MiR-627 MEDIATES THE EPIGNETIC MECHANISMS OF VITAMIN D IN SUPPRESSION OF COLON CANCER GROWTH

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Low circulating vitamin D levels have been linked to increased risks of cancer by epidemiologic studies, and preclinical research has demonstrated clear antitumor activities of vitamin D against various types of cancers. Calcitriol (1α, 25$(OH)_2$ D3), the active form of vitamin D, has been shown to inhibit proliferation, induce differentiation, and activate apoptosis in colon cancer cells. However, the mechanism of calcitriol's antitumor action remains poorly defined and the hypercalcemic side effect of vitamin D limits its use as an anti-cancer agent. To understand its mechanism of action in tumor suppression, we investigated the effects of calcitriol on microRNA expression in colon cancer cells. We identified miR-627 as the microRNA whose expression was most significantly stimulated by calcitriol. Furthermore, JMJD1A (jumonji domain containing 1A), the gene encoding a histone demethylase, was identified as the target of miR-627. It has been reported that JMJD1A is upregulated in colon cancer during hypoxia. Increased JMJD1A expression decreases the histone methylation on the promoters of target genes such as adrenomedullin (ADM) and the growth and differentiation factor 15 (GDF15) and increases their expression, which promotes tumor growth. By downregulating JMJD1A, miR-627 mediated the effects of calcitriol to increase histone H3K9 methylation and to suppress the expression of growth-promoting genes, such as GDF15. Calcitriol also induced miR-627 and downregulated JMJD1A in colon cancer xenografts in nude mice. Calcitriol significantly suppressed the growth of colon tumors in nude mice, whereas colon tumor xenografts with stable JMJD1A-UTR expression showed resistance to the Calcitriol treatment, confirming the critical role of miR-627 in Calcitriol induced tumor suppression. Overexpression of miR-627 decreased JMJD1A and suppressed colon cancer growth both *in vitro* and *in vivo*. In addition, we found that miR-627 expression was decreased in human colon adenocarcinomas compared with

controls. These results suggest that, miR-627 is a major epigenetic regulator in vitamin D induced growth inhibition. MiR-627 and JMJD1A may serve as potential targets to exploit the antitumor activity of vitamin D without eliciting its hypercalcemic side effect.

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LIST OF ABBREVIATIONS

3’-UTR …….3'–Untranslated Region

5-FU……..5-Fluoro Uracil

µg/ml…….microgram per milliliters

µM…….micro Moles

ACS……American Cancer Society

BrdU…….....Bromodeoxyuridine

Ca^{2+}……...Calcium ion

Cl^{-}……..Chloride ion

CCND1…….....CyclinD1

cDNA……..……complementary Deoxyribonucleic Acid

ChiP…….….Chromatin imuunoprecipitation

CpG islands…….……Cytosine-phosphodiester bond-Guanine islands

CYP27A1…… Cytochrome p450-27A1

CYP27B1…… Cytochrome p450-27B1

DMSO…….....Dimethyl sulfoxide

DNA…….……..Deoxyribonucleic Acid

DNMT……..DNA methyltransferase

ECL…….…....Enhanced Chemiluminescence

EDTA……Ethylenediaminetetraacetic acid

FACS.....…….....Fluorescence Activated Cell Sorter

FBS……..…..…...Fetal Bovine Serum

FITC…….....Fluorescein isothiocyanate

GDF15…….Growth and Differentiation Factor 15

GFP……..…..Green Fluorescence Protein

H3K4Me3……..…….Histone3 Lysine4 trimethyl

H3K9Me2…….....Histone3 Lysine9 dimethyl

H3K27Me2/Me3….……...Histone3 Lysine27 dimethyl / trimethyl

HAT……Histone Acetyl Transferase

HDAC…….Histone Deacetylase

HOXA1……. Homeobox A1

I.P. ……..…Intra Peritoneal

JMJD1A…….Jumonji Domain Containing 1 A

KDM3A…….….Lysine Demethylase 3A

miR, miRNA…….…….micro Ribonucleic Acid

mRNA…….messenger Ribonucleic Acid

MUT……..….Mutation

NC…….…..Negative Control

nm…….…..nanometer

nM……nanomolar

PBS…….....Phosphate Buffered Saline

PCR…….....Polymerase Chain Reaction

PI……..…...Propidium Iodide

qRT-PCR……quantitative Real Time PCR

RNA……..…..Ribonucleic Acid

RNAi…….….Ribonucleic Acid interference

RT-PCR……..……...Reverse Transcription Polymerase Chain Reaction

RXR……Retinoid X Receptor

S.C. ……..…..…...Subcutaneous

SDS-PAGE…….……..Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis

siRNA…….……..small interfering Ribonucleic Acid

UV…….……...Ultraviolet

VDR…….……..…Vitamin D Receptor

VDRE……Vitamin D Response Element

WST…….…..…Water soluble Tetrazolium

CHAPTER 1: BACKGROUND INFORMATION; OBJECTIVES AND SPECIFIC AIMS; MATERIALS AND METHODS

1.1. Background information

1.1.1. Colorectal cancer

Colorectal cancer develops in the colon or the rectum and it progresses very slowly over a period of 10 to 15 years (Kelloff G.J., et al., 2004). Colorectal cancer is the third most commonly diagnosed cancer both in men and women and the second most common cause of cancer related deaths in the United States, with an estimated 143,460 new cases and about 51,690 deaths in 2012. More than 1.1 million Americans are living with current or past diagnosis of colorectal cancer (American Cancer Society, 2011-2013). Lifetime risk of developing colon cancer is about 1 in 20 for both men and women in the US (National Cancer Institute, 2011).

Colon cancer originates from the inner lining of the colon mucosa. The tumor typically starts with a noncancerous polyp. A polyp is an abnormal growth of tissue that develops on the lining of the colon or rectum and that can become cancerous. Less than 10% of adenomas (certain types of polyps are called adenomas) progress to cancer (Levine J.S. and Ahnen D.J. 2006). As cancer progresses, it grows larger in the lumen of the colon, but also grows outward through the other layers which compose the wall of the colon and invade other structures and it can spread to lymph nodes (Figure 1). Cancer cells can also be carried away by blood to other organs such as liver or lungs. Metastasis is the process by which cancer cells travel to distant parts of the body through blood or lymphatic vessels (American Cancer Society, 2011-2013).

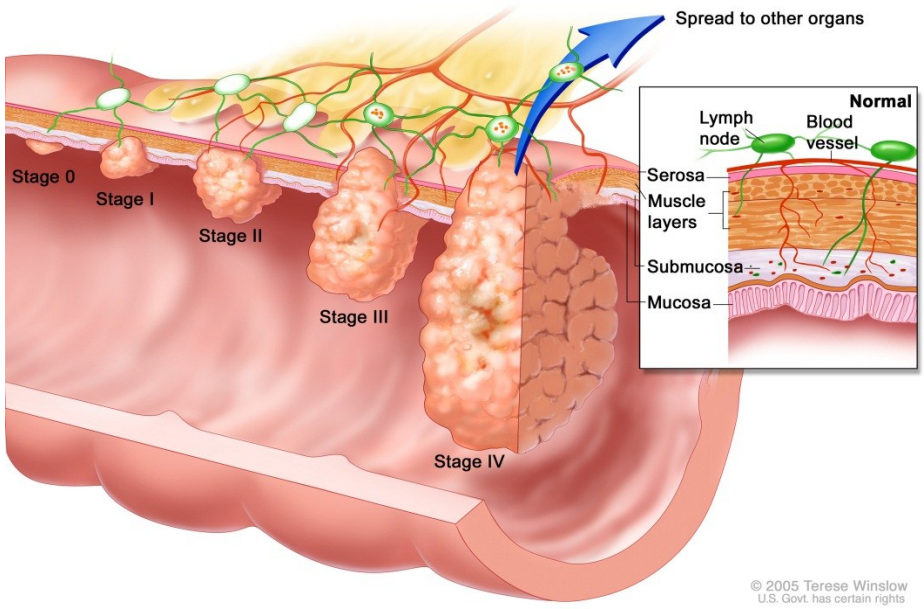


Figure 1. Colorectal cancer growth (The website of the National Cancer Institute, (http://www.cancer.gov)).

1.1.1.1. Colorectal cancer prevention

Even though the exact reason for most of the colorectal cancers is unknown, it is possible to prevent many of them**.** A minimum of 6 out of every 10 deaths due to colorectal cancer could be prevented if all men and women aged 50 years or older were screened routinely (Whitlock E.P., et al., 2008).

The American Cancer Society (ACS) recommendations for early detection, followed by prevention of colorectal cancer are:

i. Regular colorectal cancer screening is one of the most important recommendations from ACS to prevent colorectal cancer. People without any known risk factors should begin their regular screening at 50 years of age. People with a family history or other risk factors for colorectal polyps or cancer, such as inflammatory bowel disease, should

discuss with their doctor about early screening at a younger age and also screening more frequently.

ii. Healthy weight maintenance by adopting a physically active lifestyle and consuming a healthy diet with more emphasis on plant sources. Colon cancer rates are high in populations with high total fat intakes and are lower in those consuming less fat. Diets high in vegetables, fruits, and whole grains have been linked with a decreased risk of colorectal cancer. Avoiding diets high in red meats (beef, lamb) and processed meats (hot dogs) also decreases colorectal cancer risk (Rose D. P., et al., 1986).

iii. Decreasing alcohol consumption to no more than 2 drinks per day for men and 1 drink per day for women. A large cohort study reported a dose-response relationship between alcohol consumption and death due to colorectal cancer, with a Relative Risk of 1.2 (95% CI, 1.0–1.5) for four or more alcohol drinks per day compared with nondrinkers (Thun M. J., et al., 1997).

iv. Taking vitamins and calcium: Some studies have suggested that taking a daily multi-vitamin may lower colorectal cancer risk, but not all studies have confirmed this finding. More research is needed in this area (Giovannucci E., et al., 1998).

1.1.1.2. Colorectal cancer treatment (National Cancer Institute)

Different types of treatment options are available for patients with colorectal cancer, some are standard currently using treatments and some are being tested in clinical trials.

- **Standard treatment options**
  - **Surgery:** The most common treatment for all colon cancer stages is surgery. If the cancer is detected at an early stage, a local excision is performed to remove it without cutting through the abdominal wall. A polypectomy (surgical procedure

to remove polyps) is performed if the cancer is found in a polyp (early stage of colon cancer) form. If the cancer is found at a later stage, partial colectomy is performed (removing the cancer and a small amount of healthy tissue around it) followed by sewing the healthy parts of the colon together (anastomosis). Lymph nodes near the tumor area are removed and observed under a microscope to see whether the cancer has metastasized. If the two ends of the colon are not able to be sewed back together, an opening (called stoma) is made on the outside of the body for waste to pass through. This entire procedure is called a colostomy. A bag is placed around the stoma to collect the waste. Colostomy sometimes is temporary (until the lower colon has healed), and then it can be reversed. However, the colostomy may be permanent if the entire lower colon is removed due to cancer. Chemotherapy or radiation therapy (as an adjuvant therapy) is advised after surgery to kill any cancer cells that are left inside the body or to lower the risk of reoccurrence of cancer.

- **Chemotherapy:** Treatment of colorectal cancer with anti-cancer drugs to stop the growth of cancer cells. Mechanism of these anti-cancer drugs is either by killing the cells or by stopping them from dividing. Depending on the stage and type of cancer, chemotherapy is given in various ways.
    - **Systemic chemotherapy:** Where anti-cancer drugs are injected into a vein, muscle or given by mouth. These drugs enter the bloodstream and can reach cancer cells present throughout the body. This treatment is recommended for metastasized cancers. The most commonly used drugs for colorectal cancer treatment are: Oxaliplatin, 5-Fluorouracil (5-FU), Capecitabine and

Irinotecan. Mostly, 2 or more of these drugs are combined for effective treatment. Adding Oxaliplatin to a regimen of 5-FU and Lecovorin significantly improved 5-year disease free survival (DFS) and 6-year overall survival (OS) in the adjuvant treatment of stage II or III colon cancer. These results also suggested that adjuvant oxaliplatin plus FU and leucovorin therapy is useful after surgery for patients with stage III colon cancer (Andre T., et al., 2009).

- **Regional chemotherapy:** In this case, anti-cancer drugs are injected directly into the cerebrospinal fluid, an artery leading to a part of the body containing a tumor. This approach concentrates the dose of the drug and affects mainly cancer cells in those areas. It reduces severe side effects by limiting the amount of drug reaching the rest of the body. Hepatic artery infusion or chemoembolization is performed to treat cancer that has spread to the liver. This procedure is performed by blocking the hepatic artery and injecting chemotherapeutic drug between the blockage and the liver. This type of regional chemotherapy is used for colon cancer that has metastasized to the liver

- **Radiation therapy:** Radiation therapy uses high-energy X-rays or other types of radiation to destroy cancer cells. It is often given along with chemotherapy. These two treatments together are known as chemoradiotherapy. Chemoradiotherapy is more effective against some colorectal cancers. Depending on the type and stage of the cancer, either external or internal radiation therapy is performed.

- **External radiation therapy** is most commonly used in colorectal cancer patients and it uses a machine outside the body to send radiation towards the cancer site.
- **Internal radiation therapy or Brachytherapy** uses small pellets of radioactive substance placed directly into or near the cancer. The radiation travels only a short distance, limiting the adverse effects on surrounding healthy tissues.

- **Targeted therapy:** Targeted therapy uses drugs or other substances to stop the growth and spread of cancer by interfering with specific molecules, which are involved in cancer growth and progression. Targeted cancer therapy is less harmful to normal cells. Monoclonal antibody therapy is a type of targeted therapy used to treat colon cancer. Monoclonal antibodies bind to specific targets on cancer cells and stop their growth, or keep them from spreading. Bevacizumab (Hurwitz H., et al., 2004), Cetuximab (Cunningham D., et al., 2004) and Panitumumab (Gibson T. B., et al., 2006) are most commonly used targeted therapy drugs in colorectal cancer treatment. These drugs target the vascular endothelial growth factor (VEGF), VEGF stimulates angiogenesis in a variety of diseases, especially in cancer. Over the past decade, life expectancy in patients with metastatic colorectal cancer has changed dramatically with the help of molecularly targeted agents. Novel molecular targeted therapy drugs, Ziv-Aflibercept and Regorafenib were recently approved by the U.S. Food and Drug Administration (FDA) to treat metastatic colorectal cancer patients. These anti-angiogenic drugs improved the survival of metastatic colorectal cancer patients (Jitawatanarat P., Wee W., 2013).

1.1.1.3. Major risk factors of colorectal cancer (American Cancer Society, 2011-2013)

Age: The risk of developing colorectal cancer increases with advancing age, younger adults can develop colorectal cancer, but the chances increase significantly after age 50. More than 90% of colorectal cancer cases occur in people with 50 years of age or older. Many epidemiological and experimental studies suggest an association between decreased vitamin D levels and increased risk of various cancers, the evidence is more significant for colorectal cancer (Garland C.F., et al., 2009; Yin L., et al., 2009; Wu K., et al., 2007; Gorham E. D., et al., 2007; Giovannucci E., 2007; Lin J., et al., 2005; Huncharek M., et al., 2009; Bertone-Johnson E. R., et al., 2005; Garland C.F., et al., 2007; Lin J., et al., 2007; Robien K., et al., 2007; Freedman D.M., et al., 2008). Older people are at a greater risk of developing vitamin D deficiency, because of changes in lifestyle like clothing and outdoor activity (Photochemical synthesis of vitamin D is the major source of vitamin D). Older people also suffer additional mobility restrictions. Aging is also associated with a decreased ability to make precursor of vitamin D3 in the skin and renal production of calcitriol (active form of vitamin D) also decreases because of diminished renal function at old age (Holick, M.F., et al., 1989; Lau K.-H.W. & Baylink, D.J.1999).

Gender: Colorectal cancer is more predominant in males than in females, but common in both men and women (Fatime A.H., and Robin P.B. 2009).

Race and Ethnicity: African Americans have the highest colorectal cancer incidence and mortality rates, when compared to all other racial groups in the United States. It was reported earlier that the African Americans have wide spread vitamin D deficiency, due to higher degree of melatonin in their skin (Clemens T.L., et al., 1982; Marilyn T., et al., 2009; and Harri S.S. and

Dawson-Hughes B., 1998). Highest colorectal cancer risk is also observed in Ashkenazi Jews of Eastern European descent, compared to any ethnic group in the world (Feldman G.E. 2001).

Personal or family history of colorectal cancer: Personal history of adenomatous polyps, inflammatory bowel disease (Bernstein C.N., et al., 2001) or colon cancer increases the risk of colorectal cancer incidence. Most of the colorectal cancer cases are observed in people without a family history of colorectal cancer. But, about 20% who develop colorectal cancer have a close relative(s) who have been affected by this disease. People with a history of colorectal cancer or adenomatous polyps in their first-degree relatives (parents, siblings, or children) are at a greater risk in developing the disease (Lynch H.T. and de la Chapelle A., 2003).

1.1.2. Vitamin D

Vitamin D is a fat-soluble pro-hormone that is present in very few diets like cod liver oil, fish, eggs, milk and vitamin D fortified foods. Vitamin D is also produced endogenously when ultraviolet rays from sunlight converts 7-dehydroxycholesterol in the skin to cholecalciferol (vitamin D3). Vitamin D obtained from sun exposure and food sources is biologically inactive and must undergo two hydroxylation steps in the body for activation. Vitamin D is first hydroxylated in the liver by an enzyme 25-hydroxylase (CYP27A1) to produce 25-hydroxy vitamin D or also known as calcidiol. Calcidiol is further hydroxylated in the kidney by an enzyme 1α-hydroxylase (CYP27B1) to produce biologically active 1α, 25-Dihydroxyvitamin D3 or also known as Calcitriol (Feldman D., et al., 2008).

Vitamin D produces its biological effects by binding to vitamin D receptor (VDR). VDR expression has been identified in almost every human tissue. 1, 25$(OH)_2$ vitamin D3 is a flexible molecule as shown in Figure 2, and it can rotate around its single carbon bond positioned at 6,7

and generate a wide array of ligand shapes ranging from 6-s-cis (6C) to 6-s-trans (6T). Depending on the ligand configuration, it binds to either cell membrane receptor (VDR_{mem}) or cytosolic/nucleus receptor (VDR_{nuc}). 6C configuration of vitamin D preferentially mediates rapid, non-genomic (seconds-minutes-hours) effects that include the opening of voltage gated channels like Ca^{2+} and Cl^-, 6T configuration of vitamin D favors slow, genomic (hours-days) responses (Anthony W. et al., 2001 and Norman A.W. 2005). Upon binding to VDR, 1α,25-Dihydroxyvitamin D3 heterodimerizes with retinoid X receptors (RXR). This complex then binds to a small sequence of DNA called vitamin D response element (VDRE) present in the promoters of many genes it regulates. A variety of additional proteins called coactivators, complex with the activated VDR/RXR heterodimers and help in recruitment of histone acetyl transferases (HAT), and allowing transcription to proceed.

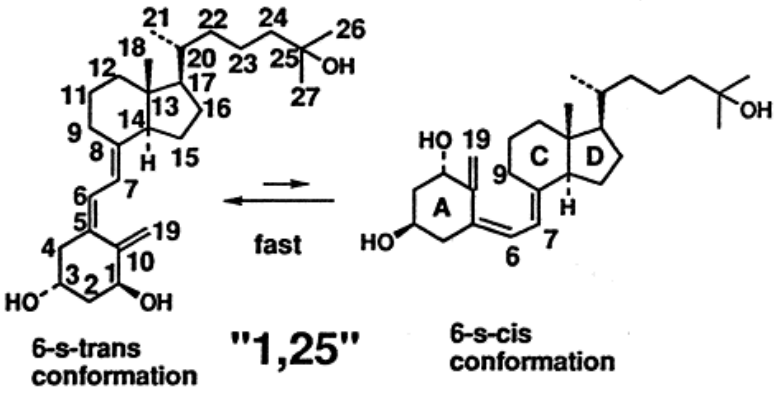


Figure 2. 6-s-*cis* conformation and 6-s-*trans* conformation of 1α,25$(OH)_2$ vitamin D_3 (Norman A.W. 2005).

In addition to coactivators there are a number of corepressors, which act by recruiting histone deacetylases (HDAC) to the gene which reverses the actions of HAT, leading to a reduction in access to the gene by the transcription process. Coactivators and corepressors are specific for different genes, so various cell types differentially express these coregulators (McKenna N.J., et al., 1999, McKenna et al., 2002 and Murayama A., et al., 2004).

1.1.2.1. Vitamin D and mortality

The World Health Organization estimates that 61% of male deaths and 65% of female deaths throughout the world are correlated with low blood levels of serum 25-hydroxyvitamin D (25(OH)D). The top 6 causes of deaths (cardiovascular disease, cancer, respiratory infection, respiratory disease, tuberculosis and diabetes mellitus) are related to low vitamin D levels (Grant W.B., et al., 2011).

1.1.2.2. Vitamin D and colorectal cancer

African-Americans have higher incidence and mortality from colorectal cancer (CRC) than white Americans, due to their significantly lower mean serum levels of vitamin D (Jemal A., et al., 2009; Irby K., et al., 2006). Colorectal cancer mortality rates in African Americans were 44% higher than in white Americans (Altekruse S., et al., 2010). Many epidemiological and experimental studies support a protective action of vitamin D against colon cancer. Vitamin D slows down the progression of colon cancer cells from premalignant to malignant states, keeping their proliferation in check (Garland C. F. and Garland F. C. 1980; Garland C.F., et al., 1989; Freedman D. M., et al., 2002). Researchers found that people with the highest pre-cancer circulating vitamin D levels (100 nM per liter of blood or higher) were nearly 40 percent less likely to develop colon cancer than those with the lowest levels (less than 25 nM per liter) (Jenab M., et al., 2010).

Several studies have reported that the anti-tumoral action of calcitriol (the active from of vitamin D) in CRC depends on various mechanisms such as inhibition of cell proliferation and angiogenesis, sensitization to apoptosis, induction of epithelial cell differentiation and cell detoxification metabolism. Calcitriol induces the expression of p21 and p27 (cyclin-dependent kinase inhibitors), and represses the expression of cyclin A and cyclin F (Palmer H.G., et al.,

2003, Fernandez-Garcia N.I., et al., 2005). Calcitriol sensitizes colorectal cancer cells to apoptosis by up-regulating the proapoptotic protein BAK1, and down-regulating the anti-apoptotic protein BAG1 (Diaz G.D., et al., 2000, Barnes J.D., et al., 2005). Calcitriol also increases the expression of occludin, E-cadherin, and plectin levels, which are essential in maintaining the epithelial phenotype (Palmer H.G., et al., 2003 and Palmer H.G., et al., 2001). The combined effect of all these mechanisms may determine the anti-tumoral action of calcitriol (Larriba M.J., et al., 2008 and Krishnan A.V., Feldman D., 2011). Colorectal cancers arise due to the accumulation of genetic and epigenetic changes, which in turn induce the malignant progression of colon epithelial cells (Pereira F., et al., 2012), the study of epigenetic mechanisms of calcitriol to suppress the colon cancer growth remains an active area of investigation.

1.1.3. Epigenetics

For many years, colon cancer research was mainly focused on genetic alterations, and changes in the DNA sequence is the best known genetic alteration. In recent years, due to major advances in understanding molecular pathogenesis of colon cancer, attention has been focused on epigenetic alterations. Epigenetics is defined as heritable changes in gene expression without a change in the DNA sequence (Egger G., et al., 2004). The term “epigenetics” was coined by Conrad H. Waddington in the year 1942 in reference to the study of “causal mechanisms by which the genes of the genotype bring about phenotypic effects” (Waddington C., 1942). However, in the year 2009 Alexander Vargas, an evolutionary developmental biologist from University of Chile suggested that Paul Kammerer's experiments, performed back in the early 1900s, revealed signs of what are now well-known epigenetic effects (Vargas A. O., 2009). Epigenetic changes include histone modifications, DNA methylation and RNA-mediated

silencing (Herceg Z. and Hainaut P., 2007). DNA methylation is a well-known epigenetic marker, and global hypomethylation was the first epigenetic change to be identified in cancer cells (Feinberg A. P., Vogelstein B., 1983).

1.1.3.1. DNA methylation

DNA methylation is a process of chemical modification used by mammalian cells to maintain a normal gene expression pattern. DNA methylation is mediated by DNA methyl transferases (DNMTs), which is an enzymatic addition of a methyl group to the 5'position of a cytosine ring (Figure 3, Takai D and Jones P. A., 2002). CG dinucleotide sequence (CpG) is the most favorable substrate for DNMTs. In mammalian cells, most of the CpGs are methylated, the unmethylated CpGs are typically present in regions of DNA that are called CpG islands. A CpG island is defined as a sequence of minimum 200 base pairs in length with more than 50% CG content and an observed to expected ratio of CpG more than 60 % (Gardiner-Garden M and Frommer M., 1987 & Lao V. V. and Grady W. M., 2011).

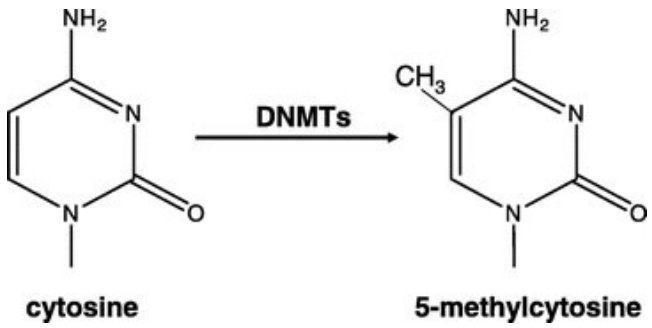


Figure 3. DNA methylation involves the addition of a methyl group onto the 5 position of a cytosine residue, mediated by the enzymes DNMTs (Takai D. and Jones P.A., 2002).

1.1.3.1.1. DNA methylation and colorectal cancer

Generally, CpG islands remain unmethylated, whereas the sporadic CpG sites in the rest of the genome are methylated. During aging, this pattern is gradually reversed and leads to sporadic methylation in the CpG islands and a global hypomethylation, but this change is more prominent during cancer development (Egger G., et al., 2004 and Bird A., 2002). Several genes affected by hypermethylation are involved in cell cycle regulation, cell adhesion, apoptosis, angiogenesis, invasion and metastasis (Table 1). CpG island methylation in promoter regions is often associated with silencing of tumor suppressor genes and there are evidences suggesting that the aberrant DNA methylation occurs in most cancers, including colorectal cancers (Toyota M., et al., 1999 and Jones P. A., Baylin S. B., 2002).

