Study of Mitochondrial Fission/Fusion Dynamics and Its Role in Colorectal Cancer Growth, Survival, Migration and Angiogenesis in Response to Short Chain Fatty Acid

Thesis submitted to the

Central University of Gujarat

In the partial fulfillment of requirement

for the award of degree of

Doctor of Philosophy

In

Life Sciences

By

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Enrol. No. CUG/2010/0079 2015
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Acknowledgements

I would like to convey my gratitude to my supervisor, Professor Rana Pratap Singh whose efforts and guidance takes a shape of this thesis. He has been supportive since the day I began working in the area of cancer biology. Prof. Singh has supported me academically and emotionally through the journey of my Ph.D with his affection and humbleness. I also thank him for his help during difficult times; he gave me moral support and freedom I needed to move on.

I would like to express my gratitude to Prof. Shivendra V. Singh, University of Pittsburgh, Pittsburgh, PA, USA for his generous support and inspiration.

With a deep sense of appreciation, I would like to remember the encouragement and support from my M.Sc. supervisor Dr. Bhavesh H. Joshi.

I would like our thank to former dean Prof. Man Singh and present dean Prof. J.P.N. Mishra, SLS, CUG for the continuous encouragement.

I would like to acknowledge Dr. Eun-Ryeong Hahm, UPitt, USA and my seniors Dr. Tariq Bhat and Dr. Dhanya Nambiar for the providing training and their continuous support.

I place on record, my sincere thanks to faculty members, SLS, CUG; Dr. Umesh Chand Singh Yadav, Dr. Achuit Kumar Singh and Dr. Rajesh Vasita for their cooperation and encouragement.

I acknowledge Prof. R. K. Kale (Former) and Prof. S. A. Bari (Present) Vice chancellor, CUG for providing me the infrastructure for doing my research work, CIF for giving instrumentation facility and University Grants Commission, Government of India for providing research fellowship through CUG, India.

I express my special thanks to my batchmates and labmates for their excellent cooperation, valuable suggestions, and great care throughout my training period. I am also indeed thankful to our laboratory staff Girijesh and Vimal for providing good support in the lab.

I acknowledge fellowship support from University Grants Commission, New Delhi, India.

I bow down on my knees and express profound regards to my parents and almighty God.

Besides this, several people have knowingly and unknowingly helped me in the successful completion of this project are respectfully acknowledged.
I would like to convey my sincere thanks to my country and its citizens for all the resources I was able to make use of during the tenure of Ph.D and thus making it a successful journey. Henceforth, I dedicate this work, which was my supervisor’s vision and effort to my mother land.

Tailor Dhanir M.
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<td>5-FU</td>
<td>5-Fluorouracil</td>
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<tr>
<td>AKT</td>
<td>Protein kinase B</td>
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<td>APC</td>
<td><em>Adenomatous Polyposis Coli</em></td>
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<td>ASR</td>
<td>Age Standardized Rate</td>
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<tr>
<td>BA</td>
<td>Butyric Acid</td>
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<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
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<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
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<td>BME</td>
<td>β-Mercaptoethanol</td>
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<tr>
<td>CDC25C</td>
<td>Cell Division Cycle 25C</td>
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<td>CDK</td>
<td>Cyclin-Dependent Kinases</td>
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<tr>
<td>CHX</td>
<td>Cycloheximide</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CRC</td>
<td>Colorectal Cancer</td>
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<tr>
<td>DCF-DA</td>
<td>Dichloro-Dihydro-Fluorescein Diacetate</td>
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<tr>
<td>DNM1L</td>
<td>Dynamin – 1 like Protein</td>
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<tr>
<td>DRP1</td>
<td>Dynamin Related Protein - 1</td>
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<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraacetic Acid</td>
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<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
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<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases 1/2</td>
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<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
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<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FIS1</td>
<td>Mitochondrial Fission 1</td>
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<td>FIT</td>
<td>Fecal Immunochemical Test</td>
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<tr>
<td>gFOBT</td>
<td>Guaiac-Based Fecal Occult Blood Test</td>
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<tr>
<td>HNPCC</td>
<td>Hereditary Non-Polyposis Colon Cancer</td>
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<td>HUVECE</td>
<td>Human Umbilical Vein Endothelial Cell</td>
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<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
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<tr>
<td>IL1β</td>
<td>Interleukin 1β</td>
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<tr>
<td>k-RAS</td>
<td>Kirsten Rat Sarcoma Viral Oncogene</td>
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<tr>
<td>LC3A/B</td>
<td>Microtubule-Associated Protein 1A/1B-light chain 3</td>
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<td>MET</td>
<td>Mesenchymal-Epithelial Transition</td>
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<td>MFF</td>
<td>Mitochondrial Fission Factor</td>
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<td>MFN</td>
<td>Mitofusin</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
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<tr>
<td>NAC</td>
<td>n-Acetyl Cysteine</td>
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<td>NCI</td>
<td>National Cancer Institute</td>
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<td>NCR</td>
<td>National Cancer Registry</td>
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<td>Propionic Acid</td>
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<td>PARP</td>
<td>Poly (ADP-ribose) Polymerase</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
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<td>RB</td>
<td>Retinoblastoma</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<td>RT</td>
<td>Room Temperature</td>
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<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
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<td>SCFA</td>
<td>Short Chain Fatty Acid</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>TGFβ1</td>
<td>Transforming Growth Factor Beta 1</td>
</tr>
<tr>
<td>TGFβR2</td>
<td>Transforming Growth Factor Beta Receptor 2</td>
</tr>
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<td>TP53</td>
<td>Tumor Protein P53</td>
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<tr>
<td>uPA</td>
<td>urinary-type Plasminogen Activator</td>
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<td>VEGF</td>
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Introduction

Eight million individuals lost their life and fourteen million new cases were reported around the world in 2012 due to cancer (Ferlay et al., 2015). In developed country like United States of America (USA) every year 22% of total deaths occur due to the different types of cancers. According to National Cancer Institute, (NCI) USA “Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems”. It cannot be claimed as a single disease but is a group of diseases and common terminology used for a range of symptoms and complications. There are wide varieties of cancer causing agents like some chemicals and viruses present in food and environment, hormonal and physiological conditions and radiation. Cancer cases are also contributed through hereditary mechanisms. Among all, colorectal cancer (CRC) is the second most commonly diagnosed cancer in females and the third in males, which caused 1.3 million new cancer cases and 0.7 million deaths in 2012 (Ferlay et al., 2015).

Abnormality in cells arises due to different types of carcinogens and genetic factors. These factors lead to accumulation of mutations in the cell and finally damages DNA, leading to deregulation of signalling pathways which control proliferation, differentiation and survival of cells. At cellular level, cancer development starts with tumor initiation in which first proliferation of single mutated cell occurs. Unregulated cell proliferation results in development of population of tumor cells. Further, tumor progression occurs with additional mutation in tumor cell population and some of these mutations provide selective advantage to tumor cells like increase in proliferation, survival and metastasis and some cells will be further selected and increase their population. This selection continues throughout lifetime leading to malignancy (Cooper and Hausman, 2007). Further, damage in various cell cycle checkpoints and repair genes impairs the repair mechanism in cancer cells and accumulation of mutation continues.

Cancer cells behave very differently from the normal cells. Normal cells sustain growth and division in controlled manner and they have particular life span after which they die. In contrast, cancer cells grow and divide uncontrollably and are
immortal. Another unique property of cancer cells is metastasis i.e. they have capacity to invade nearby or distant tissues through circulatory and lymphatic system. Cancer cells are less firmly attached to their extracellular matrix and also secrete the proteases which help them to degrade extracellular matrix and move to other sites whereas normal cells are firmly attached to their extracellular matrix. Normal cells have property of contact inhibition i.e. normal cells migrate and when they come in contact with other cell, they attach with them and further migration and proliferation is inhibited but cancer cell keeps on migrating and proliferating even after coming in contact with neighbouring cells. Cancer cells also possess property of angiogenesis i.e. they secrete growth factors which encourage the formation of new blood vessels to maintain oxygen and nutrients level when tumor grows in size (Cooper and Hausman, 2007). Hence, the properties of cancer cells are very different from normal cells which make them more ferocious.

Cancer can be classified on the basis of tissues from which they are originated as epithelial tissue cancer, cancer of blood cells, skeletal tissue cancer and neuroectodermal cancer. Cancers arising from epithelial tissues are termed as carcinomas. According to American Cancer Society report, epithelial cancers are the most common type of cancer accounting 85% of total cancers. Epithelial tissue forms protective covering outside the body and organs and, also covers inner lining of cavities. The carcinomas can be further classified into squamous cell carcinoma and adenocarcinoma. Squamous cell carcinomas (SCC) are carcinoma arising from squamous cells that form upper layer of epithelial tissue that is epidermis. Adenocarcinoma arises from epithelial cells that line cavities and secrete substances like mucous into cavity (Weinberg, 2007). Among epithelial cancers, lung, prostate, colorectum and stomach cancer in men and breast, cervix uteri, colorectum and lung in women are most frequently occurring cancer (Ferlay et al., 2010).

In earlier times, only strategies for cancer management were early detection and treatment and no concept of prevention was there. But during 1970s, interest towards cancer detection and prevention was emphasized and the research headed towards the identification of major risk factors associated with cancer (Bode and Dong, 2009). Major risk factors for cancer are as follow:
1. Age: Based on SEER’s 2006-2010 incidence report, the median age for diagnosis and death for cancer of all sites was found to be 66 and 72 years, respectively. Incidence rate of cancer in children is very low and only 1% was diagnosed under the age of 20 years, 7.9% were diagnosed between 20-44 year and 91% cancer occurs between ages of 45-85 years.

2. Poor nutrition, Obesity and Physical inactivity: Nutrition plays very important role in cancer prevention. Westernized diet like fast food which lacks fibers and contains high fat content, increases risk of almost all types of cancer.

3. Viruses and Bacteria: Infection with certain viruses known as tumor viruses are known to cause cancer in humans such as, hepatitis B and C cause liver cancer, HPV causes cervical and head and neck cancer, Epstein-Barr virus causes Burkitt’s lymphoma and T-cell lymphotropic virus causes adult T-cell leukemia. Some bacteria such as *Helicobacter pylori* also causes stomach cancer (Cooper and Hausman, 2007).

4. Sunlight and Ionizing radiations: Sunlight contains different ranges of UV radiations among which UV-B is known to be potent carcinogen. Exposure to UV-B causes formation of photoproducts which are not recognized by DNA repair machinery and finally damages the DNA leading to skin cancer (Kraemer, 1997). Ionizing radiations such as X-ray, gamma ray also contain sufficient energy which can causes DNA damage and leads to cancer.

5. Tobacco: Tobacco is the major risk factor for all types of cancer. It not only causes lung cancer in smokers but also increases risk of head and neck, stomach, pancreas, kidney, bladder, uterus cervix, colon, rectum and ovary cancer and acute myeloid leukemia (Pfeifer et al., 2002). According to International Agency for Research on Cancer (IARC) report, 55 types of carcinogens are present in cigarette smoke like polyaromatic hydrocarbons, Asz-arenes, N-nitrosoamines and various organic compounds. These carcinogens form the DNA adducts and lead to mutations. It has been found that tobacco smoke causes mutation in p53 and KRAS genes (Pfeifer et al., 2002).

6. Alcohol: Alcohol intake is major risk factor for many cancers including head and cancer, stomach, colorectal, breast and ovaries (Bagnardi et al., 2001). The major carcinogen present in alcohol is acetaldehyde which leads to cytogenetic abnormality in cells leading to cancer (Seitz and Stickel, 2007).
Avoiding the above mentioned risk factors is an important strategy to decrease the worldwide cancer burden. Apart from avoidance, early detection of cancer is also very important. Some cancers including cervical, colon, breast cancers do not show early observable clinical symptoms when they are in early pre-malignant stage and symptoms arises only when they are in late invasive stage. Hence, early screening of pre-malignant lesion is important so that they can be cured before they become highly malignant. According to GLOBOCAN report, cancer deaths decreased by 1.8% per year in males and 1.5% per year in females during recent 5 years of data. Death rate peaked in men and women in 1991 then after due to improved medical facility and screening technique, there was decrease in overall mortality rate by 20% (Siegel et al., 2013). Although, avoiding above mentioned risk factors can surely decrease the cancer burden but in today’s world nothing is safe and we cannot predict which unknown factor will act as carcinogen. Therefore, avoidance is a good strategy but chemoprevention is an additional protective mechanism to evade cancer.

Human body has at least 10 times more bacteria than the number of human cells presents in the body, and most of them are in the human gastrointestinal tract (Savage, 1977). They produce certain molecules which play crucial role in various metabolic events including digestion and homeostasis maintenance. Short chain fatty acids (SCFA) produced by microbial flora by anaerobic fermentation of dietary fibers, have active role in homeostasis regulation. SCFAs including butyric acid, propionic acid and acetic acid are present in milli-molar concentrations. One of which butyric acid, previously shown to withdraw cells from cell cycle or to promote cell differentiation, and finally to induce programmed cell death (Pajak et al., 2007).

Mitochondria are a crucial organelle, which play an important role in range of events starting from embryonic development to cell death. It also plays role in cancer, as cancer cells express metabolic instability and also show resistance to mitochondrial apoptosis. There are two approaches to regulate it, first to triumph on glycolysis to revert the Warburg’s effect and another by inducing apoptosis by targeting mitochondrial proteins and/or membranes (Kroemer, 2006). This organelle behaves very dynamically and constantly undergoes fission and fusion events. Many human disorders including neurodegenerative diseases are related with deficiencies in the
proteins which are regulating mitochondrial dynamics (Westermann, 2010). Dynamin-related protein 1 (DRP1) or Dynamin-1-like protein (DNM1L) functions as mitochondrial and peroxisomal division machinery. It facilitates the membrane fission via oligomerization into ring-like structures which wrap around the scission site to constrict and sever the mitochondrial membrane through a GTP dependent mechanism (Shin et al., 1997). This molecule plays role in normal brain development by facilitating the regulated apoptosis, which takes place during neural tube development (Chen et al., 2000; Parone et al., 2006). It is also required for cytochrome c release, activation of caspase during apoptosis, during mitosis it is required for mitochondrial fission and in addition it is also required for programmed necrosis execution and may be involved in vesicle transport. DRP1 inhibition leads to ATM-dependent G2/M arrest and aneuploidy (Qian et al., 2012). Levels of the mitochondrial fission GTPase Drp1 are highly elevated in growth factor erv1-like (Gfer)-Knockdown (KD) embryonic stem cell (ESC) and decreased in Gfer-overexpressing cells. Treatment with a specific inhibitor of Drp1 rescues mitochondrial function and apoptosis, whereas expression of Drp1-dominant negative resulted in the restoration of pluripotency marker expression in Gfer-KD ESCs. This study revealed a novel prosurvival role for Gfer in maintaining mitochondrial fission–fusion dynamics in pluripotent ESCs (Todd et al., 2010). Mice based study suggests that, mice lacking the mitochondrial fission GTPase Drp1 have developmental abnormalities, particularly in the forebrain, and die after embryonic day 12.5. Neural cell-specific (NS) Drp1(-/-) mice die shortly after birth as a result of brain hypoplasia with apoptosis (Ishihara et al., 2009). Another mice based study also suggests that modulating Fis1/Drp1 complex availability regulate the Siah2 as a key regulator of hypoxia-induced mitochondrial fission. Siah2 has physiological significance in ischemic injury and nematode life span (Kim et al., 2011). Overall, these studies suggest that mitochondrial fission-fusion dynamics could be a potential target in growth control of cancer cells.
Review of Literature

Human body is a complex system comprises trillions of cells as their building block. Each of the cells is part of an organ and member of a specific tissue system. These cells are tightly regulated by internal as well as external factors. Every cell has its accurate regulatory mechanisms. Genome of the cell has information related to life as well as death including rescue mechanisms. Cells continuously come into the contact of different environmental conditions from the outside and inside of the body. Cells try to overcome the stress conditions, and usually rescue from it or get die. Cells divide in a disciplined manner such that old and damaged cells get replaced by next generation in a manner that function of system does not get compromise. When the cells start to neglect regulation due to mutation in genome, regular cycle of cells get disbalanced. Accumulation of this kind of mutations leads to the uncontrolled cell proliferation and death. When cells proliferate without control and requirements, they create burden on the system. This uncontrolled proliferating mass of cells is called Tumor or Neoplasm. It can be differentiated in to four classes benign, in situ, malignant, and neoplasms with uncertain or unknown behaviour. In general, malignant tumors are called as cancer. Cancer is a group of diseases which is characterized by unregulated growth, uncontrolled proliferation and increased survival of abnormal cells. Global burden of cancer is increasing day by day and needs measures for its control. Cancer occurs due to slow accumulation of mutations within the cell over period of time due to different types of carcinogens or it may be inherited genetically. Mutations occurring in the region of tumor suppressor genes and proto-oncogenes slowly convert normal cells into cancer cells.

II.1. Cancer Statistics

Cancer is one of the few diseases in the modern human history that has invited a strong debate and focuses for extensive investigation in the modern medicine, and challenged the modern scientific caliber. This evil attribute (as we can say) of cancer can be linked to its complex nature, lack of timely diagnosis, poor prognosis and no cure. Although modern medicine has not come up with a cure for cancer, yet many developments in this area have been achieved that have eased many aspects like diagnosis, treatment, reduction in cancer related deaths, predicting its fate and course in the patient and improving quality of life of the patient. Nearly estimated 14 million
new cancer cases and 8 million cancer deaths occurred in 2012 worldwide; while the number of new cancer cases ranges from 3.7 million in Eastern Asia to about 1800 in Micronesia/Palynesia (Ferlay, et al. 2015). Among men, the incidence of cancer is high in Australia/New Zealand (Age Standardized Rate (ASR) 356.8 per 100,000), Northern America (ASR 334 per 100,000) and Northern and Western Europe (ASRs 288.9 and 335.3 per 100,000 respectively), and one of the main reasons is higher rates of prostate cancer in these regions. Like males, the regions with the highest cancer incidence rates in females are Australia/New Zealand (ASR 276.4 per 100,000), Northern America (ASR 274.4), and Northern and Western Europe (ASRs 257.8 and 250.5 respectively) which is because of the high rates of breast cancer in these regions. Cancer incidences are very low in African and Asian regions compared to Western populations but mortalities with respect to incidences are higher in former ones compared to later populations. Few of the various reasons for this higher mortality rates in the African and Asian regions are late diagnosis, less patient care after diagnosis and poverty (which limits access to timely healthcare facilities) as compared to Western countries (Table 1).

II.2. India and Cancer

India is a developing country with diverse populations living with diverse life styles. Cancer rates are lower in India than in Western world, but these rates increase with increased migration of the rural population to cities, changes in life style and increase in life expectancy (Gajalakshmi et al., 2001; Finlay et al., 2001). Most frequently diagnosed cancers in India are breast, cervix uteri, lip, oral cavity, lung and colorectum (Ferlay et al., 2015). In India, incidence of head and neck cancers, especially oral and oesophageal types is prominent in male mostly due to tobacco chewing and betel nut (areca nut) eating etc. (Sinha et al., 2003). Generally, the lower cancer incidences in India compared to the Western world have been associated with the vegetarian dietary habits and importance of herbs in the Indian culture. Many bioactive phytochemicals have been isolated and characterized from these vegetables that have been shown to possess strong anticancer activities against many cancers, and have been suspected to cause this disparity (Aggarwal and Shishodia, 2006; Singh and Agarwal 2006; Mehta et al., 2010).

II.3. Indian Cancer Statistics
In India, estimated numbers of new cancer cases every year are around 700,000-900,000 although survey is mostly being conducted in urban areas and more than 70% Indian population resides in rural areas (Ferlay et al., 2015; NCR, ICMR India). Compared to cancer incidences in West, Asia, particularly India has lower incidences and also less number of cancer-related deaths. But ratios of ASRs of mortality between a developed country (like USA) and India are close to 1.0. This is because of the same reasons as cited above. Cancer-related statistical data from India are mainly managed and stored by cancer registries that function on the guidelines of IARC, and are either IARC or non-IARC registries. There are total eight IARC registries in India. The all cancers (excluding non-melanoma skin cancer) facts in India are given in Tables 2. The five most common cancers in Indian men are lung, lip, oral cavity, oropharynx, oesophagus and stomach while in Indian women cervix uteri, breast, ovary, lip-oral cavity and oesophagus are common ones; and overall common cancers in India are cervix uteri, breast, lip-oral cavity, lung, and oesophagus (Table 2) (Ferlay et al., 2015).

II.4. Molecular Basis of Cancer
Oncogenes play very important role in cancer initiation and progression. Proto-oncogenes are present in all cells and they are beneficial genes since they are involved in regulation of cell division, differentiation and inhibition of apoptosis in normal cells. These proto-oncogenes turn harmful when mutation, chromosomal rearrangements and gene amplification occur in the region of proto-oncogenes and convert proto-oncogenes to oncogenes. The product of these genetically altered oncogenes can act as an activator of transcription factors, chromatin remodelers, constitutive activator of growth factor genes such as platelet derived growth factor. Mutated oncogene product can activate growth factor receptor such as mutation in ligand binding domain of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) leads to constitutive activation of receptor in many cancers. These products also activate tyrosine kinases such as ABL, SRC, serine kinases and threonine kinases like PI3K and its downstream targets such as AKT and RAF1. The altered proto-oncogene products can also activate BCL-2 protein that increases the survival of cell and prevents apoptosis (Croce, 2008). Ras oncogene also plays important role in development of cancer such as K-RAS mutation is common in lung, colon and pancreas cancer. Mutation in Ras results in continuous transduction of
receptor tyrosine kinase signaling and an increase in cell proliferation (Rajalingam et al., 2007). Myc gene amplification also plays important role in various types of cancer including lung cancer, breast cancer, cervical cancer and head and neck cancer. Myc and cyclin D1 genes are important regulators of cell cycle and mutation of these genes result in increased production of mitogenic signals and hence increased cell proliferation (Evan and Vousden, 2001).

