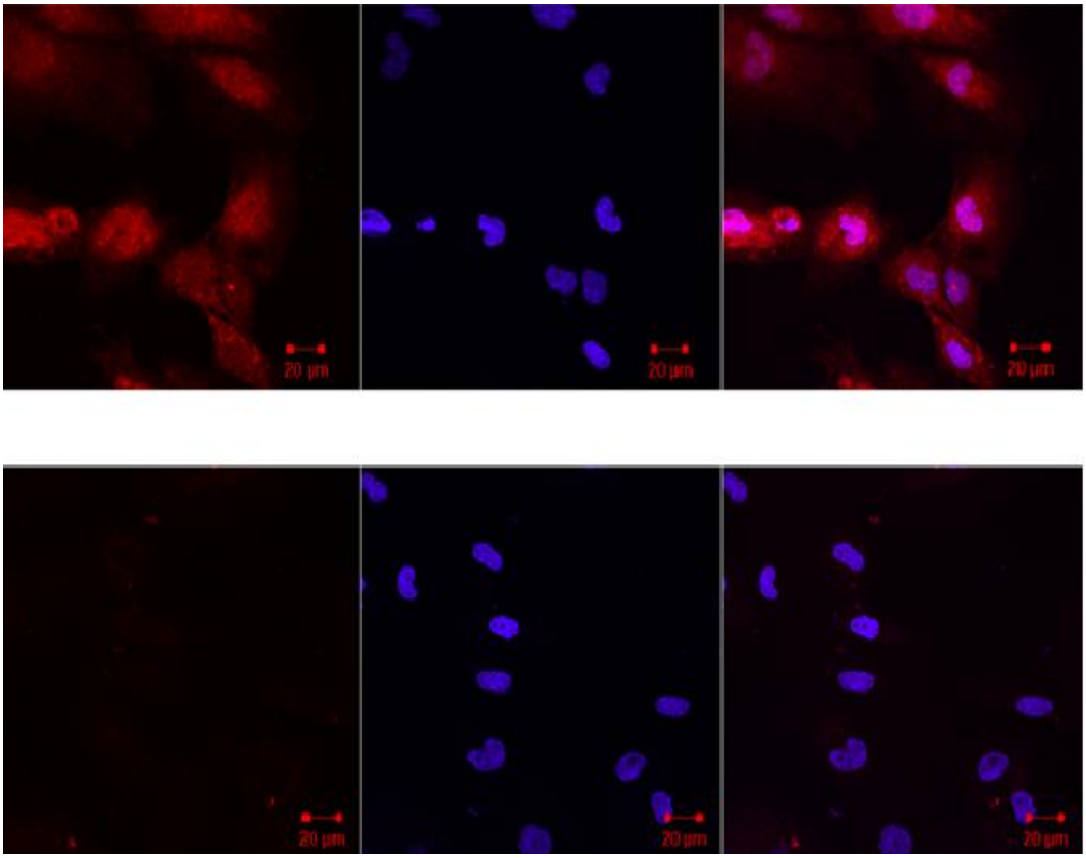


Figure 17: FHC cells stained anti-RAR alpha and DAPI. A) The first panel (red) shows RAR alpha stain and second panel shows nuclear DAPI staining. The third panel shows the merged images. B) Shows rabbit IgG in first panel and DAPI staining in second panel followed by merged image in third panel. Images were taken with a Zeiss LSM 5 LIVE High Speed Confocal microscope with a C-Apochromat 40X/1.20 W Korr-VIS-IR objective.

3.3.2 Quantification of ICC

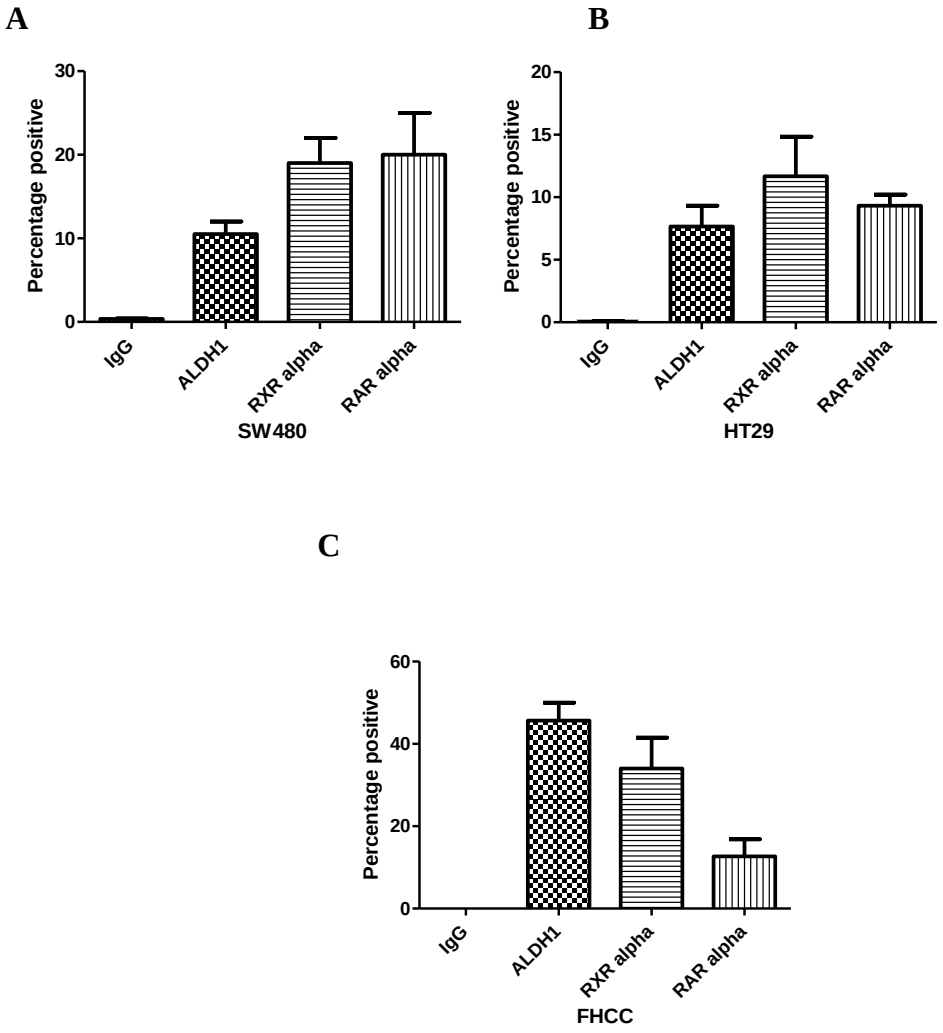
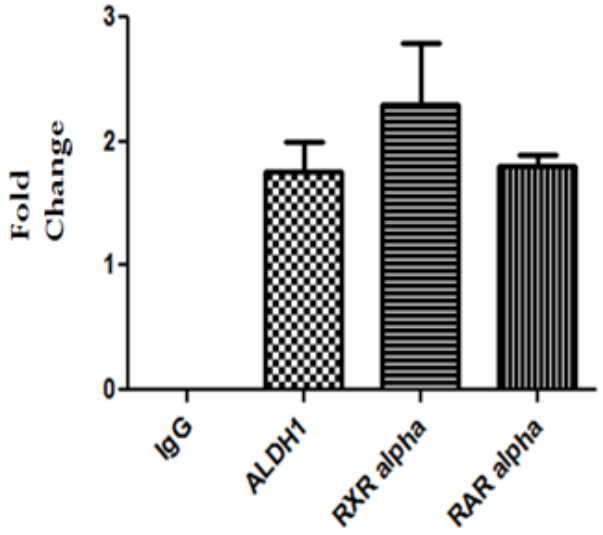


Figure 18: Relative expression of ALDH1, RXR alpha and RAR alpha in SW480, HT29 and FHC cells. A) Shows percent positive cells for anti-ALDH1, anti-RXR alpha and anti- RAR alpha in SW480 cells. Figure B and C also shows the percent positive cells for the same proteins for HT29 and FHC cell lines respectively. Normalization done with IgG control. All quantification done based on three individual experimental sets. Statistical analysis done with unpaired t-test, positive cells vs IgG for each antibody. A p-value less than 0.05 was considered significant.

Graph showing fold change of SW480 over HT29 cells from ICC staining



Fold change of SW480 over HT29 cells from ICC	
Protein	**SW480/HT29 Ratio**
ALDH1	2
RXR alpha	2.3
RAR alpha	2

Figure 19: The graph represents ALDH1, RXR alpha and RAR alpha fold change in SW480 cells over HT29 cells. Error bars represents ± SEM. The table in the right shows the fold change values for all three proteins. SW480 cells have 2 fold higher ALDH1, RXR alpha and RAR alpha expression compared to HT29 cells. Fold change calculated by SW480 positive cells over HT29 positive cells for each antibody.