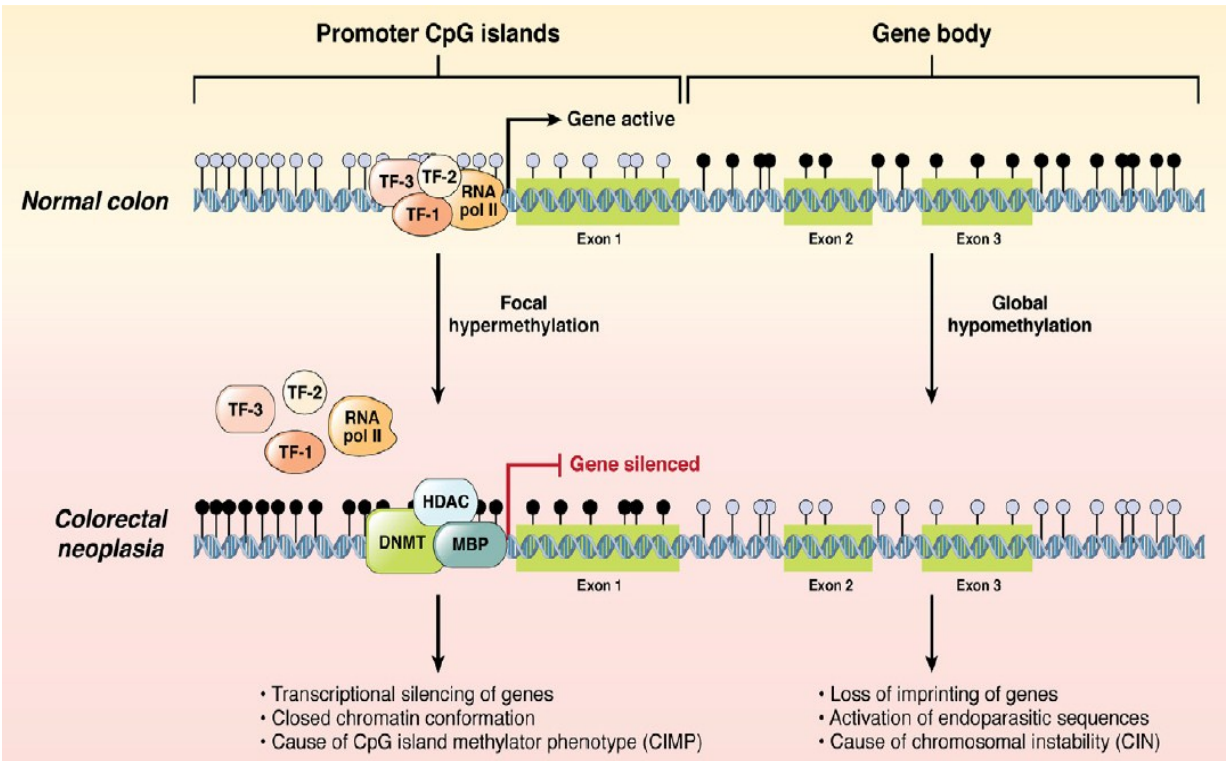


Figure 4. DNA methylation in normal colon and colorectal cancer (Goel A. and Boland C.R., 2012).

In normal colon, CpG islands within the promoter of a tumor suppressor gene are generally unmethylated (blue circles), whereas the CpG repeats within the gene body are frequently methylated (black circles). This permits easy access by transcription factors and RNA polymerase II to bind to the gene promoter and activates the gene expression. In colorectal cancer, DNA methyla transferase (DNMT), Histone deacetylase (HDAC), and methyl binding protein (MBP) complex catalyze the transfer of methyl groups to cytosines in the promoter CpG sites, resulting in the hypermethylation induced transcriptional silencing of the tumor suppressor genes (Figure 4, Goel A. and Boland C.R., 2012).

1.1.3.2. Histone modification and chromatin remodeling

The nucleosome is the basic unit of chromatin structure, composed of approximately 200 base pairs of DNA tightly wrapped around the cylindrical histone octamer. Histone octamer consists of two molecules of each of the four core histones, H2A, H2B, H3, and H4. The linker histone, H1 plays an important role in linking the nucleosome structures together to further condense the chromatin (Thomas J. O., Kornberg R. D., 1975 and Luger K., et. al 1997). The four core histones consist of a globular C-terminal domain and an unstructured N-terminal tail. Histone N-terminal tail that protrude from the histone octamer are frequently targeted by various posttranslational modifications, (Kouzarides T., 2007; Santos-Rosa H., and Caldas C., 2005; Peterson C. L., and Laniel, M. A., 2004; Bhaumik S. R., et al., 2007) and some of these modifications are enzymatically reversible (Shi Y., and Whetstine J. R., 2007). The biological significance of all these histone modifications is not clearly understood, but the modifications are known to influence transcription, DNA repair, DNA replication and chromatin condensation.

Table 1. Genes commonly methylated and suppressed in colorectal cancer.

Gene	Protein	Purpose	Function/Pathway	Colorectal neoplasia	Methylation frequency (%) colon cancer	Reference
APC	Adenomatous polyposis coli	Diagnosis	Inhibits Wnt signaling pathway	Hypermethylated	10-30	Lee et al., 2009; Deng et al., 2004; Esteller et al., 2000
HLTF	Helicase-like transcription factor	Prognosis	Chromatin remodeling factor	Hypermethylated	30-50	Wallner et al., 2006; Herbst et al., 2009
CDH1	E-cadherin	-	Cell adhesion: Invasion and Metastasis	Hypermethylated	30-55	Wheeler et al., 2001
VIM	Vimentin	Diagnosis	Cytoskeleton stabilization /	Hypermethylated	50-80	Chen et al., 2005; Ahlquist et al., 2012
CDKN2A/p16	Cyclin-dependent kinase inhibitor 2A	Diagnosis	Epithelial-Mesenchymal Transition (EMT) Cell cycle regulation	Hypermethylated	20-30	Zou et al., 2002; Nakayama et al., 2010

The impact of histone modification on chromatin structure is governed by the type of modification and the specific amino acid involved in the modification (Table 2). For example, lysine acetylation is associated with transcriptionally active DNA (euchromatin), whereas the effects of lysine and arginine methylation vary by location of the amino acid and number of methyl groups on these residues (Fischle W., 2003; Pray-Grant M.G., et al., 2005; Covic M., at al., 2005).

Table 2. Types of histone modification.

Amino Acid	Modification
Lysine	Methylation, Acetylation, Ubiquitination, Sumoylation, ADP-Ribosylation
Arginine	Methylation
Serine	Phosphorylation
Threonine	Phosphorylation

1.1.3.2.1. Histone modification in colorectal cancer

Histone methylation is recognized as an important histone modification linked to both transcriptional activation and repression depending on the specific amino acid affected (Martin C., and Zhang Y., 2005). A well-studied type of histone methylation is methylation of lysine residues, which is conferred by histone methyltransferases (HMTs). More than twenty lysine and arginine HMTs have been identified. HMTs either act alone or in complex with other HMTs in order to catalyze site-specific histone methylation. Histone methylation is not only important for

normal development and differentiation, but is also associated with tumor initiation and development. For example, histone-lysine N-methyltransferase, EZH2 (Enhancer of Zeste Homologue 2, an HMTase) was found to be overexpressed in colon cancer (Bracken A.P. and Helin K., 2009; Ougolkov A.V., et al., 2008).

There are three possible states of lysine methylation, namely, mono, di, and trimethylation. The methyl groups on the lysine residues can be removed by histone demethylases. Two families of histone demethylases have been identified, including flavin adenine dinucleotide dependent family and the jumonji C domain containing family (Lim S., et al., 2010 and Tsukada Y., et al., 2006). The JmjC family of histone demethylases consists of approximately 30 members within the human genome (Klose R., 2006). Jumonji domain containing 1A (JMJD1A) is a member of the jumonji C domain containing 1A family and responsible for demethylation of mono- and dimethylated H3K9 (Yamane K., et al., 2006). Histone demethylase JMJD1A has been shown to be upregulated in hypoxic conditions via hypoxia inducible factor-1-alpha (HIF-1α).

Hypoxia is a common characteristic of many solid tumors such as prostate cancer, breast cancer and colon cancer. Hypoxia occurs when the demands of growth and metabolism of a tissue or organ surpass the vascular oxygen supply (Wellmann S., 2008). Recent studies have shown that cellular hypoxia in colon cancer cells stabilizes HIF-1α, thereby increasing histone demethylase JMJD1A levels. Increased JMJD1A expression decreases the H3K9Me2 methylation on the promoters of target genes such as adrenomedullin (ADM) and the growth and differentiation factor 15 (GDF15), thereby modulating tumor growth (Figure 5, Krieg A. J., et al., 2010 and Mamoru U., et al., 2010).

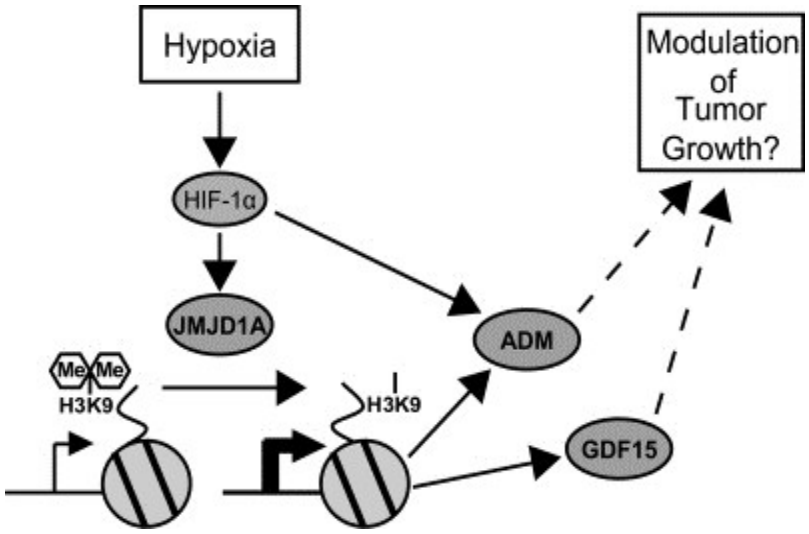


Figure 5. JMJD1A action during hypoxia (Krieg A. J., et al., 2010).

1.1.3.3. Noncoding microRNAs (miRNAs)

Changes in the expression profiles of miRNAs have been observed in a variety of human tumors, including colon cancers (Wong J. J., et al., 2007 and Slaby O., et al., 2009). MiRNAs are small non-coding RNAs about 19-25 nucleotides long. The miRNAs are transcribed from their genes by RNA Polymerase II (Pol II) as long primary miRNAs (Cai X., et al., 2004 and Lee Y., et al., 2004) and processed into 60-70 nucleotide-long precursor miRNAs by Drosha (RNase III endonuclease) and a RNA binding protein Pasha (Figure 6). The precursor miRNAs are exported to the cytoplasm by a Ran GTP/Exportin 5 – dependent mechanism (Bohnsack M. T., et al., 2004; Lee Y., et al., 2003; Zeng Y., and Cullen B. R., 2004). In the cytoplasm, they are further processed into 19-25 nucleotides long mature miRNAs by the RNase III protein Dicer (Esquela-Kerscher A., and Slack F. J., 2006; Lee Y., et al., 2002). These mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) to recognize and bind to target mRNAs (Filipowicz W., et al., 2008).

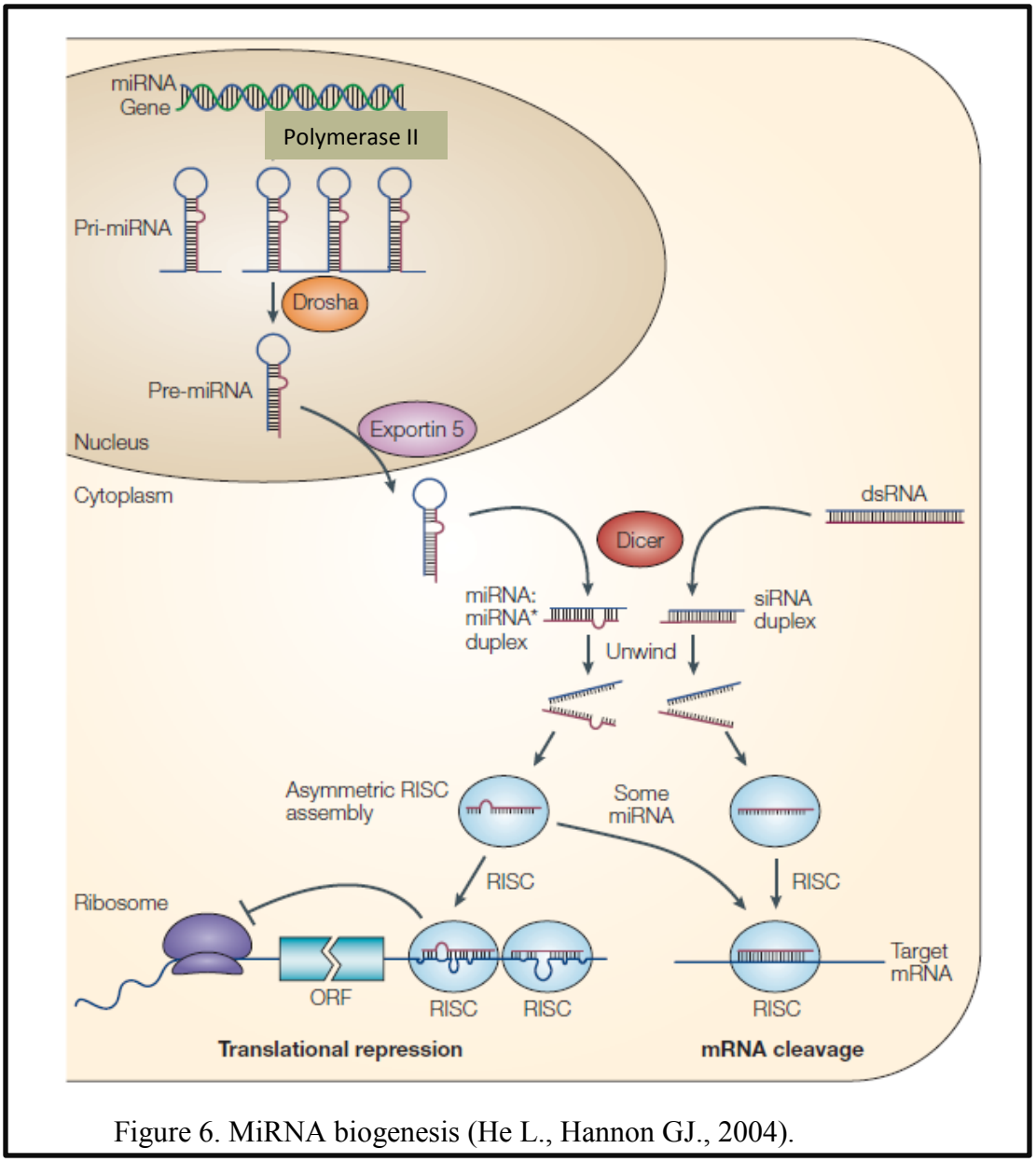


Figure 6. MiRNA biogenesis (He L., Hannon GJ., 2004).

MiRNAs regulate gene expression by targeting mRNAs and by associating with the 3'-untranslated region (UTR) through complementary base pairing (Bartel D. P., 2004), and this leads to either degradation or translational suppression of their target genes depending on the degree of complementarity (Figure 6). Each miRNA is predicted to have several targets, and each mRNA may be regulated by several miRNAs, which makes them master regulators of gene expression (Rajewsky N., 2006; Lewis B.P., et al., 2003; Lim L. P., et al., 2005). MiRNAs have

been implicated in regulation of important processes like cell proliferation (Cheng A. M., et al., 2005), apoptosis (Xu P., et al., 2004), differentiation (Karp X., and Ambros V., 2005), development (Chen C., et al., 2004), metabolism (Poy M., et al., 2004), invasion and metastasis (Ma L., et al., 2007).

1.1.3.3.1. Epigenetic dysregulation of miRNAs expression in colorectal cancer

Nearly, a total of 160 miRNAs are found to be dysregulated in colorectal cancer (Luo X., et al., 2011). The mechanisms responsible for miRNAs dysregulation in human cancers are still poorly understood. One mechanism that is best-characterized is aberrant methylation of the miRNA gene promoters in various tumors, including colorectal cancer (Lopez-Serra P., and Esteller M., 2011; Tang J.T., et al., 2011). An important study of genomic sequencing of miRNA genes revealed that nearly fifty percent of miRNAs are associated with CpG islands (Weber B., et al., 2007). MiR-34b and miR-34C, TP53 pathway components, are found to be epigenetically silenced in colorectal cancer. Treatment with 5-aza-2'-deoxycytidine (demethylating agent) has been shown to restore the expression of miR-34b and miR-34c (Toyota M., et al., 2008). The two miRNAs miR-143 and miR-145 were found to be downregulated in colorectal adenomas/cancers compared to normal colon (Michael M.Z., et al., 2003; Cummins J. M., et al., 2006) and to play the role of tumor suppressor (Bandres E., et al., 2006; Schepeler T., et al., 2008; Wang C. J., et al., 2009). The mir-143BP is a chemically modified miR-143 with improved nuclease resistance, and has been used as therapeutic drug for colorectal cancer treatment (Kitade Y., and Akao Y., 2010).

The introduction of high-throughput microarray-based miRNA expression profiling radically changed the field of miRNA expression pattern analysis. The list of differentially

expressed miRNAs is growing steadily, a list of important miRNAs and their target genes involved in colorectal carcinogenesis is shown in Table 3.

Table 3. List of microRNAs and their gene targets involved in colorectal cancer pathogenesis.

miRNA	Up-regulated / Down-regulated in CRC	Target Gene	Reference
miR-20a	Up-regulated	PTEN, RUNX1, TP53INP1	Motoyama et al., 2009
miR-21	Up-regulated	PTEN, TIMP3, PDCD4, RECK, TPM1, SPRY2	Schetter et al., 2008; Schmitz et al., 2009; Yamamichi et al., 2009; Kumar et al., 2007; Gabriely et al., 2008; Zhu et al., 2007; Sayed et al., 2008
miR-31	Up-regulated	BMP2	Motoyama et al., 2009
miR-34a/b/c	Down-regulated	CDK4, CDK6, E2F3, CyclinE2	Toyota et al., 2008; Raver-Shapira et al., 2007
miR-143	Down-regulated	KRAS, p53, DNMT3a, ERK5	Wang et al., 2009; Suzuki et al., 2009; Akao et al., 2007; Chen et al., 2009; Ng et al., 2009
miR-145	Down-regulated	cMYC, STAT1, OCT4, SOX, p53, IRS-1, YES1, KLF4	Wang et al., 2009; La et al., 2009; Suzuki et al., 2009; Sachdeva et al., 2009; Gregersen et al., 2010; Xu et al., 2009

Changes in the expression of proteins, which are involved in miRNA processing is also another possible reason for the miRNA dysregulation in colorectal cancer. For example up-regulation of the mRNA levels of Drosha, Dicer and Ago2 have been reported in certain colorectal cancer (Papachristou D. J., et al., 2011).

1.2. Objective and specific aims

1.2.1. Objective of the study

The main goal of this study was to determine the epigenetic mechanisms of how calcitriol (active form of vitamin D) suppresses the proliferation of colon cancer cells and the growth of tumor xenografts in nude mice.

Colon cancer is the second most common cause of cancer related deaths in the United States. Many epidemiological and experimental studies have supported a protective action of calcitriol against colon cancer. Researchers found that people with high circulating vitamin D levels (100 nM or higher) were nearly 40 percent less likely to develop colon cancer than those with low levels (less than 25 nM). However, the mechanism of how calcitriol suppresses colon cancer remains to be understood. We have focused on the novel epigenetic mechanisms of the antitumor activity of calcitriol in colon cancer. We have identified miR-627 as the only microRNA whose expression is induced by calcitriol in colon cancer cells *in vitro* and *in vivo* in tumor xenografts. We have also identified that histone demethylase Jumonji domain containing 1A (JMJD1A) is a potential target of miR-627. It has been recently reported that JMJD1A is upregulated in colon cancer during hypoxia (a low oxygen condition exists in the center of solid tumors). Increased JMJD1A expression decreases the histone methylation on the promoters of target genes such as adrenomedullin (ADM) and the growth and differentiation factor 15

(GDF15) and increases their expression, which promotes tumor growth. By inhibiting JMJD1A, we have shown that miR-627 suppresses colon cancer growth.

This study is important because the results will help us to understand the mechanism of action of calcitriol in colon cancer. Although calcitriol has excellent antitumor activity, its side effect of inducing hypercalcemia prevents its use in the clinical treatment of cancer. This study has the potential to identify the novel targets (miR-627 and JMJD1A) downstream of calcitriol, which can be used to design new therapy for colon cancer while bypassing the hypercalcemic toxicity. Our study is innovative because this is the first study of microRNA in the antitumor action of calcitriol, the function of miR-627 has not been studied before. The regulation of JMJD1A by calcitriol through miR-627 is also a novel mechanism.

In this study, we hypothesize that miR-627 may play an important role in growth inhibition induced by calcitriol and JMJD1A may be a potential therapeutic target to treat colon cancer.

1.2.2. Specific aims

To test our hypothesis, we propose the following specific aims:

1.2.2.1. Specific aim 1: To determine the role of miR-627 in colon cancer suppression by calcitriol *in vitro*

The working hypothesis was that calcitriol induces miR-627 expression in colon cancer cells. MicroRNA-627 would in turn inhibit JMJD1A expression and this would make colon cancer cells susceptible to calcitriol induced growth inhibition. The experimental design for this part of the study was:

a) MicroRNA expression profiling in control and calcitriol (100nM) treated HT-29 cells using the Ambion mirVana miRNA Bioarray. The upregulation of miR-627 by calcitriol was confirmed by real-time PCR in HT-29 and HCT-116 cells.
b) To determine the effect of miR-627 on HCT-116 colon cancer cell proliferation by WST-1 assay.
c) To determine the VDR requirement for activation of miR-627 expression in calcitriol treated SW-620 cells.
d) To determine the potential target of miR-627 and its expression in calcitriol treated colon cancer cells.
e) To determine the effect of JMJD1A expression on HCT-116 colon cancer cell proliferation by WST-1 assay.
f) To determine the effect of calcitriol induced miR-627, on H3K9Me2 levels on the promoters of JMJD1A targets genes, such as GDF 15.

1.2.2.2. Specific aim 2: To determine the role of miR-627 on the *in vivo* growth of colon cancer xenografts

The working hypothesis was that miR-627 overexpression in colon cancer xenografts will inhibit JMJD1A expression and this would in turn decrease the growth of the colon cancer xenografts. The experimental design for this part of the study was:

a) To determine the expression of miR-627 and JMJD1A in calcitriol treated HT-29 tumor xenografts.
b) To determine the effect of calcitriol on the growth of HCT-116 and HT-29 colon tumor xenografts.

c) To determine the effect of calcitriol on the growth of HCT-116-JMJD1A-3'UTR colon tumor xenografts, after blocking the activity of miR-627 by overexpressing the JMJD1A 3'UTR sponge.

d) To determine the effect of calcitriol on the growth of HCT-116-JMJD1A-3'UTR-MUT colon tumor xenografts, when the miR-627 binding site within the 3'UTR sequence was mutated.

e) To determine the effect of miR-627 on the growth of colon tumor xenografts: Comparing the tumor growth in HCT-116 cells stably expressing miR-627 or miR-NC.

1.2.2.3. Specific aim 3: To determine miR-627 expression in human colon cancer specimens

The working hypothesis was that miR-627 expression will be downregulated in human colon cancer specimens compared to normal colon tissues. Decreased miR-627 expression would in turn increase the JMJD1A expression in human colon cancer specimens, which will stimulate colon cancer growth. The experimental design for this part of the study was:

a) To determine the expression levels of miR-627 in human colon cancer specimens by Real-Time PCR.

1.3. Materials and methods

1.3.1. Cell lines used

The human colon cancer cell lines HCT-116, HT-29 and SW-620 were purchased from American Type Culture Collection (ATCC). HCT-116 cells were cultured in RPMI-1640 medium (Hyclone, Thermo scientific) containing 10% fetal bovine serum (FBS). HT-29 and SW-620 cells were cultured in DMEM medium (Hyclone, Thermo Scientific) containing 10% FBS.

1.3.2. Drugs and chemicals

Calcitriol (1α, 25-dihydroxy vitamin D_3, the active form of vitamin D) was purchased from Cayman Chemical (Ann Arbor, MI). Formaldehyde was purchased from Alfa Aesar (Ward Hill, MA). BrdU (5-Bromodeoxyuridine, Product No: B9285) was purchased from Sigma-Aldrich (St. Louis, MO). Propidium Iodide (PI, Cat# 10009957) was purchased from Cayman chemical company. 10mM concentration of Calcitriol was prepared in DMSO and then finally diluted 100X in media to treat colon cancer cell lines at desired concentrations.

1.3.3. Western blot analysis

Cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS). Complete protease inhibitor cocktail (Roche) was added to lysis buffer before use. Protein concentration was determined by Bio-Rad DC protein assay (Bio-Rad). Protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked in 5% non-fat milk in PBS overnight and incubated with primary antibody and subsequently with appropriate horse radish peroxidase-conjugated secondary antibody. Signals were developed with ECL reagents (Pierce) and exposure to X-ray films. Anti-JMJD1A polyclonal antibody was kindly provided by Dr. Doug Demetrick at University of Calgary. Anti-β-tubulin and anti-GFP antibodies were purchased from Santa Cruz Biotechnology. Anti-p15, p16, p21, and p27 antibodies were purchased from Cell Signaling Technology.

1.3.4. Detection of apoptosis by flow cytometry

Colon cancer cells were harvested after desired treatment and washed in cold phosphate-buffered saline (PBS). Cell density was measured using a hemocytometer and cells were resuspended in annexin-binding buffer to $1x10^6$ cells/mL. 5 μL Annexin V fluorescein conjugate (Alexa Fluor 647 purchased from Invitrogen) was added to 100 μL of cell suspension and cells

were incubated at room temperature (RT) for 15 minutes. After the incubation period, 400 µL of annexin-binding buffer was added to the cell suspension, gently mixed and cells were analyzed immediately by an Accuri C6 Flow Cytometer.

1.3.5. Labeling cells with anti-BrdU and propidium iodide (PI) for flow cytometric analysis

For *in vitro* labeling of BrdU, HCT-116 colon cancer cells were treated with calcitriol for 72 hrs, BrdU was dissolved in PBS and added to culture medium directly to achieve a final concentration of 10µM. After incubating the cells with BrdU for 30 min at 37^0C, the cells were washed with RPMI-1640 medium twice and were further incubated at 5% CO_2 and 37^0C. 0hrs, 4hrs, 8hrs, 12hrs, and 6hrs after the BrdU pulse, cells were washed with PBS and fixed in 70% ice-cold ethanol and stored overnight at -20^0C. Fixed cells were resuspended in 2N HCl-0.5% Triton X-100 solution and incubated for 30 min at RT, to separate the DNA from the cells. To remove the excess acid, the cells were washed twice with PBS and then resuspended in PBS-1% BSA-0.5% Tween 20 solution and hybridized with a mouse monoclonal anti-BrdU antibody conjugated to FITC (1:20 dilution, Enzo Life Sciences, ITEM NO: ALX-804-197L-T060) for 30 min at RT in the dark. The cells were then washed with PBS-1%BSA-0.5%Tween20 (2X), resuspended in PBS and stained with PI (5µg/mL) for 30 min at RT in the dark. The cellular DNA content and the amount of BrdU incorporated were measured simultaneously using an Accuri C6 flow cytometer. Green fluorescent light emission (FITC=BrdU incorporation) was collected using FL1 and red fluorescence (PI=DNA content) was collected in FL3 detectors. Plots of BrdU labeling vs DNA content were drawn using FLOWJO software.