Tumor suppressor genes also play important role in cancer development. The function of tumor suppressor genes in normal cells is to suppress the development of tumor by inducing cell cycle checkpoint, checking DNA damage and its repair, mitogenic signaling and apoptosis regulation. p53 is an important tumor suppressor gene which is found to be mutated in >50% of cancer. p53 is a guardian gene which guides the cells for repair or death when DNA damage occurs in the cell. When DNA damage occurs in cell, p53 induces p21/cip1 which binds to cdk-cyclin complex and causes arrest so that cells get time to repair the damaged DNA. If cells cannot repair themselves, they guide the cell for apoptosis. However, mutations in p53 can lead to cell cycle progression and inhibit repair and apoptosis and thus leading to cancer (Cooper and Hausman, 2007). Retinoblastoma (Rb) is another tumor suppressor gene which regulates cell cycle in normal cells by interacting with transcription factor E2F which halts production of cyclin E and in turn, cell cycle progression. Mutation in Rb results in release of cell from this checkpoint and hence can lead to cancer. Other tumor suppressor gene mutations which includes mutation in INK4a, ARF, APC, PTEN, ATM, ATR, BRCA1 and BRCA2 result in various types of cancer (Sherr, 2004). Hence in cancer, activation of oncogenes and repression of tumor suppressor genes release cancer cells from various types of constrains that are present in normal cells which help them to proliferate and survive continuously. Among all tissues of body, epithelial tissue is the outermost surface of body which is most likely to be exposed to various types of environmental carcinogens. Hence, tumor suppressor genes and oncogenes mutations in epithelial tissues are very common that lead to epithelial cancer of various parts.

II.5. Angiogenic and Metastatic Targets
Tumor angiogenesis as a promising target in cancer chemoprevention. Solid tumors usually grow only up to ~1-3 mm diameter in size owing to simple diffusion and
without active supply of nutrients and gaseous exchange, and remain in a dormant phase at this stage for a long time (up to several years). Tumor dormancy is broken-down once active nutrient supply and gaseous exchange is resumed via a process, called tumor angiogenesis. Angiogenesis is essentially required for cancer progression from dysplasia onwards till metastasis phases. Tumor angiogenesis is the proliferation of a network of blood vessels penetrating into the cancerous growths to supply nutrients and oxygen, and remove metabolic wastes from them. Beyond the critical size limit of tumors, simple diffusion is not sufficient to maintain growth in tumor mass, and as a result hypoxia gets created. Hypoxia is considered as one of the important factors that lead to the HIF-1α/β-mediated expression of angiogenic factors (Shih et al., 2001). It is documented that creation of hypoxia within the tumor mass due to insufficient oxygen supply beyond the critical size limit leads to the stabilization of HIF-1α protein that complexes with its β-subunit and activates VEGF expression and hence, tumor angiogenesis. Hypoxia activates prolyl hydroxylase (an oxygen sensor), which hydroxylates proline residue of HIF-1α and causes its stabilization and nuclear translocation. Tumor angiogenesis is a very complex process and involves a tight interplay of tumor cells, endothelial cells, phagocytes and their secreted factors, which may act as promoters or inhibitors of angiogenesis. The angiogenic promoters send signals to the surrounding normal tissues in which various gene activations and protein expression encourage growth and proliferation of new blood capillaries (Ferrara, 2000). Many different proteins (such as VEGF, bFGF and IL8 etc.), as well as several small molecules (such as adenosine, PGE, etc.) have been identified as angiogenic factors secreted by tumor cells to induce angiogenesis. Among these molecules, VEGF and bFGF are of prime importance for sustaining angiogenesis and hence, tumor growth and progression. These angiogenic factors are produced by tumor cells and also by certain types of normal cells such as phagocytes. VEGF signals through two tyrosine kinase receptors, VEGFR-1 and VEGFR-2 which are expressed predominantly on vascular endothelial cells. VEGFR-2 is the principal signaling receptor for vascular endothelial cells, whereas VEGFR-1 probably functions as a decoy receptor, serving to regulate the availability of VEGF in a given tissue (Risau et al., 1997).

The tumor angiogenesis process follows an orderly series of events as outlined below, each of which could be exploited for anti-angiogenesis strategy:
1. Tumor cells express and secrete angiogenic factors diffusing into nearby tissues.
2. These angiogenic GFs recognize and bind to specific receptors located on the endothelial cells of nearby pre-existing blood vessels.
3. On binding to receptors, these factors activate endothelial cells and alter expression of molecules like enzymes such as MMPs and uPA.
4. Such types of enzymes dissolve tissue matrix and sheath-like covering surrounding the existing blood vessels.
5. Endothelial cells begin to proliferate and migrate out in an orderly fashion through dissolved portion of tissues and existing vessels towards the diseased tissue such as tumor.
6. Adhesion molecules or integrins help to pull the nascent blood vessel sprout forward.
7. Sprouting endothelial cells roll up to form a tubular structure to move through the tissue dissolved by MMPs and uPA.
8. Finally, newly formed blood vessel tubes are remodelled and stabilized by smooth muscle cells and pericytes in to matured vessels.

Critical role of tumor angiogenesis in cancer progression and metastasis has been postulated more than four decades ago where antiangiogenic therapy for cancer control and prevention was proposed to be one of the most effective anticancer strategies, and this subsequently opened a new era of antiangiogenesis research (Folkman, 1971). Since then active investigation in this field has led to the clinical trials of many antiangiogenic drugs (Folkman, 1971; Gimbrone et al., 1972; Boehm et al., 1997; Kerbel, 2000; Carmeliet and Jain, 2000; Brekken et al., 2002). Since many angiogenic diseases like diabetic retinopathy, atherosclerotic plaques etc. are treated by angiogenic inhibitors, these agents could also inhibit tumor angiogenesis and hence, cancer growth and progression (Carmeliet and Jain, 2000; Liotta and Kohn, 2001; Brekken et al., 2002). However, discovery of nontoxic antiangiogenic phytochemicals could have a greater practical significance compared to non-selective cytotoxic therapies to control tumor growth and progression by targeting angiogenesis. Since, many dietary and non-dietary phytochemicals possess strong antitumorigenic activities and do not affect the survival of normal cells; therefore, they might also possess antiangiogenic activities as one of their overall anticancer
mechanisms, and further, this strategy could be a rational approach to examine their inhibitory effect on tumor angiogenesis. The blood vessels/capillaries of normal and tumor-derived tissues show considerable differences in morphological as well as physiological aspects (Carmeliet and Jain, 2000; Liotta and Kohn, 2001). Since, the blood vessels of non-pathological normal tissues are generally quiescent in adults and are intact in appearance, and tumor-derived blood vessels are highly disorganized, relatively thin walled, leaky and have irregular diameters with less supporting pericytes or smooth muscle cells; therefore, tumor-associated angiogenesis would be comparatively the sole target in antiangiogenic strategy. In addition, tumor-associated endothelial cells are abnormal in shape and grow on top of each other, and remain in an environment rich in pro-angiogenic factors and other inflammatory cytokines (Thurston et al., 1999; Etoh et al., 2001). Therefore, selective targeting of tumor vasculature by nontoxic phytochemicals could be a novel and worthy approach for cancer control and prevention and this would be associated with reduced or no harmful side effects.

II.6. Metastasis and Cancer

One of the most important aspects of cancer progression and spread is tumor metastasis, in which tumor cells acquire the ability to invade into normal tissues and organs through tissue boundaries to form new cancerous lesions at sites distinct from the primary tumor (Fidler, 1990; Bhat and Singh, 2008). The process through which cancer cells can spread within the body are varied, such as direct invasion of surrounding tissues, spread via the blood vascular system (hematogenous metastasis) and spread via the lymphatic system (lymphatic metastasis) (Fidler, 1990; Sherwood, 2006). The molecular mechanisms involved in this process are incompletely understood but those associated with cell-cell and cell-matrix adhesion, with the degradation of extracellular matrix, and with the initiation and maintenance of early growth at the new site are generally accepted to be very critical. Adhesion molecules (e.g., integrins and cadherins) play a major role in signaling from outside to inside a cell, thereby controlling how a cell is able to sense and interact with its local environment (Juliano, 2002). Proteolytic enzymes and their inhibitors (e.g., matrix metalloproteinases and TIMPs) regulate the breaking down of the components of the extracellular matrix and the release of factors which can regulate the growth of the cells positively or negatively (Imai et al., 1997). It is not only the immediate cellular
microenvironment but also the extended cellular microenvironment, such as vascular insufficiency and hypoxia in the primary tumor, which can modify cellular gene expression to enhance metastasis. Mechanisms of metastasis involve a complex array of genetic and epigenetic changes many of which are specific both for different types of tumors and for different sites of metastasis.

II.7. Colorectal Cancer

Colorectal cancer is a term used for cancer that starts in the colon or the rectum. These cancers can also be referred to separately as colon cancer or rectal cancer, depending on where they start. Colon cancer and rectal cancer have many features in common. The colon and rectum are parts of the digestive system, which is also called the gastrointestinal (GI) system. The first part of the digestive system (the stomach and small intestine) processes food for energy while the last part (the colon and rectum) absorbs fluid to form solid waste (fecal matter or stool) that then passes from the body. The small intestine joins the large intestine (or large bowel) in the right lower abdomen. Most of the large intestine is made up of the colon, a muscular tube about 5 feet long. The colon absorbs water and salt from the food matter and serves as a storage place for waste matter.

The colon has 4 sections (Fig. 1):

- The first section is called the ascending colon. It starts with a small pouch (the cecum) where the small bowel attaches to the colon and extends upward on the right side of the abdomen. The cecum is also where the appendix attaches to the colon.
- The second section is called the transverse colon since it goes across the body from the right to the left side in the upper abdomen.
Figure II.1: Anatomy of large intestine (colon & rectal).
(Source: Colorectal Surgical Associates)

- The third section, called the descending colon, continues downward on the left side.
- The fourth and last section is known as the sigmoid colon because of its "S" or "sigmoid" shape.

Most colorectal cancers develop slowly over several years. Before a cancer develops, a growth of tissue or tumor usually begins as a non-cancerous polyp on the inner lining of the colon or rectum. A tumor is abnormal tissue and can be benign (not cancer) or malignant (cancer). A polyp is a benign, non-cancerous tumor. Some polyps can change into cancer but not. The chance of changing into a cancer depends on the kind of polyp. The 2 main types of polyps are:

- Adenomatous polyps (adenomas) are polyps that can change into cancer. Because of this, adenomas are called a pre-cancerous condition.
- Hyperplastic polyps and inflammatory polyps, in general, are not pre-cancerous. However, some hyperplastic polyps can become pre-cancerous or might be a sign of having a greater risk of developing adenomas and cancer, particularly when these polyps grow in the ascending colon.

More than 95% of colorectal cancers are adenocarcinomas generated from glandular cells. Other rare groups are carcinoid tumors, gastrointestinal stromal tumors, lymphomas and sarcomas. (Source: American Cancer Society. Cancer Facts & Figures 2015 Atlanta, Ga: American Cancer Society; 2015.)

II.7.1. Colorectal Cancer Statistics
Colorectal cancer is the third most common cancer in men (7,46,000 cases, 10% of total cancer) and second most common cancer in women (6,14,000 cases, 9.2% of total cancer). Mortality wise colorectal cancer ranks second among men (3,74,397 cases, 7.8% of total cancer) and third among women (3,20,654 cases, 8.6% of total cancer) (Ferlay et al., 2015). Majorly, 55% of the cases occur in developed countries.
Mortality is lower (694,000 deaths, 8.5% of the total) with more deaths (52%) in the less developed regions of the world, reflecting a poorer survival in these regions (Table 3).

In India, during year of 2012 total 37,000 males and 27,000 females get diagnosed and 28,000 males and 21,000 females died due to colorectal cancer. Ten year predictions and data for year 2010 to 2020 suggest that except USA, incident rate of colorectal cancer is increasing which includes India too (Fig. 2) (Takiar et al., 2010).

![Scenario of Colorectal cancer](image)

**Figure II.2:** Scenario of colorectal cancer in India and USA and their compression.
(Sources: Rahib et al., 2014; Takiar et al., 2010; NCRP, India)

Colon carcinogenesis offers a huge window period of 10-15 years which could be coupled with various screening procedures for the identification of the preneoplastic lesions, making this malignancy suitable for implementation of chemoprevention strategies (Ravichandran et al., 2010).

**II.7.2. Major Risk Factors**

According to NCI, major risk factors for colorectal cancer are of two groups:

1. Lifestyle-related factors include excessive use of alcohol, cigarette smoking, obesity, physical inactivity, dietary fat, meat intake and less consumption of fibers and vegetables.

2. Other risk factors include age, personal history of colorectal polyps or colorectal cancer, inflammatory bowel disease, inherited syndromes [Familial adenomatous polyposis (FAP), Hereditary non-polyposis colon cancer...
Colorectal cancer is caused due to various genetic alterations which include mutational inactivation of tumor suppressor genes such as APC, KRAS, PTEN, SMAD4 and activation of oncogenes such as Ras and BRAF (Markowitz and Bertagnolli, 2009). Epigenetic changes also play important role in development of colorectal cancer by hypermethylation of promoter region of CpG island which results in gene silencing (Markowitz and Bertagnolli, 2009). Familial adenomatous polyposis (FAP), a hereditary CRC predisposition syndrome, is caused by mutations in adenomatous polyposis coli (APC) gene and is characterized by progressive development of numerous adenomas in colon progressing to CRC during later stages. Approximately 90% of FAP patients also develop small intestinal adenomas; overall, these patients are at ~330 times higher risk to develop small intestinal adenomas than normal population (Rajamanickam et al., 2010).

II.7.3. Molecular Basis of Colorectal Cancer
The accumulation of mutations in tumor suppressor genes or oncogenes is a key event of transformation of normal cells into cancer cells. Approximately 13,000 genes revealed mutations in the coding sequence of ~67 genes in the average colon cancer genome, out of these 12 genes were proposed to be the genes most likely to be involved with cancer formation in individual cancers (Sjöblom et al., 2006). The first identifiable histological lesion implicated in CRC formation is the aberrant crypt focus. Dysplastic aberrant crypt foci can harbor mutations in APC, one of the most frequently mutated tumor suppressor genes in CRC. Inactivation of APC leads to activation of the Wingless/Wnt pathway, a common mechanism for initiating the polyp → cancer progression sequence (Vogelstein et al., 1988).

Subsequent to mutations in APC or other genes of the Wnt signaling pathway, mutations in genes such as KRAS or TP53 occur and foster the clonal progression of the polyp cells to cancer (Vazquez et al., 2008). Progression can involve KRAS and TP53 mutations as well as mutations in genes that regulate important cell signaling pathways such as the transforming growth factor β (TGFβ1) signaling pathway. Mutations in the type II TGFβ receptor (TGFB2) gene occur in approximately 30%
of CRC (Grady et al., 1998). The type of mutation that inactivates TGFBR2 depends upon the molecular subtype of the developing cancer cell (Markowitz et al., 1995). In addition, mutations and epigenetic alterations affecting other TGFβ1 signaling pathway members, including SMAD2, SMAD4, RUNX3 and TSP1, have been identified in colon cancers (Eppert et al., 1996; Takaku et al., 1998; Wood et al., 2007). Ultimately, it is believed that although the underlying form of genomic or epigenomic instability determines the types of mutations that occur in colon cancer, the selective pressures that lead to the clonal evolution of the tumors are largely the same across all CRC.

Gene mutations and epigenetic alterations have been proposed to contribute to colon cancer formation through the activation of oncogenes and the inactivation of tumor suppressor genes that regulate signaling pathways that control hallmark behaviors of cancers (Fearon, 2011). For example, KRAS is a proto-oncogene that is a downstream effector of EGFR. It signals through BRAF to activate the MAPK pathway. Mutations in KRAS or BRAF occur in approximately 55–60% of CRCs, aberrantly activating the MAPK signaling pathway, thus inducing proliferation and suppressing apoptosis (Samowitz et al., 2005; Nosho et al., 2008). The most common signaling pathways that carry mutant genes in CRC include not only the RAS–RAF–MAPK pathway, but also the PI3K pathway, the WNT–APC–CTNNB1 pathway and the TGFβ1–SMAD pathway (Grady and Carethers, 2008). Insights into the biology of CRC have resulted from the identification of genes commonly mutated in these particular pathways in colon cancer and have led to paradigms that have informed the study of epigenetic alterations in cancer. These insights are also currently being used to develop new diagnostic and prognostic assays for CRC, as well as new therapies (Lao and Grady, 2011).

II.7.4. Signs, Symptoms, Screen and Diagnosis

Colorectal cancer is often found after symptoms appear, but most people with early colon or rectal cancer donnot have symptoms of the disease. CRC may cause one or more of the symptoms below.

- A change in bowel habits, such as diarrhea, constipation, or narrowing of the stool, that lasts for more than a few days.
• A feeling that you need to have a bowel movement that is not relieved by doing so; Rectal bleeding, and blood in the stool which may make it look dark.
• Cramping or abdominal (belly) pain.
• Weakness and fatigue, Unintended weight loss.

Colorectal cancers can bleed. While sometimes the blood can be seen or cause the stool to become darker, often the stool looks normal. The blood loss can build up over time, though, and lead to low red blood cell counts (anemia). Sometimes the first sign of colorectal cancer is a blood test showing a low red blood cell count.

One should be careful as most of these problems are more often also caused by conditions other than colorectal cancer, such as infection, hemorrhoids, irritable bowel syndrome, or inflammatory bowel disease. Regular screening can often find colorectal cancer early, when it is most likely to be curable. In many people, screening can also prevent colorectal cancer altogether. This is because some polyps, or growths, can be found and removed before they have the chance to turn into cancer. The strategies been used for screening and diagnosis include guaiac-based fecal occult blood test (gFOBT) and fecal immunochemical test (FIT), stool DNA test, sigmoidoscopy, colonoscopy, double contrast barium enema, CT colonography (virtual colonoscopy) and biopsy.

II.7.5. Treatment Strategies for Colorectal Cancer
Treatment for CRC depends on the stage at which cancer was diagnosed. Early stage CRC is best treated with surgery. Approximately 95% of Stage I and ~72% of Stage II colorectal cancers are curable with surgery. Rectal cancer however, may require additional radiation therapy to minimize the risk of recurrence. At advanced stages (Stage III and Stage IV), it is often consisted of a combination of therapies including, surgery, chemotherapy and radiation.

A number of different drugs have significant antitumour activity in metastatic CRC, including the systemic drugs 5-fluorouracil (5-FU), irinotecan, oxaliplatin, bevacizumab, cetuximab and panitumumab, and the oral drug capecitabine. Different combinations of these drugs, such as the FOLFOX regimen (leucovorin, 5-FU and
oxaliplatin), the FOLFIRI regimen (leucovorin, 5-FU and irinotecan) and the XELOX regimen (oxaliplatin and capecitabine), with or without a monoclonal antibody agent, have been shown to improve outcomes in metastatic CRC (Edwards et al., 2012).

The monoclonal antibodies bevacizumab, cetuximab and panitumumab are more recent additions to the list of systemic drugs available for the treatment of metastatic CRC. Bevacizumab, an antibody against vascular endothelial growth factor (VEGF), was first approved as a treatment for metastatic CRC in 2004, followed by cetuximab (also in 2004) and panitumumab (2006). Cetuximab and panitumumab both target the epidermal growth factor receptor (EGFR) and are effective only in patients with wild-type KRAS and metastatic CRC. Panitumumab is the only approved fully human anti-EGFR monoclonal antibody, while cetuximab is a chimeric antibody and bevacizumab is a humanized monoclonal antibody (Edwards et al., 2012).

The current indications for monoclonal antibody therapy differ in Europe and the USA and between the three monoclonal antibodies. Bevacizumab is indicated for the first- and second-line treatment of metastatic CRC in combination with fluoropyrimidine-based chemotherapy. Cetuximab and panitumumab are indicated for wild-type KRAS and metastatic CRC as monotherapy, and cetuximab is also indicated in combination with chemotherapy in Europe and in combination with irinotecan in irinotecan-refractory wild-type KRAS and metastatic CRC in the USA (Edwards et al., 2012).

II.7.6. Colorectal Cancer Chemoprevention

Global cancer burden is continuously increasing. The therapeutic approaches are also developing, however, it is not ineffective as required due to several limitations. Sporn and Wattenberg gave the concept of ‘cancer chemoprevention’, in which rather to fight against cancer, they emphasized how to avoid its courses of development and prevent its occurrence (Sporn, 1976; Wattenberg, 1985). In 2009, WHO stated that one third life can be saved out of total cancer deaths by changing the dietary habits (Bode and Dong, 2009). The results from population-based studies show that macronutrients and micronutrients from vegetables and fruits have potential to reduce the risk of cancer (Surh, 2003). Food items like vegetables, fruits, cereals, beans, and
plant-based beverages such as tea and wine are the rich sources of different phytochemicals and other nutrients (Arts and Hollman, 2005).

During the screening of chemopreventive agents, many synthetic as well as natural compounds including dietary, non-dietary phytochemicals, micronutrients including vitamins were screened, some of which are already in house hold practices. This screening postulated the association between vegetable and fruit consumption and the risk of cancer (Block et al., 1992). Base on this, USA government started “5 A Day” (1991-2006) followed by “Fruits & Veggies-More Matters” (2006-till now) program to promote the consumption of green fruits and vegetables to avoid the cancer risk. Most likely, due to this and regular screening program, USA is the only country where rate of colorectal cancer has declined by 30% during last 10 years whereas in remaining world it is constantly increasing or steady (Siegel et al., 2014).

Apart from this, use of compounds including synthetic or natural have shown potential cancer preventive efficacy. Epidemiologic studies in humans suggest that regular users of aspirin having lower risk of CRC in compare to other (Thun et al., 1991). Phytochemicals (pure as well as in combination) are getting attention as a cancer preventive agents including, silibinin (Singh et al., 2008; Kumar et al., 2014), epigallocatechin gallate (Yang et al., 1998; Hu et al., 2015), curcumin (Wang et al., 2006), etc. Microbiota and colorectal cells homeostasis play a key role in cancer development and its progression (Schwabe and Jobin, 2013). Components which are crucial for the maintenance of microbiota or inoculation of microbiota from probiotics or from food are also getting attention as a chemopreventive agent. Dietary fibres and short-chain fatty acids are also the member of this group and screened for their cancer preventive as well as anticancer potency. Over all, these studies suggest that chemopreventive agents could play an important role in cancer control and management.

II.7.7. Colorectal Cancer, Dietary Habits and Prevention
Evidence from ecological studies, migrant studies, and secular trend studies suggest that environmental risk factors are of major importance in the cause of colorectal cancer (Kono, 2004). Dietary factors have been suspected as important, but only
intakes of red and processed meat and alcohol are considered to be convincing dietary risk factors for colorectal cancer.

In the 1970s, Burkitt proposed the hypothesis that dietary fibre reduces the risk of colorectal cancer, based on the observation of low rates of such cancer among rural Africans who ate a diet with high fibre content (Burkitt, 1993). Several plausible mechanisms have been proposed to explain the hypothesis, including increased stool bulk and dilution of carcinogens in the colonic lumen, reduced transit time, and bacterial fermentation of fibre to short chain fatty acids (Lipkin et al., 1999).