3.3.3 Western blots results:

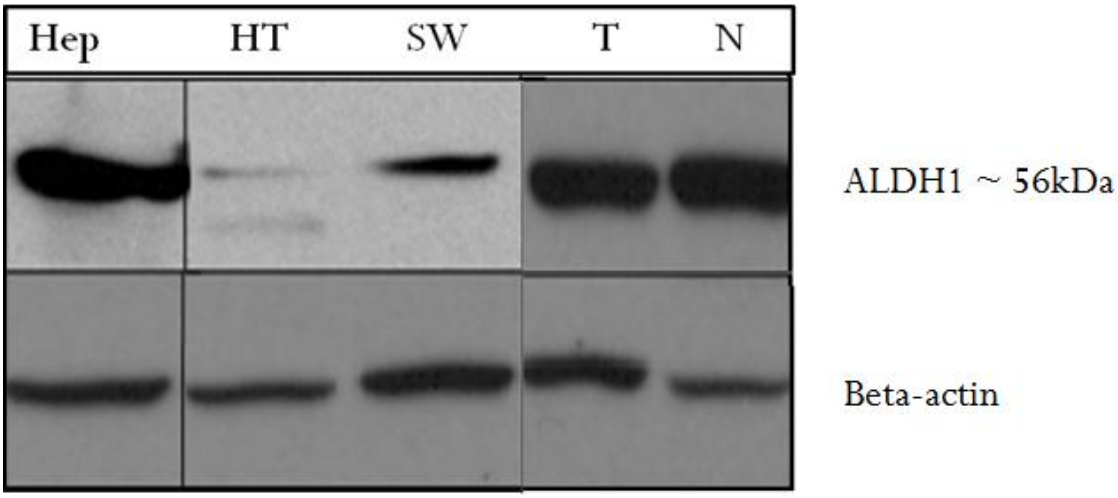


Figure 20: Western blot analysis done with a total cell and tissue extract for ALDH1. Thirty µg of protein was loaded in each well. Membranes were probed for ALDH 1. We observed a band at 55 kDa. Results show that SW480 has a higher ALDH1 alpha level than HT29 cells. Both normal and tumor tissues expressed ALDH1 protein. (Note: SW= SW480 cells, HT= HT29 cells, T= tumor, N= Normal, Hep= HepG2)

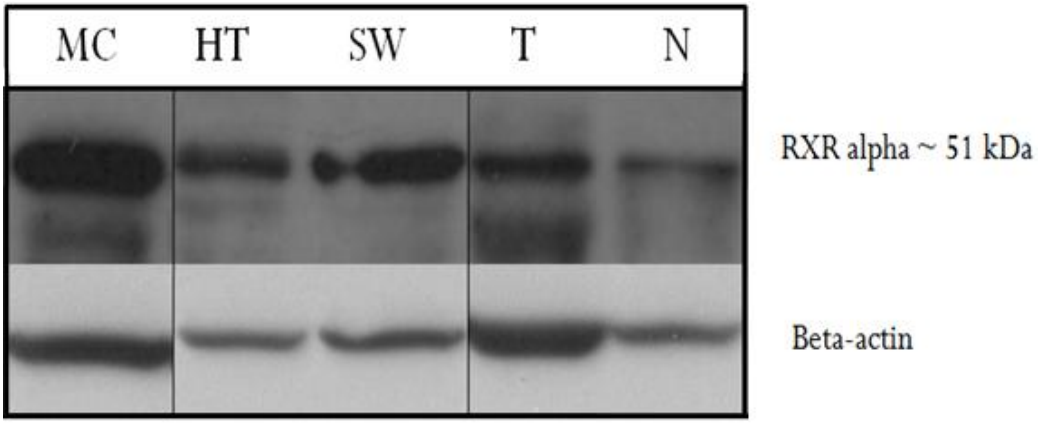


Figure 21: Western blot analysis done with a total cell and tissue extract for RXR alpha. Thirty µg of protein was loaded in each well. Membranes were probed for RXR alpha. We observed a band at 51 kDa. Results show that SW480 has a higher RXR alpha than HT29 cells. Both normal and tumor tissues expressed RXR alpha protein. (Note: SW= SW480 cells, HT= HT29 cells, T= tumor, N= Normal, MC= MCF7)

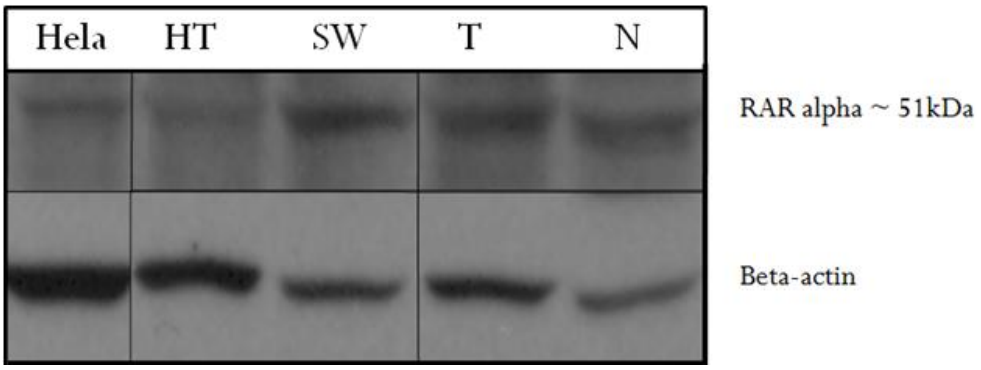


Figure 22: Western blot analysis done with total cell extract and tissue extract for ALDH1 for RAR alpha. Thirty µg of protein was loaded in each well. Membranes were probed for RAR alpha. We observed a band at approximately 51 kDa. Results show that both SW480 and HT29 cells expression RAR alpha but slightly higer in SW480 compared to HT29 cells. RAR alpha expression did not change between normal and tumor tissue. (Note: SW= SW480 cells, HT= HT29 cells, T= tumor, N= Normal)

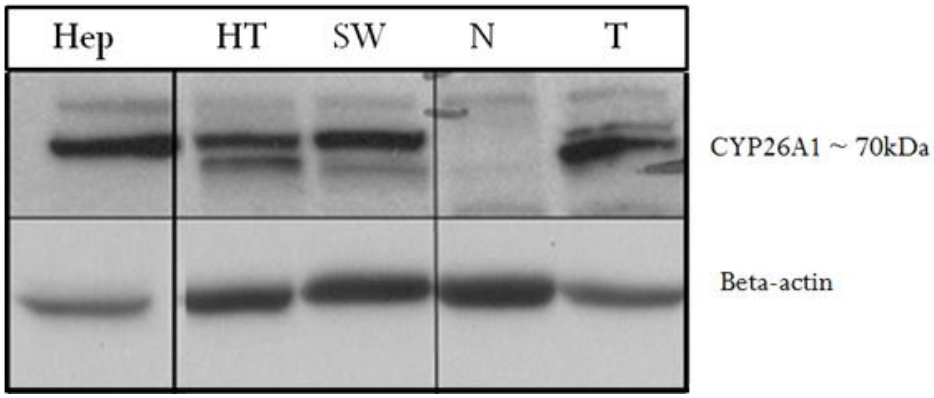


Figure 23: Western blot analysis done with total cell extract and tissue extract for CYP26A1. Thirty µg of protein was loaded in each well. Membranes were probed for CYP26A1. We observed a band at approximately 70 kDa. Results show that both SW480 and HT29 have high CYP26A1expression level. No or very minimal expression of CYP26A1 expression was observed in normal tissue, but, as expected, it was highly expressed in tumor tissue. (Note: SW= SW480 cells, HT= HT29 cells, T= tumor, N= Normal, Hep= HepG2)

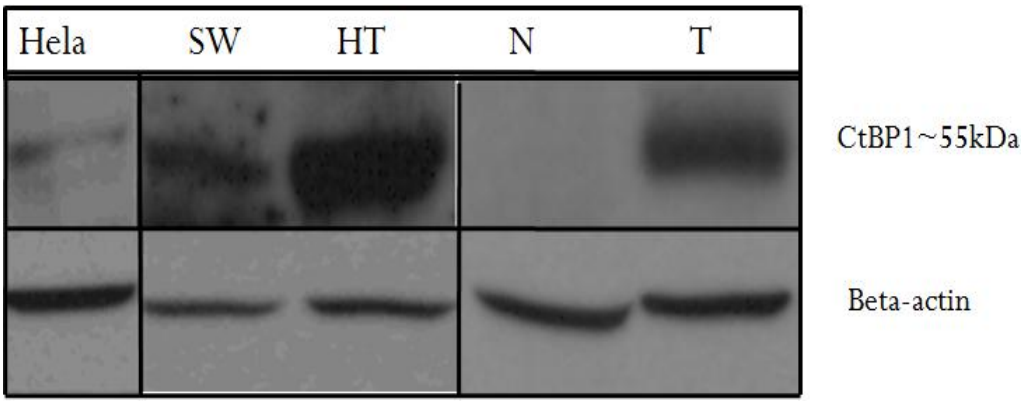


Figure 24: Western blot analysis done with total cell extract and tissue extract for CtBP1. Thirty µg of protein was loaded in each well. Membranes were probed for CtBP1. We observed a band at approximately 51 kDa. Results show that both HT29 cells have higher CtBP1expression level than SW480. Minimal expression of CtBP1 was observed in normal tissue, but, as expected, it was highly expressed in tumor tissue. (Note: SW= SW480 cells, HT= HT29 cells, T= tumor, N= Normal)

Table 5: Comparison of results from ICC and western blot analysis

Antibodies	ICC SW480/HT29 cells	Western blot SW480 / HT29 cells	Western Blot Normal N=4	Western Blot Tumor N= 4
ALDH1	↑	↑	No change	No change
RXR alpha	↑	↑	No change	No change
RAR alpha	↑	↑	No change	No change
CYP26A1	-	Comparable	No/minimal expression	↑
CtBP1	-	↓	No/minimal expression	↑

Table shows fold change results from ICC in second panel. Third panel shows ratiometric analysis of western blot analysis results on SW480 cells over HT29 cells for each antibody. Fourth and fifth panel shows western blot results to compare the level of each protein between matched normal and tumor tissue. Western blot results are obtained from densitometric analysis.