1.3.6. WST-1 cell proliferation assay

WST-1 reagent (Roche, Indianapolis, IN) was used to measure the relative proliferation rates of cells in culture medium. The principle of this assay is based on the conversion of the

tetrazolium salt, WST-1 (slightly red) into water soluble formazan dye (dark red) by mitochondrial dehydrogenase present only in viable cells. The activity of mitochondrial dehydrogenase increases proportionally with the number of viable cells, leading to an increase in the conversion of WST-1 to formazan dye. The resulting increased color intensity is in turn quantified by measuring the absorbance at 440 nm. Briefly, after treatment with calcitriol, the colon cancer cells were incubated with WST-1 for 4 hours at 37^{o} C in a humidified atmosphere maintained at 5 % CO_2 as recommended by the manufacturer. The absorbance of the formazan dye was measured at 440nm.

1.3.7. Microarray-miRNA expression profiling

In order to identify the potential microRNAs that are differentially expressed in HT-29 colon cancer cells following calcitriol treatment, we performed microarray-based miRNA expression profiling. Total RNA was isolated from the control and calcitriol (100 nM for 24 hours) treated HT-29 cells using the mirVana miRNA Isolation Kit (Ambion). MicroRNAs were further concentrated using flashPAGE^{TM} Fractionator system (Ambion). A polynucleotide tail was added at the 3'end of these concentrated miRNA samples from control and calcitriol treated HT-29 cells. These concentrated miRNAs from control and calcitriol treated HT-29 cells were labeled with the mirVana miRNA Labeling kit (Ambion) using fluorescent dye-Cy3 and Cy5 post labeling reactive dyes, respectively (Amersham Biosciences). Following this, the miRNA samples from the HT-29 cells were kept for overnight hybridization with the probes on the mirVana miRNA Bioarray slide. The array slides were scanned with an Aron 4000B Genepix Microarray Scanner and results were analyzed with Genepix software (Molecular Devices, Sunnyvale, CA, USA). To ensure accuracy, the labeled miRNA samples were hybridized in duplicate probes. The ratio of fluorescence intensity of calcitriol treated HT-29 cells over non

treated HT-29 was calculated to determine the differential expression of the miRNAs in the two treatment groups.

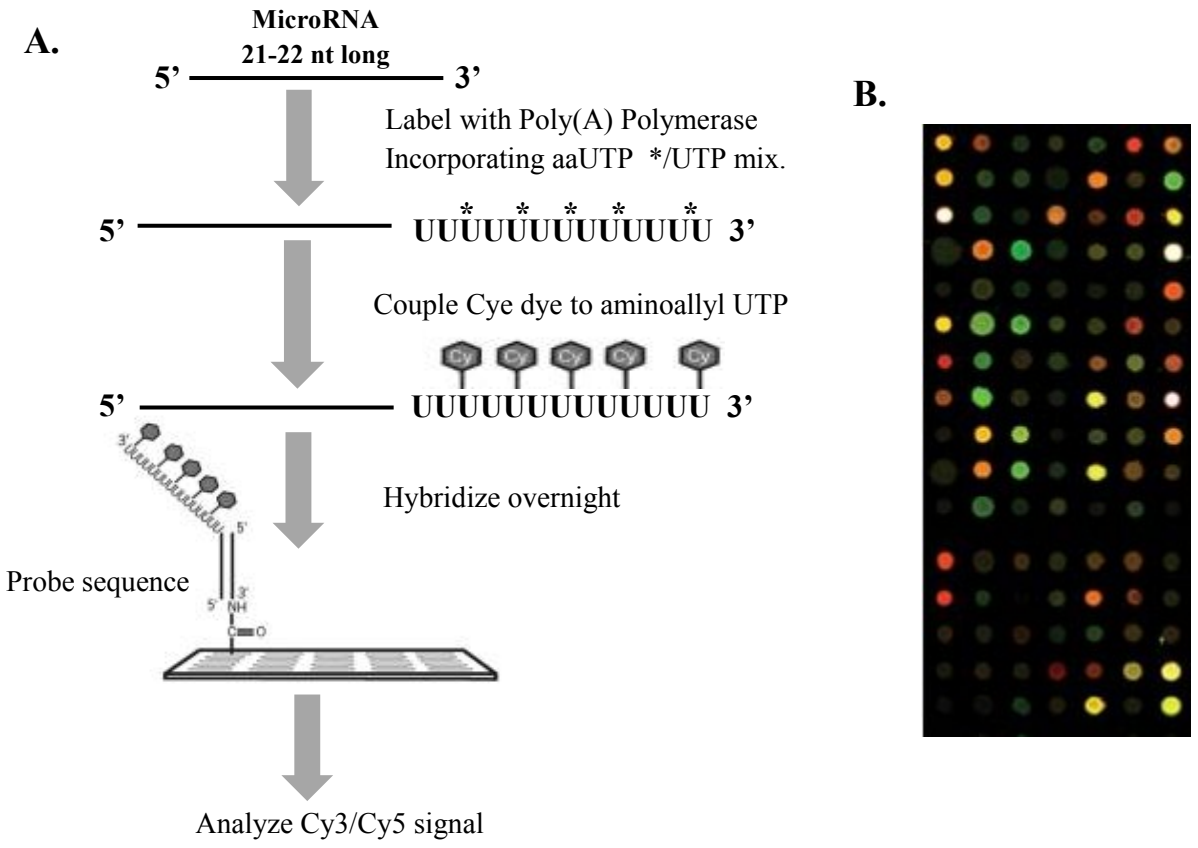


Figure 7. MiRNA array procedure. (A) Amine NTPs are incorporated into purified miRNAs during tailing with poly (A) polymerase. MiRNAs are then labeled with amine reactive dyes. After hybridization with probes on a microarray slide, dye signals are analyzed. (B) Represent--ative hybridization signals from a miRNA array procedure (Red: Cy3; Green: Cy5; nt: nucleotide; Shingara J., et al., 2005).

1.3.8. Real-time polymerase chain reaction

The miRNA expression was measured by real-time PCR using TaqMan® MicroRNA assay (Cat # RT1560 for miR-627) from Applied Biosystems (Foster city, CA). Total RNA was isolated from colon cancer cells using *mir*Vana™ miRNA Isolation Kit (Ambion). 5 µg of total

RNA was used in reverse transcription reaction. The cDNA was used as template to perform PCR on an Applied Biosystems 7500 Real-time PCR System following the manufacturer's protocol. Relative miRNA expression levels were calculated using 18S RNA as reference. GDF15 mRNA expression was measured by real-time PCR using the TaqMan® Gene Expression assay (Cat # Hs00171132_m1).

1.3.9. Transient transfection of SW-620 cells with pCEP4-Flag-VDR plasmid

We created an expression vector to over express the full length VDR gene in SW-620 cells. The VDR cDNA was cloned into the pCEP4 vector (Figure 8) to express VDR as a flag-tagged protein. Full length VDR gene was obtained by polymerase chain reaction using expressed-sequence tag clone as a template. Transient transfection was performed in SW-620 cells with this plasmid using Lipofectamine ^{TM} PLUS reagent (Invitrogen) following manufacturer's protocol. 0.5 µg/ml of plasmid was used to form complexes with transfection reagent. pCEP4-Flag-VDR transfected (24h) SW-620 cells were treated with 100 nM calcitriol for 24 hours, miR-627 expression was analyzed by real-time PCR.

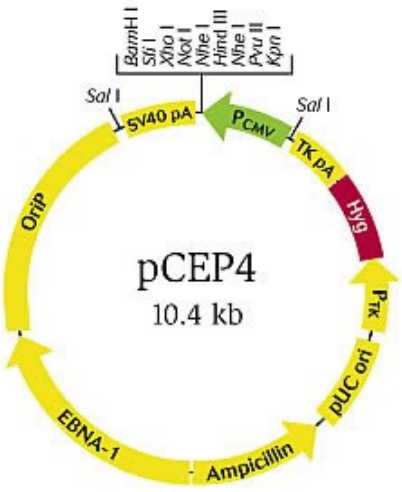


Figure 8. pCEP4 plasmid (Invitrogen Catalog # V044-50).

1.3.10. Transient transfection of HCT-116 cells with miR-627 and miR-NC mimics

Synthetic miRNA mimics (Pre-miR™ miRNA Precursors) for miRNA-627 (Catalog # AM17100) and miR-NC (Catalog # AM17110) were purchased from Ambion. Ambion® Pre-miR™ miRNA Precursor Molecules are small, chemically modified, double-stranded RNA molecules designed to mimic endogenous mature miRNAs. These miRNA mimics were transfected into HCT-116 cells using X-tremeGENE siRNA transfection reagent (Roche, Indianapolis, IN) following manufacturer's protocol. 100 nM negative control miRNA or miR-627 mimic was used for transfection into HCT-116 cells and cell growth was determined at various time points using the WST-1 assay.

1.3.11. Transient transfection of HCT-116 cells with miR-627 or miR-NC plasmids

We created an expression vector to over express miR-627 or miRNC in HCT-116 colon cancer cells. The vector used for this study was a special type of vector from Invitrogen (The BLOCK-iT™ Pol II miR RNAi expression vector, Figure 9). Double strand oligoes containing pre-miR-627 / miR-NC were cloned into pcDNA6.2-GW/EmGFP-miR plasmid (Invitrogen) by directional cloning. This plasmid has a promoter region called P_{CMV} which causes over expression of any gene placed adjacent to it. A Green Fluorescence Protein gene (GFP) is placed just before the miRNA region so that the over expression of the miRNA could be determined by the over expression of the GFP, marked by green fluorescence. Transient transfection was done in HCT-116 cells with these plasmids using Lipofectamine ^{TM} PLUS reagent (Invitrogen). 0.5 μg/ml of plasmid was used to form complexes with transfection reagent. After transfecting the HCT-116 cells with miR-627 and miR-NC plasmids, the fluorescence of the cells was observed for two days. The increase in the fluorescence showed that the gene inserted in the BLOCK-iT™ Pol II miR RNAi expression vector was being overexpressed. To confirm the overexpression of

miR-627 in HCT-116 cells, protein was isolated from cells 2 days after transfection and western blot was performed to detect JMJD1A.

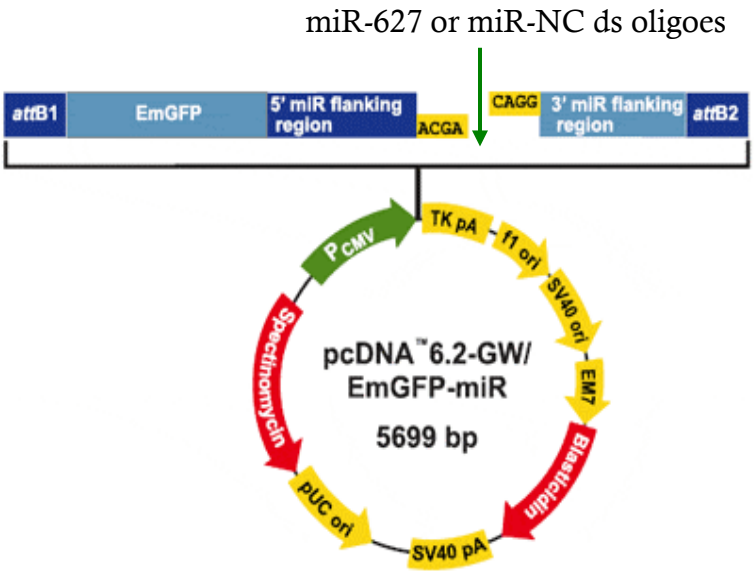


Figure 9. The BLOCK-iT™ Pol II miR RNAi expression vector, pcDNA 6.2-GW/EmGFP-miR. (Invitrogen, Catalog # K4936-00).

1.3.12. Transient transfection of HCT-116 cells with negative control or JMJD1A specific siRNAs

The Silencer® Select siRNAs for JMJD1A (Catalog # 4392420) and negative control (Catalog # AM4611) were purchased from Ambion. The Silencer® Select siRNAs are more potent than currently available siRNAs with fewer off-target effects. These siRNAs were transfected into HCT-116 cells using X-tremeGENE siRNA transfection reagent (Roche, Indianspolis, IN) following manufacturer's protocol. 100 nM negative control or JMJD1A specific siRNA was used for transfection into HCT-116 cells and JMJD1A knockdown was confirmed by western-blot. Effect of JMJD1A knockdown on cell growth was determined by WST-1 assay.

1.3.13. Transient transfection of HCT-116 cells with pCEP4-Flag-JMJD1A plasmid

We created an expression vector to over express the full length JMJD1A protein in HCT-116 cells. The JMJD1A cDNA was cloned into the pCEP4-Flag vector (Figure 7) to express JMJD1A as a flag-tagged protein. Full length JMJD1A gene was obtained by polymerase chain reaction (PCR) using expressed-sequence tag clone as a template. Transient transfection was done in HCT-116 cells with this plasmid using Lipofectamine ^{TM} PLUS reagent (Invitrogen) by following manufacturer's protocol. JMJD1A overexpression was confirmed by western blot using anti-Flag antibody.

1.3.14. Transient transfection of HCT-116 cells with miR-627 and miR-NC inhibitors

MiRCURY LNA™ microRNA Inhibitors for miR-627 (Catalog # 410399-00) and miR-NC (Catalog # 199004-00) were purchased from Exiqon. miRCURY LNA™ microRNA inhibitors are ideal for use as specific suppressors of microRNA activity. These miRNA inhibitors were transfected into HCT-116 cells using X-tremeGENE siRNA transfection reagent (Roche, Indianapolis, IN) following the manufacturer's protocol. 100 nM LNA-modified miRNA inhibitor specific to miR-627 or the negative control inhibitor was used for transfection, and cells were treated with 500 nM calcitriol for 48 hours. Total RNA and protein was isolated to analyze the miR-627 and JMJD1A expression by RT-PCR and Western blot respectively.

1.3.15. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP assay kit from Millipore. Formaldehyde was added directly to the medium containing colon cancer cells after desired vitamin D treatment, to crosslink histones to DNA. After incubating cells with formaldehyde for 10 minutes at 37^0C and 5% CO_2, media was removed as much as possible. Cells were washed twice using ice cold PBS containing protease inhibitors. Cells were scrapped

into a conical tube and centrifuged at 2000 rpm for 4 minutes at 4^0C. Cell pellet was resuspended in 200µl of SDS lysis buffer with protease inhibitors and incubated for 10 minutes on ice. We used 200µl of SDS lysis buffer per 1 X 10^6 cells. Cell lysate was sonicated on ice to shear DNA into fragments of 200 and 1000 base pairs length (Branson Digital Sonifier, 30 % power, 10 seconds pulse 3X, 1 minute interval between each pulse). After sonication, the samples were centrifuged for 10 minutes at 13,000 rpm and 4^0C, the cell supernatant was then diluted 10 times in ChIP Dilution Buffer, and protease inhibitor cocktail was added as indicated above. 1% (20µL) of diluted supernatant was stored at -20^0C, and was considered as INPUT, and loading control. To reduce nonspecific background, 80µl of Salmon Sperm DNA/Protein A Agarose-50% Slurry was added to 2ml diluted cell supernatant and incubated for 1 hour at 4^0C with agitation. Brief centrifugation (1000 rpm, 1 min at 4^0C) was performed to pellet the agarose and the supernatant fraction was collected. Immunoprecipitations were performed by incubating the supernatant fractions with anti- H3K9Me2, H3K4Me3 (Cell Signaling Technology, Danvers, MA, 1:200 dilution) and H3K27Me2/Me3 (Active Motif, Carlsbad, CA, 1:200 dilution) or control IgG antibodies for overnight at 4ºC with rotation. 60µl of Salmon Sperm DNA/Protein A Agarose Slurry was added to the immunoprecipitated complex and incubated for one hour at 4ºC with rotation to collect the antibody/histone complex. Protein A agarose /antibody/histone complexes were collected by brief centrifugation, these complexes were washed for 3 min on a rotating platform with 1 ml of each of the following buffers:

1. Low Salt Immune Complex Wash Buffer, (1X)
2. High Salt Immune Complex Wash Buffer, (1X)
3. LiCl Immune Complex Wash Buffer, (1X)
4. 1X Tris-EDTA Buffer, (2X)

After each wash, the complexes were centrifuged at 1000 rpm for 1 min at 4^0C. Histone complexes were eluted from the antibody by incubating with 250µL freshly prepared elution buffer (1%SDS, 0.1M $NaHCO_3$) for 15 min at room temperature (RT). After centrifugation at 13000 rpm for 5 min, the supernatant fractions were collected. The elution process was repeated and the eluates were combined (500µL). INPUTS as well as eluates were reverse cross-linked by treating with 20µL 5M NaCl at 65^0C for overnight. Finally, a polymerase chain reaction was performed with the primers designed from the sequences of the GDF15 promoter, (5'-CTGTCTCTGGCCGAGGCG AG-3' and 5'-CACTTACCTTCTGGCGTGAGTATCCGG-3').

1.3.16. Creation of miR-627 and miR-NC stable expressing HCT-116 cell lines

Stable cell lines expressing miR-627 or negative control miRNA were established by transfection of HCT-116 cells with BlockiT™ Pol II miR expression vectors: pcDNA6.2-GW/EmGFP-miR-627 or pcDNA6.2-GW/EmGFP-nega-control. These vectors have a blasticidin-resistance and a spectinomycin-resistance gene. XL-1 Blue competent E.Coli bacteria from Agilent Technologies were used to amplify the plasmid. The bacterial colonies overexpressing miR-627 and miR-NC were selected in spectinomycin-positive agar plates (50µg/mL spectinomycin). Both the plasmids were linearized by digesting with ApaL1. Linearization of plasmids before the transfection increases the efficiency of stable clone generation as well as target gene expression (Grant S. and Gerald M., 2010). Lipofectamin™Plus reagent (Invitrogen) was used to transfect miR-627 and miR-NC plasmids into HCT-116 cells. The transfected HCT-116 cells were treated with the optimized dose of blasticidin (8µg/mL), which selected only cells that had taken up the plasmid containing the resistance gene to blasticidin. Due to the presence of a GFP gene placed before the miRNA, the overexpression of the miRNA was determined by the green fluorescence of GFP. These GFP colonies were picked

and sub-cultured, resulting in HCT-116 stable cell lines overexpressing the miR-627 or miR-NC. Stable miR-627 expression was confirmed by checking the JMJD1A expression through western blot analysis (Table 4).

1.3.17. Creation of JMJD1A-3'UTR stable expressing HCT-116 cell lines

The 3'UTR (Un Translated Region) cDNA fragment of JMJD1A gene was obtained by PCR using EST clone as a template. The JMJD1A-3'UTR cDNA was inserted into pRNAT-CMV3.2/Puro plasmid (Figure 10, BamH1/Xho1). This vector has a puromycin-resistance and an ampicillin-resistance gene. XL-1 Blue competent E.Coli bacteria from Agilent Technologies were used to amplify the plasmid. The bacterial colonies overexpressing JMJD1A-3'UTR were selected in ampicillin-positive agar plates (100µg/mL Ampicillin).

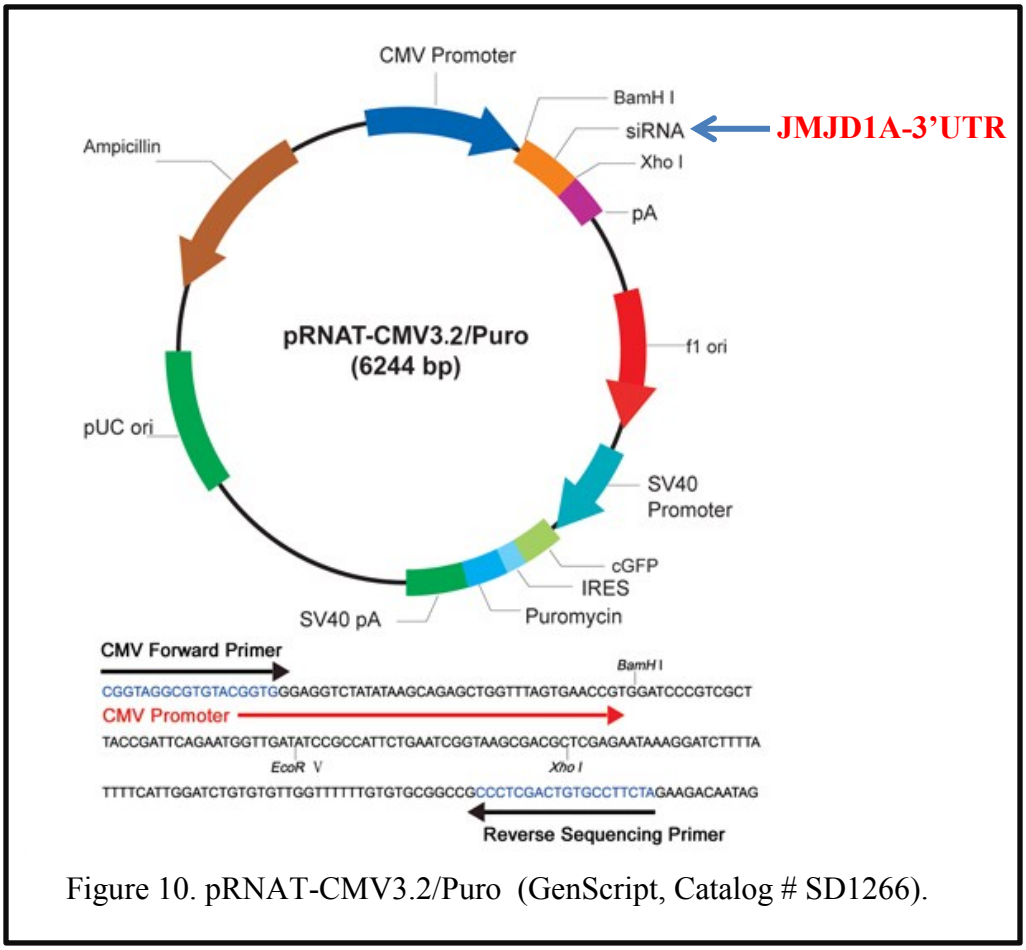


Figure 10. pRNAT-CMV3.2/Puro (GenScript, Catalog # SD1266).

Plasmid was linearized by digesting with Pvu1. Lipofectamin^{TM}Plus reagent (Invitrogen) was used to transfect JMJD1A-3'UTR plasmid in to HCT-116 cells. The transfected HCT-116 cells were treated with the optimized dose of puromycin (0.4µg/mL), and the cells that took up the plasmid continued to grow and form colonies. Expression of the JMJD1A-3'UTR was determined by the green fluorescence of GFP, marked by green fluorescence. These GFP colonies were picked and sub-cultured, resulting in HCT-116 stable cell lines overexpressing the JMJD1A-3'UTR. Expression of JMJD1A-3'UTR was determined by measuring JMJD1A protein levels in these stable cells before and after the calcitriol treatment using western blot analysis (Table 4).

1.3.18. Site-directed mutagenesis

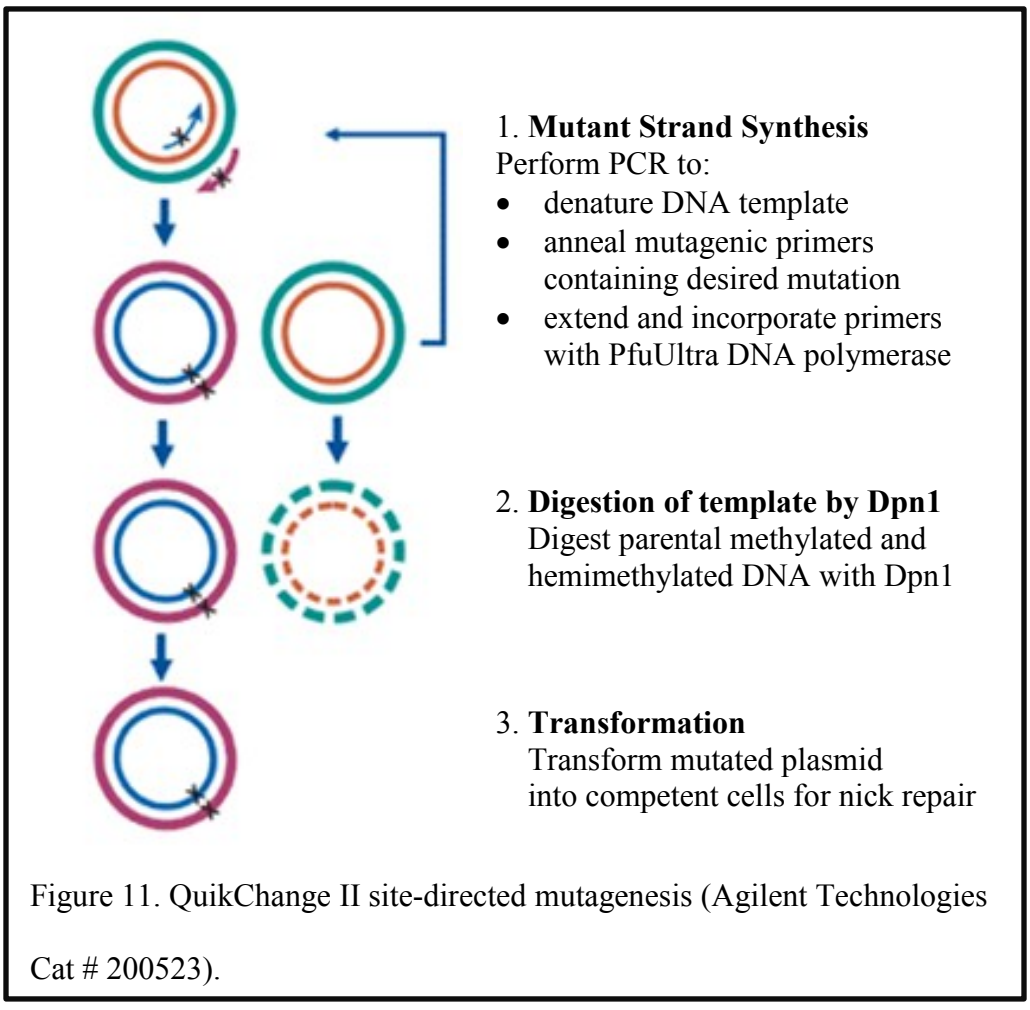


Figure 11. QuikChange II site-directed mutagenesis (Agilent Technologies Cat # 200523).