II.8. Short-Chain Fatty Acids (SCFA)

Human body has at least 10 times more bacteria than the number of human cells present in the body, and most of them are in the human gastrointestinal tract (Savage, 1977). They produce certain molecules which play crucial role in various metabolic events including digestion and homeostasis maintenance. Short-chain fatty acids produced by microbial flora, by anaerobic fermentation of dietary fibers, have active role in homeostasis regulation. Short-chain fatty acids (SCFA) including butyric acid, propionic acid and acetic acid are present in mM concentrations in gastrointestinal tract. One of which, the butyric acid, is previously shown to withdraw cells from cell cycle or to promote cell differentiation, and finally to induce programmed cell death (Pajak et al., 2007).

![Chemical structure of SCFAs](Source: PubChem)

**Figure II.3:** Chemical structure of SCFAs. (Source: PubChem)
II.9. Mitochondrial dynamics

Mitochondrion is a crucial organelle, which plays an important role in a range of events starting from embryonic development to the control of cell death. It also plays a role in cancer growth and progression, as cancer cells express metabolic instability and show resistance to mitochondrial apoptosis. There are two approaches to regulate it, first to triumph on glycolysis to revert the Warburg's effect and another by inducing apoptosis by targeting mitochondrial proteins and/or membranes (Kroemer, 2006). This organelle dynamically and constantly undergo fission and fusion events. Many human disorders including neurodegenerative diseases are related with deficiencies in the proteins that regulate mitochondrial dynamics (Westermann, 2010).

Dynamin-related protein 1 (DRP1) or dynamin-1-like protein (DNM1L) functions as mitochondrial and peroxisomal division machinery. It facilitates the membrane fission via oligomerization into ring-like structures which wrap around the scission site to

**Figure II.4: Microbiota & Short Chain Fatty Acid (SCFA)**
constrict and sever the mitochondrial membrane through a GTP-dependent mechanism (Shin et al., 1997). DRP1 plays an important role in normal brain development by facilitating the regulated apoptosis, which takes place during neural tube development (Chen et al., 2000; Parone et al., 2006). It is also required for cytochrome c release and consequently activation of caspases during apoptosis. During mitosis, it is required for mitochondrial fission and in addition, it can also be involved in programmed necrosis execution and vesicle transport. Inhibition of DRP1 is reported to cause ATM-dependent G2/M arrest and aneuploidy (Qian et al., 2012) (Fig. 5).

**Figure II.5:** Regulation of Mitochondrial fission through cyclin-CDK complex.
III.1. Hypothesis

SCFA plays role in maintenance of homeostasis, epithelial cell proliferation and apoptosis. Microbiota shows favorable effect in terms of energy metabolism, self-renewal and differentiation rate of normal stem cell. Therefore they may show their potency in controlling cancer progression as well as association with mitochondrial fission/fusion. Mitochondrial fission/fusion associated molecules including DRP1, MFF (mitochondrial fission factor) and FIS1 (mitochondrial fission 1 protein) have shown their regulatory effect on embryonic development and neuronal maintenances and disorders. Therefore these molecules may also play regulatory role in cancer cell differentiation and in Epithelial–Mesenchymal Transition (EMT) and Mesenchymal–Epithelial Transition (MET) as well as in migration and angiogenesis.

Following objectives are proposed:

III.2. Objectives

1. To study the effect of SCFA, the native microbial products of colon, on CRC cells.
2. To study the effect and regulatory mechanism of SCFA on mitochondrial fission/fusion and its role in growth, cell cycle regulation and survival in CRC cells.
3. To study the mitochondrial fission/fusion mediated regulation of EMT and MET in CRC cells.
4. To study the role of mitochondrial fission/fusion and associated molecular changes in angiogenesis in CRC.
Material and Methods

IV.1. Chemicals and Reagents
Sodium chloride, agarose, agar, glucose, acetic acid, ethylenediamine tetra acetic acid (EDTA), glycerol, hydrochloric acid, potassium acetate, potassium chloride, isopropanol, phenol, sodium hydroxide, manganese chloride, methanol, isoamyl alcohol, bromophenol blue, crystal violet and chloroform were all procured from Sigma Aldrich, USA.

Molecular biology grade DMSO, MTT \{3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide\}, Diethyl pyro carbonate (DEPC), RNase A, Trizma Base, calcium chloride, sodium dodecyl sulphate (SDS), ethidium bromide (EtBr) and \(\beta\)-mercaptoethanol (BME) were procured from Sigma Aldrich, USA.

Complete cocktails of protease and phosphatase inhibitors were from Roche Molecular Biochemicals (Indianapolis, IN, USA). Nitro-cellulose (NC) membrane and enhanced chemiluminescence (ECL) detection system was provided by Amersham Biosciences USA, Millipore USA and MDI India. Ethanol was supplied by Merck Biosciences, India.

dNTPs, Reverse transcriptase enzyme and Taq Polymerase reagents including associated buffers were purchased from Bangalore Genie (India). Gene-specific primers for semiquantitative PCR were custom synthesized by Sigma Aldrich, USA.

Mdivi-1 (mitochondrial division inhibitor) was procured from Sigma Aldrich, USA.

All other common chemicals and laboratory reagents were from S. D. fine-chem. Ltd, India, HiMedia Laboratories, India and SRL Pvt. Ltd., India.

IV.2. Cell Culture Chemicals and Reagents

IV.2.1. Cell lines: Human colorectal carcinoma HCT116 and SW480 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in well maintained CO\(_2\) incubator (Thermo Scientific) under
standard conditions of 5% CO2 and 37°C temperature in specific culture media. Human umbilical vein endothelial cell line (HUVEC), primary cells, was procured from HiMedia Laboratories, India.

**IV.2.2. Culture media and conditions:** Roswell Park Memorial Institute (RPMI)-1640 cell culture media, antibiotic-antimycotic cocktail and other culture reagents for colorectal carcinoma (CRC) cells growth were procured from GIBCO, USA. Fetal bovine serum (FBS) was procured from GIBCO, USA. Endothelial cell growth medium HiEndoXL™ Endothelial Cell Expansion Medium and all other recommended materials for HUVEC growth were procured from HiMedia Laboratories, India. Human CRC cells were grown in RPMI-1640 media supplemented with 10% FBS and 1% penicillin-streptomycin-amphotericin B cocktail in a humidified atmosphere under 5% CO2 and 37°C temperature in CO2 incubator by Thermo Fisher Scientific, USA.

HUVEC were grown in endothelial basal or complete HiEndoXL™ Endothelial Cell Expansion Medium containing recommended concentrations of growth factors and antibiotics in a humidified atmosphere in 5% CO2 and 37°C temperature. The cells were grown as adherent monolayers in specific media. Antibiotics like penicillin (100 units/ml) and streptomycin (10 µg/ml), and antimycotic amphotericin B (250 ng/ml) were added and plates incubated at 37°C in a humidified atmosphere with 5% CO2. Cells were fed every alternate day and passaged when cells reached 80-90% confluency.

**IV.2.3. Revival of cells:** Ampules of cells were retrieved from liquid nitrogen storage tank (or -80°C deep freezer) and placed immediately in water bath at 37°C for 1-2 minutes till contents thaw. Vials were wiped with 70% ethanol and contents immediately transferred to cell culture dishes containing 3 ml pre-warmed complete media. Petri plates were incubated at 37°C and 5% CO2 after observing under the phase contrast microscope. Cells were regularly examined microscopically and fed every alternate day.

**IV.2.4. Splitting of cells:** After revival of cells (or cell seeding), cultures were regularly examined microscopically for confluence and bacterial or fungal...
contamination. Once desired confluency (70-80%) was achieved, spent media was removed and trypsin-EDTA solution (1 ml for 60 mm and 2 ml for 100 mm dish) was pipetted onto the cell monolayer. Petri plates were returned to the incubator and left for 5 minutes of incubation at 37°C for trypsin to act. Microscopic examination of cells was done to ensure that all the cells were detached and floating. Plates were removed after complete trypsinization and equal volume of complete serum containing media was added to each plate to inactivate trypsin. Cell suspension was transferred into 15 ml falcon tubes and centrifuged at 1500-1800 rpm for 5 minutes at room temperature. The supernatant was removed and cells resuspended in 2-5 ml of fresh media and transferred to new 100 mm plates containing pre-warmed (37°C) media. Cells from a single 100 mm confluent plate were transferred into four 100 mm dishes, while cells from one 60 mm confluent plate were seeded onto a single 100 mm plate. Culture plates were kept for incubation in a humidified CO₂ incubator at 37°C. Cells were examined microscopically after 24 hours. Media of plates was changed alternately till desired confluency was achieved.

IV.3. Antibodies and Proteins

IV.3.1. Protein factors: IL1β was purchased from Life technologies, USA.

IV.3.2. Cell cycle-related antibodies: Anti-cyclin-dependent kinase (Cdk-2, 6) antibodies; anti-CDC25C, Cyclin D1 and p53 antibodies were purchased from Santa Cruz, USA. Anti-Cyclin B1, Cdk1, Anti-p21 & -p27 antibodies were purchased from Cell Signaling Technology, USA.

IV.3.3. Survival and cell death related antibodies: Anti-Akt, Bel-2, Bax, Total PARP antibodies were purchased from BD Biosciences, USA. Anti-phospho-/total antibodies against Erk1/2, (phospho-Tyr 202 & 204/total); anti-Survivin, PCNA, LC3A/B and Beclin-1 antibodies were purchased from Cell Signaling Technology, USA.

IV.3.4. Angiogenesis and metastasis related antibodies: Anti-VEGF antibody was procured from Abcam, USA. Anti-MMP-2 & -9, anti-uPA, anti-vimentin, anti-Snail & Slug antibodies were procured from Cell Signaling Technology, USA. Anti-E-
catherin, N-catherin and β-catenin antibodies were purchased from BD Biosciences, USA. Anti-Hif1α antibody was purchased from Pierce, USA.

**IV.3.5. Mitochondrial dynamics related antibodies:** Anti-DRP1 (phospho-Ser 616 & 637/total) and MFN2 antibodies were purchased from Cell Signaling Technology, USA. MFN1 antibody was purchased from Santa Cruz, USA.

Mouse anti-β-actin primary antibody was procured from Sigma Aldrich USA or Cell Signaling Technology, USA. Anti-rabbit and anti-mouse HRP-linked secondary antibodies were procured from Cell Signaling Technology, USA and Sigma Aldrich, USA respectively.

**IV.4. Short Chain Fatty Acids (SCFA)**

SCFA, Butyric acid and Propionic acid (sodium salt) were purchased from Sigma Aldrich, USA.

**IV.4.1. Stocks and doses:**

**Butyric acid:** Concentrations of 0, 1, 2.5 and 5 mM were used for HUVEC assays as well as CRC cell lines.

**Propionic acid:** Concentrations of 0, 2.5, 5 and 10 mM were used for the studies in CRC cell lines.

Both SCFA were dissolved in autoclaved distilled water. Stock solutions of both of the SCFA were prepared fresh.

**IV.4.2. SCFA treatments:** Cells were seeded and grown on tissue culture plates till desired confluencies were achieved. For cell growth, death assays and flow cytometry analyses, 1 x10^5 cells were seeded for 24 h and treated under specific conditions. For migration, invasion, adhesion, semiquantitative and immunoblot/IP assays, cells were seeded and treated with agents at 70% confluency. In clonogenic assays, 500 cells were seeded and treatment started next day. Cells were treated with the specific doses of agents under specific growth conditions for specific time periods. For other assays, cells were seeded as described in respective procedures in “Methods”. At the end of the treatment times, specific assays were performed for specific purposes to accomplish the proposed objectives.
IV.5. Cell Growth and Death Assay

For cell growth and death assays, two types of methods were employed:

IV.5.1. Trypan blue method

Cells at a density of $2 \times 10^4$ or $1 \times 10^5$ were seeded per 35 or 60 mm plates in specific culture media. Next day, cells were treated with indicated concentrations of SCFA for specific time periods. After incubation periods were over, cells were harvested by brief trypsinization and centrifugation at 1500-1800 rpm for 5 minutes. Supernatants were decanted and cell pellets gently washed once with ice cold 1x PBS and after centrifugation (as above) were resuspended in 1 ml ice cold 1x PBS. Ninety microliter of this cell suspension of each treatment was aliquoted into 1.5 ml conical tubes on ice and mixed with 10 µl of trypan blue dye (0.5 % solution in 1x Dulbeccos phosphate buffered saline) for incubation at room temperature (RT) for 2-3 minutes. Tubes were then placed on ice and cell counting was done using hemocytometer (counting chamber). 10 µl of trypan blue-stained cell suspension was put in counting chamber and total and dead cells were counted. Live cells were bright while dead as blue. Counting was done in duplicate for the same sample and each treatment was in triplicate. All the counting experiments were repeated 3-4 times.

IV.5.2. MTT method

Eight thousand cells were seeded per well of 96-well culture plate and grown for 24 h in specific culture media. Next day, cells were treated with specific SCFA concentrations for specific time periods. After specific time periods were over, media was aspirated and cells in each well washed once with 1x PBS gently. Then 100 µl of 5mg/ml MTT solution prepared in 1x PBS was added to each well and incubated for 4 h at $37^{\circ}$C in CO$_2$ incubator. After incubation time was over, plates were removed from incubator and MTT solution was aspirated out. Cells were gently washed with 1x PBS once and incubated at RT in 100 µl DMSO for 5 minutes to dissolve the formazan crystals. Purple color was observed after formazan crystals got dissolved in DMSO. Wells were read at 570 nm after brief pre-mixing using Synergy H1 BioTek multimode plate reader.

IV.6. Flow Cytometry Analysis for Cell Cycle Distribution (FACS)
Cells were similarly seeded and treated as in trypan blue viability assay mentioned above. At the end of each treatment, total cells were collected by brief trypsinization and centrifugation at 1500 rpm followed by processing for cell cycle analysis as reported recently (Nambiar et al., 2013). Briefly, after 2 washings with 1x PBS, total cells were collected by centrifugation as above and resuspended in 0.5 ml of saponin/PI solution (0.3% saponin (w/v), 25 mg/ml PI (w/v), 0.1 mM EDTA and 10 mg/ml RNase (w/v) in PBS), and incubated overnight at 4°C in dark. Cell cycle distribution was then analyzed by flow cytometry using BD FACS aria III flow cytometer provided by BD Biosciences, USA. Finally, percentage of cells in different phases of cell cycle was determined by ModFit LT cell cycle analysis software.

IV.7. Apoptosis Assay
To quantify apoptosis, cells were seeded and treated similarly as for the trypan blue assay above. Cells were harvested and processed for apoptosis assay using Annexin V-FITC Apoptosis Detection Kit from BD Pharmingen, USA following step-by-step protocol prescribed by the manufacturer and analyzed by flow cytometry. Briefly, at the end of treatment, both floating and attached cells were collected, washed twice with 1x PBS, and subjected to annexin V (conjugated with FITC) and PI staining. Flow cytometry was performed within 30 min with a 488-nm line of an argon-ion laser for excitation. Cells at different stages of death from early apoptosis to necrosis were scored, and data collected using the BD FACS aria III flow cytometer from BD Biosciences, USA.

IV.8. Clonogenic Assay
Materials to be prepared:
1. 1x PBS (pH 7.4)
2. 6-well-plate
3. Crystal violet (5 mg/ml in 2% ethanol and stored at RT)
4. Fixing solution (95:5 ethanol-acetic acid, ice cold)
5. Phase contrast microscope fitted with camera

Procedure:
Clonogenic assay was performed to check the effect of phytochemicals on clonal expansion potential of CRC cells on tissue culture plates in two dimensions. To carry out this assay, 300 cells were seeded and grown for 24 h in complete RPMI-1640
media onto 6-well culture plates in CO₂ incubator at 37°C. Cells were treated with the specific concentrations of SCFA for 5-7 days in complete media after which plates were removed from the CO₂ incubator and media aspirated-out. Plates were gently washed three times with 1x PBS to remove unattached cells. Cells were fixed with 2 ml/well of fixing solution (95:5 ethanol-acetic acid) for 10 min at RT Wells were washed three times with dd water and stained with crystal violet solution for 15 minutes at RT. Plates were profusely destained with tap water till background was clear and air-dried overnight. Colonies were counted under 200 x magnification employing phase contrast microscope by Carl Zeiss, Germany. Colonies of sizes 1-10, 11-20, 21-40, 41-50 and >50 were counted. Entire plate-surface area was scored for colonies. Treatments were done in duplicate and each experiment repeated at least 2 times with similar results.

IV.9. Whole Cell Lysate Preparation

Materials required:
1. 100 mm culture plates
2. Cell scrapers
3. 1x PBS (pH 7.4)
4. 1x Cell lysis buffer
5. Protease and phosphatase inhibitor cocktails (Roche, USA)
6. Refrigerated high speed centrifuge, -80°C deep freezer, water bath and vortex.

Composition of cell lysis buffer (Non-denaturing):
- 10 mM Tris (pH 7.0)
- 150 mM NaCl
- 1% Triton X-100
- 1 mM EDTA
- 1 mM EGTA
- 0.2 mM Sodium orthovanadate
- 0.5% NP-40
- 1x Complete protease inhibitor cocktail (stock 25x)
- 1x Phosphostop phosphatase inhibitor (stock 10x)
Procedure: (Note: All the steps were done on ice)

1. At end of the treatment time, aspirated out media from the culture dishes.
2. Washed cell monolayer gently with chilled 1x PBS, 2-3 times (used 6-10 ml of 1x PBS for each 100 mm dish). After putting PBS into the plates, swirl it slowly and then suck it out with vacuum gently.
3. Tilted dishes for 1 min and aspirated-out remaining PBS (to avoid sample dilution).
4. Added 200 µl of cold cell lysis buffer in each 100 mm dish (by spreading the buffer in the maximum area of dish by swirling gently).
5. Kept dishes for 20-30 min. on ice.
6. Scraped the cells with cell scrapper and collected contents in a sterile microfuge tubes, and kept these tubes on ice for additional for 20-30 min. Then stored at -20°C or -80°C freezer.
7. Freeze-thawed samples by doing four cycles of [-80°C = 37°C (water bath) = brief vortex] freeze-thaw procedure.
8. Spun samples at 14,000 rpm for 40 min at 4°C.
9. Collected supernatant which is whole cell lysate and discarded the pellets.
10. Protein estimation was done by Bradford assay and equal amounts of lysate was taken for assays like immuno-precipitation or western blotting. Protein estimation by Bradford method is given in Appendix section.

IV.10. Immunoblot Analysis

Materials required

1. Cell lysate
2. SDS PAGE buffers (stacking and running)
3. Gel running and transfer assemblies
4. Transfer, blocking and wash buffers (compositions given in the Appendix)
5. Antibodies (primary and HRP-linked secondary)
6. Nitrocellulose (NC)/ Polyvinylidene fluoride (PVDF) membrane
7. Blot developing and fixing solutions
8. ECL solution from Millipore, USA
9. Kodak developer, fixer, x-ray film and cassette
10. Stripping buffer (composition given in the Appendix)

**Procedure:**

1. After treatment with agents for specific time periods under desired conditions, whole cell lysates were prepared as mentioned above.
2. Whole cell lysates (60-80 µg) were denatured in SDS-PAGE sample buffer by mixing equal volumes of lysate and 2x sample buffer followed by brief vortexing and heating on boiling water bath for 5 min.
3. Samples were loaded onto 8, 12 or 15% denaturing SDS PAGE gels and proteins resolved at constant voltage of 80-100 volts till bromophenol blue dye-front leaked out from the gel.
4. Gels were removed from the assembly and kept in chilled 1x transfer buffer along with the NC membrane for equilibration for 10 minutes.
5. Proteins from the gel were transferred to NC membrane using transfer assembly at a constant voltage of 100 volts for 1.5 to 2 h depending on the molecular weight of protein of interest. Orientation of the assembly was kept as “blotting papers=negative electrode-SDS Gel=NC membrane-positive electrode=blotting papers” as proteins migrate towards positive electrode in SDS PAGE.
6. After transfer was complete, assembly was disassembled and NC membrane was washed once in 1x wash buffer (pH 7.4).
7. Membranes (blots) were blocked in blocking buffer for 1 h at RT or overnight at 4°C.
8. Specific primary antibody incubation with high speed shaking at RT for 1 h was followed by overnight incubation at 4°C.
9. Next day, high speed shaking was repeated for 1 h and was followed by three times washing in 1x wash buffer.
10. Blots were subsequently incubated with corresponding HRP-linked secondary antibody for 1-1.5 h at high speed shaking and followed by three time washing in 1x wash buffer and processed for ECL detection.
11. ECL was poured and layered on blot, and incubated at RT for 1-5 min.
12. Blots were dried on paper towels from one end, fixed on x-ray cassettes in transparent pouches and signals caught on x-ray film using Kodak developer and fixer in dark.

13. Different exposures were taken to get the optimum band intensity on x-ray film.

14. X-ray films were air dried and marked for molecular weight protein marker. Bands on films were scanned using high resolution scanner and films pasted on data book and stored for future reference.

IV.11. RNA Isolation from Mammalian Cells

**Materials required:**

1. 100 mm culture plates
2. Cell scrapers
3. 1x PBS (pH 7.4)
4. Trizol reagent
5. Autoclaved DEPC-treated water
6. Chloroform and Isoamyl alcohol
7. Ethanol (70%)
8. Refrigerated high speed centrifuge and UV-spectrophotometer.

**Procedure:**

1. Seeded cells onto culture plates and grown till treated at 70% confluency in specific media for specific time periods in presence or absence of agents.

2. At the end of the treatment times, cell monolayers were washed with ice cold 1x PBS. Cells lysed directly in tissue culture plates by adding 1 ml of Trizol reagent/100 mm culture dish (0.5 ml/60 mm dish) and scraping with cell scraper. Passed cell lysate several times (4-6 times) through pipette and transferred contents into 2 ml tubes. Kept tubes on ice for 2 min.

3. Added 0.2 ml chloroform/ml of Trizol used to each tube. Capped tubes and shook vigorously for 15 seconds followed by incubation at RT for 2-3 min.
4. Centrifuged samples at 10,000 rpm for 15 min at 4°C and transferred upper aqueous phase carefully without disturbing the interphase into fresh 1.5 ml tubes.
5. Precipitated RNA in aqueous phase by mixing with 0.8 volumes of isopropyl alcohol/ml of Trizol reagent used. Left at RT for 5 min.
6. Centrifuged samples at 15,000 rpm for 15-20 min at 4°C.
7. Removed all the supernatant carefully without disturbing the RNA pellet.
8. Washed pellet with 1 ml 75% ethanol (to remove organic and inorganic impurities) by resuspending it and then gently shaking tubes 2-3 times.
9. Repeated steps 6-8 and centrifuged samples as above at 4°C.
10. Removed the supernatant (75% ethanol) carefully.
11. Semi-dried the RNA pellet in air for 3-5 min at RT.
12. Dissolved RNA pellet in 20 µl DEPC-treated water by passing solution through tip several times. Incubated samples at 56°C water bath for 5 min for faster dissolution.
13. Estimated RNA concentration using UV-spectrophotometer at A260 nm wavelength and labeled tubes properly and stored at -20°C. RNA estimation method is given in Appendix.