3.4 DISCUSSION:

My research findings have relevance to tumor biology because retinoic acid signaling is a major signaling pathway that modulates cell proliferation, differentiation and apoptosis. A lot of studies done in mice reveal that retinoic acid signaling is important for fetal development. A study by Schroeder et al (1998) and Tamagawa, M. et al (1995) in fetal mouse model reported that a retinoic acid is a key role in limb formation (von Schroeder and Heersche 1998). It also helps in differentiation and growth of different types of tissues including cartilage and bones during different stages of embryogenesis (Huang, Hsuuw et al. 2006). But a high level of RA can be fatal to the embryo (Ross-Innes, Stark et al.). As mentioned in chapter 1, the retinoic acid signaling involves two types of receptors-retinoic acid receptor (RAR) and retinoic X receptor (RXR). It is through ligand binding that the receptors become activated. Different isoforms of these receptors play a major role in cancer development by homodimerizing or heterodimerizing with other types of receptors. Under pathologic condition these receptors can become mutated and function abnormally to give rise to teratomas. RAR alpha induces estrogen receptor (ER) mediated cell growth and transcription and is also a target gene of the ER in breast cancer cells. RAR beta is downregulated in colorectal cancer (Fang, Wei et al.). N-COR a RAR co-repressor is upregulated in glioblastoma multiform patients, thus decreasing neural stem cell differentiation in these patients (Park, Li et al. 2007). RXRs are associated with different types of cancer including colorectal cancer. Not only do retinoid receptors play an important role in stem cell differentiation and normal homeostasis, other members of the RA pathway also play significant roles. ALDH1, enzyme which converts retinaldehyde to ATRA, CYP26A1 an enzyme which

metabolizes ATRA and CtBP1 which inhibits RDH expression are important for proper cell function and provide a check on the cell cycle.

Colorectal normal and tumor tissues and colorectal cancer cell lines were used in my study of the RA signaling pathway in colorectal cancer development. Results show that CYP26A1 and CtBP1 are upregulated in tumor tissue compared to normal tissue, whereas RAR alpha, RXR alpha and ALDH1 expression did not change in tumor compared to normal. Among the two colorectal cancer cell lines SW480 and HT29, SW480 cells showed significantly higher ALDH1, RXR alpha and RAR alpha compared to HT29 cells, validated by both western blot analysis and immunocytochemistry.

The expression level of RXR alpha and RAR alpha does not change or decrease in colorectal tumor, suggests that it probably is inducing proliferation of CSCs. In colorectal cancer the cellular level of retinoic acid decreases which effects the RA signaling pathway. CYP26A1, the ATRA metabolizing enzyme, was found, as expected, to be higher in tumor tissues along with CtBP1, which inhibits RDH production, was also higher in tumor tissues. RDH converts retinaldehyde to ATRA. Thus, the decrease in basal level of ATRA could be due to increased metabolism of ATRA by CYP26A1 and also inhibition of RDH by CtBP1. ALDH1, the stem cell marker used for the study, expression did not change between tumor and normal which means the number of cells expressing ALDH1 increases with the total number (ALDH1 expressing+ non expressing) cells in tumor compared to normal tissue, but the proportion of number of positive cells to total number of cells remains the same in tumor and normal. Therefore, though the number of cells increases still the expression level is same for both normal and tumor.

The role of retinoic acid in the fetus has been widely studied, and its importance in fetal development is crucial (Tamagawa, Morita et al. 1995; von Schroeder and Heersche 1998). Many pathways and conditions are similar between cancer and fetal stages e.g. increase in number of stem cells etc. Hence, we used FHC cells as a control to study RA signaling in fetal stage and then compare with the results obtained from the colorectal cancer tissues and cell line. ICC results showed high expression of RXR alpha and ALDH1 in FHC cells. As FHC is a fetal line, a high percentage of stem cells were expected. Indeed 80% of cells were found to be positive for ALDH 1, a finding that was validated by western blot analysis. Retinoic acid signaling modulates stem cell expression, and 30% of cells were found to be positive for RXR alpha. It was expected that RAR alpha expression would be high too. However, only 15% of FHCs were positive for RAR which is comparatively lower than the RXR alpha. Thus probably in FHC the stem cells are modulated more via RXR alpha receptor than RAR alpha receptor. CtBP1 expression is high during embryonic development where it controls cell proliferation and differentiation. CtBP1 expression was not detected in FHC cells. Thus suggesting CtBP1 role is probably not that significant in FHC cells and proliferation and differentiation of this cell line is dependent on some other proteins.

Using both western blot analysis and ICC it has been observed that the expression of ALDH1, RXR alpha and RAR alpha expression in SW480 cells is higher than in HT29 cells. It was reported earlier that RAR alpha receptors binds to RARE (retinoic acid response element) which recruits CCAAT/ enhancer-binding protein and transactivate the promoter of ALDH1, thereby increasing ALDH1 transcription when there is a decrease in ALDH1 expression in the cell. The

expression level of ALDH1 in SW480 is higher than in HT29 cells. Thus this can be explained by RAR binding to RARE and recruitment of CCAAT/ enhancer-binding protein which increases ALDH1 transcription in SW480 cells compared to HT29 cells.

CYP26A1 and CtBP1 expression could not be determined by ICC probably because the antibody was not compatible for cell staining. Different protocols and dilutions were tried to figure out the right protocol for CYP26A1, but the antibody didn't work even for the positive control HepG2 and Hela cells respectively. The conformation of the native protein (CYP26A1 and CtBP1) could be such that, in its 3D conformation, it does not represent the same internal sequence of amino acids as an immunogen compared to the synthetic peptide that was used as an immunogen to raise the antibody. RXR alpha and RAR alpha expression could not be determined by western blot analysis in FHC cells. It could be due to the fact that both the receptor forms have undergone posttranslational modification eg. phosphorylation, prenylation etc and that's why the antibody against N-terminus of RXR alpha and C-terminus of RAR alpha could not be detected.

Thus, abnormal retinoic acid signaling might contribute to colorectal tumor development and growth via several mechanisms: 1) abnormal retinoid receptor expression, 2) heterodimerization with other receptor types, 3) becoming phosphorylated like RXR alpha is, due to activation of the MAP kinase pathway, or 4) due to a decrease in cellular retinoic acid levels. Compromised retinoic acid signaling gives rise to a situation whereby an adequate check on cell cycling cannot be maintained. Thus, all these factors make retinoic acid signaling a potential therapeutic target for increasing differentiation and apoptosis of cancerous cells and restoring normal colonic functions.

CHAPTER 4

TO EVALUATE THE EFFECTS OF RA DERIVATIVES ON GROWTH OF COLON CANCER CELL LINES.

4.1 INTRODUCTION:

Retinoic acid derivatives have been widely used in studies to evaluate ways to inhibit proliferation of malignant cells such as studies of prostate, colon and breast carcinoma cells. In our study we primarily focused on retinoid acid effects on colon carcinomas cells in vitro. Two retinoic acid derivatives studied that showed possible anti-cancer effects by treatment of SW480 and HT29 cells were all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cis RA). As mentioned in chapter 3, ATRA binds only to retinoic acid receptors and 9-cis binds to both retinoic acid and retinoic X receptors. Binding of these derivatives to retinoid acid receptors then activates retinoic acid signaling to the nucleus. Activation of retinoic acid signaling activates many downstream target genes such as P21, CDKI, P27, some *HOX* genes and many others. Retinoic acid signaling has primary, secondary and tertiary downstream targets. So, retinoic acid has different effects depending on the nature of activation of retinoic acid signaling in the target tissue, it is also dependent on the degree of retinoic acid circulating in the blood and timing of exposure of the receptors to the ligands.

ATRA binds to RAR with very high affinity, whereas its binding affinity for RXR is lower. A great number of colorectal cancer cell lines are ATRA sensitive due to silencing of the *RAR beta 2* gene by DNA hypermethylation. SW480 is resistant and HT29 is sensitive to ATRA. It has been seen earlier that prolonged exposure of ATRA to resistant cell lines like HL60 and bone marrow (BM) leukimea cell line, can change the resistance of these cell lines, making it more sensitive (Jiang, Tang et al.

2000). It has been reported that some ATRA-resistant colorectal cancer (CRC) cell lines overcome their resistance and show growth inhibition after 10 days of 1µM ATRA treatment but some CRC cell lines such as SW480 and RKO CRC cells, did not show any growth inhibition even after 10 days. When ATRA is combined with trichostatine A (TSA), a histone deacetylation inhibitor, SW480 cells recover expression of the *RAR beta 2* gene and also show growth inhibition after 5 days; the drugs separately have no effect (Youssef, Lotan et al. 2004). HT29 is an ATRA sensitive cell line. Thus ATRA induces growth inhibition in this cell line. Thus, ATRA inhibits anchorage-independent growth in HT29 cells but without any morphological changes that would suggest differentiation. This anchorage independent growth not mainly but somehow mediated by RAR alpha receptors (Nicke, Kaiser et al. 1999).

One RA derivative, 9-cis retinoic acid, is a stereoisomer of ATRA and has an ATRA-like effect; it also induces growth inhibition and apoptosis. It has high binding affinity for RXR compared to RAR. In VAD mouse testis, 9-cis RA stimulates differentiation and then proliferation in growth-arrested A spermatogonia by increasing mRNA expression of RAR beta (Gaemers, Sonneveld et al. 1998). SW480 is an ATRA resistant cell line but sensitive to 9-cis RA; HT29 is sensitive to both derivatives. It has been seen earlier that prolonged exposure of SfW480 to ATRA can change the resistance of these cells.