The QuikChange site-directed mutagenesis procedure is used to make point mutations, delete or insert single or multiple amino acids. As shown in Figure 11, this procedure requires a supercoiled double-stranded DNA vector with an insert (gene of interest, JMJD1A-3'UTR) and two synthetic oligonucleotide primers containing the desired mutation. Briefly a mutant strand was synthesized by performing a polymerase chain reaction (PCR) using a pfuUltra DNA polymerase. The original and non-mutated plasmid DNA that remained after the PCR cycles was digested with the enzyme DpnI. XL-1 Blue bacteria were used to amplify plasmid DNA containing the desired mutation (JMJD1A-3'UTR-MUT).

1.3.19. Creation of JMJD1A-UTR-MUT stable expressing HCT-116 cell lines

pRNAT-CMV3.2/Puro plasmid with JMJD1A-3'UTR-MUT insert was used to generate these stable cells. This vector has a puromycin-resistance and an ampicillin-resistance gene. XL-1 Blue bacteria (Agilent Technologies) were used to amplify the plasmid. The bacterial colonies overexpressing JMJD1A-3'UTR-MUT were selected in ampicillin-positive agar plates (100µg/mL ampicillin). The plasmids were linearized by digesting with Pvu1 prior to the transfection. Lipofectamin^{TM}Plus reagent (Invitrogen) was used to transfect JMJD1A-3'UTR-MUT plasmid in to HCT-116 cells. After 24 hours, the transfected HCT-116 cells were treated with the optimized dose of puromycin (0.4µg/mL), and the cells that took up the plasmid continued to grow and form colonies. Expression of the JMJD1A-3'UTR-MUT was determined by the green fluorescence of the GFP. These GFP colonies were picked and sub-cultured to create HCT-116 stable cell lines overexpressing the JMJD1A-3'UTR-MUT. Expression of JMJD1A-3'UTR-MUT was determined by measuring JMJD1A protein levels in these stable cells before and after the calcitriol treatment through western blot analysis (Table 4).

Table 4. Stable cell lines summary.

Objective	Vector used	Target gene tested	Section
Expression of miR-627 and/or miR-NC	pcDNA6.2-GW/EmGFP	JMJD1A	1.3.16.
Expression of JMJD1A-3'UTR and/or 3'UTR-MUT	pRNAT-CMV3.2/Puro	JMJD1A	1.3.17./1.3.19.

1.3.20. Tumor xenografts in nude mice

Six to 8-week-old female nude mice (Nu/Nu) were purchased from Charles River (Wilmington, MA). The mice were maintained in sterile conditions using the Innovive IVC system from Innovive (San Diego, CA), following the protocol approved by the Institutional Animal Care and Use Committee of North Dakota State University. Tumor xenografts were established by subcutaneous injection of 2×10^6 cancer cells in the flank region of the mice. Calcitriol and dimethyl sulfoxide (as control) were administered by intraperitoneal injection. Two axes of the tumor (L, longest axis; W, shortest axis) were measured with a digital caliper. Tumor volume was calculated as: $V = L \times W^2/2$.

CHAPTER 2: ROLE OF CALCITRIOL INDUCED MIR-627 IN SUPPRESSION OF COLON CANCER GROWTH

The working hypothesis for this part of the study was that, calcitriol induces miR-627 expression in colon cancer cells. MicroRNA-627 in turn inhibits histone demethylase, JMJD1A, resulting in the inhibition of colon cancer growth. The following experiments were performed to test our hypothesis.

2.1. Results and discussion

2.1.1. Determining the effect of calcitriol on the growth of HCT-116 and HT-29 colon cancer cells

To determine the effects of calcitriol on colon cancer cells, we incubated HCT-116 and HT-29 cells with various concentrations of calcitriol for 48 hours. At the end of the incubation, WST-1 reagent (Roche, Indianapolis, IN, USA) was added and placed in the incubator for 30 min. Finally absorbance was measured at 440 nm and the percentage of growth inhibition was reported (Figure 12), untreated colon cancer cells absorbance was considered as 100 percent cell growth. It was observed from the Figure 12, that with increasing concentration of calcitriol, the percentage of cell growth significantly decreased both in HCT-116 and HT-29 colon cancer cells.

2.1.2. Effect of calcitriol on HCT-116 cell cycle distribution

To find out the mechanism of calcitriol induced growth inhibition, we analyzed the cell cycle distribution in calcitriol treated HCT-116 cells using a simple PI staining. Cells were treated with calcitriol and stained with PI to analyze the cell cycle distribution on an Accuri C6 flow cytometry. As shown in Figure13**,** calcitriol treatment slightly increased the percentage of cells in the G1 phase of cell cycle (but not statistically significant).

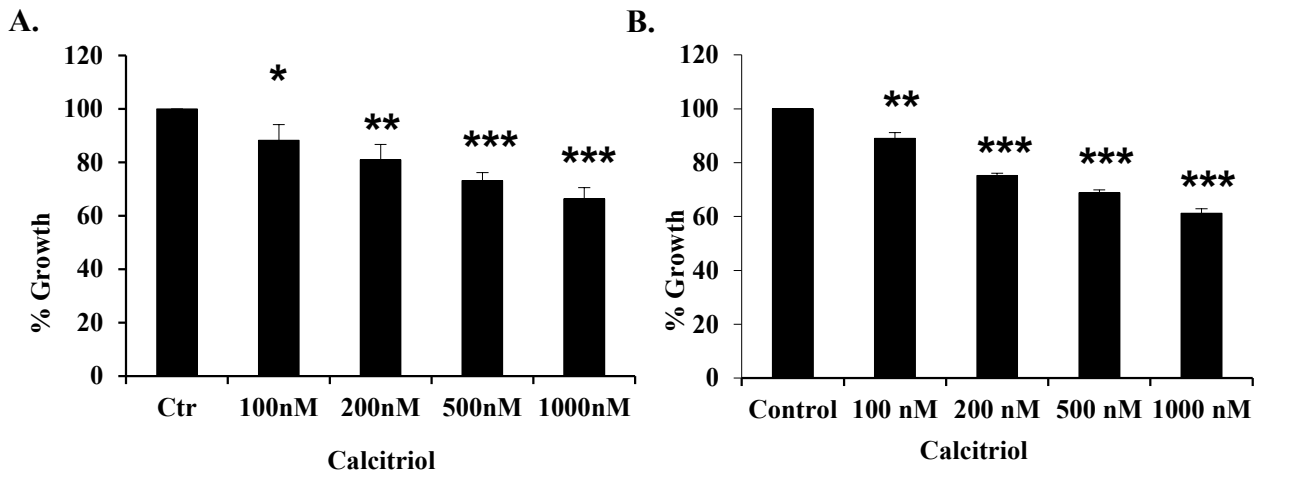
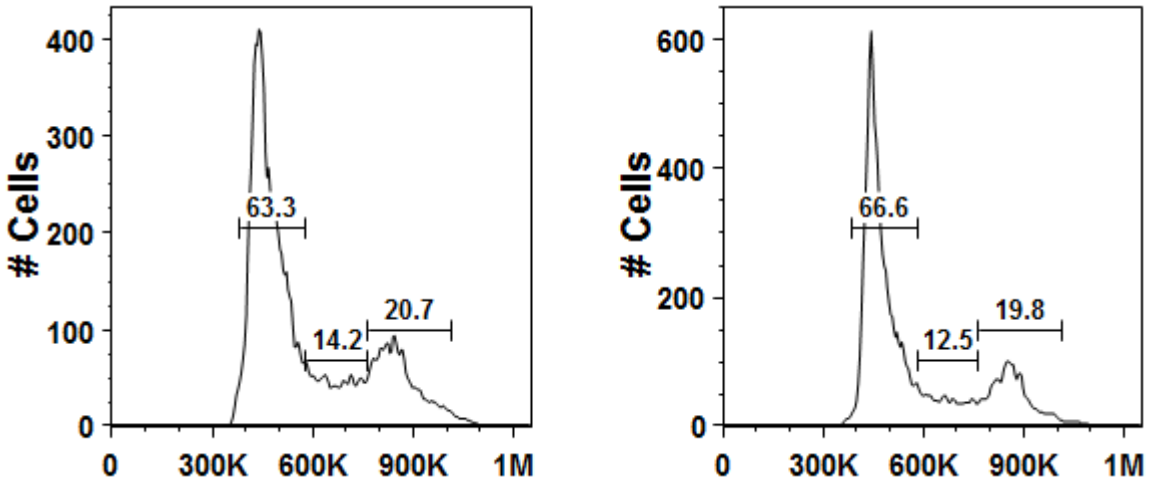


Figure 12. Calcitriol inhibits the proliferation of colon cancer cells. (A) HCT-116 and (B) HT-29 cells were treated with various concentrations of calcitriol for 48 hours and *in vitro* cell proliferation was analyzed using WST-1 assay (*p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001).



FL2-Wmod, FL2-Amod subset
Sample **Control** A01.fcs
Event Count: 12812

FL2-Wmod, FL2-Amod subset
Sample **Calcitriol** A02.fcs
Event Count: 13024

Figure 13. Calcitriol had minimal effects on the cell cycle of HCT-116 cells. HCT-116 cells were treated with 500 nM calcitriol for 48 hours and cell cycle distribution was analyzed by flow cytometry (Control: G1=63.3%, S=14.2%, G2/M=20.7% ; Calcitriol: G1=66.6%, S=12.5%, G2/M=19.8%).

2.1.3. Flow cytometric analysis of apoptosis using Annexin V in calcitriol treated HCT-116 cells

We determined if apoptosis plays a role in calcitriol induced growth suppression in colon cancer cells. HCT-116 cells were treated with calcitriol, incubated with Annexin V fluorescein conjugate and analyzed for apoptosis using an Accuri C6 flow cytometer. As shown in Figure 14, apoptosis induced by calcitriol is very minimal in colon cancer cells.

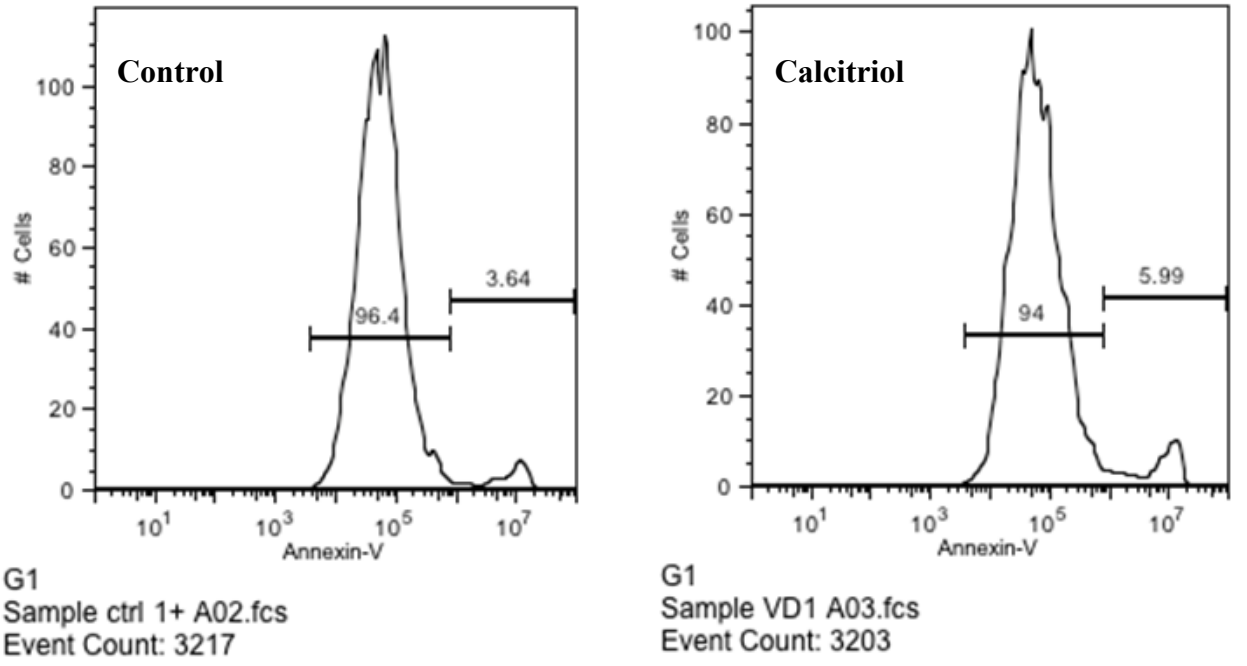


Figure 14. Calcitriol had minimal effects on apoptosis of HCT-116 cells. HCT-116 cells were treated with 500 nM calcitriol for 48 hours, apoptosis was determined by Annexin V staining and flow cytometry.

2.1.4. Western blot analysis of cyclin-dependent kinase (CDK) inhibitors in calcitriol treated HCT-116 cells

Cyclin-dependent kinase inhibitors (CDKIs) bind to and inhibit the activity of CDKs. Two major classes of CDK inhibitors have been identified. The p16 family (p15, p16, p18 and p19) binds to and inhibits the activities of CDK4 and CDK6. The p21 family (p21, p27, p28 and p57) can bind

to broad range of CDK-cyclin complexes and inhibit their activities. CDKIs are capable of suppressing the cancer growth (Bayrak A., Oktay K., 2003). We determined if cyclin-dependent kinase inhibitors play a role in calcitriol induced growth suppression in colon cancer cells. HCT-116 cells were treated with calcitriol for 48 hrs, then protein was isolated and western blot analysis was performed using p15, p16, p21, and p27 antibodies. As shown in Figure 15, calcitriol had minimal effects on the cyclin-dependent kinase inhibitors. Expressions of p15, p21, and p27 were not upregulated by calcitriol, while p16 expression was slightly increased. Previously, it was reported that calcitriol induced p21 expression in HCT-116 cells, at a significantly higher dose of 1 μM **(**Liu G., et al 2010**)**.

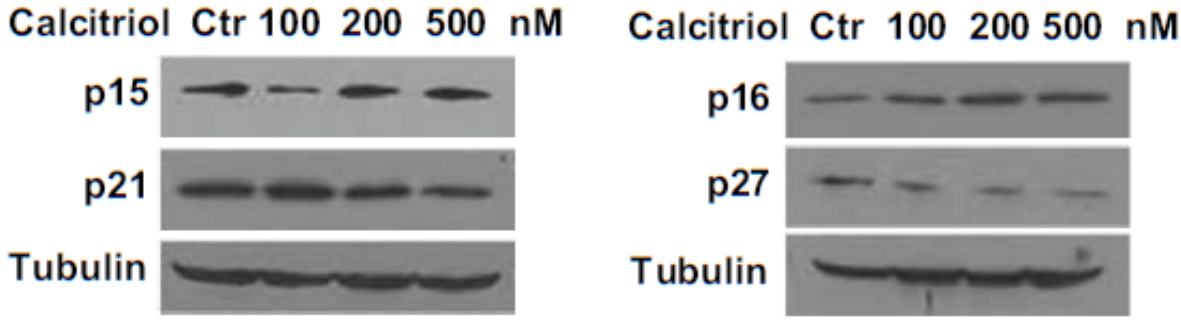


Figure 15. Effects of calcitriol on the cyclin-dependent kinase inhibitors. HCT-116 cells were treated with various doses of calcitriol (100, 200, 500 nM) for 48 hrs and the expression of cell cycle regulatory proteins (p15, p16, p21 and p27) was analyzed by western blots.

2.1.5. Effect of calcitriol on HCT-116 cell cycle progression

2.1.5.1. Cell doubling time

To determine if calcitriol caused a general slowdown of the cell cycle, we compared the cell doubling time in control and calcitriol treated HCT-116 cells. Control and calcitriol treated cells were collected at various time points. Cell number data was used to calculate the cell doubling time using an online doubling time calculator (http://www.doubling-time.com/compute.php, Table 5). As shown in Figures 16A and B, calcitriol increased the cell doubling time by about 3 hours.

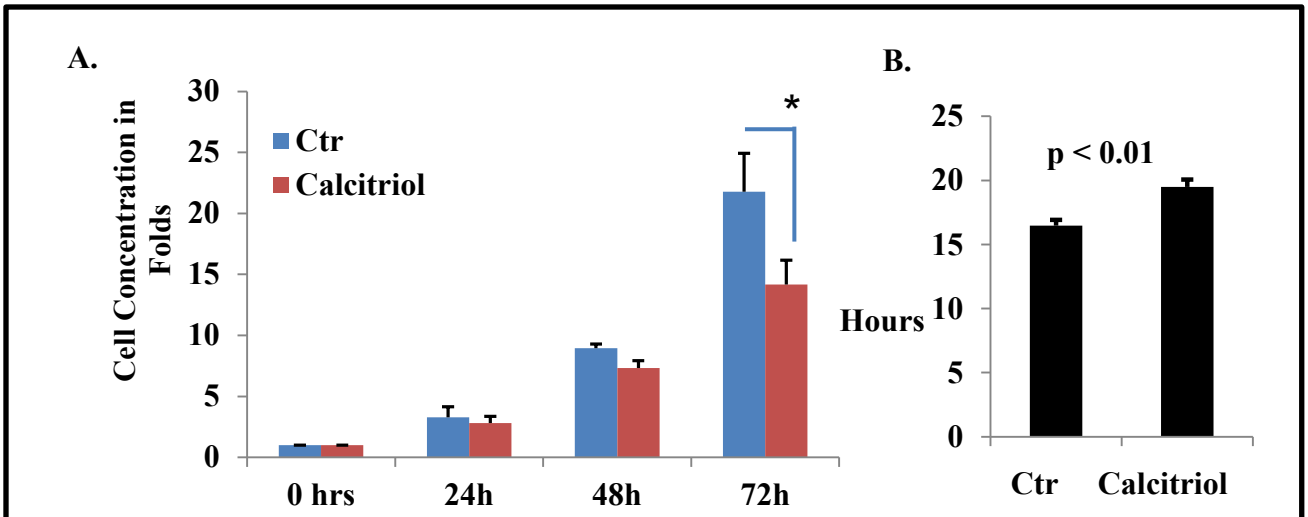


Figure 16. Calcitriol increases cell doubling time in colon cancer cells. (A) HCT-116 cells were continuously treated with 500 nM calcitriol. Control and calcitriol treated cells were collected at 0h, 24h, 48h, and 72h. Cell number was determined using hemacytometer and counting under a microscope. (B) Cell number data was used to calculate the cell doubling time (Control: 16.5h; Calcitriol: 19.47h; * p-value < 0.05).

Table 5. Various parameters of cell doubling time in calcitriol treated HCT-116 cells.

Parameter	Control	Calcitriol 500nM
Doubling Time in hours	16.5	19.47
Growth Rate	0.042	0.0356
Equation	Amount = $1.2108*e^{0.042*time}$	Amount = $1.2554*e^{0.0356*time}$
Cell Concentration at T= 0 hrs	1.2108 (Folds)	1.2554 (Folds)

2.1.5.2. Cell cycle progression by BrdU pulse chase experiment

To determine if calcitriol caused a general slowdown of the cell cycle, we also measured cell cycle progression by a pulse chase experiment using flow cytometric analaysis. HCT-116 cells were treated with calcitriol for 72 hours. BrdU was directly added to the cell culture media (control and

calcitriol treated) to a final concentration of 10µM. These cells were incubated for 30 minutes at 37^0C and 5%CO_2. After the 30 min short pulse, cells were washed with media to remove excess BrdU and incubated further in normal growth media. At various time points post BrdU pulse cells were harvested, washed, fixed in ice-cold ethanol and labeled with anti-BrdU (FITC conjugated) followed by propidium iodide (PI), as discussed in materials and methods section. As shown in Figures 17A and 17B, HCT-116 cells treated with calcitriol showed a general slowdown of the cell cycle. Compared to control, calcitriol treated samples showed more BrdU labeled cells to lag behind at all-time points.

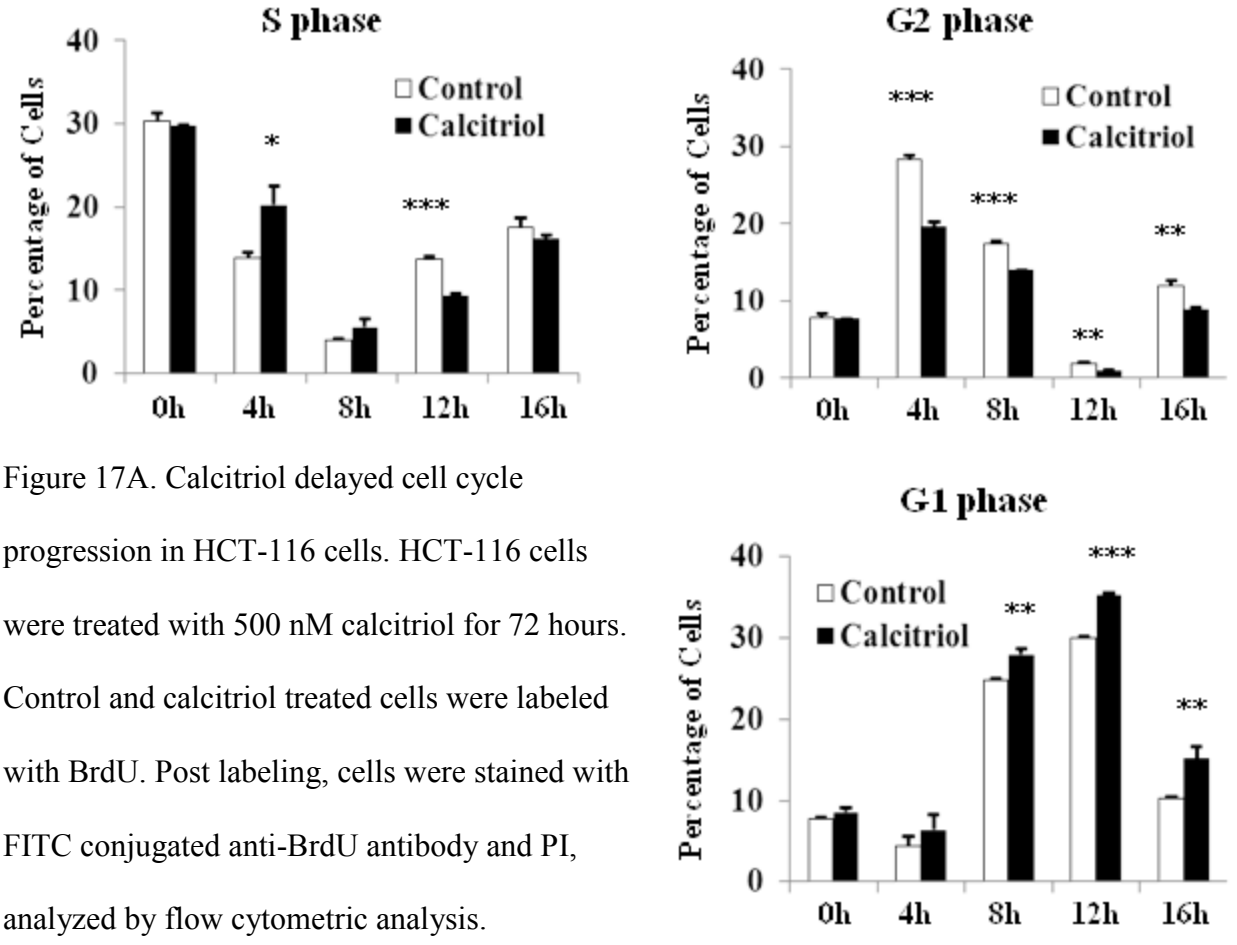


Figure 17A. Calcitriol delayed cell cycle progression in HCT-116 cells. HCT-116 cells were treated with 500 nM calcitriol for 72 hours. Control and calcitriol treated cells were labeled with BrdU. Post labeling, cells were stained with FITC conjugated anti-BrdU antibody and PI, analyzed by flow cytometric analysis.

(* $p < 0.05$, **$p < 0.01$, *** $P < 0.001$).

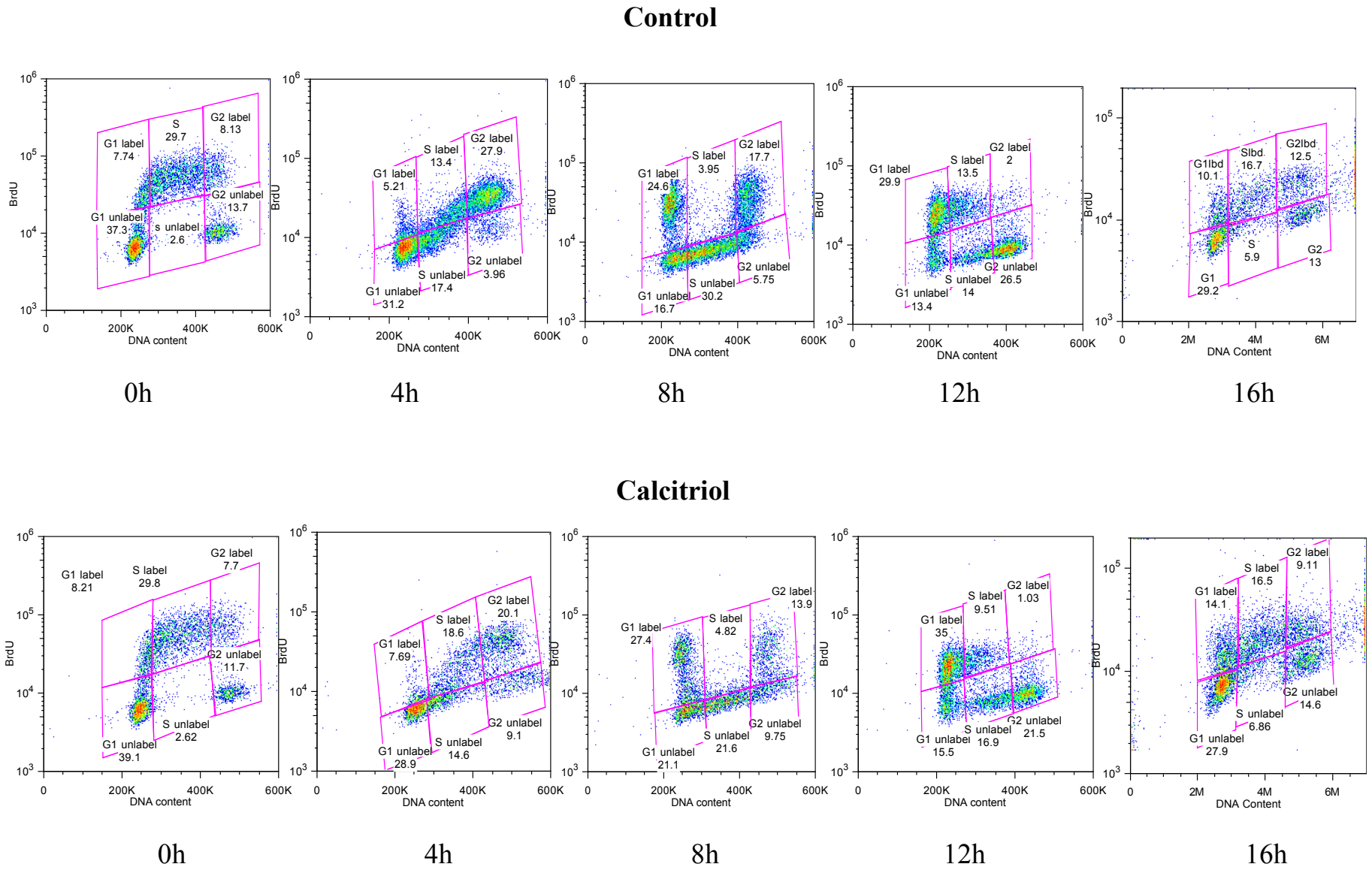


Figure 17B. Flow cytometric analysis of control and calcitriol treated HCT-116 cells labeled with anti-BrdU and PI.