IV.12. Reverse Transcriptase PCR (RT-PCR)

RT Primers utilized to amplify specific gene sequences:
1. Oligo(dT) primer
2. Survivin
   Forward Primer: 5’-GCCCAGTGTGTTTCTTCTGCTT-3’
   Reverse Primer: 5’-GACAGAAAGGAAAGCGCAAC-3’
3. Bcl-2
   Forward Primer: 5’-AAGCGGTCCCGTGGATAG-3’
   Reverse Primer: 5’-TCCGGTATTCGCAGAAGTCC-3’
4. GAPDH
   Forward primer: 5’-GCCTTCCGTCTCCCCACTGC-3’
   Reverse primer: 5’-CAATGCCAGCCCCAGCGTCA-3’

Step 1: 1st strand cDNA synthesis
The following 20 µl reaction volume was used for 5 µg of total RNA isolated.

1. Denatured RNA before use at 65°C for 10 min and then immediately kept on ice for 5 min.
2. Added following components to a nuclease-free microcentrifuge tube:
   - 1 µl of Oligo(dt)18 bp Primer (0.5µg/ml)
   - 5 µg of total RNA
   - 1 µl of 10 mM dNTP mix (dATP, dGTP, dCTP & dTTP)
   - Added sterile DEPC-treated H₂O to make up the final volume to 14.0 µl
3. Heated the mixture to 65°C for 5 min and incubated samples on ice for at least 1 min.
4. Collected contents of the tubes by brief centrifugation at 10,000 rpm for 5 seconds and added the following components in order:
   - 4 µl of 5x First-Strand Buffer
   - 1 µl of RNaseOUT (Recombinant RNase Inhibitor) 40 units/µl
   - 1 µl of MuLV Reverse transcriptase (200 units/µl)
   **Total reaction volume = 20 µl**
5. Mixed contents by gently pipetting up and down and collected by brief centrifugation at 10,000 rpm for 5 seconds.
6. Incubated tubes at 42°C for 90 min.
7. After reaction was over, it was inactivated by heating at 70°C for 15 min.

**Step 2: Regular PCR reaction cycles**

**Preparation of primers:** Specific primers provided as lyophilized powder were dissolved in T₁₀E₁ so that the final concentration of master stock becomes 1nmol/µl. Working primer stocks were made by diluting the master stock 100 times to get working stock of 10 pmol/µl.

The following 25 µl reaction was set to carry out 20-25 cycles of regular PCR reaction in Eppendorf Thermocycler. Components were added in order after indicated volume of autoclaved double distilled water was aliquoted initially into the 0.5 ml tube.

1. Template (cDNA) 3.0 µl
2. Forward primer (working stock) 1.0 µl
3. Reverse primer (working stock) 1.0 µl
4. dNTP mix 10 mM (dATP, dGTP, dCTP & dTTP) 1.25 µl
5. 10x PCR buffer containing Mg++ 2.5 µl
6. Taq DNA polymerase 0.5 µl
7. Autoclaved ddH\textsubscript{2}O 15.75 µl

**Total reaction volume** 25.0 µl

**PCR set up:**

**Step 1:** Initial Denaturation 95\degree C 3.0 min

**Step 2:** (20-25 cycles) Annealing temperature was adjusted +/- depending on primers

- Denaturation 94\degree C 30 seconds
- Annealing 55\degree C 30 seconds
- Elongation/extension 72\degree C 1.30 min

**Step 3:** Final Elongation 72\degree C 5-7 min

Stored at 4\degree C

PCR products were analyzed on 1% agarose gel electrophoresis and visualized under GelDoc system (Applied Biosystems).

**IV.13. Determination of Mitochondrial Mass**

Cells (1 \times 10^5) were seeded in 60-mm dishes, allowed to attach by overnight incubation, and then treated with desired concentrations of BA for 12 and 24 h. After completion of the treatment time periods, cells were exposed to 200 nM MitoTracker Red CMXRos for 60 min. Thereafter, cells were collected by brief trypsinization and washed with PBS. Pelleted cells were resuspended in 0.5 mL PBS and mitochondrial degradation was determined by FACS Aria III (Tal et al., 2009).

**IV.14. Mitochondrial Staining**

Cells were plated on coverslips and allowed to attach by overnight incubation. Cells were then treated with BA (2.5 mM) followed by incubation with 100 nM MitoTracker Red CMXRos for 30 min at 37\degree C. After washing with PBS, cells were fixed with 2% paraformaldehyde for 1 h at room temperature and examined under an Olympus fluorescence microscope at 100\times objective magnifications.
IV.15. Mitochondrial Reactive Oxygen Species (ROS) Determination

IV.15.1. Flow cytometric analysis
ROS production was measured by flow cytometry following staining of cells with MitoSOX Red. Cells were treated with desired concentration of BA (2.5 mM) for 24 h and then incubated with 5 µM MitoSOX Red for 30 min. Cells were collected, washed with PBS and fluorescence was detected using FACS Aria III (Hahm et al., 2011; Mukhopadhyay et al., 2007).

IV.15.2. Immunofluorescence microscopy
Cells were plated on coverslips and allowed to attach by overnight incubation. Cells were then treated with BA (2.5 mM) followed by incubation with 2.5 µM MitoSOX Red for 30 min at 37°C. After washing with PBS, cells were fixed with 2% paraformaldehyde for 1 h at room temperature and examined under an Olympus fluorescence microscope at 100× objective magnifications (Hahm et al., 2011).

IV.16. Sub-cellular Fractionation

Materials required
1. Isotonic mitochondrial buffer
2. Dounce homogenizer

Procedure
Mitochondrial and cytosolic fractionation was carried out using protocol described earlier (Jan et al., 2004).
1. SW480 cells were collected after treatment with BA (2.5 mM) for 12 and 24 h using cell lifter.
2. Cells were washed with PBS and resuspended in 1 mL isotonic mitochondrial buffer (250 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES [pH 7.5]).
3. Suspension was homogenized using Dounce homogenizer with 50 strokes.
4. Nuclei and unbroken cells were removed by centrifugation at 500 × g for 5 min, this was followed by centrifugation at 10,000 ×g for 30 min at 4°C to collect mitochondrial pellet.
5. Supernatant was collected as cytosolic fraction.
6. The pellet was resuspended in 50 µL isotonic mitochondrial buffer.
7. These fractions were resolved on SDS-PAGE followed by immunoblotting.

IV.17. Construction of HCT116-DRP1 Overexpression Cell Line

IV.17.1. Vector Preparation

Materials required
1. pLVX-Puro Vector (Clontech, Cat no.: 632164)

2. EcoR1 Restriction enzyme (Fermentas)
3. BamH1 Restriction enzyme (Fermentas)
4. cDNA
5. Hi-fidelity polymerase enzyme (Roche Life Science)
6. DRP1 full length primers
7. T4 DNA Ligase enzyme (Fermentas)
8. Plasmid isolation kit (Fermentas)
9. Gel extraction kit (Fermentas)
10. DH5α E.coli strain
11. Ampicillin (100 µg/ml)
12. Luria Agar medium
13. Dry bath
14. Circulating water bath
15. Agarose gel electrophoresis
16. UV illuminator

**Primers**

**DRP1 Forward primer:** GAATTCATGGAGGCGCTAATTCTGT (with EcoR1)

**DRP1 Reverse primer:** GGATCCCTCACAAAGATGAGTCTCCCG (with BamH1)

**Procedure**

1. Total RNA was isolated from HCT116 cells and converted to cDNA as described above.
2. Full length DRP1 cDNA containing EcoR1 and BamH1 restriction sites was amplified by PCR using DRP1 full length primers and Hi-fidelity polymerase enzyme.
3. PCR product was resolved by agarose gel electrophoresis.
4. Desired product was cut from gel and eluted from gel using gel extraction kit as per the manufacturer’s protocol.
5. DRP1 Full length cDNA and pLVX-Puro empty vector were digested using EcoR1 and BamH1 as per the manufacturer’s instructions. Digested products were resolved on agarose gel and eluted from gel.
6. Both elutes mixed (1:3, vector: insert) and ligated using T4 DNA ligase enzyme as per the manufacturer’s instructions.
7. After 16 h of ligation incubation at 16°C, ligated vector was transformed in to *E.coli* DH5α strain using standard transformation protocol. Transformed cells were plated on Luria Agar (LA) medium containing 100 µg/ml Ampicillin and incubated for 12-16 h at 37°C.
8. Positive colonies were screened by isolating plasmids (from LB colony inoculum) followed by double restriction digestion.
9. Digested products were resolved by agarose gel electrophoresis. Plasmids which show desired fallout (2112-2262 bp) were selected and further confirmed by sequencing for DRP1 cDNA.
10. Clone containing desired plasmid was cultured in LB broth and stored in -80°C freezer in the form of glycerol stock.

IV.17.2. Lentivirus particle production

Materials required

1. Lenti-X 293T cell line (Clontech)
2. DMEM/F-12 Medium (Gibco)
3. Lenti-X HTX Packaging Mix (Clontech)
4. Xfect Polymer (Clontech)
5. Lenti-X GoStix (Clontech)

Procedure

Lentivirus production was carried out by following the manufacturer’s protocol.

1. Approximately 24 h before transfection, 5 x 10^6 Lenti-X 293T cells/100 mm plate were seeded in 10 ml of growth medium. Make sure that the cells are plated evenly. Incubated at 37°C, 5% CO₂ overnight. The cells were 80–90% confluent at the time of transfection.

2. Thoroughly vortexed Xfect Polymer.

3. For each transfection sample, prepared two microcentrifuge tubes by adding reagents in the order listed below:

<table>
<thead>
<tr>
<th>Tube 1 (Plasmid DNA)</th>
<th>Tube 2 (Polymer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>557 µl Xfect Reaction Buffer</td>
<td>592.5 µl Xfect Reaction Buffer</td>
</tr>
<tr>
<td>36 µl Lenti-X HTX Packaging Mix</td>
<td>7.5 µl Xfect Polymer</td>
</tr>
<tr>
<td>7 µl Lenti-X Vector DNA (1 µg/µl)</td>
<td>600 µl Total Volume</td>
</tr>
<tr>
<td>600 µl Total Volume</td>
<td></td>
</tr>
</tbody>
</table>

4. Vortexed each tube well to mix.

5. Added the Polymer solution (Tube 2) to the DNA solution (Tube 1) and vortexed well at a medium speed for 10 sec.

6. Incubated each DNA-Xfect mixture for 10 min at RT to allow nanoparticle complexes to form.

7. Added the entire 1200 µl of DNA-Xfect solution from Step 5 drop wise to cultured cells from Step 1. Rocked the plate gently back and forth to mix.

8. Incubated the plate at 37°C.
9. After 4 h to overnight, replaced the transfection medium with 10 ml fresh complete growth medium (containing Tet System Approved FBS) and incubated at 37°C for an additional 24–48 h. Viral titers were generally highest at 48 h after the start of transfection.
10. Harvested the lentiviral supernatants and pooled similar stocks, when desired.
11. Verified virus production with Lenti-X GoStix™ then used the virus to transduce target cells, or aliquot and store at –80°C.

IV.17.3. Transducing Target Cells with Lenti-X Viruses

Materials required
1. HCT116 cell line
2. 6-well plate
3. Polybrene (Sigma)
4. Puromycin (Clontech)

Procedure
Transducing target cells with Lenti-X Viruses was carried out by following the manufacturer’s protocol.
1. Plated target cells in their complete growth medium, 12–18 h before transduction.
2. Thawed aliquots of your cleared and titrated lentiviral stock, or used cleared virus stock freshly prepared from packaging cells (Section VI). Mixed gently, avoided vortexing. Note that each freeze-thaw cycle will decrease titer by ~2–4-fold.
3. Adjusted the volume of medium in the target cell cultures to accommodate the addition of virus and polybrene. Used sufficient polybrene to obtain the desired final concentration during the transduction step (e.g. 4 µg/ml).
4. Diluted the lentiviral stock with medium to obtain the desired MOI. For unknown titer values, used serial dilutions of the virus stock or supernatant such that the total volume of virus represented no more than 1/3 the final volume of culture medium used for transduction.
5. Added viral supernatant to the cells and transduced for 8–24 h. Centrifuged the cultures to improve infection efficiency. Care was taken to adjust the time of transduction as the exposure to either the polybrene or to the viral
supernatant (which contains medium conditioned by the packaging cells) could adversely affect the target cells.

6. Removed and discarded the virus-containing transduction medium and replaced it with fresh growth medium.

7. Continued to incubate the cells for 24–48 h to allowed gene product to accumulate in the target cells.

8. Harvested the cells for analysis or proceeded with selection using the Puromycin antibiotic.

IV.18. Co-Immunoprecipitation (Co-IP)

Materials required

1. Cell lysate
2. Protein A/G PLUS agarose beads
3. Antibodies (primary and HRP-linked secondary)
4. Nutator (Rocker)
5. SDS PAGE buffers (stacking and running)
6. Gel running and transfer assemblies
7. Transfer, blocking and wash buffers
8. Nitrocellulose membrane
9. Blot developing and fixing solutions
10. ECL solution
11. Developer, fixer, x-ray film and cassette

Procedure:

Co-IP is a technique to study protein-protein interactions. In present situation, it was performed to assess the effect of phytochemicals on interaction of cell cycle regulatory proteins. To execute this assay, cells were grown to 70% confluency onto 100 mm tissue culture plates in complete RPMI-1640 media and treated with specific concentrations of drugs for the desired time periods in a humidified CO₂ incubator at 37°C temperature. After treatment times were over, whole cell lysates were prepared as mentioned above and following steps were followed to accomplish Co-IP.

1. Whole cell lysates (200 to 500 µg) were taken and kept on ice.
2. Volume was made up to 0.6-1.0 ml with cell lysis buffer.
3. Protein A/G PLUS agarose beads (15 µl) were added to the above protein solution (vial was shaken gently to disperse beads).
4. Samples were pre-cleared by gently rotating on nutator at 4°C for 1 h to remove non-specific binding to beads and were centrifuged at 4000 rpm for 5 min at 4°C.

5. Supernatant was collected into new 1.5 ml conical tubes and beads were discarded.

6. Appropriate primary antibody was added (as recommended by manufacturer) to each sample and gently rotated at 4°C for 4-6 h using nutator.

7. Protein A/G PLUS agarose beads (20 µl) were added to each sample and rotated at 4°C overnight using nutator.

8. Samples were centrifuged as before and supernatants were taken out gently without disturbing the bead pellet to which immuno-complexes are attached.

9. Beads were washed by adding 800 µl lysis buffer with gentle shaking.

10. Samples were centrifuged at 4000 rpm for 5 min at 4°C.

11. Repeated steps 9-10 twice.

12. Supernatants were taken out and discarded carefully to retain the beads.

13. 1x Gel loading buffer was added to each bead sample and vortexed for 5 seconds, heated on boiling water-bath for 7 min. and centrifuged at 14,000 rpm for 2-5 min.

14. Samples were loaded (without disturbing the bead-pellets) and run on 12% or 15% SDS PAGE. Gels were run at a constant voltage of 100 volts till tracking dye leaked-out. While loading samples, care was taken so as not to load any bead.

15. Immunoblot analyses using specific primary and HRP-linked secondary antibodies were performed as described above.

IV.19. DCFDA Assay for Detection of ROS Generation and Scavenging

Materials required

1. Cell suspension at 10,000 cells/200 µl density in complete media/well
2. 96-well plate
3. 2’,7’-Dichlorofluorescin diacetate (DCFDA) (Sigma)
4. N-Acetyl-L-cysteine (NAC) (Sigma)
5. Multi-mode plate reader (Fluorometric)

**Procedure**

This assay was also performed in 96-well culture plate.

1. 10,000 cells were seeded per well and allowed to grow for 24 h in complete culture medium. Next day, when the cells confluence was 80% the media was removed.

2. 100 µl of 20 µM DCFDA in complete media was added to each well and incubate the cells for 45 min. at 37°C & 5% CO₂ incubator.

3. After 45 min incubation over, DCFDA solution was aspirated from each well and 100 µl of desired concentration of BA in complete media was added to each well.

4. Similarly, for ROS scavenging 100 µl of NAC (5 mM) was dispensed in a different set of wells and incubated for 2 h at 37°C in CO₂ incubator.

5. Now, in this new set of wells 100 µl of 20 µM DCFDA in KRB solution was added and incubated for 45 min in the incubator.

6. After 45 min incubation is over, the DCFDA solution was aspirated from each well and 100 µl of desired concentration of drug with NAC (5 mM) in complete media was added to this set of wells.

The fluorescence at excitation 485 nm and emission at 538 nm through multimode ELISA plate reader was noted for each hour for the 1st 6 h and then after 6 h intervals till 24 h.

**IV.20. Acidic Vesicular Organelles (AVO) Staining**

Transduced HCT116 cells (empty vector & DRP1) ($1 \times 10^5$) were seeded on 6-well plate, allowed to attach by overnight incubation, and then exposed to BA for 6 or 12 h at 37°C. Subsequently, cells were stained with 1 µg/mL acridine orange for 15 minutes, washed with PBS, and cells were collected, again washed with PBS and fluorescence was detected using FACS Aria III (Prajapati et al., 2015).

**IV.21. CRC cell migration assay using Boyden chamber**

**Materials to be prepared:**

1. Cell suspension at the density of 30,000 cells/500 µl serum free media
2. 24-well migration transwells (Boyden chambers with 8 micron pore size)
3. Complete and serum-free media
4. Fixing solution (chilled 95:5 ethanol-acetic acid)
5. Crystal violet (5 mg/ml in 2% Ethanol)
6. Glass slides, cover slips, scalpel and DPX mountant for mounting insert membranes on glass slides for observation/cell counting
7. Phase contrast microscope fitted with camera

Procedure:

1. Cells grown in regular serum conditions and treated at 70% confluency with specific SCFA concentrations for specific time periods.
2. After treatment time was over, cells were harvested as in trypan blue assay and washed twice in serum-free media and counted using hemocytometer.
3. For the assay, 30,000 cells in 500 µl serum-free media were seeded in upper chamber of migration transwells and incubated for 16-20 h. Lower chambers were filled with 500 µl of complete media containing 10% FBS.
4. After assay time was over, media was aspirated-out and inserts swabbed with cotton swab to remove non-migrated cells.
5. Migrated cells were fixed in chilled ethanol: acetic acid solution (95:5) and stained with 0.5% crystal violet solution for 15 minutes at RT.
6. Cells were destained with ddH₂O till background was clear.
7. Inserts were kept on paper towels overnight till air dried.
8. Insert membranes were carefully removed with scalpel and mounted on glass slides using DPX mountant and cover slips. As minimum mountant was used as possible and air bubbles were avoided.
9. Slides were air-dried overnight.
10. Cells were counted at 400x magnification under phase contrast microscope (Carl Zeiss, Germany). Minimum 5 microscopic fields were counted in each slide and treatments were done in duplicate.

IV.21.1. HUVEC migration assay following procedure was followed

Conditioned media preparation
1. Transduced HCT116 cells were seeded in 60 mm dishes and treated with SCFA at 70% confluency for 12 h.
2. After 12 h of treatment time, treatment media were removed and plates were washed with autoclaved 1X PBS.
3. Cells were further incubated in 0.2% FBS containing media for 24 h.
4. After 24 h of incubation media was collected in sterile 15 ml conical tube and centrifuged at 3500 rpm for 10 min to remove floating cells.
5. Supernatant media was used as conditioned media directly or stored at -80°C for further use.
6. Ratio of conditioned media was normalized with number of live cells present on plate.

**HUVEC migration assay**

1. For HUVECs, 30,000 cells were seeded in upper chamber of Boyden inserts (with 8 micron pore size) in serum-free medium.
2. Cells were allowed to migrate for 16 h towards lower chamber filled with 50% conditioned medium.
3. After migration time was over, inserts were gently swabbed with cotton-swab to remove non-migrated cells.
4. Migrated cells on the bottom side of membrane were fixed in methanol, stained with crystal violet solution and de-stained with ddH2O.
5. Cell counting was performed under the phase-contrast microscope at 200x magnification scoring 5 microscopic fields on each membrane.
6. Photographs were taken by inverted microscope equipped with digital camera by Carl Zeiss, Germany.

**IV.22. CRC cell invasion assay using matrigel-coated Boyden chamber**

**Materials to be prepared:**

1. Cell suspension at the density of 40,000 cells/500 µl serum-free media
2. 24-well invasion matrigel-coated transwells (Boyden chambers with 8 micron pore size)
3. Complete and serum-free media
4. Fixing solution (chilled 95:5 ethanol-acetic acid or methanol)
5. Crystal violet (5 mg/ml in 2% ethanol)
6. Glass slides, cover slips, scalpel and DPX mountant for mounting insert membranes on glass slides for observation/cell counting
7. Phase contrast microscope fitted with camera

**Procedure:**

1. Cells were grown in regular serum conditions and treated at 70% confluency with specific SCFA concentrations for specific time periods.
2. After treatment time was over, cells were harvested as done in trypan blue assay and washed twice in serum-free media and counted using hemocytometer.
3. For the assay, 40,000 cells in 500 µl serum-free media were seeded in upper chamber of invasion transwells and incubated for 16-20 h. Lower chambers were filled with 500 µl complete media containing 10% FBS.
4. After assay time was over, media was aspirated-out and inserts swabbed with cotton swab to remove non-invaded cells and matrigel.
5. While in wells, invaded cells were fixed in chilled ethanol: acetic acid solution (95:5) and stained with 0.5% crystal violet solution for 15 minutes at RT.
6. Cells were destained with ddH₂O till background was clear.
7. Inserts were kept on paper towels overnight till air dried.
8. Insert membranes were carefully removed with scalpel and mounted on glass slides using DPX mountant and cover slips. As minimum mountant was used as possible and air bubbles were avoided.
9. Slides were air dried overnight.
10. Cells were counted at 400x magnification under phase contrast microscope (Carl Zeiss, Germany). Minimum 5 microscopic fields were counted in each slide and treatments were done in duplicate.