Literature on ATRA sensitivity or resistance of colorectal cancer cell lines vary based on the set of experiments performed. Nicke, B. et al. (Nicke, Kaiser et al. 1999) reported that ATRA inhibits HT29 cells in a dose response manner. 1µM concentration of ATRA induced growth inhibition in HT29 cells followed by 10 µM

to 10,000 µM concentrations of ATRA. On the other hand Liu, P.L. et al. (Liu, Wei et al.) reported that 1µM or 10 µM concentration of ATRA did not induce any growth inhibition in HT29 cells. Lee, M.O. et al. (Lee, Han et al. 2000) and Liu, P.L. et al. (Liu, Wei et al.) reported HT29 cells as ATRA resistant cell line whereas Nicke, B et al. (Nicke, Kaiser et al. 1999) and few other papers reported HT29 cells as ATRA sensitive cell line. Liu, P.L. et al. (2009) have also reported that SW480 cells are also resistant to 1µM or 10 µM concentrations of ATRA.

ATRA induces apoptosis via TRAIL (TNF-Related Apoptosis-Inducing Ligand) and its receptors (TRAILRs) in mitochondria. There is synergy in the way ATRA activates TRAILRs. ATRA activates interferons and together they activate caspase8 and TRAILRs. This leads to the release of CytoC (Cytochrome-C) from mitochondria. Binding of TRAIL to TRAILRs and ATRA mediated activation recruits a regulator of apoptosis, FADD (Fas-Associated via Death Domain). Caspase 8 undergoes cleavage of BID (BH3 interacting Domain death) and tBID (truncated BID). These two then translocate to mitochondria and induce cytochrome C release. CytoC then associates with APAF1 (Fas-Associated via Death Domain) and activates another caspase (caspase 9). Caspase 9 then cleaves all the proteins required for cell viability, resulting in a death cascade and apoptosis (Gottlieb 2000). Retinoic acid-mediated differentiation and apoptosis offers a promising therapeutic approach and has been or can be helpful in treating diseases like Alzheimer Disease (Ding, Qiao et al. 2008), Schizophrenia (Wan, Yang et al. 2006), and Cancer (Tang and Gudas).

One major clinical drawback of retinoid treatment is the high dose needed to achieve successful treatment. High doses can lead to "hypervitaminosis A syndrome” in patients and make them resistant to retinoids. So, new derivatives of

retinoic acid are being developed to decrease this toxic effect and achieve better results with lower doses and without ATRA resistance. One of the latest derivatives found is IIF which is an ATRA derivative. Its effects have been tested in CRC cells (HT29 and CaCo-2 cells). IIF showed better results than ATRA in terms of growth inhibition and cellular differentiation (Bartolini, Ammar et al. 2004).

It has been reported that retinoic acid signaling triggers a feedback mechanism. Increase in intracellular retinoic acid concentrations lead to feedback inhibition of ALDH1 levels and activity thus decreasing the conversion of retinaldehyde to retinoic acid. ATRA then becomes reduced in the cell and lowers activation of the RA receptors. A drop in cellular levels of ATRA increases ALDH transcription which then increases retinaldehyde conversion to ATRA, thus generating a positive feedback effect on ATRA signaling (Elizondo, Corchero et al. 2000), (Elizondo, Medina-Diaz et al. 2009).

Considering all the above background knowledge, I propose the following hypothesis: "***:*** *Retinoic acid signaling induces inhibition of cell growth in colorectal cancer cell lines*." The following aim is designed to test my hypothesis "*To evaluate the effects of RA derivatives on growth of colon cancer cell lines."*

4.2 MATERIALS AND METHODS:

We used two colon adenocarcinoma cell lines, SW480 and HT29 cells, to study the effects of retinoic acid treatment. SW480 cells are an undifferentiated cell line and one of the best characterized colorectal cancer cell lines. These cells were originally isolated from a 50 year old patient with Duke's B colon adenocarcinoma. They have a wide range of properties including production of TGF (transforming

growth factor receptor), inhibition and activation of plasminogen, binding to collagen via a non-integrin like receptor, mutation at codon 273 of the p53 gene (thus elevating p53 levels), loss of the *APC* gene and c-myc amplification etc (Tomita, Jiang et al. 1992). HT29 cells can also grow as undifferentiated phenotype under standard conditions and forms multiple layers of non-polarized cells. The phenotype of HT29 cells can be modulated to follow growth inhibition with appropriate treatment and culture conditions (Le Bivic, Hirn et al. 1988). HT29 cells have large mitochondria with dark granules and secondary and few primary lysosomes. The cells do not express plasminogen activator but do express urokinase receptors. They also have p53 mutations and thus increased p53. They also express c-myc, K-ras and H-ras etc. HT29 cells have mutation in codon 273 of the p53 gene (www.atcc.org **).**

4.2.1 Retinoic acid derivatives (ATRA and 9-cis RA) for dose response

ATRA (R 2625) and 9-cis RA (catalog no. R 4643) were purchased from Sigma Aldrich. 9-cis was also purchased from Enzo life sciences (catalog no. 5300-03-8). ATRA was dissolved in DMSO and 9-cis RA was dissolved in ethanol. Both cell lines were exposed to different doses of ATRA and 9-cis RA to determine the IC_{50} (half maximal inhibitory concentration).

4.2.2 Dose response experiment:

Each of the cell lines were plated in 24 well tissue culture plates with a starting concentration of 30,000 cells per well for SW480 and 25,000 cells for HT29.

Two to three days after cell plating, when the cells had reached 75-80% confluence, they were serum starved with serum free media for 24 hours to bring all the cells into the same cell cycle. After 24 hours of serum starvation, cells were treated with different doses of ATRA (10^{-9} to 10^{-3} M) (M=Molar concentration) and incubated at 37 degrees for 48 hours. Doses of 9-cis RA from 10^{-10} to 10^{-5} M were given to SW480 and HT29 cells. SW480 cells were incubated for 48 hours and HT29 cells were incubated for 72 hours. The difference in incubation time between SW480 and HT29 cells is due to, no inhibition was observed in HT29 cells after 24 or 48 hours of 9-cis RA treatment. After incubation, cells were trypnised with 0.25% trypsin. After detachment, cell suspensions were tirturated by pipetting to break any clumps and then stained with trypan blue to enumerate live and dead cells. Cells counted with hemacytometer.

4.2.3 Cell counting

Cells were trypsinized and media was added. After detachment, cell suspensions were tirturated by pipetting to break any clumps and then stained with trypan blue to enumerate live and dead cells. Cell counting was done using a hemacytometer. Dead cells took up trypan blue dye and live cells did not. Four blocks in the hemacytometer were counted and then averaged and multiplied by the amount of liquid (trypsin + media) added to make the cell suspension for the count. An untreated control was used to normalize the treated counts. Each experiment had 3 replicas for each concentration of drug and the final dose response curve was calculated based on three individual experiments.

4.2.4 Time course experiment with ATRA and 9-cis RA.

4.2.4.1 Time course experiment done with ATRA in SW480 and HT29 cells

We plated 100,000 cells in 6 well tissue culture plates. Cells were kept there for 2-3 days to allow attachment and spreading until they were 75-80% confluent. Cells were then serum starved with serum free media. SW480 cells were treated with $1*10^{-5}$ M ATRA and HT29 cells were treated with $1*10^{-3}$ M ATRA. These concentrations were determined from the dose response curves for each cell line for each drug. These are the concentrations at which approximately 50% of cells are killed. SW480 cells were treated for 2, 6 and 8 days; HT29 cells were treated for 1, 2 and 4 days. DMSO (0.1%) was used as a vehicle control for both SW480 and HT29 cells. Cells were counted at each time point using a hemacytometer and trypan blue. Drug was changed every 2 days. Each experiment had three replicates of the same drug or vehicle. The final time course graph was plotted based on the average of three individual experimental sets.

4.2.4.2 Time course experiment done with 9-cis RA in SW480 and HT29 cells

Cells (100,000 per well) were plated in 6 well tissue culture plates. They were incubated at 37 degrees for 2-3 days for attachment and spreading until they were 75-80% confluent. Then cells were serum starved with serum free media. SW480 cells were treated with $5*10^{-7}$ M 9-cis RA and HT29 cells were treated with $5*10^{-5}$ M 9-cis RA. These concentrations were determined from the dose response curve for each cell line for each drug. These are the concentrations at which approximately 50% of cells are killed. SW480 cells were treated for 2, 4 and 6 days and HT29 cells were treated for 3, 6 and 8 days. Ethanol (0.1%) was used as a vehicle

control for both SW480 and HT29 cells. Cells were then counted after each time point using a hemacytometer and trypan blue. Drug was changed every 2 days. Each experiment had three replicates of the same drug or vehicle. The final time course graph was plotted based on the average of three individual experimental sets.

4.3 RESULTS:

4.3.1 Differential Effects of retinoid derivatives on cell growth of two colorectal cancer cell lines SW480 and HT29.

4.3.1.1 Dose responses for ATRA treatment of SW480 and HT29 cells.

To investigate the effect of the retinoid derivative ATRA, the cell lines were treated with a series of concentrations ranging from 10^{-3} to 10^{-9} M. SW480 is an ATRA resistant cell line while HT29 cells are ATRA sensitive. ATRA elicited antiproliferative responses in SW480 cells with 50% inhibition at 10^{-5} M. HT29 cells, an ATRA sensitive cell line, on the other hand, showed fifty percent growth inhibition at 10^{-3} M ATRA concentration.