2.1.6. Effect of calcitriol on differential expression of miRNAs in colon cancer cells

In order to reveal the possible mechanism of how calcitriol inhibits cancer cell growth, we investigated the effects of calcitriol on the expression of the microRNAs in HT-29 colon cancer cells. We used the mirVana miRNA Bioarray kit from Ambion, to compare the microRNA expression profiles in HT-29 cells before and after calcitriol (100nM, 24 hours) treatment. Among the 471 human miRNAs examined, miR-627 was the only microRNA whose expression level was significantly increased by calcitriol treatment. List of dysregualted miRNAs are shown in Tables 6 and 7. It was observed that the miR-627 expression was up-regulated most significantly in calcitriol treated HT-29 cells as compared to control HT-29 cells. Therefore, miR-627 was selected to investigate its potential role in colon cancer treatment. MiR-627 is one of the miRNAs identified in a recent genome-wide miRNA expression study in colon cancer cells (Cummins J.M., et al., 2006).

Table 6. List of miRNA that are increased by calcitriol treatment in HT-29 cells.

Name of miRNA	Average fluorescence ratio of calcitriol/control	Standard deviation
let-7g	1.279	0.073
miR-28	1.342	0.091
miR-346	1.345	0.256
miR-627	6.458	1.239

NOTE: The fold of changes in miRNA levels in calcitriol treated cells compared to control cells is indicated by the ratio of fluorescence of the two samples. For example, a ratio of 6.458 indicates 545.8% increase of the miRNA in the calcitriol treated cells.

Table 7. List of miRNA that are decreased by calcitriol treatment in HT-29 cells.

Name of miRNA	Average fluorescence ratio of calcitriol/control	Standard deviation
miR-125a	0.486	0.021
miR-133b	0.585	0.1
miR-139	0.837	0.1
miR-154	0.366	0.061
miR-196a	0.669	0.021
miR-26a	0.715	0.057
miR-296	0.686	0.071
miR-30b	0.47	0.071
miR-33	0.709	0.044
miR-491	0.471	0.093
miR-499	0.553	0.042
miR-517a	0.648	0.017
miR-518a	0.37	0.06
miR-532	0.667	0.148
miR-549	0.526	0.058
miR-553	0.834	0.016
miR-557	0.365	0.004
miR-571	0.62	0.147
miR-579	0.627	0.139
miR-591	0.57	0.055
miR-595	0.613	0.079
miR-597	0555	0.062
miR-600	0.57	0.155
miR-608	0.456	0.004
miR-637	0.533	0.015
miR-646	0.73	0.158
miR-662	0.8	0.234
miR-9-AS	0.452	0.008

NOTE: The fold of changes in miRNA levels in calcitriol treated cells compared to control cells is indicated by the ratio of fluorescence of the two samples. For example, a ratio of 0.365 indicates 63.5% decrease of the miRNA in the calcitriol treated cells.

2.1.7. Confirmation of miRNA expression by real-time PCR

In order to confirm the results from the microarray-based miRNA expression profiling and to quantify the relative levels of miR-627, we performed Real-Time Polymerase Chain Reaction (RT-PCR) in control and calcitriol treated HCT-116 and HT-29 colon cancer cell lines. As evident from Figure 18, miR-627 levels were significantly upregulated in calcitriol treated HCT-116 and HT-29 colon cancer cell lines. These results indicate that miR-627 may be a major epigenetic regulator in the calcitriol induced growth inhibition in colon cancer cells.

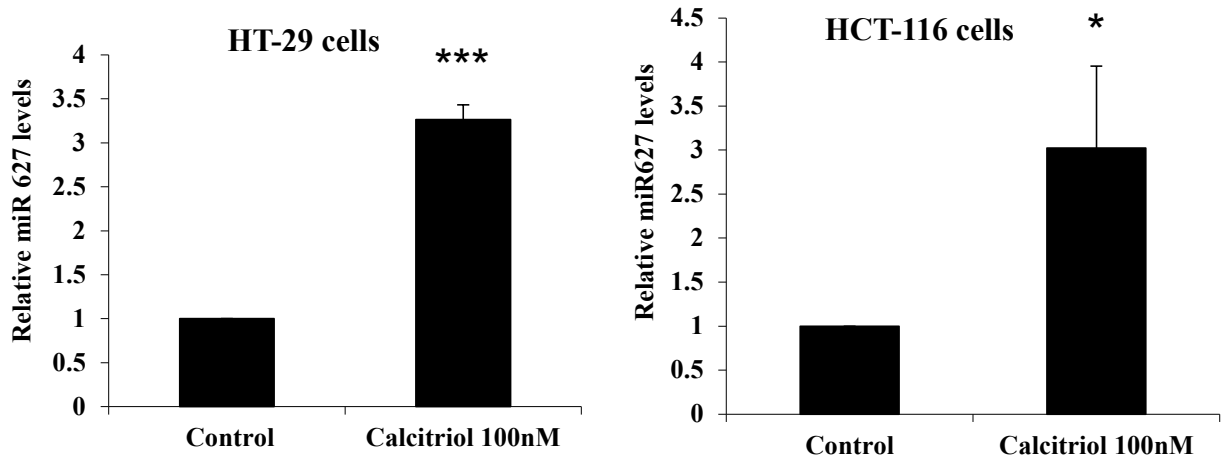


Figure 18. Calcitriol induces miRNA-627 expression in colon cancer cells. HCT-116 and HT-29 cells were treated with 100 nM calcitriol for 24 hours and total RNA was isolated from the cells and analyzed by RT-PCR (* $p < 0.05$; *** $p < 0.001$).

2.1.8. Vitamin D receptor and miR-627 expression in SW620 cells

In order to determine the involvement of vitamin D receptor (VDR) for the activation of miR-627 expression, we observed the effects of calcitriol in SW620 cells. SW620 is a colon cancer cell line which expresses extremely low level of vitamin D receptor VDR, not detectable by Northern Blot (Palmer H.G., et al., 2001). The VDR cDNA was cloned into the pCEP4-Flag vector to express VDR as a Flag-tagged protein. Real-time PCR was performed in control and calcitriol

treated SW620 cells to measure the miR-627 expression levels. As shown in the Figure 19**,** calcitriol induced miR-627 expression at a level relatively lower than that in HT-29 or HCT-116 cells (comparing with Figure 18). However, after transfection of SW620 cells with a plasmid to overexpress full length VDR, calcitriol induced miR-627 expression to a level similar to that in HT-29 or HCT-116 cells (Figure 19). Therefore, the results indicated that both genomic (VDR-dependent) and non-genomic mechanisms may be required for calcitriol to induce miR-627 expression in colon cancer cells.

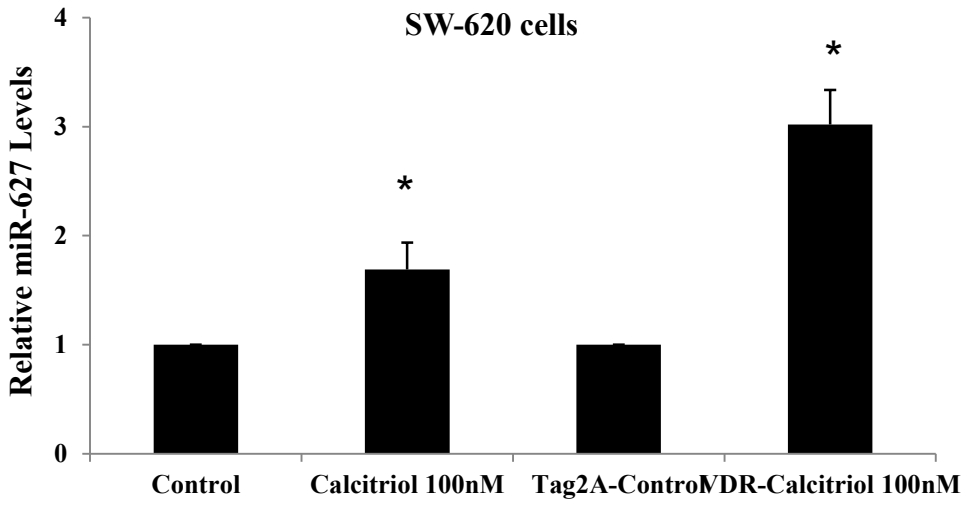


Figure 19. Calcitriol induces miR-627 expression in SW-620 cells, after VDR overexpression. Untransfected or pCEP-Flag-VDR transfected SW-620 cells were treated with 100 nM calcitriol for 24 hrs, total RNA was isolated and analyzed by RT-PCR (* $p < 0.05$).

2.1.9. Effect of miRNA-627 on colon cancer cell proliferation

To determine the effects of miR-627 on colon cancer cell proliferation, we transfected synthetic miRNA-627 and miR-negative control (miR-NC) mimics into HCT-116 cells using X-tremeGENE siRNA transfection reagent following manufacturer's protocol. At the end of 24, 48, and 72 hours after transfection WST-1 reagent was added and placed in the incubator for 30 min.

Finally absorbance was measured at a wavelength of 440 nm and growth curves were reported. As shown in Figure 20, synthetic miR-627 significantly inhibited colon cancer cell growth while the negative control miRNA had no effect. Thus, miR-627 may mediate the anti-proliferative activity of calcitriol.

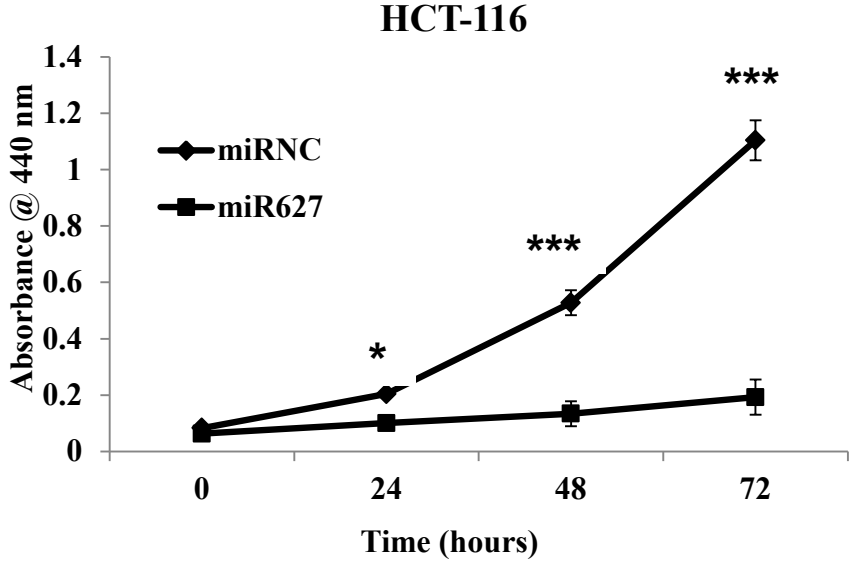


Figure 20. MiR-627 overexpression significantly inhibited the growth of HCT-116 cells. HCT-116 cells were transfected with 100 nM negative control miRNA or miR-627 mimic. Cell growth was determined at various time points after transfection by WST-1 Assay (* $p < 0.05$; *** $p < 0.001$).

2.1.10. JMJD1A is the potential target of miR-627

Once we confirmed the inhibitory effect of miR-627 on the growth of HCT-116 colon cancer cells, we searched online databases (www.microrna.org and miRBase) to found out the gene which is potentially targeted by miR-627 and we found that miR-627 may repress the expression of the histone demethylase, JMJD1A (Jumonji Domain containing 1A) by binding to its 3' untranslated region (UTR). Figure 21, shows the partial complementarity between the miR-627 and JMJD1A-3'UTR. JMJD1A is an iron- and 2-oxoglutarate-dependent dioxygenase which

specifically catalyzes the demethylation of mono and dimethylated Lys-9 of histone H3, with a preference for dimethylated residue, thereby playing a crucial role in histone code (Yamane K., et al., 2006). JMJD1A has recently been shown to promote colon cancer growth by mediating the effects of HIF-1. (Krieg A.J., et al., 2010) The growth-promoting activity of JMJD1A makes it an interesting target for miR-627.

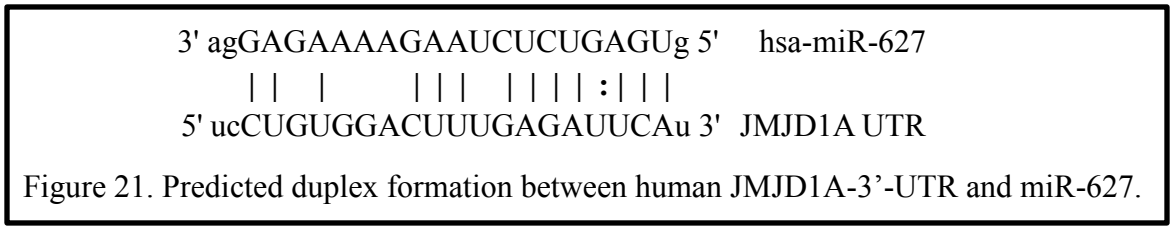


Figure 21. Predicted duplex formation between human JMJD1A-3'-UTR and miR-627.

To confirm that the JMJD1A-3'UTR is the target of miR-627, a cDNA fragment containing the 3'UTR sequence of JMJD1A was inserted downstream of the GFP gene in the pEGFP-C1 plasmid and the plasmid was transfected into HCT-116 cells together with pcDNA6.2-GW-miR-627 (to overexpress miR-627), GFP expression (western blot analysis) was significantly reduced comparing with cells transfected with pEGFP-JMJD1A-3'UTR and pcDNA6.2-GW-negative-control plasmids (Figure 23A). The action of miR-627 was dependent on the miRNA binding site within the JMJD1A 3'UTR, because GFP expression was not reduced by miR-627 when the JMJD1A-3'UTR binding site was mutated (Figure 23B) by the QuickChange II site-directed mutagenesis procedure. Mutation was performed within the miR-627 recognition site (AUUC to UGAG mutation, Figure 22) of JMJD1A 3'UTR. For co-transfection of pEGFP-JMJD1A-3'UTR and miRNA expression vectors, EmGFP was removed from pcDNA6.2-GW/EmGFP-miR-627 using Dra I digestion to create pcDNA6.2-GW-miR-627, prior to the transfection.

3' agGAGAAAAGAAUCUCUGAGUg 5' hsa-miR-627
| | | | | | | | | |
5' ucCUGUGGACUUUGAGUGAGAu 3' JMJD1A UTR-MUT

Figure 22. Compromised complimentarity between human miR-627 and JMJD1A-3'UTR-MUT, after Site-Directed Mutagenesis.

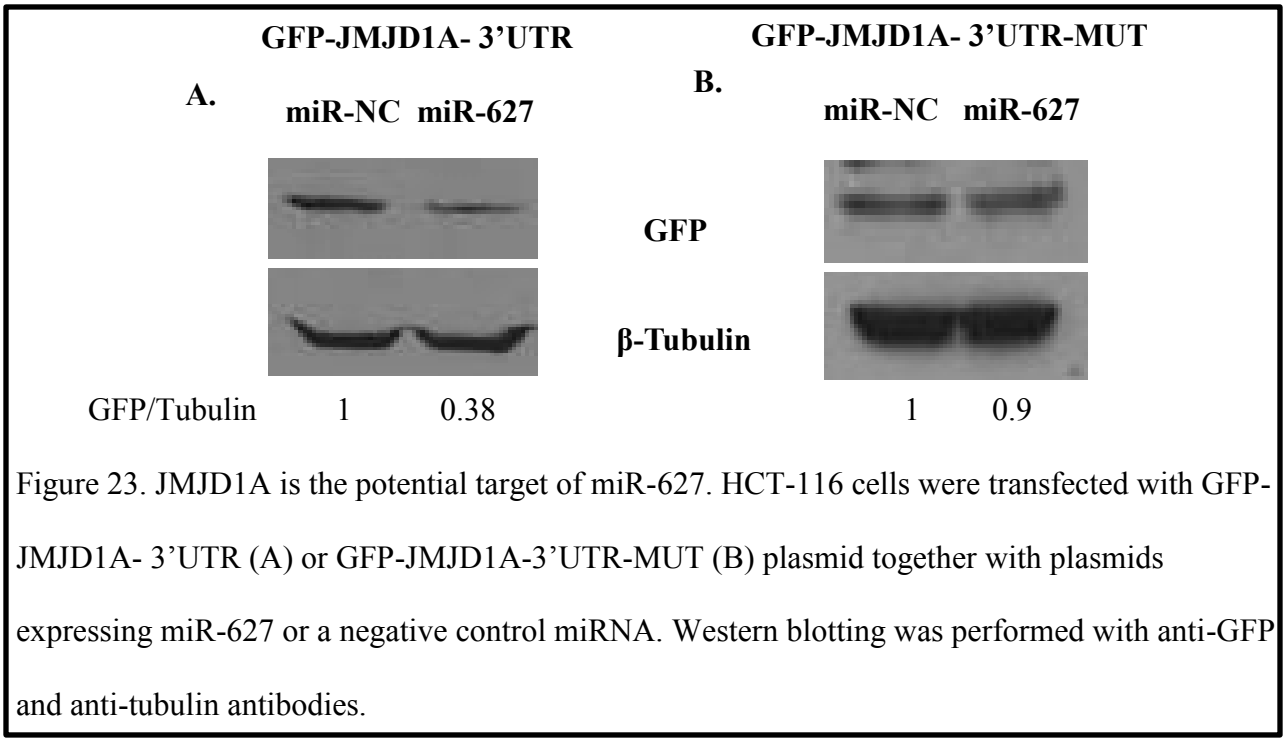


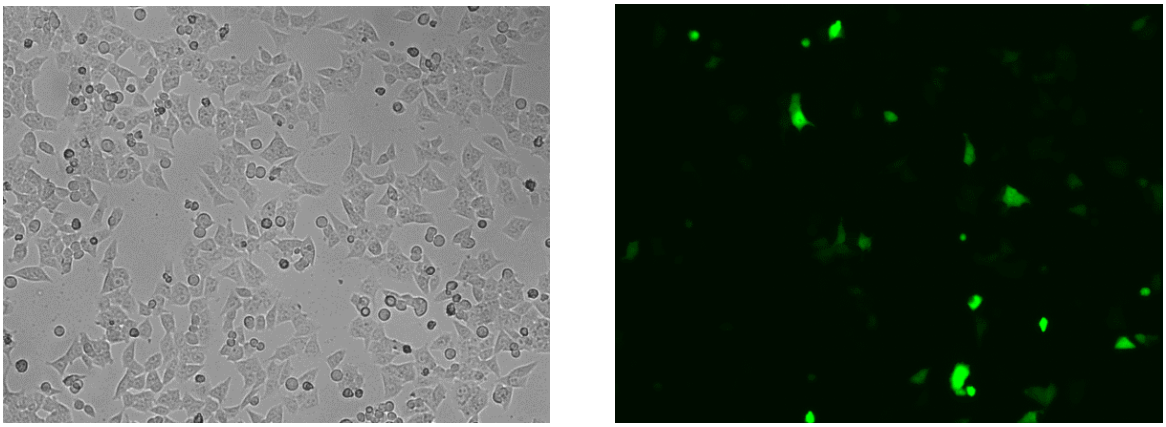
Figure 23. JMJD1A is the potential target of miR-627. HCT-116 cells were transfected with GFP-JMJD1A- 3'UTR (A) or GFP-JMJD1A-3'UTR-MUT (B) plasmid together with plasmids expressing miR-627 or a negative control miRNA. Western blotting was performed with anti-GFP and anti-tubulin antibodies.

2.1.11. Transient over expression of miR-627 in HCT-116 colon cancer cells

To further confirm that miR-627 targets JMJD1A, we used the BLOCK-iT™ Pol II miR RNAi expression vector to over express miR-627 in HCT-116 colon cancer cells. It has a P_{CMV} promoter region which causes over expression of any gene placed adjacent to it. Green Fluorescence Protein gene (GFP) was placed just before the miRNA region so that the over expression of the miRNA could be determined by the over expression of the GFP, marked by green fluorescence. HCT-116 cells were transfected with pcDNA6.2-GW/EmGFP-miR-NC or miR-627 plasmids using Lipofectamine reagent and fluorescence was monitored for 48 hours. As shown in Figure 24, the

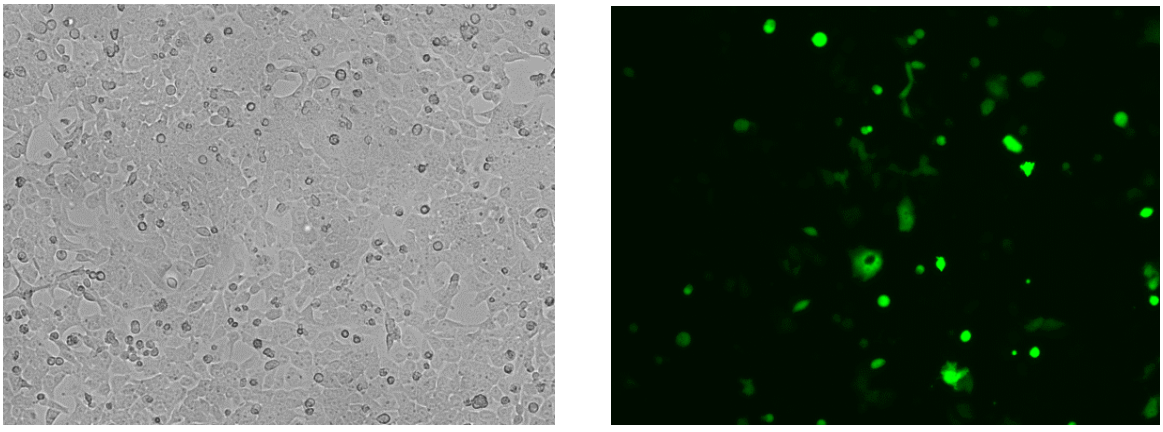
increase in fluorescence confirmed that the miR-627 or miR-NC gene inserted in the expression vector was being overexpressed. To check the effect of miR-627 overexpression in HCT-116 cells, western blot analysis was performed. It was found that histone demethylase, JMJD1A levels were significantly downregulated due to the overexpression of miR-627 (Figure 25)**,** and this could make the colon cancer cells susceptible to the calcitriol induced growth inhibition.

Bright Field **Green Fluorescence**



After **24 hours** of pcDNA6.2-GW/EmGFP-miR-627 Transfection

Bright Field **Green Fluorescence**



After **48 hours** of pcDNA6.2-GW/EmGFP-miR-627 Transfection

Figure 24. Fluorescence microscopy images showing the gradual increase in GFP expression in HCT-116 cells from day 1 to day 2 (10X magnification).

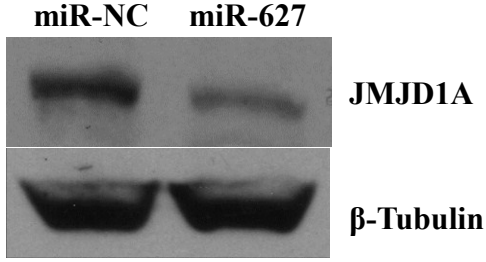


Figure 25. MiR-627 overexpression significantly decreased JMJD1A expression. HCT-116 cells were transfeted with pcDNA6.2-GW/EmGFP-miR-NC or miR-627 plasmids. 48 hours after transfection, cell lysates were analyzed by western blots using anti-JMJD1A and anti-tubulin antibodies.

2.1.12. Calcitriol inhibits JMJD1A expression in HT-29 and HCT-116 cells

If miR-627 targets JMJD1A, then calcitriol should decrease JMJD1A because it induces miR-627. The expression level of the histone demethylase gene, JMJD1A was compared in the control and calcitriol treated HT-29 and HCT-116 colon cancer cell lines. It was hypothesized that since the miR-627 levels were elevated in calcitriol treated colon cancer cells, so the expression of the respective histone demethylase gene should be repressed in these cells and the reverse should be true for the control colon cancer cells. To test this hypothesis, western blot was performed with control and calcitriol treated HT-29 and HCT-116 cells. Indeed we observed a gradual decrease in JMJD1A expression after calcitriol treatment (Figure 26) in HCT-116 and HT-29 cells as compared to the level of JMJD1a in untreated colon cancer cells.

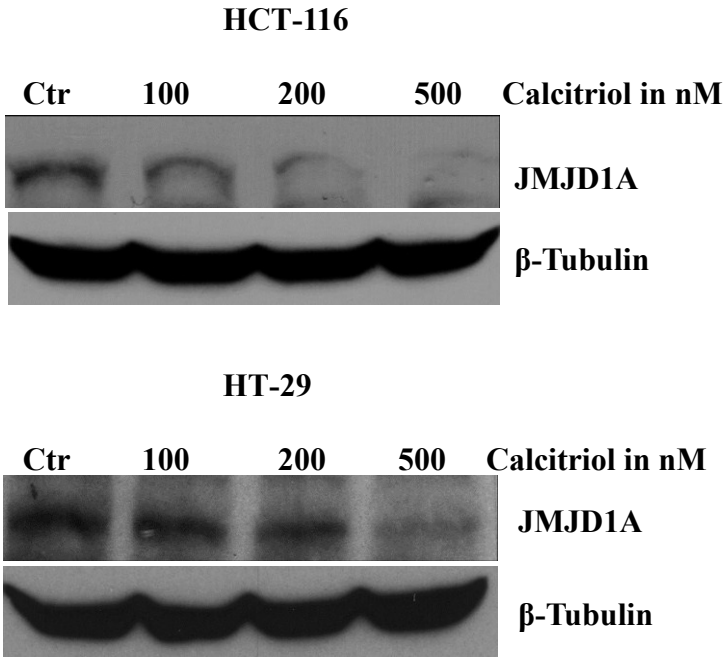


Figure 26. Calcitriol decreases histone demethylase, JMJD1A expression in colon cancer cells. HCT-116 and HT-29 cells were treated with calcitriol (100, 200, and 500 nM) for 48 hours, and cell lysates were analyzed by western blot using anti-JMJD1A and anti-tubulin antibodies.

2.1.13. Effect of JMJD1A expression on HCT-116 cell proliferation

2.1.13.1. JMJD1A knockdown

To further determine the role of miR-627 dependent suppression of JMJD1A in colon cancer growth, we specifically knocked down JMJD1A using small interfering RNA (siRNA). The Silencer® Select siRNAs for JMJD1A and negative control were transfected into HCT-116 cells. Forty eight hours after transfection, proteins were isolated and JMJD1A knockdown was confirmed by western-blot analysis (Figure 27B).