**IV.23. MMP gelatin zymography for MMP-2 and -9 activities**

**Materials required:**

1. Sample Buffer (2X)

   - 0.5 M Tris-HCl, pH 6.8: 2.5 ml
   - Glycerol: 2.0 ml
10% (w/v) SDS 4.0 ml
0.1% Bromophenol Blue in DDH$_2$O (w/v) 0.5 ml
Added distilled water to make 10.0 ml

2. Renaturing buffer (10X)
Triton X-100 (to make 25% v/v) 25.0 ml
Added distilled water to make 100.0 ml

3. Developing Buffer (10X)
Tris–HCl (pH 8.0) 0.05 M
CaCl$_2$ 5 mM
ZnCl$_2$ 5 µM
Added distilled water to make 1000.0 ml

4. Coomassie R-250 staining solution: (0.5% w/v) in methanol: acetic acid: water (50: 10: 40)
5. Coomassie R-250 destaining solution: (methanol: acetic acid: water (50: 10: 40)
6. High resolution scanner

**Procedure:**
1. Cells were grown in 60 mm plates in complete media to confluence.
2. Cells were treated with the specific concentrations of SCFA in specific culture media for specific time periods.
3. After treatment time was over, conditioned medium was collected.
4. Cells were also collected for equilibrating the volume of conditioned media for equal number of live cells in each treatment.
5. 10% SDS PAGE gels were prepared according to the standard procedure with the only difference that running gels contained 0.1% (1 mg/ml) gelatin.
6. Mixed one part sample (~20 µg protein) with one part tris-glycine SDS sample buffer (2x) and incubated for 10 min at RT Do not heat.

7. Loaded samples and run the gel with 1x tris-glycine SDS running buffer according to the standard running conditions (~125V, constant voltage for 1 h). Gel running was stopped once bromophenol blue tracking dye reached the bottom of the gel.

8. To remove SDS from the gel, zymogram renaturing buffer (10x) was diluted 1:9 with the deionized water and SDS PAGE gel was incubated in 1x renaturing buffer (100 ml for one or two mini-gels) with gentle agitation shaking for 30 min at RT.

9. Step 8 was repeated one more time to completely remove SDS.

10. Decanted zymogram renaturing buffer and replaced with 1x zymogram developing buffer (100 ml for one or two mini-gels). Equilibrated the gel for 30 min at RT with gentle agitation then replaced with fresh 1x zymogram developing buffer and incubated at 37°C overnight for maximum sensitivity.

11. Gels were stained with coomassie blue R-250 for 30-60 min. For maximum contrast, stain concentration of 0.5% (w/v) was used instead of the usual concentration of 0.1%.

12. Gels were destained with destaining solution. Areas of protease activity appeared as clear bands against a dark blue background, where the protease has digested the substrate (in this case gelatin is degraded by MMP-2/-9).

13. Gels were scanned with high resolution scanner from Amersham Biosciences, USA.

14. The densitometric analysis of zymogram bands was done using NIH ImageJ software intensity measurement tool.

**IV.24. Cycloheximide (CHX) Chase Assay**

Transduced HCT116 cells (empty vector & DRP1) were seeded into a 6-well plate at a density of 1–2.5 × 10^5 cells per well. After overnight culture, the cells were treated with 50 µg/ml CHX and BA. Total proteins were collected at different time points and subjected to immunoblotting for SNAIL and β-actin (Liu et al., 2009).
IV.25. Colonosphere Formation Assay

Materials required

1. Ultra-low attachment 6-well plates (Corning)
2. DMEM/F-12 media (Gibco)
3. 1% penicillin/streptomycin solution (Gibco)
4. B27 (Gibco)
5. Insulin (Sigma)
6. Epidermal growth factor (Sigma)
7. Basic fibroblast growth factor (Gibco)
8. 2-mercaptoethanol (Sigma)

Procedure

Colonosphere formation assay was performed as described by Kim et al., 2013.

1. 1 x 10^5 Transduced HCT116 cells (empty vector & DRP1) were plated in 60 mm dishes in triplicate. Cells were allowed to attached and then treated with BA for 12 h. Cells were trypsinized and single cell suspension was prepared by passing it through a 40 µm strainer.

2. Single cells were plated in ultra-low attachment plates at a density 500–5,000 cells/well in serum-free DMEM/F-12 media supplemented with 1% penicillin/streptomycin, B27 (1:50), 5 µg/mL insulin, 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor and 2-mercaptoethanol.

3. After 5 days, the primary colonosphere were harvested, dissociated with trypsin, and then passed through a 40 µm strainer. Single cells were then re-plated in ultra-low attachment plates for second (5 days) and third generation (7 days) colonosphere formation assays without further treatment with BA. The colonosphere were counted under an inverted microscope.

IV.26. Analysis of CD44^high/CD133^high Population of Cells

Transduced HCT116 cells (empty vector & DRP1) were trypsinized, washed with phosphate-buffered saline (PBS) twice, and stained with 20 µL of anti-CD133 (PE-conjugated, Miltenyi Biotec) and anti-CD44 (FITC-conjugated, BD Biosciences) antibodies. Cells were incubated in dark for 30 min at room temperature followed by washing with PBS. The cells were analyzed using FACS Aria III (Kim et al., 2013).
IV.27. \textit{In vitro} angiogenesis assay (Tube formation assay)

\textbf{Materials to be prepared:}

1. 48/24-well matrigel-coated tissue culture plates
2. Conditioned media from transduced HCT116 cells.
3. Complete and serum-free HIENDOXL\textsuperscript{TM} endothelial cell expansion medium
4. Phase contrast microscope fitted with a camera

\textbf{Procedure:}

\textit{Conditioned media preparation}

1. Conditioned media from transduced HCT116 cells were prepared as described for HUVEC cell migration assay.
2. Ratio of conditioned media was normalized with number of live cells present on plate.

\textit{Tube formation}

3. HUVEC (4 x10^4 cells/well) were simultaneously seeded with the different concentrations of the agent in 48/24-well matrigel-coated culture plates in HIENDOXL\textsuperscript{TM} endothelial cell expansion medium containing 5\% FBS and other growth factors and supplements.
4. In ligand-induced tube formation experiments, 20,000 HUVECs were seeded onto each well of 12-well matrigel-coated plates. After overnight starvation, treatment with the agent and VEGF (1 nM) was done for 16 h.
5. Tube formation was observed and quantified periodically over a specific time period under the phase contrast microscope.
6. Tubular network was photographed at 100x magnification using inverted microscope equipped with Digital camera.
7. Number of tubes was scored by counting the number of polygons in one microscopic field made by at least three independent cells.
8. Three independent areas per well were scored for counting the number of tubes.

IV.28. Densitometric Analysis of PCR and Western Blot Bands
To quantify PCR products or western blot bands, relative intensities were measured wherever necessary by employing ImageJ analysis software tool of NIH, USA after following step by step procedure as per their guidelines. All band intensities were compared with control band intensity which was taken as 1 for all the experiments. Relative band intensities were plotted in bar diagram for depiction or written above respective bands to show fold changes.

**IV.29. Statistical Analysis**

The data were statistically analyzed using the GraphPad Prism 5 software. Student's *t*-test and one-way ANOVA followed by Dunnett's test or Bonferroni test or Tukey’s test were employed to assess the statistical significance of difference between control and treatment groups. A statistically significant difference was considered to be present at *P* < 0.05. All experiments were repeated 2-3 times and similar results were obtained.
Results

V.1. PA decreased proliferation and survival of human CRC cells
The effect of PA treatment on cell viability of SW480 cells was determined by trypan blue dye exclusion assay (Fig. 1A). The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not (Strober, 2001). The PA concentrations used in this study are within the physiological range in normal human GI tract. PA treatment 2.5-10 mM concentration for 12 and 24 h decreased viability of SW480 CRC cell line in a concentration- as well as time-dependent manner. This accounted for 15-60% (P<0.005-0.001) decrease in viability in SW480 (Fig. 1A). This result suggests that PA decreases the survival of CRC cells.

V.2. PA treatment caused G2-M phase cell cycle arrest in human CRC cells
Graphical representation of the cell cycle distribution without or with treatment with 2.5-10 mM PA showed that it causes G2-M (8-10%, P<0.01) arrest at 12 h in SW480 cells which is accompanied by a decrease in G0/G1 phase (Fig. 1B). We did not observe any significant effect at 6 h of treatment. In SW480 cells, the percentage of cells arrested in G2-M phase showed decline at 24 h time point with an increase in sub-G1 cell population (4-10%, P<0.01) (Fig. 1B).

V.3. BA decreased proliferation and survival of human CRC cells
The effect of BA treatment on cell viability of CRC cell lines SW480, HCT116 and Colo320DM was determined by trypan blue dye exclusion assay (Fig. 2A, 3A and 4A). The BA concentrations used in this study are within the physiological range in normal human GI tract. BA treatment at 1-5 mM concentrations for 12 and 24 h decreased viability of all the CRC cell lines in a concentration- as well as time-dependent manner. This accounted for 47-93% (P<0.005-0.001), 40-61% (P<0.005-0.001) and 57-65% (P<0.001) decrease in viability in SW480, HCT116 and Colo320DM cells, respectively (Fig. 2A, 3A and 4A). We assessed the level of survivin, an endogenous apoptosis inhibitor, following BA treatment. It decreased the expression of survivin at both mRNA level (Fig. 5A) as well as protein level in SW480 and HCT116 cells (Fig. 5B). These results suggest that BA decreases the survival of CRC cells which may involve the down-regulation of survivin.
V.4. BA treatment caused G2-M phase cell cycle arrest in human CRC cells

The arrest of deregulated cell cycle in cancer cells at one or more of its phases is an important approach to inhibit tumor growth and progression including that of CRC (Singh et al., 2002). Graphical representation of the cell cycle distribution without or with treatment with 1-5 mM BA showed that it causes G2-M arrest at 12 h in both SW480 and HCT116 cells which was accompanied by a decrease in cell population in both G0/G1 and S phases (Fig. 2B and 3B). This effect is followed by significant (32-41%, P<0.001) increase in sub-G0/G1 population at the 24 h time which is point reflective of apoptosis. We did not observe any significant effect at 6 h of treatment. In SW480 cells, the percentage of cells arrested in G2-M phase showed decline with an increase in sub-G1 cell population (Fig. 2B). Similarly, in HCT116 cells, the G2-M phase cell cycle arrest mostly disappeared at 24 h time point and was taken over by sub-G0/G1 phase, an indicator of cell death (Fig. 3B).

In Colo320DM cells BA treatment did show mild G2-M phase arrest at 12 h time which was followed by 21-32% cells accumulation in G2-M phase as compared to 10% cells in control cells in response to 2.5-5 mM BA treatment. But at 1 mM concentration BA caused G0/G1 phase arrest at 24 h time (Fig. 4B).

V.5. BA altered the levels of proteins involved in regulation of G2-M transition

Eukaryotic cell cycle progression involves sequential activation of cyclin-dependent kinases (CDKs) whose association with corresponding regulatory cyclins is necessary for their activity (Molinari, 2000). The G2-M is regulated by a complex between cyclin B1 and CDK1. BA treatment (1-5 mM for 12 and 24 h) caused a marked decrease (especially at 24 h) in protein levels of cyclin B1 and CDK1 in both the CRC cells. These results indicated that BA mediated cell cycle arrest in G2-M phase in SW480 and HCT116 cells could be mediated via a decrease in protein levels of cyclin B1 and CDK1. The CDK1 is dephosphorylated by the phosphatase, CDC25C that results the entry of cells in M phase. BA treatment decreased the level of CDC25C as early as 6 h after treatment (Fig. 6).

V.6. BA treatment induced apoptosis in human CRC cells
Because the flow cytometric analysis of cell cycle distribution suggested apoptosis induction due to accumulation of sub-G0-G1 cells, we proceeded to characterize proapoptotic response to BA. BA treatment for 12 and 24 h showed a significant increase (10-17%, P<0.05-0.001) in apoptotic cells in both the CRC cell lines (Fig. 7A and 7B). SW480 cells showed 1.5 fold increase in apoptotic cells at 12 h time point which was increased by 3-4 fold after 24 h treatment (Fig. 7A). Similarly, the HCT116 cells showed 1.5 and 3 fold enrichment of apoptotic cells at 12 h and 24 h after BA treatments, respectively (Fig. 7B). To further confirm the proapoptotic effect of BA, western blot analysis was performed for the levels of total PARP, cleaved-PARP, total caspase-3, cleaved-caspase-3 and Bcl-2. BA increased the level of cleaved-PARP and cleaved-caspase-3 and decreased the level of Bcl-2 (Fig. 8 & 9), suggesting the possible involvement of mitochondrial apoptosis.

V.7. BA caused caspase-mediated apoptosis in CRC cells
The role of caspase-mediated apoptosis was addressed by using z-VAD-FMK, a pan-caspase inhibitor, which causes irreversible inhibition to caspases and inhibits mitochondrial apoptosis. The quantitative apoptotic cell death assay using annexin V/PI staining of both of the cell lines showed that in caspase inhibiting condition, BA induced apoptosis (28% increase over that of control, P<0.05) was strongly reduced (11.6% increase over that of z-VAD-FMK treatment, P<0.05) in SW480 cells (Fig. 10A). Similar effects were observed in HCT116 cells in which BA induced apoptotic cells i.e. 15.7% (P<0.05) was reduced to 4.1% (P<0.05) in caspase inhibiting condition (Fig. 10A). The protein level of cleaved caspase-3 was analyzed. BA showed an increase in cleaved caspase-3 level in both the cell lines which was drastically reduced in the presence of caspase inhibitor (Fig. 10B). These results indicated that BA mediated apoptosis is mainly mediated by caspases possibly involving mitochondria-mediated apoptotic pathway.

V.8. BA decreased active mitochondrial mass and enhanced ROS production in CRC cells
The effect of BA on mitochondrial mass was investigated using MitoTracker. MitoTracker is a fluorescent dye which gets accumulated in active mitochondria in live cells and it is dependent upon mitochondrial membrane potential (Chazotte, 2011). BA (1-5 mM) caused 15-27% reduction (P<0.001) in mitochondrial mass after
12 h of treatment in CRC cells (Fig. 11A and 11B). A further decrease (25-47%, P<0.001, in SW480 cells; 22-35%, P<0.001, in HCT116 cells) in mitochondrial mass was observed after 24 h of BA treatment (Fig. 11B). These results indicated that BA can cause the lysis or fusion of mitochondria accompanied with the reduction of total surface area. This observation indicated for inhibition of mitochondrial fission and induction of potential mitochondrial fusion by BA treatment.

Mitochondria-mediated ROS generation was determined by MitoSOX which specifically detects mitochondrial superoxides. Superoxide is also an indirect marker of hypoxia (Gao and Wolin, 2008; Vanden Hoek et al., 1998). BA (2.5 mM) treatment for 24 h showed 5 fold (P<0.001) increase in mitochondrial superoxide/ROS production in SW480 cells, whereas this increase was 2 fold (P<0.001) in HCT116 cells (Fig. 12). This observation indicated for induction of ROS generation by BA treatment.

V.9. BA caused DRP1-mediated mitochondrial fusion in human CRC cells

DRP1 is the key player in regulation of mitochondrial fission and fusion during cell cycle progression (Yamano and Youle, 2011). To examine the effect of BA on mitochondrial fusion, first we assessed SW480 cells under confocal microscope for the mitochondrial structure treated with BA (2.5 mM) for 12 h followed by MitoTracker staining (Fig. 13A). In presence of BA (2.5 mM), mitochondria appear fused near nuclear region. The protein level of DRP1 decreased in both the CRC cells. BA (1-5 mM) treatment for 12 and 24 h showed both time- and dose-dependent decrease in DRP1 in CRC cells (Fig. 13B). Next, using SW480 cell lysates, we observed that BA strongly decreased the phosphorylation of DRP1 at serine 616 position (Fig. 13C), which is required for its translocation into the mitochondrial membrane. On other hand, BA treatment induces the expression of MFN2 (Mitofusin-2), which is the mediator of event of mitochondrial fusion (Fig. 13C). Further, the localization of DRP1 was assessed in mitochondrial and cytosolic fractions of SW480 cells following 2.5 mM BA treatment for 12 and 24 h. At early time point, the decrease in DRP1 localization was noticed only in mitochondrial fraction which later became predominant in both mitochondrial as well as cytosolic fractions (Fig. 14A). 

Cyclin B1-CDK1 complex is associated with the activating phosphorylation of DRP1 (pDRP1-ser616) during mitotic division (Yamano and Youle, 2011), which was
examined by co-immunoprecipitation and looking at the complex formation of cyclin B1 with CDK1, known for its kinase activity. BA reduced the level of cyclin B1-CDK1 complex formation (Fig. 14B), and thereby it can inhibit DRP1 activation and its mitochondrial localization. In another experiment, we examined the level of DRP1 in caspase inhibiting condition. Pan-caspase inhibitor treatment showed an increase in DRP1 level and it also reversed BA-caused decrease in DRP1 level (Fig. 14C). This observation suggests for a novel mechanism of DRP1 regulation in caspase-dependent manner.

V.10. DRP1 overexpression in CRC cells
Above results indicate that BA treatment regulates cell survival and cell death in CRC cells and DRP1 could be one of its major targets. BA treatment reduced DRP1 phosphorylation at ser 616 site as well as its total protein level. Next, we overexpressed DRP1 gene in CRC cells. Prelich stated that “Overexpression or misexpression of a wild-type gene product, however, can also cause mutant phenotypes, providing geneticists with an alternative yet powerful tool to identify pathway components that might remain undetected using traditional loss-of-function analysis” (Prelich, 2012). DRP1 full-length cDNA sequence (2211 bp) was cloned in pLVX-Puro vector containing cytomegalovirus (CMV) constitutively active promoter.

After the puromycin selection screening for vector integration, positive clone was selected and analyzed for its morphology, integration of desired gene and its protein expression. Upon the transduction, DRP1 gene gets integrated in the genome of CRC cells (Fig. 16C). This leads to the overexpression DRP1 gene and increases the level
of DRP1 protein in whole cell lysate up to 3.4 fold as compared to the parent cell line (Fig. 16B). During morphological examination, it was observed that physical appearance of transduced cells is different than the parental cells and showed mesenchymal morphology (Fig 16A).

V.11. BA reverted the enhanced clonogenic potential of DRP1 overexpressing CRC cells
The clonogenic assay enables an assessment of the differences in reproductive viability (capacity of cells to produce progeny; i.e. a single cell to form a colony of 50 or more cells) between control untreated cells and cells that have undergone various treatments such as exposure to ionising radiation, various chemical compounds (e.g. cytotoxic agents) or in other cases genetic manipulation (Rafehi et al., 2011). DRP1 overexpression enhanced the clonogenic potential of HCT116 cells. Frequency of <50 cells colony is 35% (P<0.001) higher in DRP1 overexpressed cells than the empty vector. BA (1 mM) potently inhibited colony forming potential in both empty vector as well as DRP1 overexpressed cells. Compared to empty vector control, BA treatment reduced the frequency of 26-49 cells colonies (47%, P<0.001) and ≥50 cells colonies (64%, P<0.001), and increased the 11-25 cells colonies (62%, P<0.001). In DRP1 overexpressed cells, BA treatment reduces the frequency of ≥50 cells colonies (62%, P<0.001) and increased the 1-10 cells colonies (58%, P<0.001), 11-25 cells colonies (33%, P<0.01) and 26-49 cells colonies (45%, P<0.001). Overall, BA (1 mM) treatment reduced the frequency of colony formation (including all size of colonies) by 24 and 23% respectively in empty vector and DRP1 transduced HCT116 cells (Fig. 17).

V.12. BA reverted the increased cell viability of DRP1 overexpressing CRC cells
DRP1 overexpression in CRC cells, HCT116 and SW480 increased the cell viability by 16 and 17% (P<0.001) respectively. The effect of BA treatment on cell viability of DRP1 overexpressing CRC cell lines, HCT116 and SW480, was determined by trypan blue dye exclusion assay (Fig. 18A and 18B). BA treatment at 1 mM concentration for 12 h decreased the viability of both transduced CRC cell lines. This accounted for 50% (P<0.001) and 33% (P<0.001) decrease in viability in empty vector and DRP1 transduced HCT116 cells, respectively (Fig. 18A) and 55% (P<0.001) and 45% (P<0.001) decrease in empty vector and DRP1 transduced SW480
cells, respectively (Fig. 18B). Cell death induction in transduced cells in similar conditions after 12 h of BA treatment was 11.4% (P<0.001) and 8% (P≤0.001) in empty vector and DRP1 transduced HCT116 cells, respectively (Fig. 18A) and 12.7% (P<0.001) and 6.6% (P<0.001) in empty vector and DRP1 transduced SW480 cells respectively (Fig. 18B). These results suggest that DRP1 increases cell viability which is decreased by the BA.

V.13. BA treatment caused G1 phase cell cycle arrest in DRP1 overexpressing CRC cells
Anti-tumor agents act at multiple steps in the cell cycle, and their effects may be cytostatic or cytotoxic, depending on the cell cycle status of the target cells (Shapiro & Harper, 1999). Graphical representation of the cell cycle distribution without or with 1 mM BA treatment showed that it causes G2-M arrest (P<0.001) at 12 h in both empty vector transduced HCT116 and SW480 cells which is accompanied by a decrease in S phase cell population (Fig. 19). In contrast to the empty vector containing cells, DRP1 expressing cells showed G0/G1 phase arrest in presence of 1 mM BA (P<0.001). 62% and 70% cells get arrested in G0/G1 phase respectively in DRP1 overexpressed HCT116 and SW480 cells as compared to 53% and 61% cells in their respective controls (Fig. 19).

V.14. BA altered the levels of proteins involved in regulation of G1 phase arrest and cell survival
Since, BA induced G1 arrest in both of the transduced cells; we examined its effect on various cell cycle regulated molecules governing these events during cell cycle progression in transduced HCT116 cells. Upon the overexpression of DRP1 gene, levels of p53 and p21/Cip1 proteins down regulated up to 40%. These are the key regulators of cell cycle. On the other hand, Akt and phosphorylation of ERK1/2 up regulated up to 78% and 62%, respectively. Akt and Erk1/2 molecules mediate pro-survival and mitogenic processes in cells (Fig. 20). CDKI-CDK interaction is determining factor for G1 phase progression and its inhibition has been shown to induce G1 arrest; therefore, we examine the effect of BA on this interaction. After 12 h of BA (1 mM) treatment in transduced HCT116 cells, BA caused the G2-M phase arrest in empty vector cells which is followed by cell death. Accordingly, the levels of p53 and p21 up-regulated, as well as the levels of Cyclin D1, Akt and pERK1/2 were
also increased. In case of DRP1 transduced cells, BA treatment caused the G1 phase arrest as its drivers, the levels of Cyclin D1 and CDK6 reduced and its regulators p53 and p21 showed increase in expression (Fig. 20). These results suggest that DRP1 induces the cell survival which is decreased by BA.