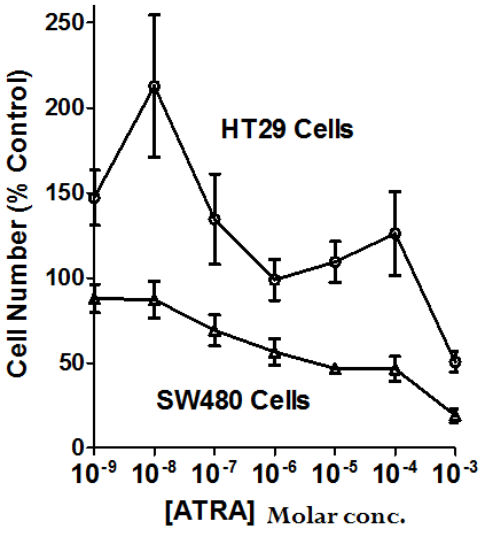


Figure 25: This figure depicts dose response curves for SW480 and HT29 cells treated with10^{-3} to 10^{-9} M concentrations of ATRA. On the X-axis are plotted the different ATRA concentrations and on the Y-axis the number of live cells. The points on the graph represent an average of three individual experiments.

4.3.1.2 Dose response of SW480 and HT29 cells on 9-cis RA treatment

To investigate the effects of the retinoid derivative 9-cis RA on the cell lines, cells were treated with a series of concentrations ranging from $5*10^{-5}$ to $5*10^{-10}$ M. 9-cis RA failed to induce a significant proliferative response in HT29 and inhibited HT29 growth at a very high dose. SW480 cells showed a biphasic response to 9-cis RA treatment. A maximum dose of $5*10^{-5}$ M of 9-cis RA induced 50% inhibition of HT29 cell survival. 9-cis RA induced a proliferative response in SW480 cells at a very low dose of $5*10^{-10}$ M. SW480 cell growth was inhibited by $5*10^{-8}$ M. There was both proliferative and inhibitory response in SW480 cells. Inhibition of SW480 cells with $5*10^{-8}$ M was followed by proliferative response at $5*10^{-7}$M and then another drop in proliferation at $5*10^{-6}$ and $5*10^{-5}$ M shown in Figure 2. To determine the concentration at which SW480 cells would 50% growth inhibition, a narrow range of 9-cis RA doses were administered to the cells. IC50 was $5*10^{-7}$ M (Figure 3).

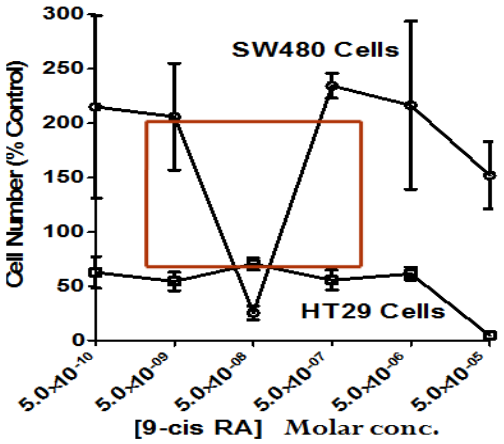


Figure 26: The figure depicts the dose response curves for SW480 and HT29 cells treated with $5*10^{-5}$ to $5*10^{-10}$ M concentrations of 9-cis RA. The graph represents the average of three individual experiments**.**

4.3.1.3 Narrow range dose response of SW480 cells to 9-cis RA.

SW480 cells had a biphasic response to 9-cis RA. To develop this biphasic curve, a narrow range of 9-cis doses (between $5*10^{-7}$ M to $5*10^{-9}$ M) were given to SW480 cells. Day 2 untreatred cells were used as a normalizing control. SW480 cells showed proliferation with the day 2 untreated control. The cell count decreased when 9-cis RA was given. $5*10^{-9}$ M elicited 60% inhibition followed by slight inhibition with $2.5*10^{-8}$ M. More than 50% inhibition was caused with $5*10^{-8}$ M 9-cis RA. At higher doses of 9-cis RA, $2.5*10^{-7}$ and $5*10^{-7}$ M, the cells started to proliferate again.

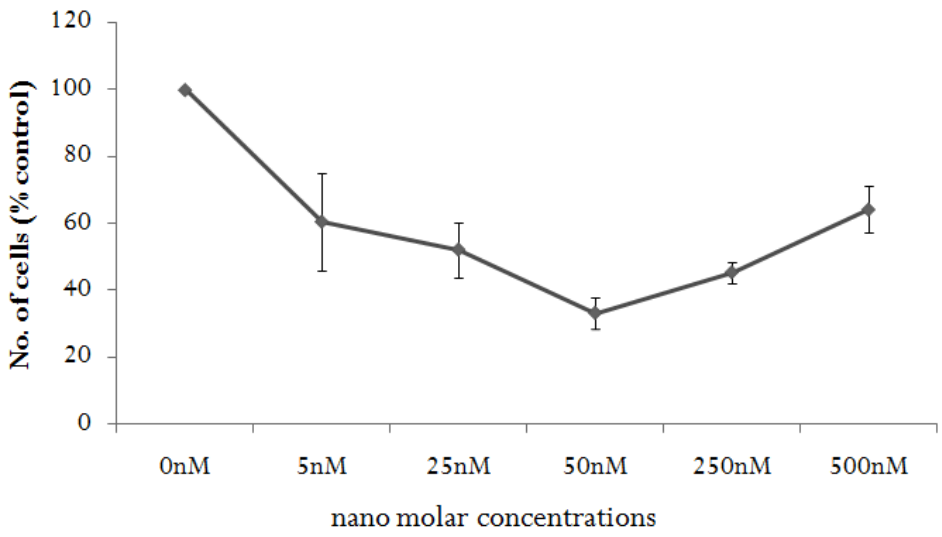


Figure 27: The figure shows the narrow range of doses given to SW480 cells to determine the IC_{50}. The day 2 vehicle control was used for normalization. The X-axis shows the different concentrations of drug used and Y-axis the percentage of live cells. Error bars represent SEM (standard error of the mean).

4.3.2 Time course for growth of SW480 and HT29 cells.

4.3.2.1 Time course experiment with ATRA in SW480 and HT29 cells

To determine the time course for measuring the effect of ATRA on SW480 and HT29 cells, we used the IC_{50} dose of ATRA. SW480 cells were treated with 10^{-5} M ATRA for 2, 4 and 8 days and HT29 cells were treated with 10^{-3} M ATRA for 1,2 and 4 days. Fresh drug was added every two days. ATRA significantly inhibited the growth of SW480 cells in a time dependent manner at days 6 and 8. In the case of HT29 cells, a high concentration of ATRA showed significant inhibition at day 1 followed by measurement at day 2 and day 4. DMSO treated cells were used as a normalizing control for both the cell lines. DMSO induced a proliferative response in both cell lines.

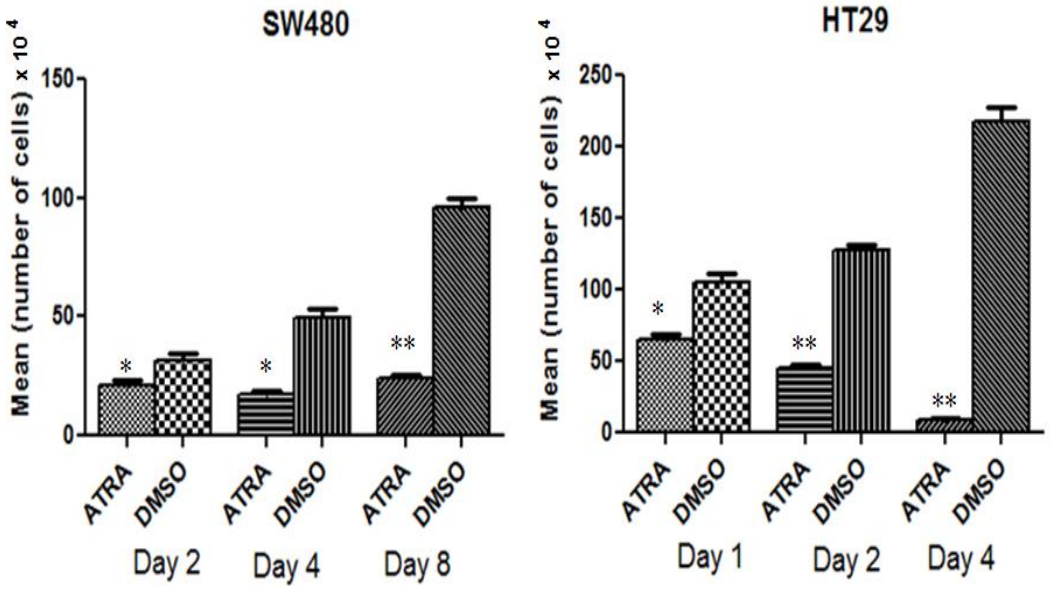


Figure 28: Response of SW480 cells to ATRA treatment. The X-axis denotes different days and the Y-axis denotes cell number. Cells treated with DMSO were used as a vehicle control for each day. Each data point represents an average of 2 experiments (N=2) for SW480 cells and an average of 3 experiments (N=3) for HT29 cells; Error bars represents ± SEM. * $p < 0.05$

4.3.2.2 Time course experiment with 9-cis RA in SW480 and HT29 cells

To see the time course for the effects of 9-cis on SW480 and HT29 cells, we used the IC_{50} dose as determined in dose response experiments. SW480 cells were treated with $5*10^{-7}$ M 9-cis RA for 2, 4 and 6 days and $2.5*10^{-5}$ M 9-cis RA was given to HT29 cells for 3, 6 and 8 days. Fresh drug was added every two days. 9-cis RA elicited significant growth inhibition in SW480 cells compared to the ethanol treated control cells at all three days (2, 4 and 6). HT29 cells were given a high dose of 9-cis RA to observe any inhibitory effect on their proliferation. 9-cis RA failed to induce any growth inhibition in HT29 cells; rather it stimulated growth after day 3. On day 4, the 9-cis RA treated cells were greater in number than the ethanol treated cells. Day 8 showed a maximum cell growth of HT29 cells and was equal to the cell growth of the ethanol treated control cells.