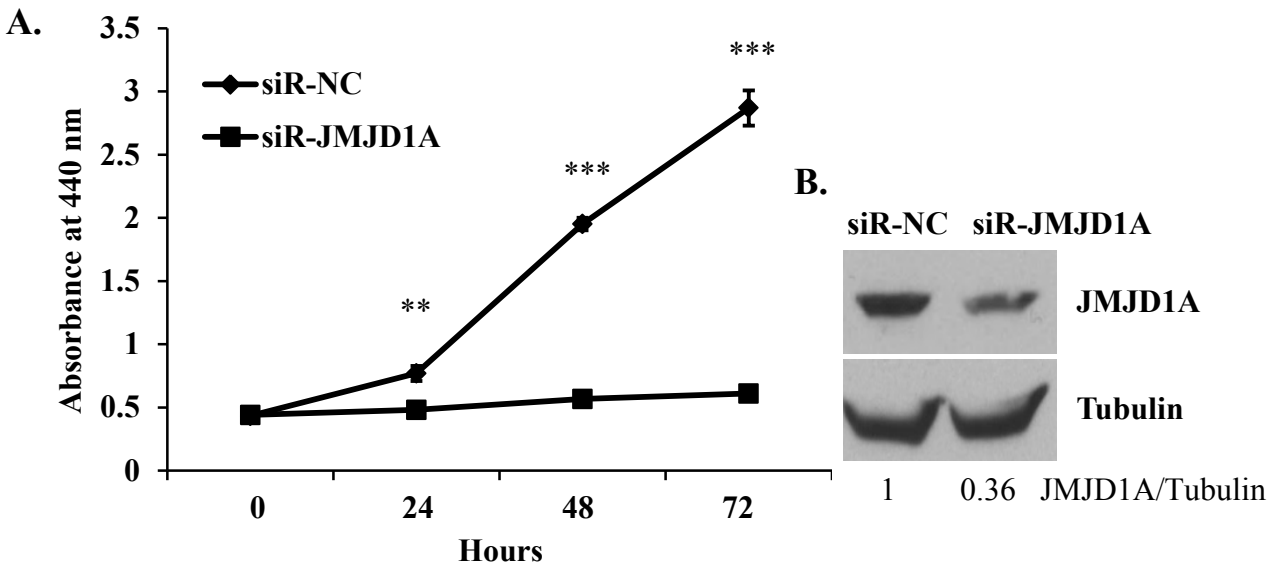


Figure 27. JMJD1A knockdown significantly decreased the proliferation of colon cancer cells. HCT-116 cells were transfected with 100 nM negative control or JMJD1A specific siRNAs. (A) Cell growth was determined at 0h, 24h, 48h, and 72h after transfection by WST-1 assay. (B) Knockdown of JMJD1A was confirmed by western blot (** $p < 0.01$; *** $p < 0.001$).

To determine the effect of JMJD1A knockdown on colon cancer growth, we transfected siR-NC or siR-JMJD1A into HCT-116 cells. At various time points after transfection, WST-1 reagent was added to the media and placed in the incubator for 30 min. Finally absorbance was measured at a wavelength of 440 nm. As shown in Figure 27A, knockdown of JMJD1A significantly inhibited the proliferation of HCT-116 cells.

2.1.13.2. JMJD1A overexpression

We created an expression vector to overexpress the full length JMJD1A gene in HCT-116 cells. The JMJD1A cDNA was cloned into the pCEP4-Flag vector to express JMJD1A as a Flag-tagged protein. Transient transfection was done in HCT-116 to overexpress JMJD1A, two

days after transfection, proteins were isolated and JMJD1A expression was confirmed by western blot analysis. (Figure 28B)

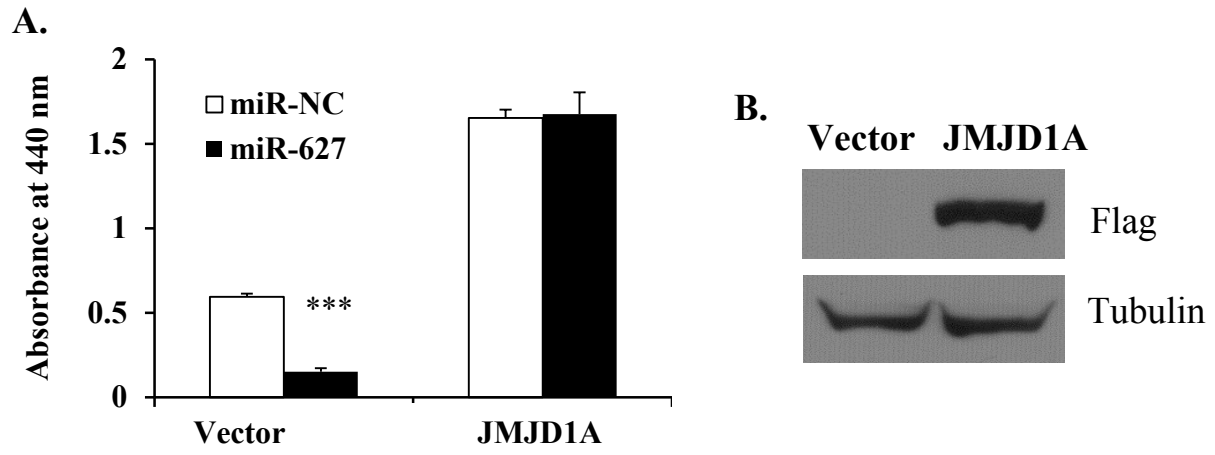


Figure 28. JMJD1A overexpression abolished the suppressive effect of miR-627 on cell proliferation. HCT-116 cells were transfected with empty vector or pCEP4-Flag-JMJD1A. 24 hours after plasmid transfection, cells were transfected with 100 nM negative control miRNA or miR-627 mimic. (A) Cell growth was determined 48 hours after miRNA transfection by WST-1 assay. (B) JMJD1A overexpression was confirmed by western blot using anti-Flag and anti-tubulin antibodies (*** $p < 0.001$).

To determine the effect of JMJD1A overexpression on colon cancer growth, HCT-116 cells were transfected with pCEP4-Flag-JMJD1A to overexpress Flag-tagged JMJD1A. After 24 hours of transfection, cells were transfected with negative control miRNA or miR-627 mimic. Cell growth was determined after 48 hours of miRNA transfection by WST-1 assay. As shown in the Figure 28A, JMDJ1A overexpression stimulated the cell proliferation in HCT-116 cells. More importantly, JMJD1A overexpression significantly abolished the suppressive effect of miR-627 on cell proliferation.

2.1.14. Calcitriol induced miR-627 mediates the growth inhibitory activity of calcitriol in colon cancer cells

To further test the hypothesis of miR-627 may mediate the anti-proliferative activity of calcitriol, we stably overexpressed a cDNA fragment containing a miR-627 target sequence (a part of JMJD1A-3'UTR sequence) in HCT-116 cells. The overexpressed mRNA containing the miR-627 target sequence (placed adjacent to a strong CMV promoter) served as a "sponge" to sequester and neutralize the activity of miR-627. We used this miRNA sponge strategy to create HCT-116 stable cell lines (please refer to sections 1.3.17 and 1.3.19) for *in vitro* and long term *in vivo* growth studies (discussed in the next chapter).

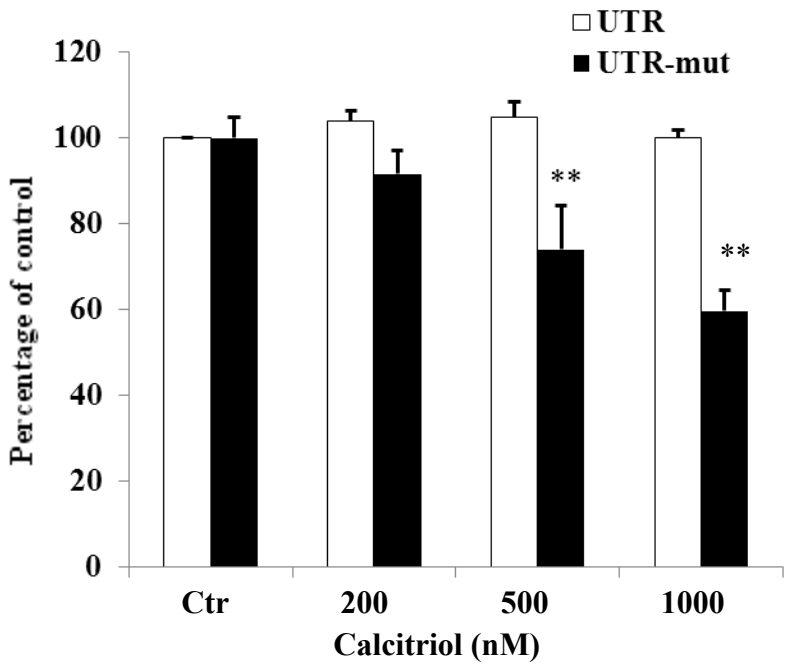


Figure 29. Mir-627 mediates the growth inhibitory activity of calcitriol in colon cancer cells. HCT-116 cells stably expressing the JMJD1A 3'UTR sponge (to block miR-627) or the JMJD1A 3'UTR sponge with miR-627 binding site mutation (ATTC to TGAG) were treated with various doses of calcitriol for 48 hours and *in vitro* cell growth was analyzed using WST-1 assay (** p-value <0.01).

When miR-627 was blocked (Figure 29; white bars), calcitriol failed to suppress the growth of HCT-116 cells, indicating that induction of miR-627 is critical to the action of calcitriol. In contrast, when the miR-627 binding site was mutated (Figure 29; black bars) calcitriol was still able to suppress cancer cell proliferation**.**

2.1.15. Calcitriol induced miR-627 mediates the JMJD1A expression in HCT-116 cells

To further establish the link between calcitriol, miR-627, and JMJD1A expression, we blocked the activity of miR-627, by transient transfection of a specific LNA (locked nucleic acid)-modified synthetic inhibitor that recognizes miR-627 (please refer to the section 1.3.14).

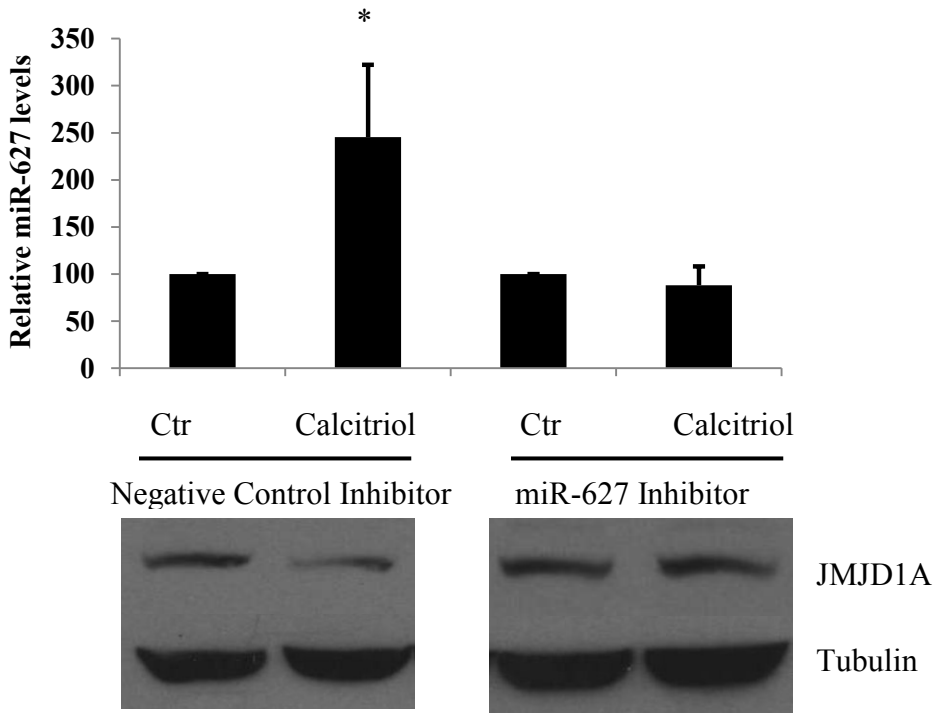


Figure 30. Calcitriol induced miR-627 mediates the JMJD1A expression in colon cancer cells. HCT-116 cells were transfected with 100 nM LNA-modified miRNA inhibitor specific to miR-627 or the negative control inhibitor for 24 hours, and then treated with calcitriol. Total RNA and cell lysates were analyzed by RT-PCR (100 nM calcitriol, 24 hours) for miR-627 and western blots (500 nM calcitriol, 48 hours) with anti-JMJD1A and anti-tubulin antibodies (* $p < 0.05$).

The effect of these inhibitors on calcitriol induced miR-627 and the decreased JMJD1A expression were determined by RT-PCR and Western blot analysis respectively. As shown in Figure 30, miR-627 inhibitor effectively blocked calcitriol-induced miR-627 and the decrease of JMJD1A expression.

2.1.16. Effect of calcitriol on histone methylation on GDF15 promoter

Histone demethylase, JMJD1A demethylates both di- or mono-methylated histone H3 lysine 9, which are commonly associated with repressed gene promoters. By decreasing JMJD1A expression, calcitriol induced miR-627 may increase H3K9 methylation on the promoters of JMJD1A target genes, such as the GDF15 gene (Krieg A.J., et al., 2010). We performed chromatin immunoprecipitation (ChIP) assay to determine the effects of calcitriol on histone methylation on the promoter region of the GDF15 gene. Immunoprecipitations were performed using anti-dimethyl-histone H3 lysine 9 (H3K9Me2), and control IgG antibodies. ChiP assay follwed by a PCR analysis, we measured the H3K9Me2 levels on the promoter region of GDF15 gene in control and calcitriol treated HCT-116 colon cancer cells. As shown in Figure 31, calcitriol induced a significant increase of H3K9Me2 on the promoter of the GDF15 gene. As a result, GDF15 mRNA expression was suppressed by calcitriol.

Similarly, by decreasing JMJD1A, miR-627 may increase H3K9 methylation on the promoter of GDF15. To determine the effects of miR-627 on histone methylation, we performed chromatin immunoprecipitation (ChIP) assay on the promoter region of the GDF15 gene. HCT-116 cells were transfected with a plasmid to overexpress miR-627. MiR-627 overexpressing cells were captured by a CherryPicker-specific antibody bound to IgG-coated magnetic beads using CherryPicker Cell Capture & Separation System. Overexpression of miR-627 induced a significant increase of H3K9Me2 on the promoter of GDF15 (Figure 32).

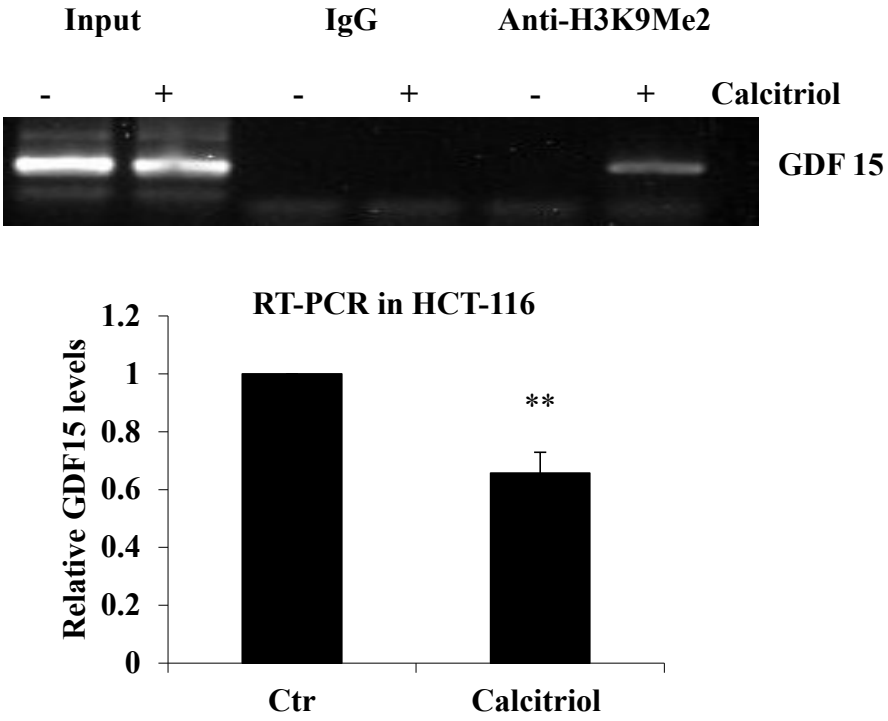


Figure 31. Calcitriol increases histone methylation, H3K9Me2 levels on GDF15 promoter. HCT-116 cells were treated with 500 nM calcitriol for 48 hours. ChIP assay was performed using primers specific for the GDF15 promoter and the indicated antibodies. Total RNA was isolated and analyzed for GDF15 expression by RT-PCR (Input-negative control; IgG-background control; ** $p < 0.01$).

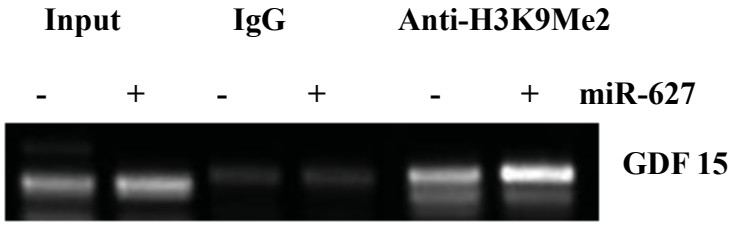


Figure 32. MiR-627 overexpression increases histone methylation, H3K9Me2 levels on GDF15 promoter. HCT-116 cells were transfected with pCherryPicker2-miR-627 plasmid to overexpress miR-627. ChIP assay was performed on miR-627 overexpressing cells using primers specific for the GDF15 promoter and the indicated antibodies.

We also determined the effects of calcitriol on two additional histone methylation markers. Calcitriol decreased histone H3K4 methylation (Figure 33A) and increased histone H3K27 methylation (Figure 33B) on the promoter of GDF15 gene. Since H3K27 methylation is associated with repression of gene expression and H3K4 methylation is associated with active transcription (Peterson C.L. and Lanoel M.A 2004; Mosammaparast N. and Shi Y. 2010; Niu X. et al., 2012), the observed changes in these two markers may also collectively influence the GDF15 expression.

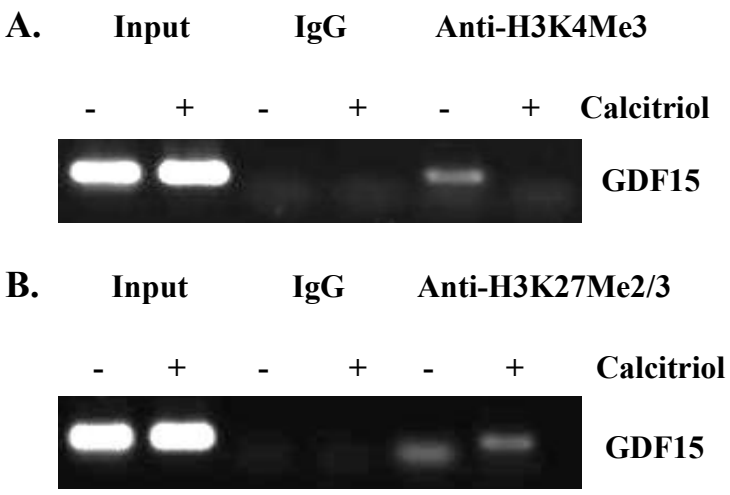


Figure 33. Chromatin immunoprecipitation assay to detect the histone methylation (H3K4Me3 and H3K27Me2/Me3) levels on GDF15 promoter. HCT-116 cells were treated with 500 nM calcitriol for 48 hours. ChIP assay was performed as described in Materials and Methods using primers specific for the GDF15 promoter and the indicated antibodies.

2.1.17. Effect of calcitriol on HOXA1 and CCND1 expression in HCT-116 cells

Recently, it has been shown that JMJD1A promotes cell cycle progression through the G1/S transition by activating HOXA1 and CCND1 (encoding cyclin D1) expression (Figure 34, Cho H.S., et al., 2012). As shown in the Figure 26, calcitriol treatment significantly decreased the JMJD1A expression in HCT-116 cells.

Based on these results, we hypothesized that the expression of HOXA1 and CCND1 will be downregulated in HCT-116 cells treated with calcitriol. Indeed our Real-time PCR data (Figure 35) showed a significant decrease in the expression of JMJD1A target genes, HOXA1 and CCND1 in calcitriol treated cells.

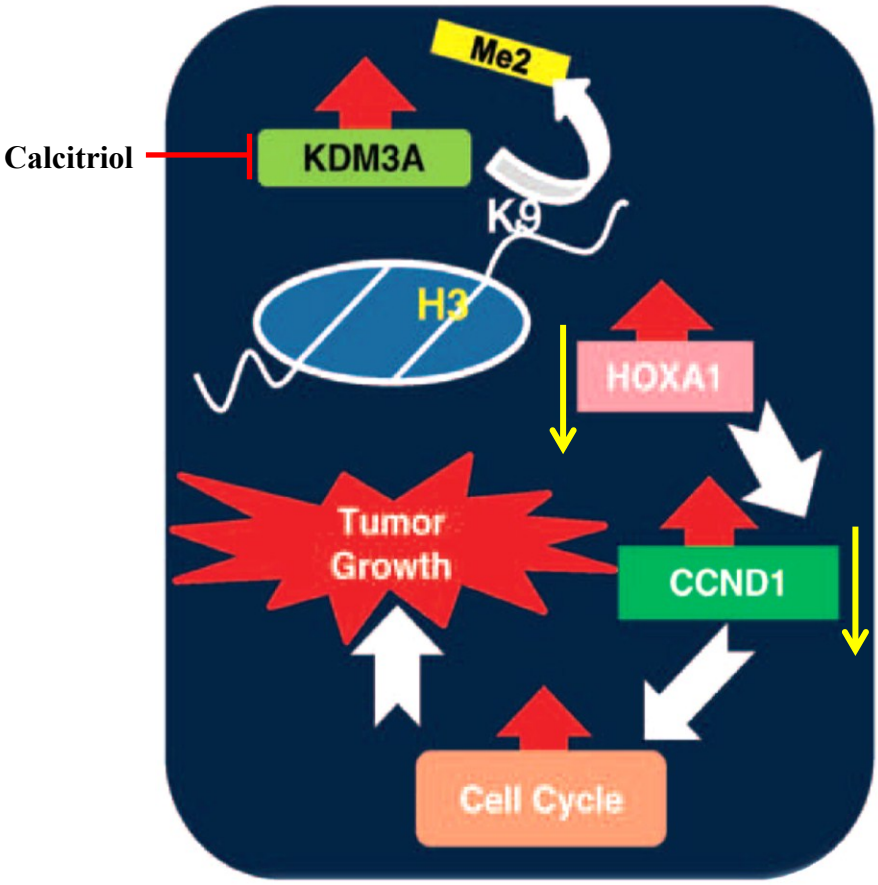


Figure 34. A proposed model of KDM3A (JMJD1A) mediated enhancement of HOXA1dependent CCND1 transcriptional activation through demethylation of histone H3K9Me2 (Cho H.S., et al., 2012).

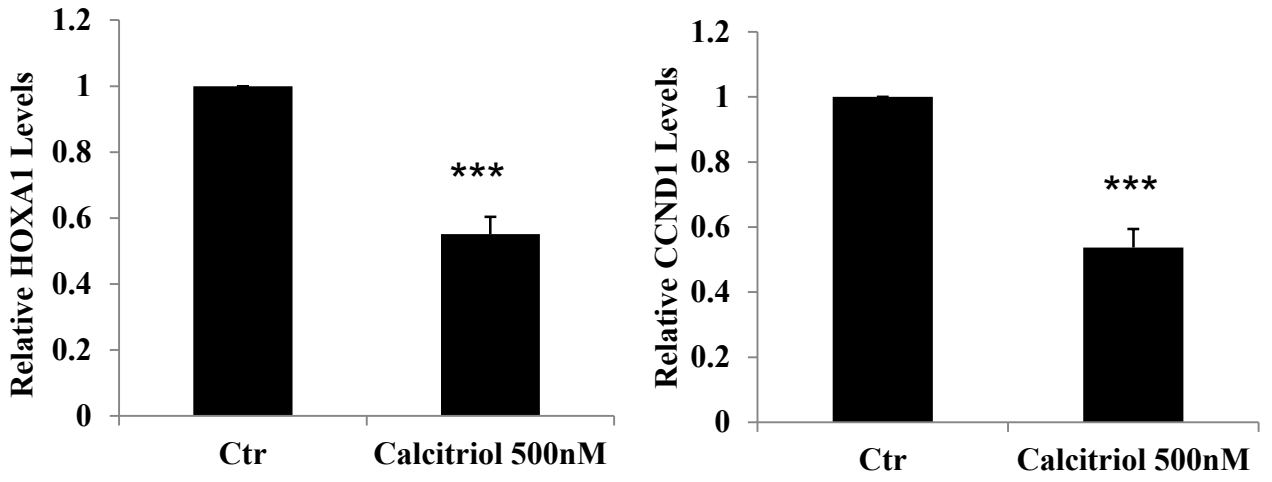


Figure 35. Calcitriol decreases the expression of HOXA1 and CCND1 in colon cancer cells. HCT-116 cells were treated with 500 nM calcitriol for 48 hours. Total RNA was isolated and analyzed for HOXA1 and CCND1 expression by RT-PCR. (*** p-value < 0.001).

CHAPTER 3: CALCITROL INDUCES MIR-627 EXPRESSION IN TUMOR XENOGRAFTS; MIR-627 MEDIATES THE ANTI-TUMOR ACTIVITY OF CALCITRIOL

The working hypothesis for this part of the study was that calcitriol induced miR-627 expression in colon cancer xenografts will inhibit JMJD1A expression and this would in turn decrease the growth of the colon cancer xenografts. The following experiments were performed to test our hypothesis.

Nude mice (Nu/Nu) were maintained in sterile conditions using the Innovive IVC system, following the protocol approved by the Institutional Animal Care and Use Committee of North Dakota State University.

3.1. Results and discussion

3.1.1. Calcitriol induces miR-627 expression in HT-29 tumor xenografts

To determine if calcitriol induces miR-627 expression *in vivo*, we established human colon cancer xenografts in nude mice by injecting $2x10^6$ HT-29 colon cancer cells subcutaneously into the flank regions of nude mice on both sides. The mice developed tumor xenografts within 5 days and were treated with calcitriol for 2 days. For control group the same volume of DMSO was injected. It has been reported that, the i.p. injection of 0.5 µg calcitriol per mouse is safe to administer without inducing any hypercalcemia (Muindi J.R., et al., 2004)**.** Tumor samples (n=3) were collected 24h after the second dose and total RNA was isolated from control and calcitriol treated tumor xenografts. Relative miR-627 expression was analyzed by RT-PCR using 18S RNA as reference. As shown in Figure 36, calcitriol induced miR-627 expression in the tumor xenografts.