**V.15. BA treatment reverted the pro-oxidative activities of DRP1 overexpressing CRC cells**

Reactive oxygen species (ROS) are important regulators of apoptosis. BA treatment induces the mitochondrial superoxide in both CRC cells (Fig. 12). The empty vector transduced HCT116 cells also showed the mitochondrial superoxide (4-8%, P<0.001) (Fig. 20A & B) and over all cellular ROS (DCF-DA assay) (35-41%, P<0.001) (Fig. 21C) production after the treatment of BA (1 mM) for 24 and 48 h. In cancer cells, mitochondrial ROS amplify the tumorigenic phenotype and accelerate the accumulation of additional mutations that lead to the metastatic behavior (Sabharwal & Schumacker, 2014). DRP1-cells showed increase in mitochondrial superoxide (7-20%, P<0.001) (Fig. 21A & 21B) and overall cellular ROS (DCF-DA assay) (24-73%, P<0.01-0.001) (Fig. 21C) as compared to empty vector control. When DRP-cells were treated with BA (1 mM) for 24 and 48 h treatment scavenged the mitochondrial superoxide (4-12%, P<0.001) (Fig. 21A & 21B) and overall cellular ROS (DCF-DA assay) (18-43%, P<0.001) (Fig. 20C). These results suggest that DRP1 prompts ROS formation which is scavenged by BA.

**V.16. NAC pretreatment enhances antioxidant activities of BA on DRP1 overexpressing CRC cells**

N-acetyl-L-cysteine (NAC) is an antioxidant and commonly used to identify and assess ROS inducers. 6 h pretreatment of NAC (5 mM) scavenged the ROS induced by BA (1 mM) treatment for 12 h (63%, P<0.001) and 24 h (72%, P<0.001) in empty vector cells (Fig. 22A). This NAC pretreatment also reverted the reduction of cell viability induced by 12 h (59% increase in cell viability, P<0.001) and 24 h (44% increase in cell viability, P<0.001) of BA treatments (Fig. 22B). When the DRP1 transduced cells were pretreated with NAC (5 mM) followed by the BA (1 mM) for 12 and 24 h, NAC enhanced the antioxidant activity of BA treatment at 24 h (24%, P<0.01) time point (Fig. 22A). This scavenging activity add up in reduction of cell viability caused by BA treatment for 24 h (17%, P<0.001) (Fig. 22B). These results
suggest that NAC pretreatment added in ROS scavenging activity of BA in DRP1-cells.

V.17. BA treatment in NAC pretreatment condition caused G2-M phase cell cycle arrest in DRP1 overexpressing CRC cells

BA (1 mM) treatment caused the G1 phase arrest at 12 h time point in DRP1 overexpressing HCT116 cells (Fig. 19A) instead of G2-M phase arrest in empty vector cells. These transduced cells were pretreated with NAC (5 mM) for 6 h followed by BA (1 mM) treatment for 12 h and analyzed for their cell cycle distribution. Effect of BA reverted by NAC pretreatment in empty vector cells (Fig. 23). On other hand, in NAC pretreated DRP1-cells BA treatment caused G2-M phase arrest (10%, P<0.001) instead of G1 phase (Fig. 19A & 23).

V.18. BA treatment in combination with Mdivi-1 caused G2-M phase cell cycle arrest in Colo320DM

Colo320DM is an advance stage (Duck type III) CRC cell line in which we observed a higher expression of DRP1 protein as compared to other cell lines (Fig. 24A). CaCo-2 is an early stage cell line whereas HCT116 (APC positive) and SW480 (APC negative) are the second-stage CRC cell lines. Colo320DM is metastatic stage cell line. BA treatment at 1 mM treatment caused G0/G1 phase arrest at 24 h time (Fig. 4B & 24B). When cells were co-treated with 10 μM (nontoxic dose, 50 μM caused G2-M phase arrest) Mdivi-1 (DRP1 inhibitor) and 1 mM BA then they caused 26% (P<0.001) G2-M phase arrest as compared to control showing 15% cells in G2-M phase. Whereas 10 μM Mdivi-1 alone didn’t show any effect and 1 mM BA caused G1 phase arrest. This result states that most likely, DRP1 inhibition via BA caused G2-M phase arrest (Fig. 24B).

V.19. BA treatment induced autophagic cell death in DRP1 overexpressing CRC cells

BA treatment (1-5 mM) caused the apoptotic cell death in CRC cells at 12 and 24 h time point (Fig. 7). Further, we analyzed the DRP1 transduced HCT116 cells for its cell death mechanisms. Empty vector cells showed apoptotic cell death whereas DRP1-cells they did not show apoptosis. To explore its cell death mechanism, cells were stained with acridine orange which is a cell permeable metachromatic cationic
dye. This dye changes emitted fluorescence from yellow, to orange, to red as the pH drops in acidic vacuole of the living cell. This flow cytometry based test is usually been used for the detection of autophagic vacuoles generated during autophagy. Autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome (Mizushima, 2007). BA (1 mM) treatment induced acid vacuoles formation in DRP1 cells in 6 (7%, \( P<0.001 \)) and 12 h (10%, \( P<0.001 \)) as compared to un-treated cells (Fig. 25A & 25B). As an indicator of autophagy LC3A/B (I/II) (microtubule-associated protein 1A/1B-light chain 3) and Beclin-1 was analyzed. BA treatment to DRP1-cells increased the LC3-phosphatidylethanolamine conjugate (LC3-II) forms as well as expression of Beclin-1 as compared to un-treated cells (Fig. 25C). These results suggest that DRP1 drive toward autophagic cell death instead of apoptosis in presence of BA.

V.20. BA treatment induced mesenchymal-epithelial transition in human CRC cells

The transdifferentiation of epithelial cells into motile mesenchymal cells, a process known as epithelial–mesenchymal transition (EMT), is integral in development, wound healing and stem cell behaviour, and contributes pathologically to fibrosis and cancer progression (Lamouille et al., 2014). SW480 and HCT116 cells were treated with 1 and 2.5 mM BA for 6, 12 and 24 h and cells were analyzed for E-cadherin expression. E-cadherin is one of the crucial types of cell-cell adhesion molecule to hold the epithelial cells tight together which is an indicator of return to an epithelial state (Polyak & Weinberg, 2009). BA treatment induced the expression of E-cadherin at 12 and 24 h (Fig. 26A) in both of CRC cells. Vimentin is a type III intermediate filament protein and a marker of mesenchymal cells. Treatment of BA (1-2.5 mM) for 12 h down-regulated the vimentin expression as well as Snail and Slug which are the transcription regulators of EMT (Fig. 26B).

V.21. BA treatment reverted the migration and invasion activities of DRP1 overexpressing CRC cells

The ability to migrate allows cells to change their position within tissues or between different organs. In pathology, invasion of carcinomas is defined as the penetration of tissue barriers, such as passing the basement membrane and infiltration (intrusion) into the underlying interstitial tissues by malignant tumor cells (Kramer et al., 2013).
The transwell assay was originally developed by S Boyden to analyze the chemotactic responses of leukocytes, it is also called Boyden chamber assay. The principle of this assay is based on two medium containing chambers separated by a porous membrane through which cells transmigrate (Boyden, 1962). DRP1 overexpression increases the migration property by 29% (P<0.001) and 38% (P<0.001) (Fig. 27) and invasion by 44% (P<0.001) and 64% (P<0.001) (Fig. 27) in DRP1 transduced HCT116 and SW480 cells, respectively. BA (1 mM) treatment for 12 h reduced the migration by 80% (P<0.001) in both HCT116 and SW480 cells transduced with empty vector (Fig. 27). In DRP1 overexpressing HCT116 and SW480 cells, BA treatment reduced the migration by 75% (P<0.001) and 50% (P<0.001), respectively (Fig. 27).

In invasion assay, in addition to transwell assay the porous filter is coated by a thin layer of ECM on which the cells are seeded into the top chamber. BA (1 mM) treatment for 12 h reduced the invasion property by 91% (P<0.001) and 83% (P<0.001) in both HCT116 and SW480 cells transduced with empty vector, respectively (Fig. 28). In DRP1 overexpressing HCT116 and SW480 cells, BA treatment reduced the ECM invasion by 90% (P<0.001) and 54% (P<0.001), respectively (Fig. 28). These results suggest that DRP1 increases cell migration and invasion in CRC cells which is decreased by the BA.

**V.22. BA treatment modulated EMT makers and regulators in DRP1 overexpressing CRC cells**

Matrix metalloproteases (MMPs) have been identified to break down the ECM which is a key requirement for cell invasion. Gelatin zymography is a method to identify proteolytic enzymes capable of degrading gelatin; it is mainly used for the activity assessment of matrix metalloproteinase family. DRP1 overexpression increases the secretion of MMP in media around 70% (Fig. 29A) in DRP1 transduced HCT116 cells. BA (1 mM) treatment reduces the MMP level by 40% and 30% in empty vector and DRP1 cells respectively (Fig. 29A). DRP1 overexpression rise the expression of EMT molecules including Vimentin, Slug, Snail, uPA, MMP-2 and β-catenin and reduces the expression of E-cadherin (Fig. 29B). BA (1 mM) treatment for 12 h was able to revert all the EMT molecules except β-catenin and induces E-cadherin in DRP1 cells (Fig. 29B). These results suggest that DRP1 led cells toward EMT which is inhibited by BA.
V.22. BA treatment inhibited SNAIL protein stability in DRP1 overexpressing CRC cells

Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *Streptomyces griseus*. It is used as an experimental tool in molecular biology to determine the half-life of a protein. Its treatment provides the ability to observe the half-life of a protein without confounding contributions from transcription or translation. Snail (Zinc finger protein SNAI1) is a family of transcription factors that promotes the repression of the adhesion molecule E-cadherin to regulate EMT. DRP1 overexpression maintained 90% stability of SNAIL protein up to 6 h in contrast to 50% in empty vector cells (Fig. 30A & 30B). Cells with cotreatment of BA (1 mM) with cycloheximide (50 µg/ml) lost the stability of SNAIL protein by up to 92% and 70% in empty vector and DRP1-cells, respectively.

V.24. BA treatment restricted the spheroid formation frequency and self-renewal in DRP1 overexpressing CRC cells

Spherical aggregates of malignant cells or multicellular tumor spheroids, may serve as an in vitro model of tumor microregions and of an early, avascular stage of tumor growth. The similarities between the original tumor and the respective spheroids include volume growth kinetics, cellular heterogeneity, e.g. the induction of proliferation gradients and quiescence, as well as differentiation characteristics, such as the development of specific histological structures or the expression of antigens (Mueller-Klieser, 1987). Transduced HCT116 cells were used to determine the in vitro effect of BA on therapy-resistant cancer stem cells (bCSC) population. Fig. 31A shows representative first generation spheroid resulting from empty vector and DRP1 transduced HCT116 cells. There is no significant difference between empty vector and DRP1 cells in terms of number of spheroid. BA (1 mM) treatment reduces the number of spheroid form by 21% (P<0.001) and 34% (P<0.01) in empty vector and DRP1 transduced HCT116 cells (Fig. 31B). Size of spheroid which were formed by DRP1 cells, was larger (48% in terms of area, P<0.01) than the empty vector cells in control condition (Fig. 29C). BA (1 mM) treatment decreases the size of spheroid form by 43 % (P<0.05) and 52 % (P<0.01) in empty vector and DRP1 transduced HCT116 cells (Fig. 31C).
DRP1 overexpression significantly increased the number of second (22%, P<0.05) and third (78%, P<0.01) generation spheroids. These findings indicated elevation of self-renewal of therapy-resistant cancer stem cells by DRP1 overexpression (Fig. 32A). BA treatment resulted in a statistically significant decrease in the number of first and second generation spheroid in both empty vector and DRP1-cells (Fig. 32A). These results indicated inhibition of therapy-resistant cancer stem cells self-renewal by BA treatment. Inhibitory effect of BA treatment on therapy-resistant cancer stem cells fraction was confirmed by flow cytometric analysis of CD44\textsuperscript{high} / CD133\textsuperscript{high} fraction. In comparison with control, the CD44\textsuperscript{high} / CD133\textsuperscript{high} fraction was significantly lower in the BA-treated empty vector (26%, P<0.001) and DRP1 (32%, P<0.001) cells (Fig. 33A). In comparison with empty vector control, DRP1 control showed 38% (P<0.001) elevation in CD44\textsuperscript{high} / CD133\textsuperscript{high} fraction (Fig. 33A). Expression of stemness and differentiation related molecules, including LGR5 and Keratin-8 was determined after 12 h of BA (1 mM) treatment. Level of LGR5 increased ~20% and differentiation marker keratin-8 decreased ~70% in DRP1-cells as compared to empty vector cells in control condition (Fig. 33B). BA treatment induced the cell differentiation and increased the level of keratin-8 as well as lead to 20% reduction of LGR5 level in DRP1-cells (Fig. 33B).

Self-Renewal capacity of both empty vector and DRP1-cell after BA (1 mM) treatment for 12 h was determined by MTT cell viability assay for consecutive 24 and 48 h without BA. After 24 h of incubation, BA treated both the cells remained for proliferation arrested and no significant growth was observed in without BA condition (Fig. 32B). After 48 h of incubation, empty vector cells treated with BA remained arrested and no significant growth was observed. But in DRP1-cells treated with BA showed 35% (P<0.001) decrease in proliferation as compared to empty vector cells treated with BA (Fig. 32B). These results suggest that DRP1 promotes stemness in CRC cells which is decreased by BA.

V.25. BA treatment and angiogenesis markers in DRP1 overexpressing CRC cells
Tumors need a dedicated blood supply for the delivery of oxygen and other essential nutrients required in order to grow beyond a certain size (generally 1–2 mm\textsuperscript{3}) (McDougall et al., 2006). These tumors secrete various growth factors including
VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor) in their surrounding media to induce blood vessel growth. DRP1 overexpressed HCT116 cells showed around 3-fold increase in cellular VEGF level and around 2 fold increase in its secreted level in media as compared to empty vector cells. BA (1 mM) treatment for 12 h was not able to revert the level of VEGF in cells (Fig. 34A) as well as in media (Fig. 34B). In contrast, when empty vector cells were treated with BA (1 mM) for 12 h, the level of VEGF in cells and media got reduced up to 80% (Fig. 34A & 34B). HIF1α regulates a wide array of genes involved in tumor angiogenesis, chemoresistance and metastasis. Cellular level of HIF1α was determined to examine its role in angiogenesis in DRP1 overexpressing HCT116 cells. HIF1α got elevated in DRP1-cells up to the 7-fold and which also could not be reverted by BA treatment (Fig. 34A). BA treatment for 12 h in empty vector cells also showed the increase in HIF1α level by upto 2-fold.

**V.26. BA inhibited DRP1 overexpressing HCT116 cells conditioned medium induced proliferation of endothelial cells**

Human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) have been the standards for cell-based assays in the field of angiogenesis research and in antiangiogenic drug discovery (Bagley et al., 2003). HUVEC cells were plated on 96-well plate and allowed to attach for 24 h followed by the treatment of conditioned media (CM). Volume of CM mixed (maximum 50% of total volume) with HUVEC cell expansion media with 0.2% serum. CM volume was normalized with equal number of live DRP1-transduced HCT116 cells at the time of collection of CM. CM from empty vector control cells induced the HUVEC cell proliferation by up to 78% (P<0.001) but HUVEC cells viability remain equals to the blank in BA treated empty vector cells CM at 12 h of incubation (Fig. 35). HUVEC cells treated with CM from control DRP1 cells for 12 h, it induces 141% (P<0.001) increase in cell proliferation as compared to blank and 113% (P<0.001) in presence of CM of BA treated DRP1 cells. This result is also supported by the PCNA expression in different CM treatment for 12 h. Only HUVEC cells treated with CM from BA treated empty vector cells showed down-regulation of PCNA (30%), all others remain equal to control (Fig. 38). These results suggest that DRP1 overexpressing cells secreting more growth factors which promote the rapid proliferation of endothelial cells which is only partially decreased by BA.
V.27. BA inhibited DRP1 overexpressing HCT116 cell CM-induced capillary formation of endothelial cells

Capillary formation assay is *in vitro* assay that can be employed to investigate the effect of endothelial cells differentiation inhibitors through assessment of their effects on capillary tube formation by endothelial cells on Matrigel (McGonigle & Shifrin, 2008). We used conditioned media (CM) from transduced HCT116 cells treated with or without BA. A HUVEC suspension was then prepared in this CM and plated onto a Matrigel-coated plate. Images were taken after 6 hours of incubation; results are presented in Figure 34A, and pooled results from three independent wells are shown in Figure 34B. Volume of CM mixed (maximum 50% of total volume) with HUVEC cell expansion media with 0.2% serum. CM volume was normalized with equal number of live DRP1 transduced HCT116 cells at the time of collection of CM. CM from empty vector control cells induced the HUVEC cell capillary formation up to 17% (P<0.001) but HUVEC capillary formation remain equals to the blank in BA treated empty vector cells CM at 12 h of incubation (Fig. 36). HUVEC treated with CM from control DRP1 cells for 12 h, it induced 41% (P<0.001) HUVEC capillary formation as compared to blank and 19% (P<0.001) in presence of CM of BA treated DRP1 cells. These results suggest that DRP1 overexpressing cells secreting more growth factor which promote the capillary formation of endothelial cells which is partially decreased by BA.

V.28. BA inhibited DRP1 overexpressing HCT116 cell CM induced migration of endothelial cells

Tumor angiogenesis is a complex multistep process driven by local positive and negative signals within the tumor. This involves the degradation of the extracellular matrix (ECM) around a local venule, the proliferation and migration of capillary endothelial cells and their differentiation into functioning capillaries (Folkman, 1995). A HUVEC suspension was prepared in 0.2 % media and plated onto Boyden chamber and in lower chamber media containing CM was added. Volume of CM mixed (maximum 50% of total volume) with HUVEC expansion media with 0.2% serum. CM volume was normalized with equal number of live DRP1 transduced HCT116 cells at the time of collection of CM. Images were taken after processing and crystal violet staining after 12 h of migration; results are presented in Figure 37A, and pooled
results from three independent wells are shown in Figure 37B. CM from empty vector control cell induced the HUVEC migration up to 7 fold (P<0.001) and 2 fold (P<0.01) presence of CM of BA treated empty vector cells CM at 12 h incubation (Fig. 35). HUVEC treated with CM from control DRP1-cells for 12 h, induced 14 fold (P<0.001) HUVEC migration in compared to blank and 8.7 fold (P<0.001) in presence of CM of BA treated DRP1 cells. Level of N-cadherin got elevated in presence of CM of BA treated and un-treated DRP1 cells and remain unchanged in presence of BA. But in presence of CM from empty vector cells, BA un-treated condition, expression of N-cadherin was negligible and no expression was detected in BA treated condition (Fig. 38) in HUVEC. These results suggest that CM from DRP1 overexpressing CRC cells has capability to induce migration of endothelial cells.
Discussion
Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, with an estimated 1.4 million cases and 0.7 million deaths occurring in 2012. The increase in several Asian and Eastern European countries may reflect an increased prevalence of risk factors for colorectal cancer, including unhealthy diet, obesity, and smoking (Torre et al., 2015). Dietary fiber has a protective effect on the pathophysiology of colorectal cancer. Diets rich in vegetables and high fiber grains demonstrated significant protection against fatal colorectal cancer, as revealed in a prospective study (Aune et al., 2011). Fiber appears to have a number of mechanisms responsible for its protective effects: it decreases fecal transit time by increasing stool bulk; it appears to dilute the concentration of other colonic constituents, which tends to minimize contact between carcinogens and colon epithelium; and it is not digested or absorbed in the small intestine but undergoes fermentation in the presence of the colonic flora, which reduces fecal pH and generates short-chain fatty acids. Certain short-chain fatty acids can protect isolated colonic epithelial cells (Aune et al., 2011). Diet appears to play a significant role in determining the incidence of colorectal cancers in the general population. Although the international incidence of colorectal cancer varies widely, groups migrating from low-risk to high-risk regions experience an increase in the incidence of the disease. Diets high in fat and low in fiber have consistent associations with increased colorectal cancer risk.

Short chain fatty acids (SCFAs; butyric, propionic and acetic acid) are formed during bacterial fermentation of carbohydrates in the colon. They are mostly produced by anaerobic fermentation of dietary fibers by microbial flora in GI tract. They play an important role in normal homeostasis maintenance of epithelial lining of colon and intestine (Vanhoutvin et al., 2009). The colonic mucosa gets their energy by oxidizing SCFAs in the order of butyric>propionic>acetic acid (Clausen and Mortensen, 1994). Butyrate is the primary source of energy for the colonocytes, however, its absence causes apoptosis, but when these colonocytes get transformed into cancer cells, the presence of butyrate can cause cell death in these cancer cells (Cuff et al., 2005; Pajak et al., 2007). Propionic acid is mainly metabolized in the liver and has been shown its role in lowering plasma cholesterol (Chen et al., 1984). Currently, it is poorly understood, especially with respect to mitochondria, how SCFAs (mainly butyrate)
induce apoptosis in cancer cells of GI tract. In present study, we explored the relationship of mitochondrial dynamics and apoptosis after butyric acid exposure to human colorectal cancer (CRC) cells.

Mitochondrial dynamics, which is regulated by fission and fusion, is a key event during mitotic division for segregation of equal number of mitochondria in each daughter cell. This is regulated by fusion to fission ratio and strongly associated with normal embryonic development to neurodegenerative disorders (Detmer and Chan, 2007). Mitochondrial fission is also resulted by cyclin B1-CDK1 mediated phosphorylation of DRP1 at S616 and its localization on outer mitochondrial membrane (Taguchi et al., 2007).

VI.1. BA, cell cycle regulation and apoptosis in CRC cells

SW480, HCT116 and Colo320DM human CRC cell lines were used in the study. Treatment with Propionic acid (PA) upto 10 mM (only SW480 cells) and butyric acid (BA) (all three) upto 5 mM concentration showed reduction in cell viability starting as early as from 6 h in a time- and dose-dependent manner and showed ~60% and ~90% decrease in cell survival by 24 h respectively. This observation was consistent with earlier reported effects of BA on cancer cells (Wang et al., 2009). When both of the cell lines were treated with PA and BA and analyzed for cell cycle distribution they showed G2-M phase arrest. Normally, physiologically both SCFAs are observed in the range of 10-20 mM concentrations depending upon population and their dietary habits (Topping and Clifton, 2001). Concentrations used in this study are in the physiological range and to reduce the 50% cell viability of both the CRC cells the SCFA required are 50% and 10% concentrations of PA and BA from the highest concentration observed in physiologically. Therefore, we have selected BA for our further studies in 10 fold lesser concentration as compared to highest concentration present in colorectal region which was sufficient to inhibit CRC cells viability.