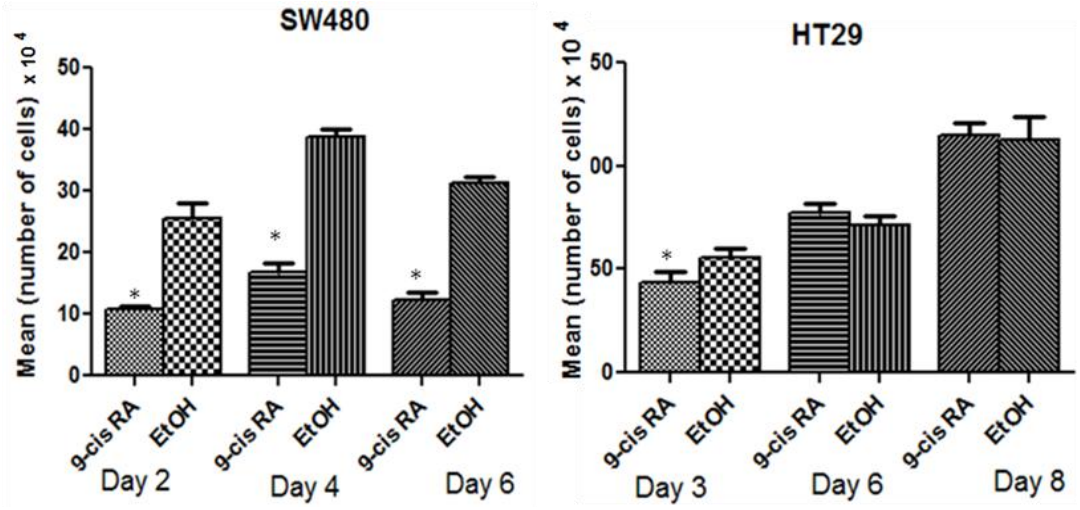


Figure 29: Response of SW480 and HT29 cells to 9-cis RA treatment. The X-axis denotes different days and the Y-axis denotes cell number. Ethanol was used as a vehicle control for each day. Each data point represents an average of 2 experiments (N=2) for SW480 cells and an average of 3 experiments (N=3) for HT29 cells; error bars represent ± SEM. * $p < 0.05$. SW480 cells were treated for day 2, 4 and 6 because we saw significant inhibition in all days including day6. HT29 cells on the other hand did not show any inhibition after day 6. Therefore the cells were treated for longer period (day 8).

4.3.3 Morphologic changes in SW480 cells and HT29 cells after treatment.

4.3.3.1 Morphological changes in SW480:

ATRA inhibited SW480 cell growth at day 2 and day 6 but there was no significant morphological change in ATRA treated cells vs DMSO treated control cells. After day 6, day 8 also showed inhibited growth of SW480 cells with morphological changes in both ATRA treated and untreated cells. SW480 cells lost their usual undifferentiated morphology after ATRA treatment. The DMSO treated control cells also lost their undifferentiated morphology. ATRA treated cells looked more spread out than DMSO treated cells.

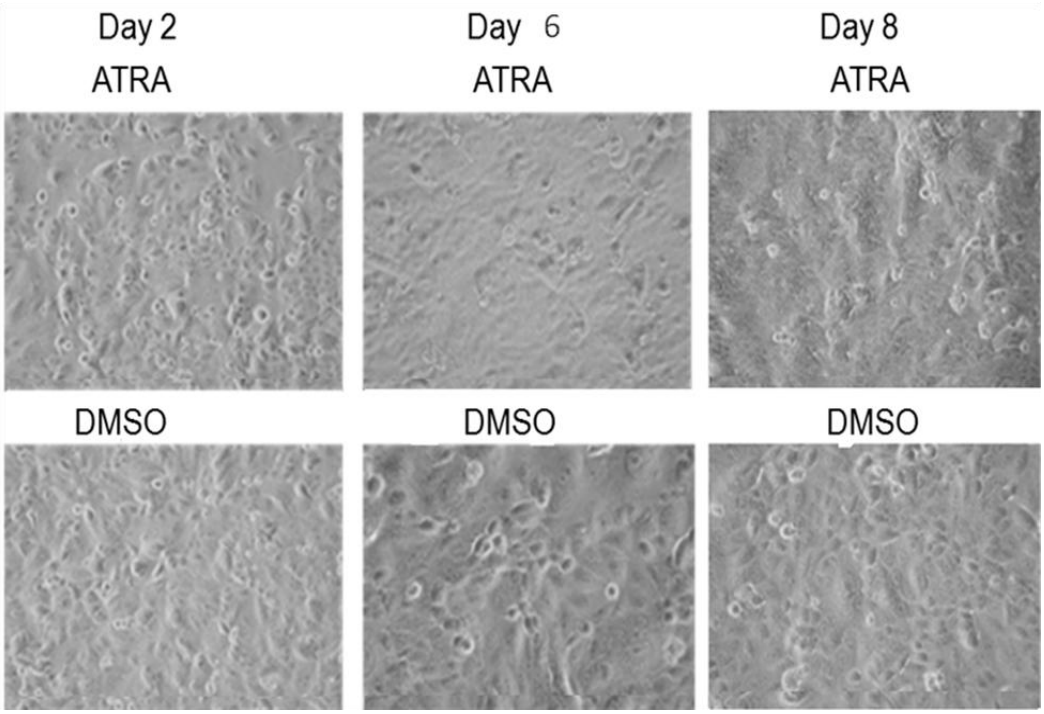


Figure 30: Bright field images of SW480 cells taken after 2, 6 and 8 days of ATRA treatment and their respective DMSO controls taken with a 20X objective.

4.3.3.2 Morphological changes in HT29 cells:

Day 1 treatment of HT29 cells with ATRA and DMSO controls did not show any morphological changes. HT29 cells maintained their undifferentiated morphology. On day 2, ATRA inhibited HT29 cell growth significantly. There was a lot of cell rounding and detachment from the plate. Also, the Day 2 ATRA treated HT29 cells were extended with sharp edges compared to DMSO treated ones. At day 4 of ATRA treatment, more than 90% of cells showed rounding and detachment from the plate. The DMSO treated HT29 cells continued to have an undifferentiated morphology with few cells becoming elongated or having sharp edges.

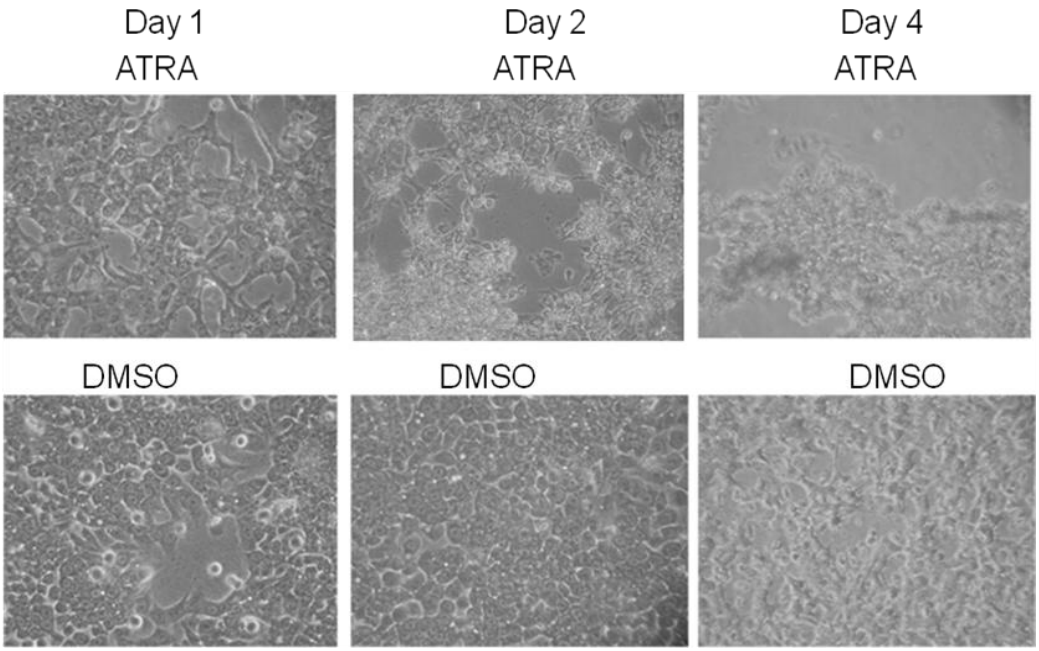


Figure 31: Bright field images of HT29 cells taken after 1, 2 and 4 days of ATRA treatment and their respective DMSO controls taken with a 20X objective.

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4.3.3.3 Morphological changes in SW480 cells:

9-cis RA induced significant growth inhibition in SW480 cells on all three days (2, 4 and 6), but there was no morphological change on day 2 in 9-cis RA treated or untreated SW480 cells. Day 4 showed cell spreading with 9-cis RA and ethanol, but the cells were more spread and longer in 9-cis RA treated SW480 cells at day 6, compared to day 4. Ethanol increased proliferation in SW480 cells on all three days.