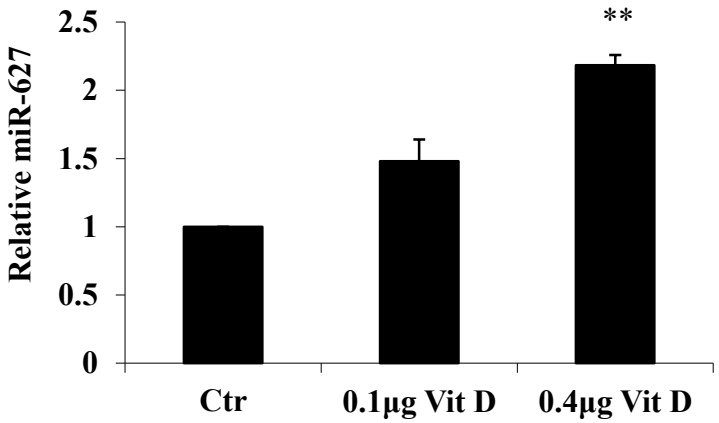


Figure 36. Calcitriol induces the miR-627 expression in HT-29 colon tumor xenografts. Nude mice bearing HT-29 tumor xenografts were treated with calcitriol at intraperitoneal doses of 0.1 µg and 0.4 µg daily for 2 days. Tumor samples were collected 24 hours after the second dose. Total RNA was isolated and miR-627 expression was analyzed by RT-PCR (**p-value < 0.01).

3.1.2. Calcitriol inhibits JMJD1A expression in HT-29 tumor xenografts

It was hypothesized that since the miR-627 levels were elevated in calcitriol treated colon tumor xenografts, so the expression of the histone demethylase, JMJD1A should be repressed in these tumors and the reverse should be true for the control colon tumors.

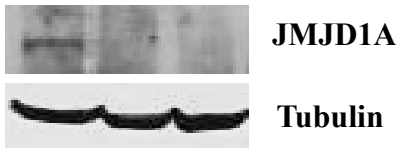


Figure 37. Calcitriol decreases histone demethylase, JMJD1A levels in HT-29 colon tumor xenografts. Nude mice bearing HT-29 tumor xenografts were treated with calcitriol at intraperito-neal doses of 0.1 µg and 0.4 µg daily for 2 days. Tumor samples were collected 24 hours after the second dose. Cell lysates were collected and analyzed by western blot using anti-JMJD1A and anti-tubulin antibodies.

To test this hypothesis, western blot was performed with control and calcitriol treated HT-29 tumor xenografts. As shown in Figure 37, the expression of JMJD1A was decreased in calcitriol treated HT-29 tumor xenografts as compared to control.

3.1.3. Calcitriol suppressed the growth of HT-29 and HCT-116 colon tumor xenografts

To determine the effect of calcitriol on *in vivo* tumor xenografts growth, we established HT-29 and HCT-116 colon tumor xenografts by injecting 2 million cells SC into the flank regions of nude mice (n=5 per each cell line) and waited for 4-6 days for the tumors to develop. Nude mice bearing HT-29 and HCT-116 tumor xenografts were treated with calcitriol. The same volume of DMSO was administered to the control group. Tumor volumes and body weights were measured at indicated time points.

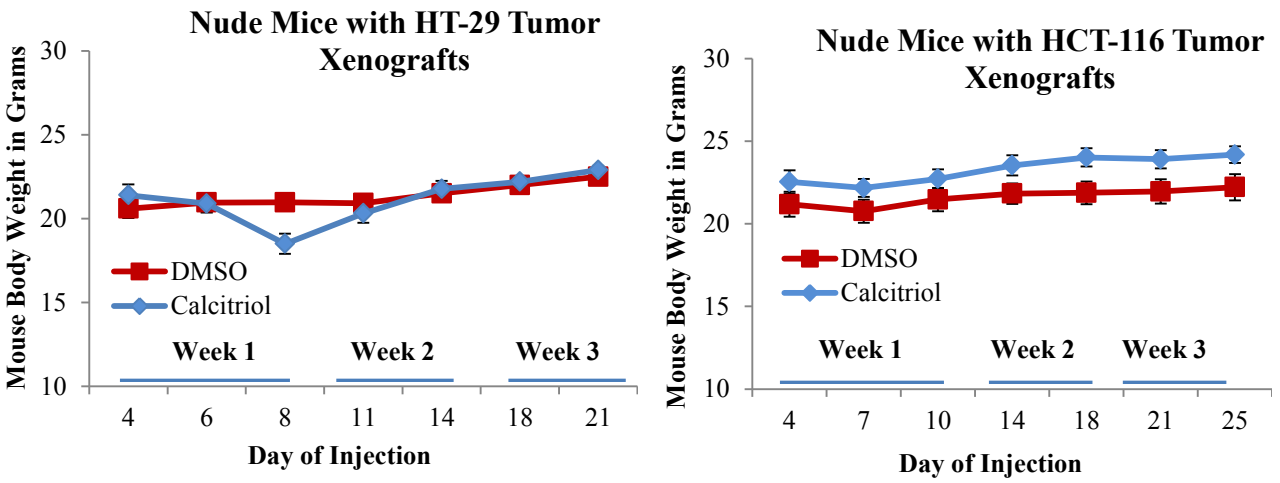


Figure 38. Calcitriol showed no significant toxicity in the nude mice with HT-29 and HCT-116 tumor xenografts. Nude mice bearing HT-29 (n=9) and HCT-116 (n=7) tumor xenografts were treated with calcitriol (i.p. dose of 0.4 µg, 3 injections: 1st week, 2 injections: 2nd and 3rd weeks), the same volume of dimethyl sulfoxide (DMSO) was administered to the control groups. Body weight was measured as described in Materials and Methods.

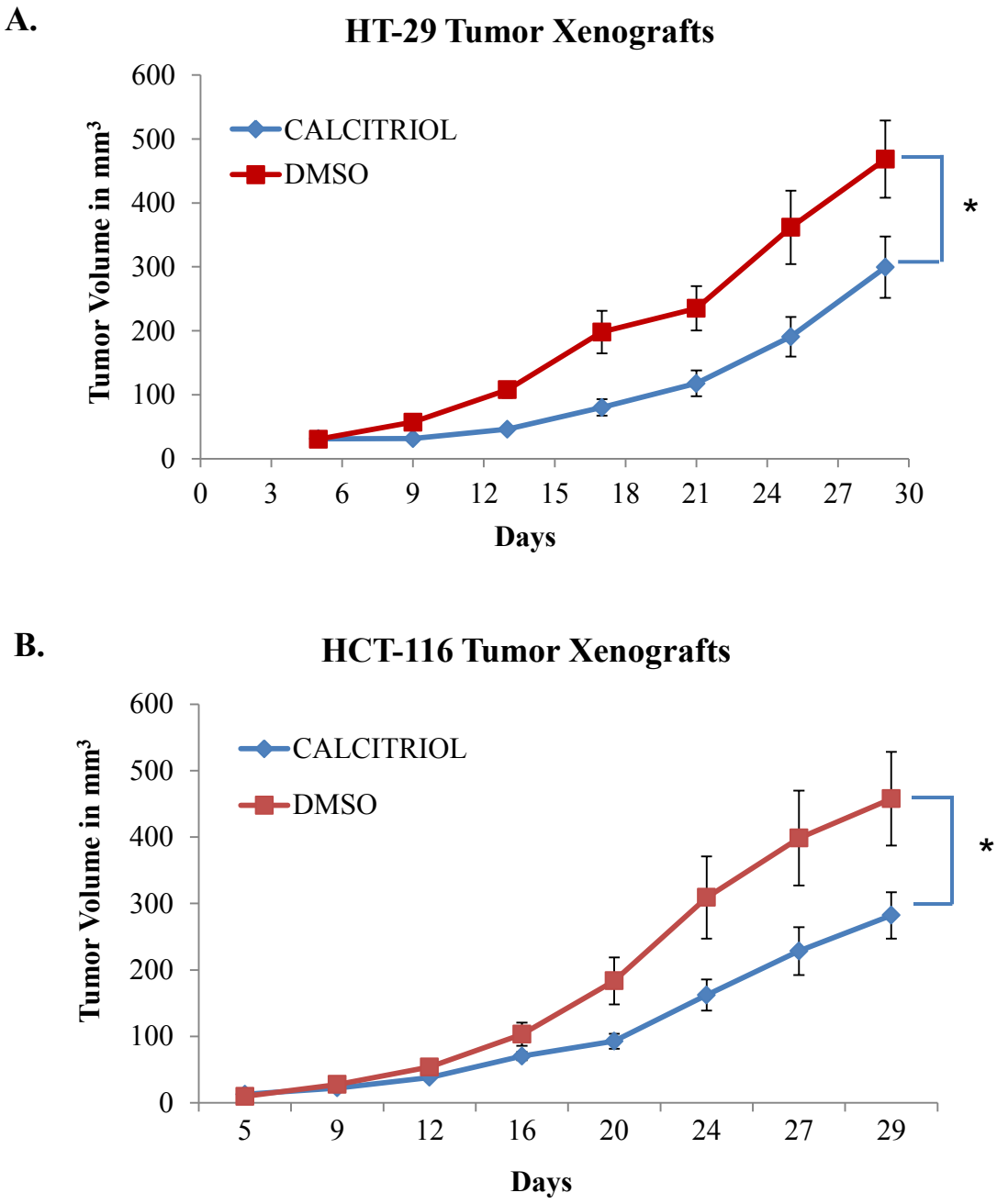


Figure 39. Calcitriol slowed down the growth of colon tumor xenografts growth in nude mice. Nude mice bearing HT-29 (n=9) and HCT-116 (n=7) tumor xenografts were treated with calcitriol (i.p. dose of 0.4 µg, 3 injections: 1st week, 2 injections: 2nd and 3rd week), the same volume of DMSO was administered to the control groups. Tumor volume was meaasured as described in materials and Methods (*$p < 0.05$).

Calcitriol significantly reduced HT-29 and HCT-116 tumor xenografts growth in the nude mice (Figures 39 A & B), without inducing any toxicity (measured by the loss of body weight), as shown in Figures 38. We did not observe any other symptoms of toxicity (lethargy, decreased feeding).

3.1.4. MiR-627 is the major epigenetic regulator of calcitriol and mediates the tumor xenografts growth inhibition caused by calcitriol

3.1.4.1. Effect of calcitriol on the growth of HCT116-JMJD1A-3'UTR-SC tumor xenografts

The JMJD1A-3'UTR cDNA was inserted into pRNAT-CMV3.2/Puro plasmid (BamH I and Xho I) to create a pRNAT-CMV3.2/Puro-JMJD1A-3'UTR for stable transfection into HCT-116 cells. After 48 hours of transfection, cells were treated with the optimized dose of puromycin (0.4µg/mL). Cells that take up the plasmid and start expressing the JMJD1A-3'UTR and anti-puromycin genes in it, continued to grow and formed colonies. Fresh media with an optimized puromycin concentration was replaced after every 3 days. Green Fluorescence Protein gene (GFP) in the plasmid was used to track the expression of the JMJD1A-3'UTR. These GFP colonies were picked and sub-cultured, resulting in HCT-116 stable cell lines overexpressing the JMJD1A-3'UTR as well as the GFP (Figure 44).

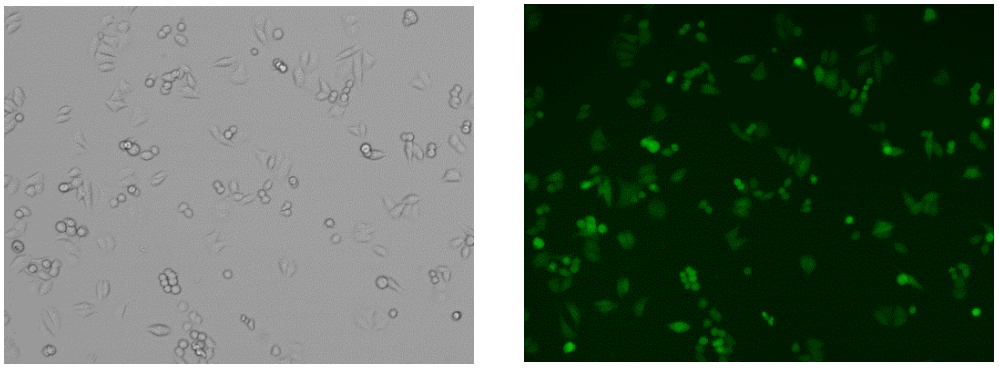


Figure 40. HCT116-JMJD1A-3'-UTR-SC-8, bright field and FITC (10X) images.

We blocked the activity of miR-627 by overexpressing the JMJD1A 3'UTR sponge which can sequester miR-627, thus calcitriol was no longer able to decrease JMJD1A (Figure 42A, HCT-116-JMJD1A-3'UTR-SC-8) . We used HCT116-JMJD1A-3'UTR-SC-8 cells to generate tumor xenografts in nude mice. Nude mice with xenografts were separated into control and calcitriol groups. Tumor volumes and body weights were measured at indicated time points. There was no significant change in the body weight (Figure 41) among the DMSO and calcitriol treated groups. As shown in Figure 42B, calcitriol failed to suppress tumor growth in the nude mice when miR-627 was blocked by 3'UTR sponge. We did not observe any other symptoms of toxicity (lethargy, decreased feeding).

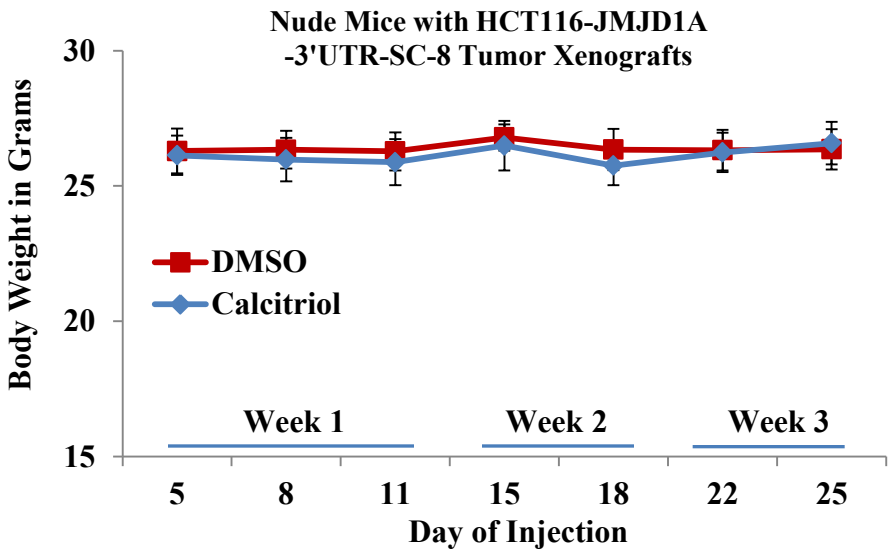


Figure 41. Calcitriol showed no significant toxicity in the nude mice with HCT116-JMJD1A-3'UTR-SC-8 tumor xenografts. Nude mice bearing HCT116-JMJD1A-3'UTR-SC-8 (n=5) tumor xenografts were treated with calcitriol (i.p. dose of 0.4 μg, 3 injections: 1st week, 2 injections: 2nd and 3rd weeks), the same volume of dimethyl sulfoxide (DMSO) was administered to the control groups. Body weight was measured as described in Materials and Methods.

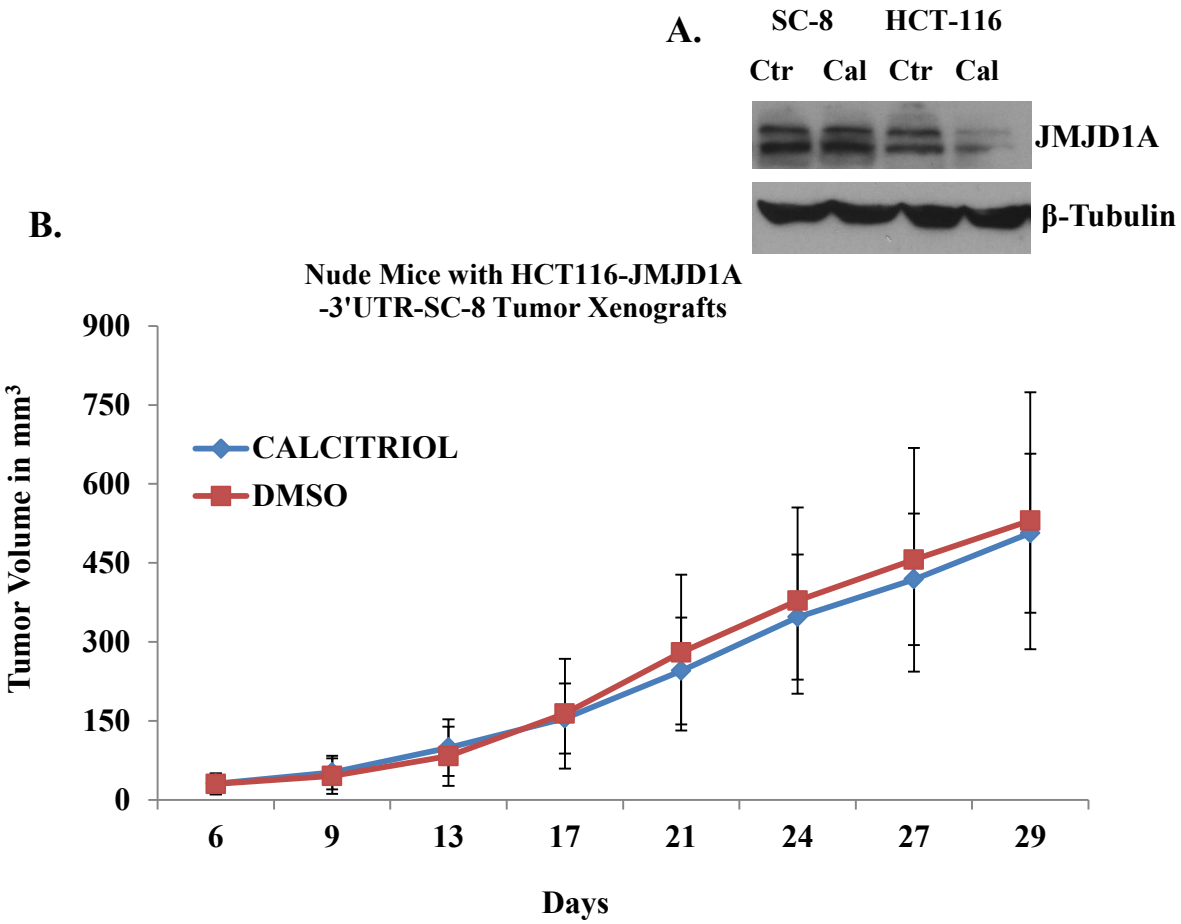


Figure 42. Calcitriol failed to suppress tumor growth in the nude mice with HCT-116-JMJD1A-3'UTR-SC-8 tumor xenografts. (A) HCT-116 and HCT-116-JMJD1A-3'UTR-SC-8 cells were treated with 500 nM calcitriol (Cal) for 48 hours, and cell lysates were analyzed by western blot using anti-JMJD1A and anti-tubulin antibodies. (B) Nude mice bearing HCT116-JMJD1A-3'UTR-SC-8 (n=5) tumor xenografts were treated with calcitriol or DMSO, the same schedule as that in Figure 41. Tumor volume was measured as described in Materials and Methods.

3.1.4.2. Effect of calcitriol on the growth of HCT116-JMJD1A-3'UTR-MUT-SC-4 tumor xenografts

The pRNAT-CMV3.2/Puro-JMJD1A-3'UTR-MUT, containing a mutation within the miR-627 recognition site (ATTC to TGAG in the JMJD1A-3'UTR) was created by PCR using the QuickChange II site-directed mutagenesis procedure. Similar protocol was followed to create

HCT116-JMJD1A-3'UTR-MUT stable cells. Green Fluorescence Protein gene (GFP) in the plasmid was used to track the expression of the JMJD1A-3'UTR-MUT. These GFP colonies were picked and sub-cultured, resulting in HCT-116 stable cell lines overexpressing the JMJD1A-3'UTR-MUT as well as the GFP (Figure 43).

We used HCT116-JMJD1A-3'UTR-MUT-SC-4 to generate tumor xenografts in nude mice. Nude mice with xenografts were separated into control and calcitriol groups, treated with DMSO or calcitriol. Tumor volumes and body weights were measured at indicated time points. There was no significant change in the body weight (Figure 44A) among the DMSO and calcitriol treated groups of nude mice.

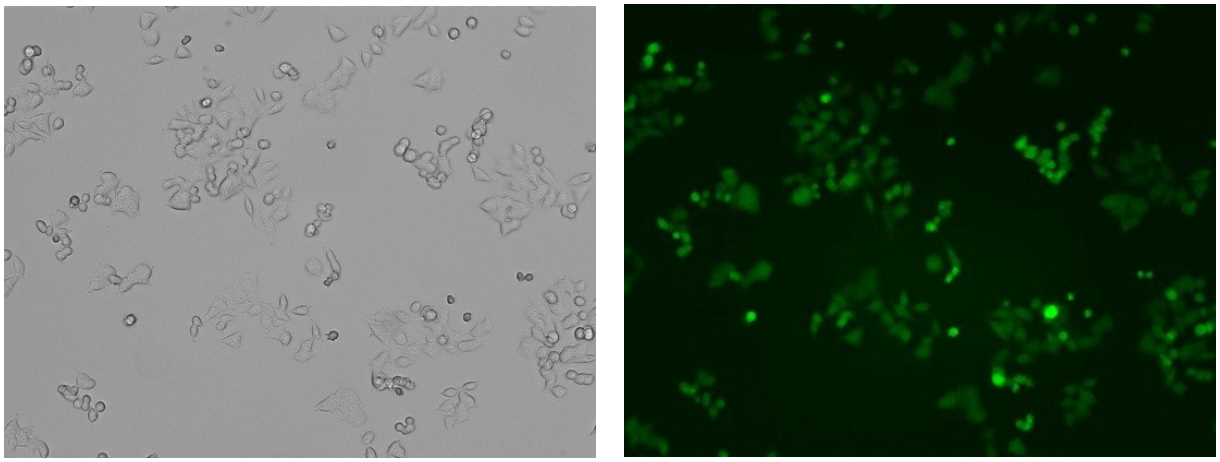


Figure 43. HCT116-JMJD1A-3'UTR-MUT-SC-4, bright field and FITC (10X) images.

In contrast to the above results from Figure 42, when the miR-627 binding site within the JMJD1A-3'UTR sequence was mutated, calcitriol was still able to decrease JMJD1A in HCT-116-JMJD1A-3'UTR-MUT-SC-4 cells and suppress tumor growth in nude mice (Figure 44B and C).

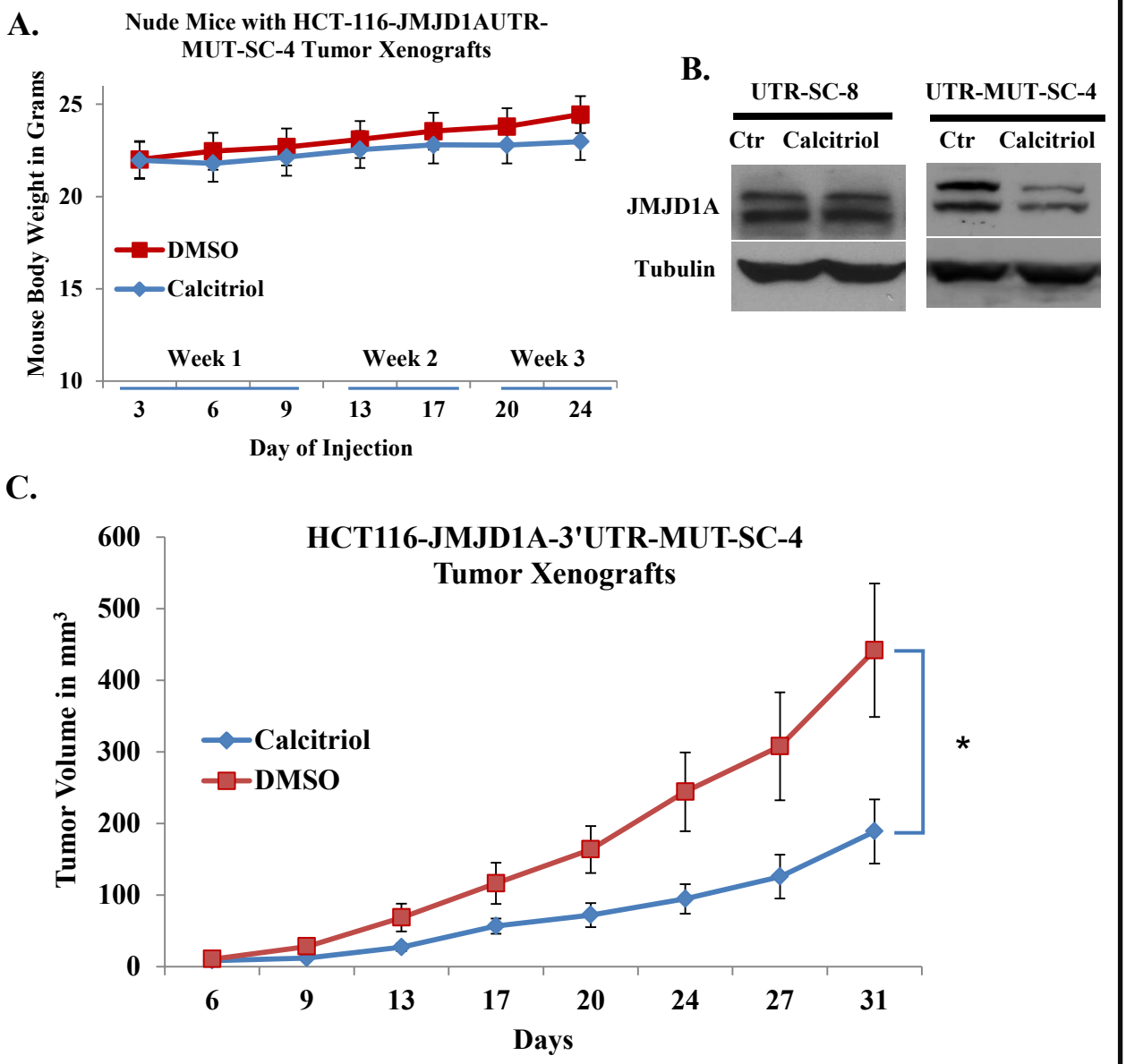


Figure 44. Calcitriol suppresses tumor growth in the nude mice with HCT-116-JMJD1A-3'UTR -MUT-SC-4 tumor xenografts. (A,C) Nude mice bearing HCT116-JMJD1A-3'UTR-MUT-SC-4 (n=7) tumor xenografts were treated with calcitriol or DMSO, followed the same schedule as that in Figure 41. Body weight and tumor volume were measured as described in Materials and Methods. (B) HCT-116-JMJD1A-3'UTR-SC-8 and HCT-116-JMJD1A-3'UTR-MUT-SC-4 cells were treated with 500 nM calcitriol for 48 hours, and cell lysates were analyzed by western blot using anti-JMJD1A and anti-tubulin antibodies (* $p < 0.05$).