Usually, induction of apoptosis is linked with cell cycle arrest which is also a means to provide cells enough time to adjust with the external environmental as well as intracellular changes in the cells. Thus, cell cycle check-points play an important role in cell survival and proliferation. Interestingly, BA treatment to CRC cells first showed an increase in G2-M phase cell population which later substituted by an
increase in sub-G0/G1 phase cell population. The increase in sub-G0/G1 is an indication of cells going under cell death involving apoptosis. The cyclin B1 and CDK1 are key players of G2-M transition in which CDK1 gets activated by a phosphatase CDC25C (Sanchez et al., 1997). In our study, BA caused G2-M phase arrest was accompanied by the decrease in protein levels of cyclin B1, CDK1 as well as CDC25C providing an evidence of molecular changes associated with the biological effect. Survivin is a small molecular weight antiapoptotic protein which is overexpressed in cancer cells, and known to provide surviving capability by inhibiting the activation of apoptotic machinery (Altieri, 2008). Our studies showed that BA decreases both mRNA as well as protein levels of survivin supporting that decrease in CRC cell survival by BA could be mediated via apoptosis in our culture conditions.

We further explored the extent of apoptotic cells and associated molecular changes following BA treatment. As expected, dose- and time-dependent increases in the number of apoptotic cells were observed. The increase in apoptosis was accompanied by an increase in PARP and caspase-3 cleavage and down-regulation of Bcl-2 expression. The Bcl-2 is anti-apoptotic mitochondrial protein and known for its survival response whereas the cleavage of PARP at Asp214 that helps in cellular disassembly is an indicator of apoptosis (Oliver et al., 1998). The role of caspase pathway in apoptosis induced by BA treatment was investigated by using pan caspase inhibitor, z-VAD-FMK that irreversibly binds to the catalytic site of caspase proteases and inhibits apoptosis. The results of this study suggested that decrease in CRC cells survival is mostly mediated via caspase-mediated apoptosis which was validated by measuring the level of cleaved caspase 3. Together, the cell cycle arrest at G2-M phase and caspase mediated apoptosis by BA indicated for the role of mitochondria in cell death.

VI.2. BA, DRP1 and mitochondrial dynamics

G2-M phase cell cycle transition is also associated with mitochondrial division which can be assessed by the active mitochondrial mass in live cells (Margineantu et al., 2002). The result of MitoTracker experiment showed the decrease in overall population/mass of active mitochondria in CRC cells and that could also be an indicator of mitochondrial fusion or inhibition of fission. Decrease in active mitochondrial mass is associated with fusion of mitochondria because of overall
surface area of mitochondria gets decreased. In neuronal disorders when mitochondrial division gets blocked, it leads to elongation of organelles due to imbalanced and excess fusion (Kageyama et al., 2012). Accumulation of these elongated tubules leads to oxidative damage, which causes swelling of mitochondria accompanied with instability in the electron transport chain leading to a decrease in respiratory competence. Eventually, this decline in respiration causes neuronal cell death (Kageyama et al., 2012). Consistent with this, our MitoSOX data showed an increase in mitochondrial superoxide production confirming the mitochondria-mediated ROS generation which could be leading to oxidative damage and cell death.

The BA-induced mitochondrial fusion was further investigated by DRP1 level which is key regulator of mitochondrial fission and fusion process. Its higher level and specifically in mitochondrial membrane favors mitochondrial fission or otherwise its lower level promotes mitochondrial fusion (Youle and van der Bliek, 2012). BA down-regulated the level of DRP1 in both the CRC cell lines. Further, we also observed that it strongly reduces the localization of DRP1 to the mitochondrial membrane. Further, it also decreased the complex formation of cyclin B1 with CDK1, the active complex of which is required for the DRP1 Ser616 phosphorylation which is needed for its translocation to mitochondrial membrane (Taguchi et al., 2007). Together these findings suggest that DRP1 could be a potential molecular target to induce mitochondrial fusion or inhibit mitochondrial fission mediated by BA treatment in CRC cells. Importantly, we observed that DRP1 protein level is also regulated in a caspase-dependent manner.

Previous studies suggest that treatment of BA to CRC cells induces the apoptosis via p53 activation (Wei et al., 2008). Localization of p53 on the surface of mitochondria is the key event during apoptosis and cell death (Marchenko, et al., 2000). Based on these facts we examined the interaction between p53 and DRP1 as this both were playing role in programmed cell death. Here, two different and opposite conditions were used; first when BA induces p53 level and second when cells are induced with IL1β (after starvation), the level of p53 goes down. Results of immunoprecipitation suggested that, when cells were treated with BA, the level of interaction between p53 and DRP1 increased and reduced when cells were induced with IL1β.
BA treatment reduces the level of DRP1 and its phosphorylation which gives evidence that DRP1 can be a potential target for cancer regulation. Level of DRP1 expression is found elevated in human breast invasive carcinomas and metastases as compared to lymph nodes (Zhao et al., 2013) and when we compare different CRC cell lines for DRP1 expression, its expression was increased in advance CRC cells. Based on this, we decided to do the overexpression of DRP1 in CRC cells. This lead to the change in cellular morphology, cell clonogenicity and cell viability. DRP1 overexpression increases the clonogenic property of cells as these cells are producing larger colonies as compared to empty vector transduced cells. This can be interpreted as an increase in clonogenicity as their ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone (Franken et al., 2006). This result is further supported by the observations of cell viability assay of both CRC cells in DRP1-overexpressed condition. Overexpression of DRP1 increases the cell viability of both CRC cells and this is effectively reverted by BA treatment.

Inhibition of mitochondrial fission protein DRP1 causes an unexpected delay in G2/M cell cycle progression and aneuploidy. Inhibiting DRP1 triggers replication stress, which is mediated by a hyperfused mitochondrial structure and unscheduled expression of cyclin E in the G2 phase (Qian et al., 2012). In accordance to this when we overexpressed DRP1 in CRC cells, in response to BA treatment cells get arrested in G1 phase instead of G2-M phase as observed in empty vector condition.

Overexpression of cyclin D1 promotes tumor cell growth and confers resistance to cisplatin-mediated apoptosis in an elastase-myc transgene-expressing tumor cell line (Biliran et al., 2005; Musgrove et al., 2011). In present study overexpression of DRP1 led to the elevation of cyclin D1 and AKT levels, which were correlated, with clonogenicity and cell viability data. The AKT-mediated phosphorylation of glycogen synthase kinase 3β on serine 9 decreases its kinase activity for Thr286 of cyclin D1, which inhibits the nuclear export and the cytoplasmic proteasomal degradation of cyclin D1. Thus, the activation of the AKT pathway leads to nuclear accumulation of cyclin D1 resulting in cell proliferation (Manning and Cantley, 2007; Shimura et al., 2012). Here, DRP1 downregulated the p53 and p21 expression opposite to AKT. AKT mediates negative control of p53 levels through enhancing MDM2 (murine
double minute 2)-mediated targeting of p53 for degradation (Abraham and O'Neill, 2014). With AKT, DRP1 also induces the phosphorylation of ERK1/2, which is mitogenic response. DRP1-mediated mitochondrial division is required for RAS induced transformation and inhibition of oncogenic MAPK signaling mediates mitochondrial fusion via DRP1 loss (Serasinghe et al., 2015). BA treatment in DRP1 overexpressed cells reduces the level of Cyclin D1, CDK6, AKT and ERK1/2 phosphorylation in accordance with G1 phase arrest and p53 and p21 levels get elevated in presence of BA to inhibit cell cycle progression. Level of AKT and phosphorylation of ERK1/2 also get induced in empty vector cells treated with BA which is in correlation with oxidative stress and p21 induction by BA treatment (Kaur et al., 2011). Role of DRP1 in cell cycle progression was further confirmed by using DRP1 inhibitor Mdivi-1 which added in the effect of BA and caused G2-M phase arrest in Colo320DM cells.

Here, DRP1 overexpression led the generation of mitochondrial superoxide and cellular ROS. Mitochondrial metabolism and mitochondrial ROS generation are essential for Kras-induced cell proliferation and tumorigenesis (Weinberg, et al., 2010). BA treatment in empty vector cells is probably acting as pro-oxidant which induces the ROS and led to cell death but in DRP1 overexpressing cells, BA treatment act as anti-oxidant and could reduce the cell viability. This result is advocated by pretreatment of N-acetyl-l-cysteine (NAC), in empty vector cells, NAC pretreatment overcome the BA treatment but in DRP1 cells it enhances the effect of BA treatment.

Inhibition of pro-oxidant event with an antioxidant NAC inhibited the progression of cells from G1 to S. NAC-induced inhibition of entry into S phase was associated with an increase in MnSOD activity and a decrease in cyclin D1 protein levels (Menon et al., 2007). Similar results were observed in both transduced CRC cells in only NAC pretreated condition but when this pretreatment is followed by BA treatment in empty vector condition no significant cell cycle arrest was observed where as in NAC pretreated DRP1 cells, BA induced G2-M phase arrest as opposed to only BA treated cells.

Mitochondria play an important role in pro-inflammatory signaling. Autophagic turnover is mainly associated with mitochondria (mitophagy) to eliminates the
dysfunctional or damaged mitochondria, therefore it also counteracts degeneration, dampening of inflammation, and preventing of unwarranted cell loss (Green et al., 2011). DRP1 overexpressed cells in presence of BA induces autophagy instead of apoptosis. Which states that, DRP1 overexpression could drive these cells towards recovery and survival via autophagy. As mitochondrial fission is a crucial event to led mitophagy.

VI.3. BA, DRP1 and EMT
Activation of invasion and metastasis is a key hallmark of cancer. Event of metastasis is regulated by epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET) event (Hanahan and Weinberg, 2011). In present study, we studied the effect of BA treatment on EMT property of CRC cells and observed that BA induces E-cadherin and reduces the expression of vimentin, Snail and Slug. These results suggest that BA led the CRC cells towards MET and restricted EMT. Cancer invasion is initiated and maintained by signaling pathways that control cytoskeletal dynamics in tumor cells and the turnover of cell-matrix and cell-cell junctions, followed by cell migration into the adjacent tissue (Friedl and Alexander, 2011). BA inhibits cell invasion and migration up to a major extend in CRC cells. In a breast cancer study, compared to non-metastatic cells, mitochondria were more fragmented in metastatic breast cancer cells that express higher levels of total and active DRP1 and less mitochondrial fusion protein 1 (MFN1). Silencing of DRP1 resulted in mitochondria elongation or clusters, respectively, and significantly suppressed metastatic abilities of breast cancer cells (Zhao, et al., 2013). Here, when we overexpressed DRP1 in CRC cells then migration and invasion property of these cells significantly got increased. In accordance with this, DRP1 lead the induction of EMT molecules and suppression of MET. As a part of invasion process, DRP1-cells were able to secrete more proteolytic enzymes in their surrounding as well as activation of β-catenin and transcription factors including Snail and Slug. BA treatment was able to revert this effect up to large extent.

Snail expression is also induced by ROS that increases both its mRNA and protein stability as well as Snail1 expression also activates the ROS pathway vice versa (Diaz et al., 2014). Based on this fact, we studied the effect of DRP1 and BA on Snail protein stability. In accordance with ROS and cell migration data of DRP1-cells,
stability of Snail protein is higher as compared to empty vector cells and that got reverted by BA treatment up to a greater extent.

Cancer cells that undergo EMT also acquired stem cell-like properties which are called as cancer stem cells (Mani et al., 2008). Based on this, we studied the effect of DRP1 and BA on stemness of CRC cells. During spheroid culture experiment, we didn’t observe any change in number between empty vector and DRP1 cells but size of spheroid generated by DRP1 cells were larger than empty vector in control. This is an indication toward the resistance mechanism. Resistance toward different therapeutic conditions is indicated by sizes of human tumor spheroids (West, 1989). On the other hand, in both transduced cells, BA treatment reduces the spheroid number as well as size. In a generation assay of spheroid up to three generations showed that DRP1 cells have more stemness than the empty vector cells. This is further advocated by the presence of CD44^{high}/CD133^{high} population in both transduced cells which is also high in DRP1-cells. This was further elucidated by elevation in LGR5 level and reduction in differentiation marker keratin-8. LGR5 positivity is defined as stem-like cells in colorectal cancer (Hirsch et al., 2014). Spheroid assay and stemness marker expression of DRP1 cells pointed out towards the resistance to therapeutic agents. We also examined this resistance from BA treatment as it was reducing stemness markers. In this experiment both transduced cells were incubated in BA free media after BA treatment and the proliferation was allowed for prolonged period of time. BA treatment restricts both of the cells up to 24 h but after 48 h DRP1-cells started proliferation up to some extent as compared to empty vector cells which were still in arrested phase. This result suggested that due to overexpression of DRP1 gene CRC cells might have gain some stemness as well as resistance property.

VI.4. BA, DRP1 and angiogenesis

Like normal tissues and organs, tumors also require sustenance in the form of nutrients and oxygen as well as removal of metabolic wastes and carbon dioxide. This need of tumors is full filled by the induction of angiogenesis (Hanahan and Weinberg, 2011). Solid tumors grow up to size limit of ~1-3 mm diameter owing to the simple diffusion of nutrients and gaseous supply before they go into a period of dormancy ranging from years to decades. This phenomenon of long period of dormancy is
broken-down by the onset of active nutrient supply and gaseous exchange to the tumor which is made possible by the recruitment of new blood vessels/capillaries to the tumor through a process called as tumor angiogenesis. Solid tumor growth and metastatic progression heavily rely on the angiogenesis process which is mediated by tumor cell and microenvironment-derived pro-angiogenic factors that induce blood vessel/capillary formation in the tumor mass (Folkman, 1971, 2002). VEGF is a key ligand for the growth of new blood vessel and for homeostatic survival of endothelial cells. Expression of VEGF is upregulated by both hypoxia and oncogene signaling. This can be regulated by expression of HIF1α. Here, we have studied the cellular level of VEGF and HIF1α as well as secreted level of VEGF in both transduced cells. DRP1-cells show strong elevation in cellular level of HIF1α and VEGF as well as secreted level as compared with empty vector cells. BA treatment in empty vector cells reduces the VEGF, cellular and secreted levels, but there is no effect on DRP1 cells. Empty vector cells treated with BA show induction of HIF1α level, and this might be a hypoglycaemia induced HIF1α which ultimately led to apoptosis (Carmeliet et al., 1998).

Endothelial cells are the primary responder of angiogenic signals coming from neovascularature of tumors. We applied the conditioned media (CM) collected from both transduced CRC cells on HUVEC cells. We observed that CM from empty vector cells without any treatment induces the HUVEC cell proliferation as compared to blank but there is no significant difference between cell growth in blank and cell treated with CM from empty vector cell treated with BA. In case of HUVEC cells treated with CM from DRP1-cells without BA treatment are more proliferating as compared to empty vector cells-CM. When HUVEC cells are treated with CM from DRP1-cells with BA treatment they were more proliferating as compared to blank as well as empty vector control but there was a significant reduction as compared to DRP1 cells in control condition. These results suggest that DRP1 cells are secreting more amount of angiogenic factor as compared to empty vector cells. BA treatment does not allow the secretion of angiogenic factor from empty vector cells but the condition is not same in case of DRP1-cells. This result is also supported by the PCNA expression in HUVEC cells with same CM treatments.
During angiogenesis process, blood capillary tube formation is an important event that leads to vessel organization; therefore, inhibitory targeting of capillary tube formation can halt angiogenesis process and hence, tumor growth and progression. We observed that CM from untreated DRP1-cells induces significant tube formation as compared to empty vector cells. CM from BA treatment empty vector cell does not allow the tube formation when compared with blank but CM from DRP1-cells treated with BA was able to induce tube formation however it was lower to control. Endothelial cell migration and invasion are essential requirements during angiogenesis process and are rate limiting events in capillary growth and organization. In HUVEC cell migration assay with CM from different cells and treatment condition shows analogous results as compared to tube formation assay. N-cadherin is the key molecule playing a role in cell mobility. When CM treated HUVEC cells were analyzed for N-cadherin expression, CM from DRP1 cells irrespective to BA treatment induces the expiration of N-cadherin as compared to empty vector control as well as blank. Over all, angiogenesis experimental data suggest that DRP1 overexpression might be inducing angiogenesis, however it is not reverted completely by BA treatment.
Summary and Conclusions

The findings in the present study suggest that Butyric acid (BA) has better efficacy as compared to Propionic acid (PA). Further, BA-caused decrease in survival of CRC cells is associated with down-regulation of survivin and Bcl-2, and G2-M phase cell cycle arrest and increase in apoptosis. Further, it is mediated by a decrease in mitochondrial mass owing to inhibition of mitochondrial fusion and an increase in mitochondrial fusion. BA down-regulated DRP1 as well as its mitochondrial translocation likely via a decrease in activity of cyclin B1-CDK1 complex as their interaction as well as expression levels were decreased by BA treatment. BA caused an activation of caspase pathway which was linked to a decrease in DRP1 level. BA treatment increased p53 interaction with DRP1 and reduced its localization to mitochondria. Reduction of p-DRP1 ser616 and induction of MFN suggest decrease in mitochondrial fission and an increase in their fusion followed by cell death (Illustration 1).

Illustration 1: Proposed mechanisms for the role of DRP1 in mitochondrial fusion in response to BA treatment of CRC cells.

Furthermore data suggest that, DRP1 gets upregulated sequentially in advanced CRC cell lines. DRP1 overexpressing cells are having more cell proliferative capacity and show induction in the levels of Cyclin D1, Akt and phosphorylation of ERK1/2, and reduction of p53 and p21/cip1. BA causes G1 phase arrest in DRP1 overexpressing cells rather G2-M phase arrest. This G1 phase arrest was caused by BA was associated with ROS production due to DRP1 overexpression, and scavenging of this
ROS returned the cells in G2-M phase arrest. BA causes mostly autophagic cell death in DRP1-cells instead of apoptosis.

DRP1 overexpressing cells (DRP1-cells) have more stemness represented by the high CD44\textsuperscript{High}/CD133\textsuperscript{High} population, increased level of LGR5 and reduction in differentiation marker Keratin-8 in concurrence with larger spheroid formation. BA treatment was able to rescue the stemness of DRP1-cells.

DRP1-cells are more migratory and invasive and show the induction of Vimentin and reduction in E-cadherin expression indicating for EMT in these cells. BA treatment reduces the migratory and invasive property in CRC cells as well as in DRP1-cells.

DRP1 led angiogenic secretions from CRC cells, DRP1-cells showed higher cellular as well as secreted level of VEGF. Conditioned media of DRP1-cells are also able to induce the cell proliferation, tube formation capacity and migratory property in human umbilical vein endothelial cells (HUVEC). DRP1-cells showed higher endogenous level of HIF1α. BA treatment was not able to completely rescue from the pro-angiogenic activity due to DRP1 overexpression.

Overall, this study further advances the understanding of mitochondrial dynamics and associated biological events. Specifically, the identification of a novel molecular target of BA, the DRP1, by which it can inhibit the survival and migration of cancer cells. This study represents butyrate as an anticancer agent, however, it should be also noted that butyrate is rapidly utilized and metabolized by normal cells (half-life 6 min in blood). Thus, it is more suitable to deliver BA via fiber-rich diet involving its microbial degradation directly at site of colorectal tissue which happens naturally as well. Furthermore, DRP1 has shown pro-survival, pro-migratory, pro-stemness and pro-angiogenic activities in CRC cells, thus it could be a potential target for cancer prevention and therapeutics. This study is the first molecular and cell culture based evidence which has illumined the role of DRP1 in CRC cancer growth, survival and progression.
References


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Appendix I. Recipes of Buffers and Other Solutions

Stock solutions of drugs/agents/proteins

<table>
<thead>
<tr>
<th>Agents</th>
<th>Stock conc.</th>
<th>Working conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric acid (BA)</td>
<td>0.5M</td>
<td>1-5 mM</td>
</tr>
<tr>
<td>Propionic acid (PA)</td>
<td>0.5M</td>
<td>2.5-10 mM</td>
</tr>
<tr>
<td>Propidium Iodide (PI)</td>
<td>5 mg/ml</td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>Gelatin</td>
<td>1% (10 mg/ml)</td>
<td>0.1% (1 mg/ml)</td>
</tr>
</tbody>
</table>

10x PBS

1. Dissolve the following in 800ml distilled H₂O.
   - 80g of NaCl
   - 2.0g of KCl
   - 14.4g of Na₂HPO₄
   - 2.4g of KH₂PO₄

2. Adjust pH to 7.4.

3. Adjust volume to 1litre with additional double distilled H₂O.

4. Sterilize by autoclaving at 15 psi for 15-20 minutes.

Crystal violet solution
- 5 mg/ml in 2% Ethanol (store at R.T.)

2x Zymography sample buffer

<table>
<thead>
<tr>
<th>Reagents required</th>
<th>Conc./Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>0.1% Bromophenol Blue in DDH₂O (w/v)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Added DD water to make</td>
<td>10.0 ml</td>
</tr>
</tbody>
</table>
10x Zymography renaturation buffer

<table>
<thead>
<tr>
<th>Reagents required</th>
<th>Conc./Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>DD water to make</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

10x Developing buffer

<table>
<thead>
<tr>
<th>Reagents required</th>
<th>Conc./Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris–HCl (pH 8.0)</td>
<td>0.05 M</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>5 mM</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>5 µM</td>
</tr>
<tr>
<td>Added DD water to make</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>

Coomassie R-250 staining solution

<table>
<thead>
<tr>
<th>Reagents required</th>
<th>Amount/Conc./Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie R-250</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Methanol</td>
<td>50 ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>10 ml</td>
</tr>
<tr>
<td>Water</td>
<td>40 ml</td>
</tr>
</tbody>
</table>

Coomassie R-250 destaining solution

<table>
<thead>
<tr>
<th>Reagents required</th>
<th>Amount/Conc./Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>50 ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>10 ml</td>
</tr>
<tr>
<td>Water</td>
<td>40 ml</td>
</tr>
</tbody>
</table>

Semiquantitative PCR component composition (1x reaction)

<table>
<thead>
<tr>
<th>Reagents required</th>
<th>Amount/Conc./Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved ddH$_2$O</td>
<td>15.75 µl</td>
</tr>
<tr>
<td>Template (cDNA)</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>Forward primer (working stock)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Reverse primer (working stock)</td>
<td>1.0 µl</td>
</tr>
</tbody>
</table>
**1x Whole cell lysis buffer (100 ml)**

<table>
<thead>
<tr>
<th>Reagents required</th>
<th>Amount/Conc./Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (pH 7.0)</td>
<td>10 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1%</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>EGTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>Sodium orthovanadate</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>NP-40</td>
<td>0.5%</td>
</tr>
<tr>
<td>Complete protease inhibitor cocktail from Roche, USA (stock 25x)</td>
<td>1x</td>
</tr>
<tr>
<td>Phosphostop phosphatase inhibitor (stock 100x)</td>
<td>1x</td>
</tr>
</tbody>
</table>

**SDS Gel composition**

**A: 12% Resolving gel composition**

<table>
<thead>
<tr>
<th>Components</th>
<th>Total volume (10 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.D. Water</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>30% Acrylamide:Bis Mix</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10%SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>10%APS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004 ml</td>
</tr>
</tbody>
</table>

**B: 5% Stacking Gel Composition**

<table>
<thead>
<tr>
<th>Components</th>
<th>Total volume (3 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.D. Water</td>
<td>2.1 ml</td>
</tr>
<tr>
<td>30% Acrylamide:Bis Mix</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>
1.5 M Tris (pH 6.8)  |  0.38 ml
10% SDS           |  0.03 ml
10% APS           |  0.03 ml
TEMED             |  0.003 ml

10x Gel running buffer

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount/Volume required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>29 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>144 g</td>
</tr>
<tr>
<td>SDS</td>
<td>10 g</td>
</tr>
<tr>
<td>DD H2O</td>
<td>1000.00 ml</td>
</tr>
</tbody>
</table>

2x Sample (loading) buffer (final volume 10ml)

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount/Volume required</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Tris buffer (pH 6.8)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Beta-mercaptoethanol</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>4.0 mg</td>
</tr>
<tr>
<td>Total Volume (make up with ddH2O)</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Sample preparation:

1. After protein estimation, mixed equal volumes of protein samples and 2x sample buffer and vortexed.
2. Heated samples at boiling water bath for 5-7 minutes followed by vortexing for 5 seconds. Centrifuged samples at 14,000 for 5 minutes.
3. Loaded supernatant into wells carefully to avoid any debris.