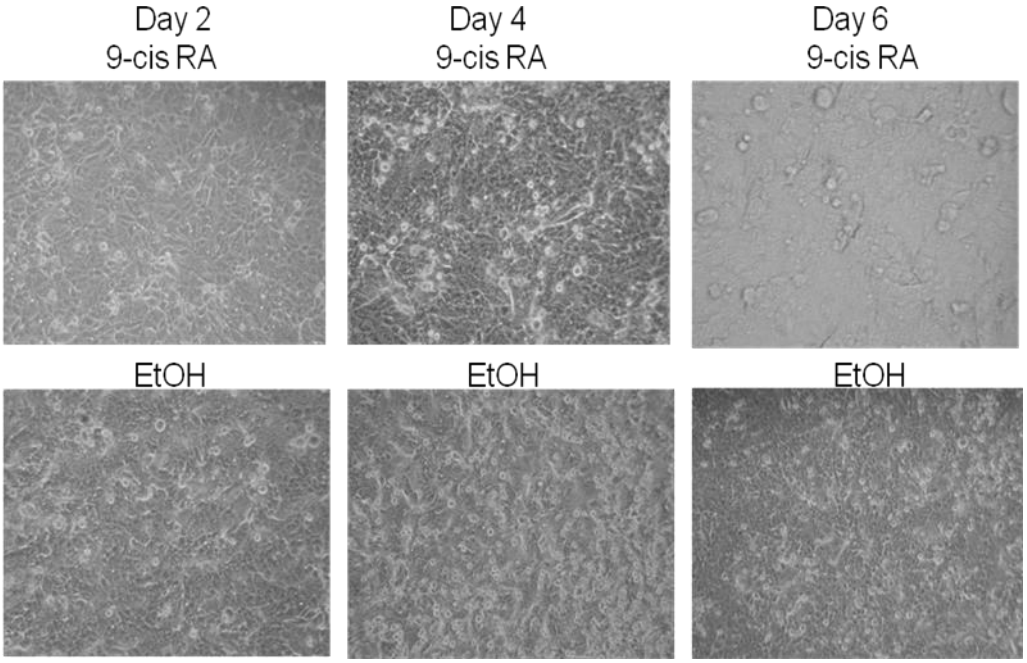

Figure 32: Bright field images of SW480 cells taken after 2, 4 and 6 days of 9-cis RA treatment and their respective ethanol controls taken with a 20X objective.

4.3.4.4 Morphological changes in HT29 cells:

9-cis RA induced some cell growth in HT29 cells on day 3 but no significant morphological change was noticed in 9-cis RA treated or untreated cells. Day 6 and day 8 treatments of HT29 cells with 9-cis RA did not induce any inhibition; rather a proliferative response was observed. The cells were more spread compared to day 3 treated cells, but there was no difference in morphology between day 6 and day8 treated and untreated cells.

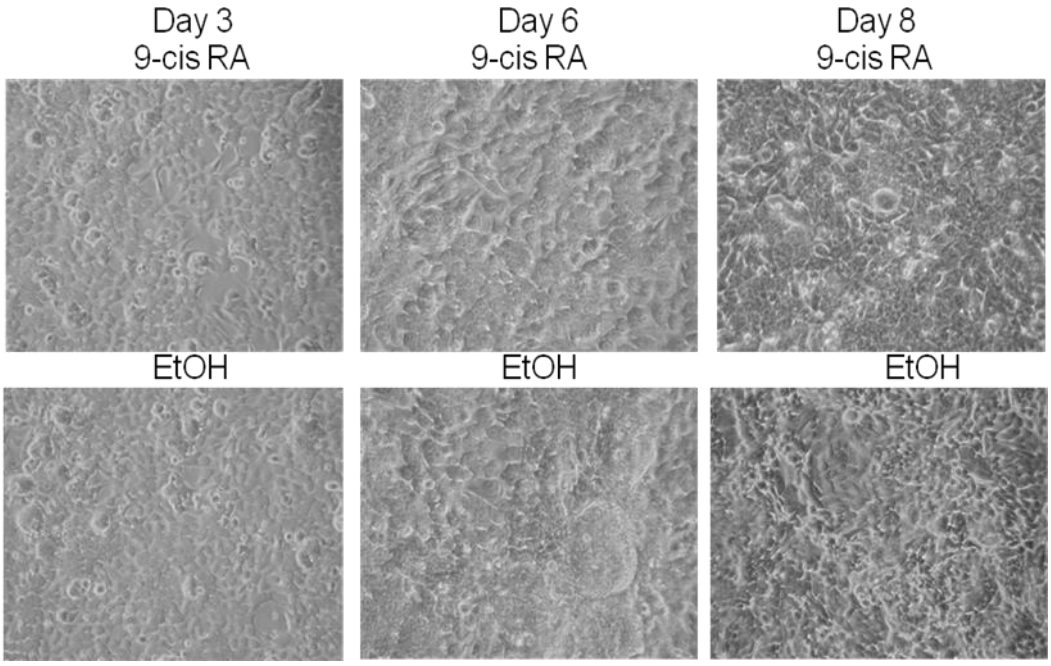

Figure 33: Bright field images of HT29 cells taken after 3, 6 and 8 days of 9-cis RA treatment and their respective ethanol controls taken with a 20X objective.

4.4 DISCUSSION:

My research findings have relevance to the emerging concept in oncology that CSCs have an important role in tumorigenesis. CSCs are a small population of undifferentiated cells that appear to drive tumor growth (Al-Hajj and Clarke 2004). So, to successfully treat CSCs, irreversible differentiation has to be induced so that CSCs become apoptotic or so CSCs can be killed directly. It would be more effective to treat cancer if these CSCs could be preferentially targeted using the unique sets of genes and antigens they express. For example, some cancer CSCs express high CD133 levels whereas other cancer types express more CD44 protein. Due to the anti-proliferative mechanism that retinoids possess, they are becoming important molecules to be studied for treating malignancies. Retinoids regulate differentiation in stem cells (Purton 2007). Therefore they might be useful for differentiating CSCs. Indeed, retinoids have been successfully used in treating APL (Acute Promyelocytic Leukemia) (Warrell et al., 1991). Different derivatives of retinoids such as ATRA, 9-cis RA and 13-cis RA have been studied as potential anti-cancer treatments. But, retinoids have a substantial toxicity profile (i.e., they have many side effects and toxicities). In some clinical studies, retinoids did not show any differentiating effects until a high dose of retinoid was used. Because cancer cells can develope resistance to retinoids, high doses of retinoids would be required for treatment and higher doses have significant toxicity including death. This makes clinical studies difficult and more research is needed towards developing synthetic forms of retinoids that would have the same pharmacological effects - differentiation of CSCs - but would be less toxic. Some synthetic retinoid derivatives that have been developed include 6-OH-11-

O-hydroyphenantrene (IIF), a RXR alpha agonist that has antitumorigenic effects in many cancer cell lines (Gottlieb 2000).

Our study focused on the effects that two types of retinoids have on the proliferation and differentiation of SW480 and HT29 cells. Due to difference in literature on ATRA sensitivity and resistance of these two cell lines, we did our own ATRA dose response experiment study with these two cell lines. Interestingly, we found that HT29 cells which were thought to be sensitive, showed growth inhibition only at a very high dose of ATRA (10^{-3} M), whereas SW480, the ATRA resistant cell line showed growth inhibition with 10^{-5} M of ATRA. Thus, clearly the sensitivity and resistance of these two cell lines reported by Nicke, B et al is questionable and our results goes along with Liu, P.J. et al. findings. The difference in literature about ATRA sensitivity and resistance probably depends on how cells were passaged, how many passages were used for the study, the conditions in which the cells were grown. When these cell lines were treated with the IC_{50} dose of retinoid drugs, their response was either proliferative or antiproliferative. In SW480 cells, an ATRA resistant cell line, retinoids had antiproliferative effects. Moreover, SW480 cells showed significant inhibition only with higher doses of ATRA at days 4 and 8, that is, 10^{-5} M ATRA. From Aim 2 results, it was observed that 22% of SW480 cells express RAR alpha receptor, but this does not mean that the receptor is active or is functioning normally. The absence of ATRA does not allow conformational changes in RAR alpha receptors to occur. This leads to downregulation of the receptor function. Thus, binding of ATRA is able to rescue cells from retinoid resistance. ATRA induces differentiation via its binding to RAR receptors. RAR receptors, mostly RAR alpha and beta, have been mutated in many cancers. Thus, cells lose their anti-proliferative mechanisms

and tumor growth is promoted. It was reported that RAR beta receptor expression is dramatically lower in colorectal cancer and may have a crucial role in colorectal tumor formation (Fang, Wei et al.). Inhibition of SW480 cell growth due to ATRA probably involves restoration of RAR beta receptor function. There was a morphological change in SW480 cells after day 6 and day 8 treatments with ATRA. The cells looked more spread out and differentiated than normal SW480 cells. Again this could be due to restoration of RAR beta receptor activation of RAR alpha receptor. Treatment with ATRA leads to homodimerization of RAR alpha with RAR beta and thereby induces differentiation. In HT29 cells, a retinoid sensitive cell line, retinoids induced a proliferative response at lower doses of ATRA and growth inhibition with higher doses. HT29 cells showed growth inhibition on day 1, day 2 and day 4 of ATRA treatment. By day 2 and day 4, the cells changed their morphology and looked more rounded. From aim 2 results, it has been found out that, HT29 cells express RAR alpha receptor. RAR alpha can promote transcription of cell cycle arrest genes, thus restricting further proliferation, and inducing differentiation in HT29 cells. Nine-cis RA has a higher affinity for binding to RXR alpha than RAR alpha. When 9-cis RA was given to SW480 cells, which has higher RXR alpha receptor expression (from aim 2) than HT29 cells, the cells immediately responded to treatment even with a low dose of $5*10^{-9}$ M. SW480 cells showed a huge drop in cell number with $5*10^{-8}$ M 9-cis RA and then an increase in cell number with a high dose of 9-cis RA. This type of response is classified as a biphasic or non-monotonic dose response. Receptor ligand complex formation can be denoted by R+L=RL (where R= receptor and L= ligand). The first interaction in RL formation is usually monotonic (linear) i.e. there will be a linearly increased or linearly decreased response. However for complex

biological systems, nonmonotonic dose responses have been seen, where the response is decreased at low doses and increased at high doses, or vice versa. This type of response has been seen in many cases of cancer including in APL (Acute Promyelocytic Leukemia). One of the reasons for this biphasic response is a difference in binding affinity among subtypes or similar receptor types. 9-cis RA binds with higher affinity to the RXR receptor family than RAR. Differences in binding affinity of 9-cis RA to different RXR subtypes or RAR receptors probably is responsible for the biphasic response seen in SW480 cells (Conolly and Lutz 2004). SW480 cells also showed morphological changes after 4 days of treatment with 9-cis RA. The cells looked more elongated and spread out. This, again, could be due to a biphasic response of 9-cis RA to SW480 cells, which leads to activation of RAR and RXR receptors, which induces cell differentiation.