3.1.4.3. Effect of miR-627 expression on the growth of colon tumor xenografts

To determine the effects of miR-627 on *in vivo* tumor xenograft growth, we established HCT-116 cell lines stably expressing negative control miRNA or miR-627. Stable cell lines expressing miR-627 or negative control miRNA were established by transfecting HCT-116 cells with Block-iTTM Pol II miR expression vectors, pcDNA6.2-GW/EmGFP-miR-627 or pcDNA6.2-GW/EmGFP-negative-control. Overexpression of miR-627 was confirmed by western blot analysis of decreased JMJD1A expression. (Figure 45B)

Two million HCT116-miR627-SC-1 or HCT116-miRNC-SC cells were injected S.C. into the flank regions of nude mice to develop tumor xenografts. Tumor volumes and body weights were measured at indicated time points. As shown in Figures 45C and D, tumors stably expressing miR-627 exhibited significantly slower growth compared to tumors expressing negative control miRNA. There was no significant change in body weight among the two groups of mice (Figure 45A).

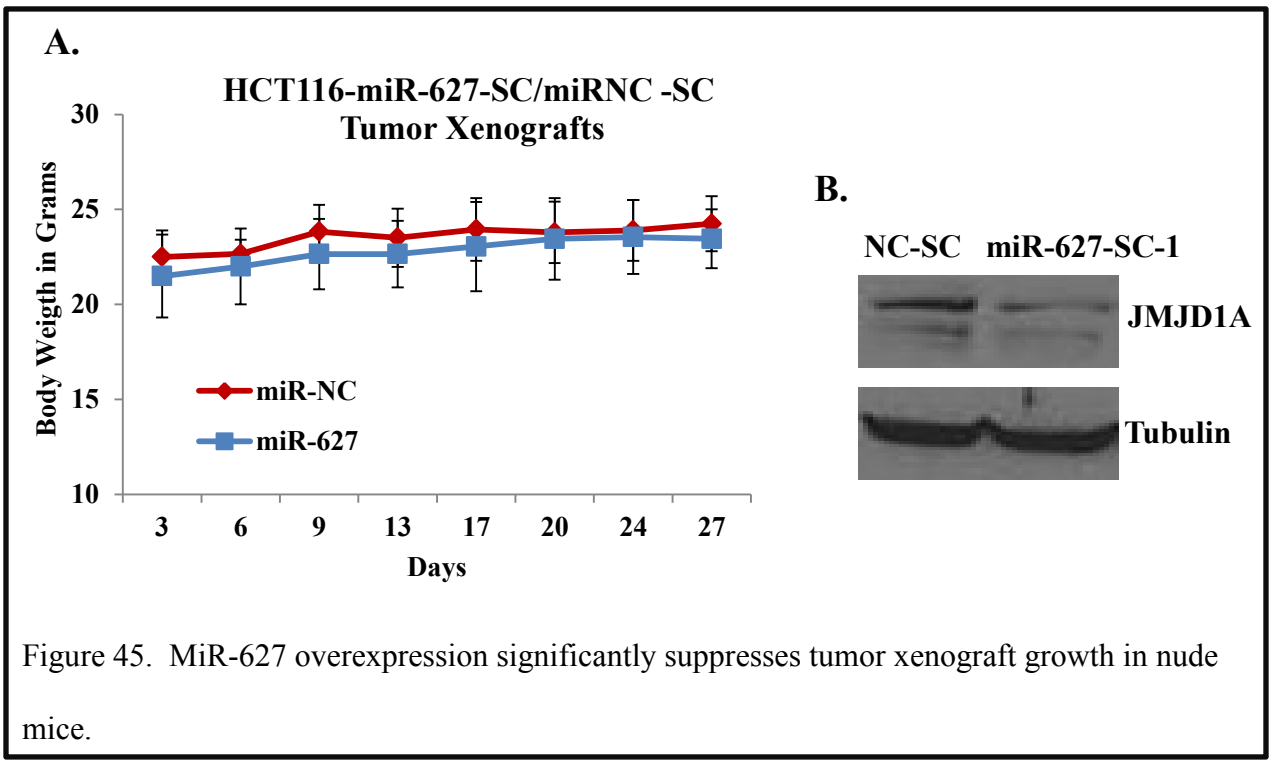


Figure 45. MiR-627 overexpression significantly suppresses tumor xenograft growth in nude mice.

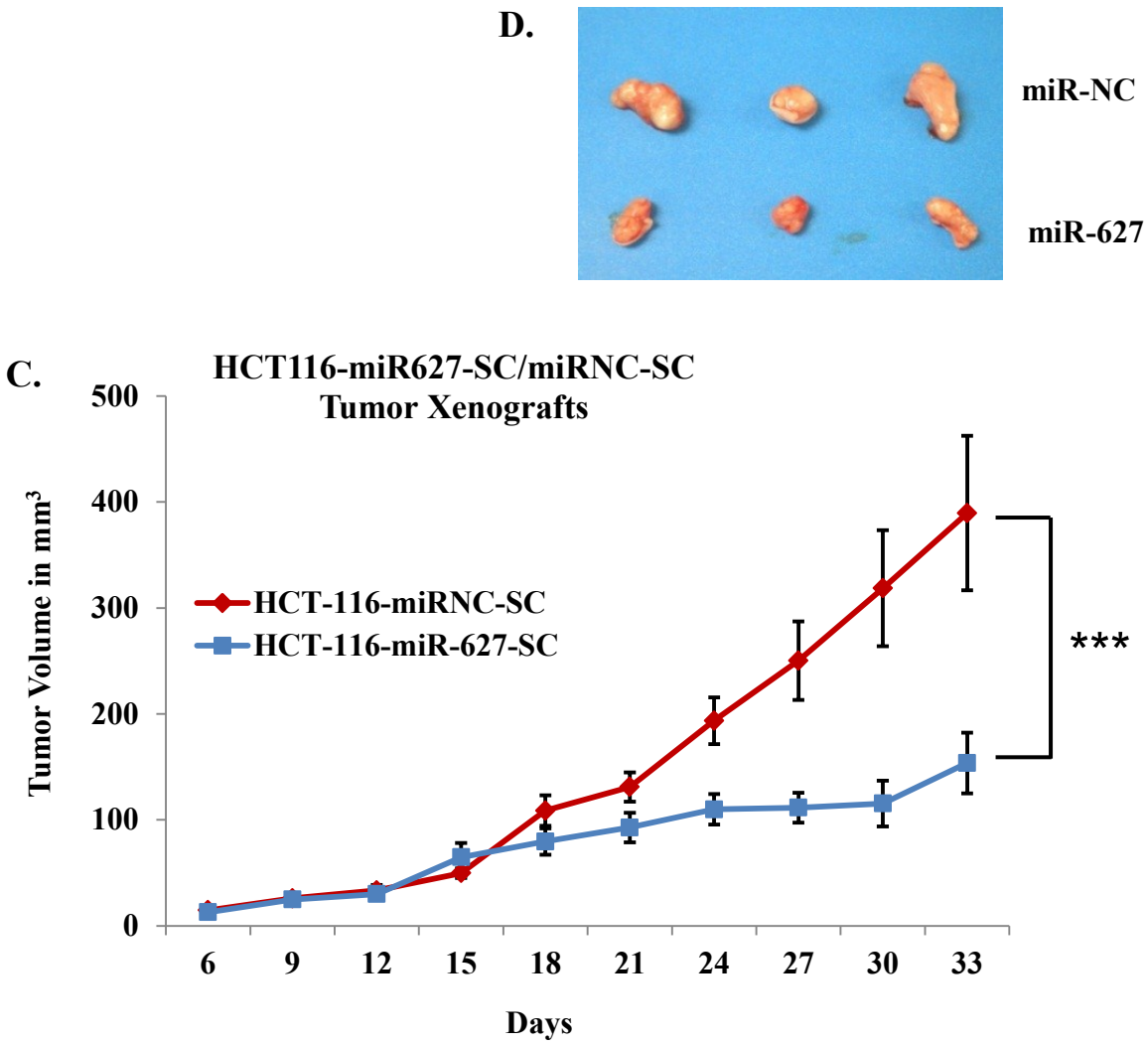


Figure 45. MiR-627 overexpression significantly suppresses tumor xenograft growth in nude mice (continued). (A,C) Negative control miRNA or miR-627 expressing stable cell lines were transplanted into nude mice to establish tumor xenografts (5 mice per group). Tumor volume and body weight were measured as described in Materials and Methods. (B) Cell lysates collected from HCT-116 cell lines stably expressing miR-627 or negative control miRNA were analyzed by western blot using anti-JMJD1A and anti-tubilin antibodies. (D) Digital image showing the size of tumor xenografts stably expressing miR-NC or miR-627 (*** p-value < 0.001).

CHAPTER 4: MIR-627 EXPRESION IN HUMAN COLON CANCER SPECIMENS

The working hypothesis for this part of the study was that miR-627 expression will be downregulated in human colon cancer specimens compared to normal colon tissues. Decreased miR-627 expression would increase the JMJD1A expression in human colon cancer specimens, which will stimulate colon cancer growth. The following experiment was performed to measure the expression levels of miR-627 in human colon specimens.

4.1. Results and discussion

4.1.1. MiR-627 expression in human colon cancer specimens

To determine the expression levels of miR-627 in human colon cancers, we analyzed RNA samples from human colon adenocarcinoma specimens as well as non-tumor colon mucosa tissues (Table 8) by real-time PCR. Total RNAs isolated from human colon adenocarcinomas and nontumor colon mucosa tissues were obtained from the Department of Pathology, Roswell Park Cancer Institute, and approved by the Institutional Review Board. All RNA samples were isolated using Trizol and checked for RIN on Bioanalyzer. The relative miR-627 levels in individual samples (Figure 47) were shown compared with one of the normal colon mucosa tissue. As shown in Figure 46, miR-627 levels in the tumor specimens were significantly lower than those in the normal tissues (Student’s test). Using Pearson's correlation coefficient test, we found no association between miR-627 expression levels and tumor stages ($r= -0.277$, $p=0.133$) or nodal metastases ($r=0.181$, $p=0.236$). There were also no correlation between miR-627 expression and tumor grade of differentiation or tumor sites (left or right colon). It is possible that miR-627 expression is decreased during the early stage of carcinogenesis in the colon.

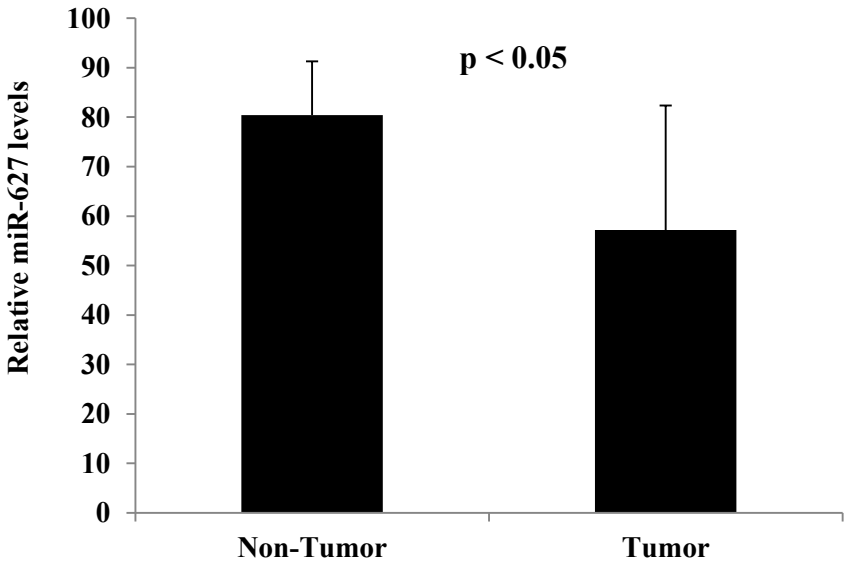


Figure 46. Expression of miR-627 decreased in human colon cancer specimens. Total RNA samples isolated from 18 human colon adenocarcinomas and 6 nontumor colon mucosa were analyzed for miR-627 expression by RT-PCR.

Table 8. List of human colon specimens (NT-Non-tumor; T-Tumor).

ID	Tumor or Non-tumor	Grade	Path stage	Nodes positive	Nodes exam	Left or Right colon
NT1	non tumor colon mucosa					Left Colon
NT2	non tumor colon mucosa					Left Colon
NT3	non tumor colon mucosa					Right Colon
NT4	non tumor colon mucosa					Left Colon
NT5	non tumor colon mucosa					Colon NOS
NT6	non tumor colon mucosa					Left Colon
T1	Adenocarcinoma	Moderately differentiated	2	0	25	Left Colon
T2	Adenocarcinoma	Moderately differentiated	2	0	30	Left Colon
T3	Adenocarcinoma	Moderately differentiated	3	10	11	Right Colon
T4	Adenocarcinoma	Poorly differentiated	3	3	18	Transverse Colon
T5	Adenocarcinoma	Moderately differentiated	4	4	20	Right Colon
T6	Adenocarcinoma	Moderately differentiated	4	9	55	Right Colon
T7	Adenocarcinoma	Moderately differentiated	3	1	15	Right Colon
T8	Adenocarcinoma	Moderately differentiated	4	0	14	Left Colon
T9	Adenocarcinoma	Moderately differentiated	1	0	7	Left Colon
T10	Adenocarcinoma	Moderately differentiated	4	0	13	Right Colon
T11	Adenocarcinoma	Moderately differentiated	4	0	19	Right Colon
T12	Adenocarcinoma	Moderately differentiated	3	6	20	Left Colon
T13	Adenocarcinoma	Moderately differentiated	4	2	14	Left Colon
T14	Adenocarcinoma	Poorly differentiated	2	0	11	Left Colon
T15	Adenocarcinoma	Moderately differentiated	4	9	55	Right Colon
T16	Adenocarcinoma	Moderately differentiated	4	9	55	Right Colon
T17	Adenocarcinoma	Moderately differentiated	2	0	33	Transverse Colon
T18	Adenocarcinoma	Moderately differentiated	4	0	22	Left Colon

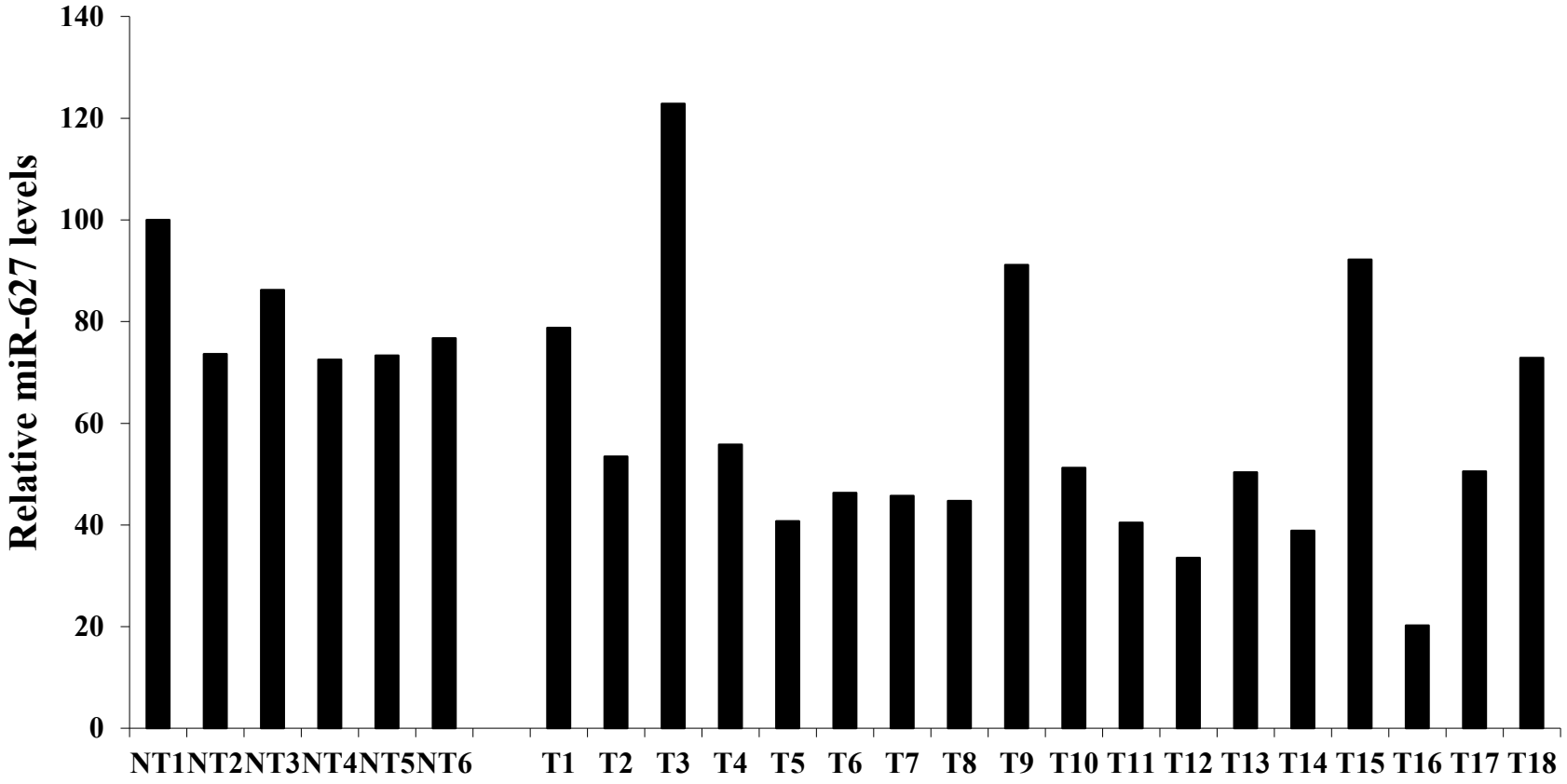


Figure 47. RT- PCR to measure the relative miR-627 levels in individual human colon cancer specimens compared with the ones in normal colon tissue (NT- Non-tumor; T- Tumor).

CHAPTER 5: SUMMARY AND CONCLUSIONS; CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

5.1. Summary and conclusions

MicroRNAs have been indicated to play important roles in the regulation of cancer cell functions including proliferation (Cheng A.M., et al., 2005), apoptosis (Xu p., et al., 2004), differentiation (Karp X. and Ambros V., 2005), invasion and metastasis (Ma L. 2007). Microarray based miRNA expression profiling indicated that among the 471 human miRNAs examined, miR-627 was the only microRNA whose expression level was significantly increased in HT-29 cells by calcitriol treatment. The upregulation of miR-627 by calcitriol was confirmed by real-time PCR in HT-29 and HCT-116 cells. Synthetic miR-627 significantly inhibited cancer cell growth while the negative control miRNA had no effects, indicating that the miR-627 may mediate the anti-proliferative activity of calcitriol. Using the computational methods available at www.microrna.org and miRBase (http://microrna.sanger.ac.uk/), we identified JMJD1A as a potential target for miR-627. We selected JMJD1A for further studies because of its known involvement in promoting colon cancer growth. JMJD1A has recently been shown to promote colon cancer growth by mediating the effects of HIF-1α (Krieg A.J., et al., 2010). The growth-promoting activity of JMJD1A makes it an interesting target for miR-627.

Overexpression of miR-627 decreased JMJD1A protein in HCT-116 cells, confirming that miR-627 targets JMJD1A. Conversely, transfection of HCT-116 cells with an LNA-modified synthetic inhibitor anti-miR-627 effectively blocked calcitriol-induced miR-627 and the decrease of JMJD1A. Calcitriol, by decreasing JMJD1A induced a significant increase of H3K9Me2 on the promoter of GDF15. As a result, GDF15 mRNA expression was suppressed by

calcitriol. Other JMJD1A target genes (such as HOXA1, ADM, and EDN1 25) may also be downregulated and together contribute to tumor suppression by calcitriol. (Figure 48)

Calcitriol induced miR-627 expression *in vivo* in the tumor xenografts while the level of JMJD1A was decreased. At the dose of 0.4 μg, calcitriol suppressed tumor growth in the nude mice, without inducing significant toxicity (measured by the loss of body weight). When the activity of miR-627 was blocked by overexpressing the JMJD1A 3'UTR fragment (which can sequester miR-627), calcitriol was no longer able to decrease JMJD1A and also failed to suppress tumor growth in the nude mice. In contrast, when the miR-627 binding site within the 3'UTR sequence was mutated, calcitriol was still able to decrease JMJD1A and suppress tumor growth in nude mice. Tumor xenografts stably expressing miR-627 exhibited significantly slower growth compared to tumors expressing negative control miRNA, indicating that the miR-627 suppresses *in vivo* colon cancer growth. Our data also showed that miR-627 expression was lower in human colon cancer specimens than that in normal colon tissues.

Finally, we have established novel epigenetic mechanisms of calcitriol in suppression colon cancer growth. This study has also identified miR-627 and JMJD1A as novel targets for an effective treatment of colon cancer.

5.2. Clinical implications and future directions

Although vitamin D has excellent antitumor activity, its side effect of inducing hypercalcemia prevents its use in the clinical treatment of cancer. Our study was able to identify the potential novel targets like miR-627 and JMJD1A, downstream of vitamin D, which can be used to design new therapy for colon cancer while bypassing the hypercalcemic toxicity. A

recent study has demonstrated that the circulating blood vitamin D concentration is inversely associated with colorectal cancer risk (Lee J.E., et al., 2011).

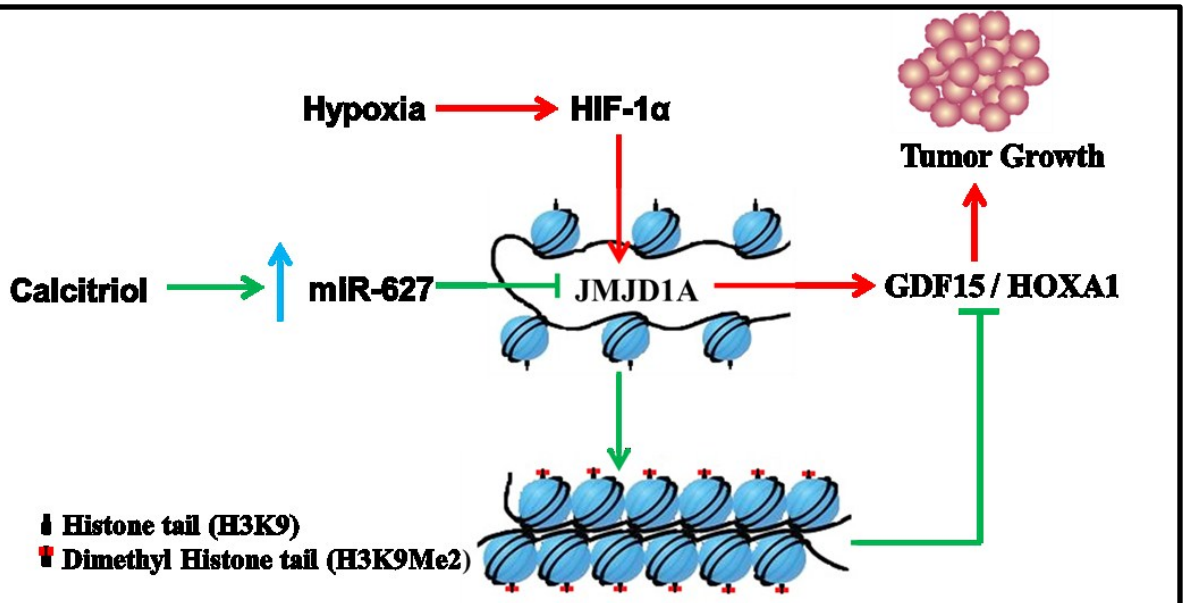


Figure 48. A model to describe the role of calcitriol in regulation of miR-627 and JMJD1A levels in colon cancer cells (Activation ⟶; Inhibition ⟞).

Our data showed that miR-627 expression is lower in colon cancer specimens than that in the normal colon tissues. Thus, lower blood levels of vitamin D may cause the decrease of miR-627 expression, which may serve as an important mechanism to promote colon cancer growth. So, strategies to over express miR-627 or directly inhibit JMJD1A may prove to be effective against colon cancer without eliciting hypercalcemia.

The future directions of this project include: (1) Synthesis of novel small molecule JMJD1A inhibitors, (2) Systemic delivery of stable and synthetic miR-627, and (3) Use of synthetic vitamin D analogues with high anticancer efficacy while being non-toxic.

1. **Synthesis of novel small molecule JMJD1A inhibitors**: JMJD demethyases have been shown to be overexpressed and plays an importance role in cell proliferation in prostate (Wissmann M., et al., 2007), breast (Liu G., et al., 2009), and esophageal cancers (Cloos

P.A., et al., 2006). JMJD inhibitors have demonstrated an inhibition of prostate and colon cancer cell growth (Hamada S. et al., 2010).

2. **Systemic delivery of stable and synthetic miR-627**: In gene therapy, the primary challenge is to develop an ideal method to deliver the synthetic miRNA to target cells. An ideal delivery method should be non-toxic to cells, protect the RNA from degradation by nucleases, and allow efficient miRNA entry into the target cells (Rajagopal N. A. 2013). Liposomes are most commonly used to deliver DNA and RNA into the cells, due to their long half-lives in systemic circulation as compared to naked oligonucleotides. Liposomes also protect the oligonucleotides from degradation and nuclease digestion. It has been reported that, neutral liposomes administered systemically were able to deliver the synthetic miR-34a and let-7 into the lung tissues of mice and inhibited tumor growth (Wiggins J.F., et al., 2010 and Trang P., et al., 2011). Biodegradable polyethanolimine based polymers (Pereira D.M., et al., 2013) (alternative to liposomes) carrying miR-34 and miR-145 mimics also showed strong inhibition in tumor growth in mouse models of colon cancer (Ibrahim A.F., et al., 2011), glioblastoma (Yang Y.p., et al., 2012), and adenocarcinoma of lungs (Chiou G.Y., et al., 2012). Atelocollagen (Takeshita F., et al., 2005) based nanoparticles carrying mir-34a and LNA-miR-135b were effectively inhibit colon cancer and lymphoma in mice, respectively (Tazawa H., et al., 2007 and Matsuyama H., et al., 2011)
3. **Use of synthetic vitamin D analogues with high anticancer efficacy while being non-toxic:** Vitamin D analogues reduce the proliferation of colon cancer cells *in vitro* and also reduce the tumor xenografts growth *in vivo.* Several epidemiologic studies show that vitamin D deficiency promotes colon cancer growth (Akhter J., et al., 1997 and Evans S.R., et al., 2000).

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