HT29 cells did not show much response to 9-cis RA treatment. No change in response was observed with a range of 9-cis RA doses on day 1 and day 2. Only after 72 hours of incubation of HT29 cells with 9-cis RA, was there a drop in cell number and only after a very high dose of 9-cis RA ($2.5*10^{-5}$ M). It was also reported earlier that 9-cis RA does not elicit an antiproliferative response in HT29 cells, or a very high dose of 9-cis RA is required to have any effect (Kane, Langman et al. 1996). HT29 cells showed a proliferative response even after days 6 and 8. It has been reported that 9-cis RA influences cells responding to 1,25 (OH) 2D3 by its catabolizing enzyme, 24-hydroxylase. 1, 25(OH) 2D3 induces antiproliferative responses in cells like CaCo2 cells. It was found that HT29 cells express 24-hydroxylase mRNA, which catabolizes 1, 25(OH) 2D3 in HT29 cells. This explains why HT29 cells do not show any inhibition of cell growth and have a high

proliferative response to 9-cis RA treatment (Kane, Langman et al. 1996). Due to this proliferative response, HT29 cells formed multiple layers of cells on top of each other thus making it hard to detect any morphological changes in the cells.

Therefore, retinoic acid derivatives can induce differentiation based on cell type. But for them to be used more successfully in cancer therapy, we need a better understanding of mechanisms by which retinoid pathways are altered in precancerous and cancerous cells and how, in many cancers, the $RAR\beta_2$ gene is mutated. There have been a tremendous number of studies using tumor samples and cell lines, but more studies need to investigate RAR expression and retinoid metabolism involving mouse models in order to study various types of cancer development and which genes are involved and in what way, and what promotes their becoming mutated. It is also important to understand the pharmacokinetics of retinoids to understand what doses should be given during clinical trials of cancer therapies, because sometimes a higher dose of retinoid is required to get an inhibitory response. But the higher dose can be toxic. Also, a better understanding of different molecular markers for differentiation would be helpful and effective for developing better treatments of cancer.

CHAPTER 5

FUTURE WORK

There have been a lot of studies done to identify stem cells using different molecular markers, but not much has been done using a histologic approach to study the structure of stem cells, their nuclei, or the presence of nonconventional pathways for stem cell division in normal colon or in colon cancer. Dr.Gostjeva's work on identification of stem cells based on nuclear morphology has produced evidence suggesting that fetal stem cells may be metakaryotic cells, but more scientific evidence is needed to confirm the existence of bell-shaped nuclei. We found bell-shaped nuclei using the Gostjeva technique, but they could not be identified with the other techniques tried in our lab. Fetal mouse intestinal tissue and fetal colon cell lines were used as a positive control because the number of colonic stem cells in the fetus is higher than in the adult human. Therefore, the chances of locating a bell shaped nuclei increases. We used an immunoflourescence technique with paraformaldehyde fixed and Carnoy's fixed tissue. ALDH1, a stem cell marker, was used to identify stem cells at the base of the crypt. However, there were not any cells that contained bell-shaped nuclei. Bell-shaped nuclei were absent in fetal human colon cell lines as well. Therefore, to establish the existence of bell-shaped nuclei in stem cells more work needs to be done. First, it is important to validate the presence of cells with bell shaped nuclei. Second, if such nuclei are found, we need to determine what properties they have. Third, do they have any properties similar to stem cells? Fourth, if their existence is validated, then what makes them different from normal cells which don't have bell shaped nuclei. Fifth, what different techniques (both histologic and

molecular) can be used to validate the presence of bell-shaped nuclei? Sixth, what is the molecular explanation of their existence? Some of the questions have been partially answered by Dr. Gostjeva. These cells are thought to have a stem cell phenotype because they have been found in all three germ layers. Why do they have bell shaped nuclei? Because they divide amitotically. However, all these answers are based on experiments using Carnoy's fixation and Giemsa staining. Others have used acridine orange staining to show the chromosome condensation at the mouth of bell shaped nuclei and to show the presence of double stranded DNA and single stranded DNA. But for these experiments employing acridine orange staining, the tissue preparation still involved mechanical force (maceration), which again raises the question as to whether metakaryotic cells might simply be a result of the Gostjeva method. Some of the techniques which can be used to study bell shaped nuclei are: 1) live cell staining, which would help us to detect any cell undergoing amitotic cell division and to see if cells have bell shaped nuclei without our having to use harsh protocols, 2) staining with different stem cell markers to see if we can locate any metakaryotic cells.

In my aim 2, the expression of retinoid receptors and of members of retinoic acid signaling pathways were analyzed with western blot analysis and immunocytochemistry. The study was done using two colorectal cancer cell lines, SW480 and HT29, one fetal human colon cell line (FHC), and four pairs of matched normal and tumor tissue samples obtained from colorectal cancer patients. My western blot analysis results and immunocytochemistry results yielded similar results. However, two proteins, CYP26A1 and CtBP1, could not be identified using immunocytochemistry. To identify these two proteins by ICC, another antibody

(preferably a monoclonal one) from some other company should be tried. Moreover, western blot analysis of RXR alpha and RAR alpha expression in FHC cells did not detect these proteins. This might be due to posttranslational modification of these proteins that affect their immunoreactivity. The antibody used for RXR alpha or RAR alpha was not raised against the phosphorylated form or any other transformed form of RXR alpha or RAR alpha. Thus, it might not identify these proteins if they are phosphorylated in FHC cells. Antibodies against the phosphorylated form of RXR and RAR alpha also should be studied in the future for FHC cells and SW480 and HT29 cells. Other than these technical issues that need to be resolved, the project can be made more interesting if we do some co-localization studies using co-staining by immunocytochemistry (ICC), and studies on the regulation of stem cells by retinoic acid signaling. This would also help us understand which type of receptor and isoform is more involved in regulation of stem cells. Colocalization studies can be performed by ICC using two different antibodies e.g. ALDH1 and RXR alpha to see the ratio of cells positive for both ALDH1 and RXR alpha and positive for ALDH1 and RXR alpha alone.

Retinoic acid signaling can also affect the mRNA levels of certain genes. Therefore, mRNA expression of each protein should be done as an independent method and compared with the results on protein level determined by western blot analysis. This can be done using quantitative PCR. It would also be interesting to study some additional primary target genes of RA signaling pathways, which would expand our understanding of the relation between colorectal cancer development and downstream RA signaling genes, entities which can be targeted for cancer therapy.

In Aim 3, ATRA and 9-cis RA treatment of SW480 and HT29 cells showed differential responses to these derivatives. Both SW480 and HT29 cells were inhibited by ATRA treatment. HT29 cells were inhibited significantly on day 2 but SW480, which is ATRA resistant, showed inhibition of proliferation in a time dependent fashion. On the other hand, there was no inhibition of proliferation of HT29 cells after 9-cis RA treatment, but 9-cis RA induced inhibition in HT29 cells in a time dependent manner. SW480 cells showed a biphasic response to different doses of 9-cis RA. Further work to understand the differences affect of these two drugs in colorectal cancer cells will be important to understand the underlying mechanisms. Studying the association of proliferation and differentiation in these cell lines is the most important part. For future work, cell lysates should be obtained after each treatment to study the effects of drug treatment on proteins expression of the components in the retinoic acid signaling pathway. Western blot analysis should be performed to detect any change in the expression of the stem cell marker ALDH1, retinoic acid receptors or other proteins which mediate differentiation or inhibition in both cell lines. HT29 cells were unaffected by 9-cis RA treatment whereas SW480 cells showed a biphasic response. Therefore, it would be interesting to compare the effects of stimulatory and inhibitory doses of 9-cis RA on the aforementioned proteins. It is also important to check mRNA levels by real time PCR. Western blot analysis and real time PCR results would be helpful in understanding what results might be predicted for other retinoic acid derivatives.

It was reported earlier that retinoic acid treatment restores RAR beta receptors. So, it would be very interesting to study responses of SW480 and HT29 cells to this drug to see if the cellular effect of retinoic acid contributes to restoration

of RAR beta receptor or whether it is due to another mechanism. Restoration of RAR beta receptors has been found to inhibit tumor formation.

Additional studies could be done to determine if retinoic acid derivatives can modulate the ability of cancer stem cells to differentiate. This could be done by determining if these drugs affect the proportion of ALDH+ cells in colon cancer cell lines. Additionally it will be important to determine if retinoids can change the degree of stemness of cancer stem cells. This could be done by determining if retinoic acid treatment of colon cancer stem cells decreases their ability to form spheres in non-adherent culture conditions. Ultimately it will also be important to see if retinoid drugs can reduce the ability of colon cancer stem cells to form tumors in mouse xenograft assays. The ability to decrease tumor initiating ability in vitro and in vivo would be very useful toward understanding ways to eliminate stem cells in colon cancer. Such scientific evidence could prove vital in developing effective cancer therapeutics.

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