10x Wash buffer for western blotting (final volume 1 liter (pH 7.5))

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount/Volume required</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount/Volume required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base (100 mM)</td>
<td>12.114 g</td>
</tr>
<tr>
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<td>Add 500 ml DD H2O and adjust pH with HCl</td>
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<tr>
<td>Add ddH2O for volume make-up</td>
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1x Wash buffer for western blotting (final volume 1 liter (pH 7.5))

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1x Blocking buffer for western blotting (final volume 100 ml) (pH 7.5)

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10x Transfer buffer (final volume 1 liter)

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Appendix II. Course Work and Publications

- **Course Work**
  - Semester 1: CGPA-8.25
  - Semester 2: CGPA-8.25
  - FGPA: 8.25

- **Publication Records**
  - **Research articles**
  - **Book chapters**
  - **Conference presentations**
    - Tailor D, Hahm ER, Kale RK, Singh SV and Singh RP. Title: Sodium butyrate, a product of microbial commensalism induces mitochondrial fusion and apoptosis in human colorectal cancer cells. International Conference on Recent Advances in Cancer Prevention and Therapeutics at Central University of Gujarat, Gandhinagar, India. (Oral presentation) (November 19-20, 2013).
- **Tailor D**, Hahm ER, Singh SV and Singh RP. Title: Butyric acid reverts DRP1-induced cell migration, epithelial–mesenchymal transition and stemness in colorectal cancer cells. International Symposium on Current Advances in Radiobiology, Stem cells and Cancer Research at School of Life Sciences, Jawaharlal Nehru University (JNU), New Delhi, India. (Poster, February 19-21, 2015).

Figure 1: Effects of propionic acid (PA) on survival of human CRC SW480 cells. (A) For the studies assessing the effect of PA on survival of exponentially growing SW480 cells, $10^5$ cells were plated in 60-mm dishes and next day, treated with vehicle (distilled water) alone or 1-5 mM of PA in fresh medium. After 6, 12 and 24 h of these treatments, viable cells were counted using trypan blue staining and hemocytometer. Represents the viable cell number for SW480 cells. (B) PI-stained cells were analyzed for cell cycle phase distribution as detailed in Materials and Methods. The cell number data shown are mean ± SD of three independent plates; each sample was counted in duplicate. Effect of PA on cell cycle distribution and cell cycle regulators in human CRC SW480 cells. Data was analyzed using one-way ANOVA Dunnett’s test. *, P<0.05, significantly different compared with corresponding control.
Figure 2: Effects of butyric acid (BA) on survival of human CRC SW480 cells. (A) For the studies assessing the effect of BA on survival of exponentially growing SW480 cells, $10^5$ cells were plated in 60-mm dishes and next day, treated with vehicle (distilled water) alone or 1-5 mM of BA in fresh medium. After 6, 12 and 24 h of these treatments, viable cells were counted using trypan blue staining and hemocytometer. Represents the viable cell number for SW480 cells. (B) PI-stained cells were analyzed for cell cycle phase distribution as detailed in Materials and Methods. The cell number data shown are mean ± SD of three independent plates; each sample was counted in duplicate. Effect of BA on cell cycle distribution and cell cycle regulators in human CRC SW480 cells. Data was analyzed using one-way ANOVA Dunnett's test. *, P<0.05, significantly different compared with corresponding control.
Figure 3: Effects of butyric acid (BA) on survival of human CRC HCT116 cells. (A) For the studies assessing the effect of BA on survival of exponentially growing HCT116 cells, 10^5 cells were plated in 60-mm dishes and next day, treated with vehicle (distilled water) alone or 1-5 mM of BA in fresh medium. After 6, 12 and 24 h of these treatments, viable cells were counted using trypan blue staining and hemocytometer. Represents the viable cell number for HCT116 cells. (B) PI-stained cells were analyzed for cell cycle phase distribution as detailed in Materials and Methods. The cell number data shown are mean ± SD of three independent plates; each sample was counted in duplicate. Effect of BA on cell cycle distribution and cell cycle regulators in human CRC HCT116 cells. Data was analyzed using one-way ANOVA Dunnett’s test. *, P<0.05, significantly different compared with corresponding control.
Figure 4: Effects of butyric acid (BA) on survival of human CRC Colo320DM cells. (A) For the studies assessing the effect of BA on survival of exponentially growing Colo320DM cells, $10^5$ cells were plated in 60-mm dishes and next day, treated with vehicle (distilled water) alone or 1-5 mM of BA in fresh medium. After 6, 12 and 24 h of these treatments, viable cells were counted using trypan blue staining and hemocytometer. Represents the viable cell number for Colo320DM cells. (B) PI-stained cells were analyzed for cell cycle phase distribution as detailed in Materials and Methods. The cell number data shown are mean ± SD of three independent plates; each sample was counted in duplicate. Effect of BA on cell cycle distribution and cell cycle regulators in human CRC Colo320DM cells. Data was analyzed using one-way ANOVA Dunnett’s test. *, P<0.05, significantly different compared with corresponding control.
Figure 5: Effect of butyric acid (BA) on survivin in human CRC cells. After treatment with butyric acid (BA) for 12 and 24 h, RNA and protein were extracted as detailed in Materials and Methods. (A) Semiquantitative reverse transcription (RT)-PCR and (B) western blot analysis were done for survivin in both the cell lines along with the loading controls, GAPDH and beta-actin, respectively. Band intensity was analyzed by densitometry in each case and was represented as fold change to that of their respective control, and shown at the top of each band (A-B).
**G2/M phase check point regulators**

**Figure 6:** Effect of butyric acid (BA) on cell cycle regulators in human CRC cells. SW480 and HCT116 cells were treated with vehicle (distilled water) or 1, 2.5 and 5 mM concentrations of BA for 6, 12 and 24h. Immunoblotting was done for cyclin B1, CDK1 and Cdc25C using lysates from SW480 and HCT116 cells treated with BA as detailed in Materials and Methods. Membranes were stripped and re-probed with anti-beta actin antibody to ensure equal protein loading. Numbers on top of the bands represent fold changes in protein levels as determined by densitometric analysis of the immunoreactive bands and corrected for beta-actin loading control.
Figure 7: The apoptotic effect of butyric acid (BA) on human CRC cells. SW480 cells (A) and HCT116 cells (B) cells were treated with 1, 2.5 and 5 mM BA for 12 and 24 h. (A-B) At the end of treatments, total cells were collected and stained with annexin V/PI and analyzed for apoptotic cell population as mentioned in Materials and Methods. Data are mean ± SD of triplicate samples in each case. *, P<0.05, significantly different from control by one-way ANOVA followed by Dunnett's test.
Figure 8: Effect of butyric acid (BA) on apoptotic regulators in human CRC cells. SW480 and HCT116 cells were treated with 1, 2.5 and 5 mM BA for 12 and 24 h. Total cell lysates were prepared as described in Materials and Methods. SDS–PAGE and western blot analysis were performed for Bcl-2 and total as well as cleaved PARP. Membranes were stripped and re-probed with anti-beta-actin antibody to ensure equal protein loading. Numbers on top of the bands represent fold changes in band intensity as compared to control as determined by densitometric analysis of the bands and corrected for beta-actin loading control.
Figure 9: Effect of butyric acid (BA) on caspase in human CRC cells. SW480 and HCT116 cells were treated with 1, 2.5 and 5 mM BA for 12 and 24 h. (A) Semiquantitative RT-PCR was done for mRNA levels of Bcl-2 and GAPDH in both cell lines as detailed in Materials and Methods. (B) Effect of BA treatment on cleavage of caspase-3 and its total level were analyzed in both the cells at 24 h using specific antibody by SDS–PAGE and western blot analysis and membrane was stripped and re-probed with anti-beta-actin. Numbers on top of the bands represent fold changes in band intensity as compared to control as determined by densitometric analysis of the bands and corrected for beta-actin or GAPDH loading control for western blot or PCR band, respectively.
Figure 10: Effect of butyric acid (BA) on caspase dependent apoptosis in human CRC cells. SW480 and HCT116 cells were first either pre-treated for 1 h with 25 μM z-VAD-FMK or left untreated and were then either left untreated again or were treated with 2.5 mM BA for 24 h. (A) Then cells were stained with annexin V and PI and analyzed for number of apoptotic cells by flow cytometric analysis. Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different as compared with control by one-way ANOVA followed by Bonferroni’s multiple comparison test. (B) In similar treatments as detailed above, SDS–PAGE and western blot analysis were performed for cleaved-caspase-3, and membranes were stripped and reprobed with anti-beta-actin to ensure equal protein loading. Numbers on top of the bands represent fold changes in band intensity as compared to control as determined by densitometric analysis of the bands and corrected for beta-actin.
**Figure 11:** Effect of butyric acid (BA) mitochondrial mass in human CRC cells. Cells were treated with 1, 2.5 and 5 mM BA for 12 h and 24 h. At the end of treatments, total cells were collected and stained with MitoTracker Red CMXRos and analyzed by flow cytometry as mentioned in Materials and Methods. (A) Representative histograms, (B) Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by one-way ANOVA followed by Dunnett's test.
Figure 12: Effect of butyric acid (BA) on mitochondrial Superoxide generation in human CRC cells. Cells were treated with 2.5 mM BA for 24 h and analyzed by flow cytometry for MitoSOX Red fluorescence in both SW480 and HCT116 cells as detailed in Materials and Methods. (A) Representative histograms, (B) Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
Figure 13: Effect of butyric acid (BA) on mitochondrial dynamics and DRP1 in human CRC cells. (A) SW480 cells were treated with 2.5 BA for 12 h. At the end of treatments, cells were stained with MitoTracker Red CMXRos and visualize under confocal microscope as mentioned in Materials and Methods. (B) SW480 and HCT116 cells were treated with 1, 2.5 and 5 mM BA for 12 and 24 h, and cell lysates were analyzed by SDS-PAGE and western blot analysis for DRP1 levels. Membranes were stripped and reprobed with anti-beta-actin. (C) Cell lysate from SW480 cells treated with 1, 2.5 and 5 mM BA for 12 h was analyzed for phospho-DRP1(serine 616) and MFN2 using specific antibody through western blotting.
Figure 14: Effect of butyric acid (BA) on DRP1 localization in human CRC cells. (A) Mitochondrial and cytosolic fractions for control and 2.5 mM BA treated SW480 cells for 12 and 24 h were prepared as described in Materials and Methods. SDS–PAGE and western blot analysis were performed for DRP1 and membrane was stripped and reprobed with anti-beta-actin. (B) Effect of BA treatment on cyclin B1-CDK1 complex was analyzed in SW480 cells by co-immunoprecipitation with anti-cyclin- B1 followed by immunoblotting for CDK1 and cyclin B1 as described in Materials and Methods. (C) Effect of pan-caspase inhibitor and BA on DRP1 expression in SW480 cells was analyzed by immunoblotting. Membrane was probed with anti-DRP1 and anti-beta-actin. Numbers on top of the bands represent fold changes in band intensity as compared to control as determined by densitometric analysis of the bands and corrected for beta-actin.
Figure 15: Effect of butyric acid (BA) on DRP1 and p53 interaction in human CRC cells. (A) Effect of BA treatment on DRP1-p53 complex was analyzed in SW480 cells by co-immunoprecipitation with anti-p53 followed by immunoblotting for DRP1 and p53 as described in Materials and Methods. (B) Effect of IL1β induction (as a positive control) on DRP1 and p53 interaction in human CRC cells. Effect of IL1β induction on DRP1-p53 complex was analyzed in 24 h starved SW480 cells by co-immunoprecipitation as described in Materials and Methods.
Figure 16: Human CRC HCT116 cell were transduced with pLVX-puro empty vector and pLVX-puro vector containing full length DRP1 cDNA sequence. Stable cell line was created via puromycin resistance screening as described in Materials and Methods. (A) Microscopic observation of stable cell lines containing pLVX-puro Empty vector (Empty vector) and pLVX-puro DRP1 vector (DRP1) representing their morphology. (B) Effect transduction on DRP1 level were analyzed in both transduced cells at 12 h using specific antibody by SDS–PAGE and western blot analysis and membrane was stripped and re-probed with anti-beta-actin. Numbers on top of the bands represent fold changes in band intensity as compared to control as determined by densitometric analysis of the bands and corrected for beta-actin loading control for western blot. (C) Confirmation of stable cells for DRP1 cDNA insertion in genome of the cells. Whole genomes were isolated from both of the stable cells and interrogated through PCR for DRP1 as described in Materials and Methods.
Figure 17: Effect of butyric acid (BA) treatment on colony forming ability of Empty vector and DRP1 transduced HCT116 cells. Cells were treated with 1 mM BA and incubated for 7 days and then number of colonies were counted. (A) Representative figure for single well of each treatment of the triplicates showing density of colonies per well. (C) Quantitative data for transduced cells colony count are shown. Data is represented as total number of colonies and number of colonies having more than 50 cells.
Figure 18: Effects of butyric acid (BA) on survival of DRP1 transduced HCT116 and SW480 cells. For the studies assessing the effect of BA on survival of exponentially growing cell, 10^5 cells were plated in 60-mm dishes and next day, treated with vehicle (distilled water) alone or 1 mM of BA in fresh medium. After 12 h of treatment, viable cells were counted using trypan blue staining and hemocytometer. The cell number data shown was mean ± SD of three independent plates; each sample was counted in duplicate. Data was analyzed using student’s t-test. *, P<0.05, significantly different compared with corresponding control. (A) Represents the viable cell number and dead cells for HCT116 cells and (B) for SW480 cells.
Figure 19: Effects of butyric acid (BA) on cell cycle progression of DRP1 transduced HCT116 and SW480 cells. For the studies assessing the effect of BA on survival of exponentially growing cell, 10^5 cells were plated in 60-mm dishes and next day, treated with vehicle (distilled water) alone or 1 mM of BA in fresh medium. After 12 h of treatment, cell were collected and stained with PI followed by FACS analysis for cell cycle phase distribution as detailed in Materials and Methods. The cell number data shown was mean ± SD of three independent plates; each sample was counted in duplicate. Data was analyzed using one-way ANOVA Dunnett’s test and student t-test. *, P<0.05, significantly different compared with corresponding control. (A) Represents the viable cell number and dead cells for HCT116 cells and (B) for SW480 cells.
Figure 20: Effects of butyric acid (BA) on cell survival markers of DRP1 transduced HCT116. Cells were treated with vehicle (distilled water) or 1mM concentrations of BA for 12 h. Immunoblotting for cyclin D1, CDK2, CDK6, p53, p21, Akt, Bax, p-ERK1/2 and Erk1/2 using lysates from transduced HCT116 cells treated with BA as detailed in Materials and Methods. Membranes were stripped and re-probed with anti-beta actin antibody to ensure equal protein loading. Numbers on top of the bands represent changes in protein levels as determined by densitometric analysis of the immunoreactive bands and corrected for beta-actin loading control.
Figure 21: Effect of butyric acid (BA) on mitochondrial Superoxide generation and ROS in DRP1 transduced HCT116 cells. (A) Cells were treated with 1 mM BA for 24 h and analyzed by confocal microscope for MitoSOX Red fluorescence in both transduced cells as detailed in Materials and Methods. (B) Cells were treated with 1 mM BA for 24 and 48 h and analyzed by Flow cytometer for MitoSOX Red fluorescence in both transduced cells as detailed in Materials and Methods. (C) Cells were treated with 1 mM BA for 24 and 48 h and analyzed by multimode plate reader for DCF-DA fluorescence in both transduced cells as detailed in Materials and Methods. Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
Figure 22: Effect of butyric acid (BA) on ROS production and cell viability of DRP1 transduced HCT116 cells. (A) Cells were treated with 1 mM BA or 5 mM NAC (6h pre-treatment) or in combination for 12 and 24 h and analyzed by for DCF-DA fluorescence in both transduced cells as detailed in Materials and Methods. (B) Cells were treated with 1 mM BA or 5 mM NAC or in combination for 12 and 24 h and analyzed for cell viability viz MTT assay as detailed in Materials and Methods. Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
Cell cycle analysis of NAC pretreated HCT116 cells

Figure 23: Effect of butyric acid (BA) and ROS on cell cycle distribution of DRP1 transduced HCT116 cells. Cells were treated with 1 mM BA or 5 mM NAC (6h pre-treatment) or in combination for 12 h and stained with PI and analyzed for cell cycle distribution using flow cytometer in both transduced cells as detailed in ‘Materials and Methods’. Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
(A) Comparative analysis of DRP1 expression in different CRC cells

![Image](image.png)

**Cell Lines**
1 - CaCo-2
2 - HCT116
3 - SW480
4 - Colo320DM

(B) Cell cycle analysis of Colo320DM in combination of Mdivi-1

![Image](image.png)

**Figure 24:** Comparative analysis of DRP1 expression in CRC cell lines and effect of DRP1 level on cell cycle progression. (A) Different CRC cell lines were plated for 24 h and cell lysate was collected. Immunoblotting for DRP1 and β-actin using lysates was performed as detailed in Materials and Methods. (B) Cells were treated with 1 mM BA or 10 µM Mdivi-1 (M, DRP1 inhibitor) or in combination (M + B) for 24 h and stained with PI and analyzed for cell cycle distribution using flow cytometer in both transduced cells as detailed in Materials and Methods. Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
Figure 25: Effect of butyric acid (BA) on autophagic cell death of DRP1 transduced HCT116 cells. Cells were treated with 1 mM BA for 12 h and (A-B) stained with acridine orange and analyzed for Fluoresce of it using flow cytometer in both transduced cells as detailed in Materials and Methods. (A) Representative histogram, (B) Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test. (C) Cells were treated with vehicle (distilled water) or 1mM concentrations of BA for 12 h. Immunoblotting for LC3A/B (I & II) and Beclin-1 using lysates as detailed in Materials and Methods. Membranes were stripped and re-probed with anti-beta actin antibody to ensure equal protein loading. Numbers on top of the bands represent changes in protein levels as determined by densitometric analysis of the immunoreactive bands and corrected for beta-actin loading control.
Figure 26: Effect of butyric acid (BA) on EMT markers and regulators in human CRC cells. SW480 and HCT116 cells were treated with vehicle (distilled water) or 1, 2.5 and 5 mM concentrations of BA for 6, 12 and 24 h. Immunoblotting for E-cadherin, Vimentin, Snail and Slug using lysates from SW480 and HCT116 cells treated with BA as detailed in Materials and Methods. Membranes were stripped and re-probed with anti-beta actin antibody to ensure equal protein loading. Numbers on top of the bands represent changes in protein levels as determined by densitometric analysis of the immunoreactive bands and corrected for beta-actin loading control.
Figure 27: Effect of butyric acid (BA) on cell migration of DRP1 transduced HCT116 and SW480 cells. Representative images of invaded transduced HCT116 cells and quantitation of transduced HCT116 and SW480 cells migration after 12 h of 1mM BA treatment, followed for migration for 12 h Boyden chambers respectively. Five independent areas were scored in each sample and data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
**Figure 28:** Effect of butyric acid (BA) on cell invasion of DRP1 transduced HCT116 and SW480 cells. Representative images of invaded transduced HCT116 cells and quantitation of transduced HCT116 and SW480 cells invasion after 18 h of 1mM BA treatment, followed for invasion for 18 h on matrigel-coated (invasion) Boyden chambers respectively. Five independent areas were scored in each sample and data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
**Figure 29**: Effect of butyric acid (BA) on EMT markers and regulators of DRP1 transduced HCT116 cells. Cells were treated with vehicle (distilled water) or 1mM concentrations of BA for 12 h. (A) MMP-2 activity in media of transduced HCT116 cell was measured after 12 h of treatment as described in Materials and Methods. (B) Immunoblotting for E-cadherin, Vimentin, uPA, β-Catenin, MMP-2 Snail and Slug using lysates from transduced HCT116 cells treated with BA as detailed in Materials and Methods. Membranes were stripped and re-probed with anti-beta actin antibody to ensure equal protein loading. Numbers on top of the bands represent changes in protein levels as determined by densitometric analysis of the immunoreactive bands and corrected for beta-actin loading control.
(A) Cycloheximide (CHX) chase assay (WB)

Figure 30: Effect of butyric acid (BA) on protein stability of SNAIL in DRP1 transduced HCT116 cells. (A) Cells were treated with CHX (50 µg/ml) and BA for the indicated hours, and Western blotting (WB) was performed. (B) the level of remaining Drp1 at different time points was quantified as the percentage of initial DRP1 level (0 hour of CHX treatment) using ImageJ software.
Figure 31: Effect of butyric acid (BA) on Frequency of spheroid formation of DRP1 transduced HCT116 cells. (A) Representative images depicting primary (first generation) spheroid after 5 days of treatment BA (100x magnification). (B) Quantitation of first generation (after 5 days) spheroid. The bar graph shows the frequency of spheroid formation. (C) Size of spheroid was measured in pixel using ImageJ software. Five independent areas were scored in each well. (B-C) Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
Figure 32: Effect of butyric acid (BA) on Frequency of spheroid formation of DRP1 transduced HCT116 cells. (A) Quantitation of first generation (after 5 days), second generation (after 5 days), and third generation (after 7 days) spheroids. The bar graph shows the frequency of spheroid formation normalized with control. (B) Cells were treated for 12 h and treatment media was removed from plate and replace with fresh (BA free) media. After desired time point MTT assay was performed as detailed in Materials and Methods. (A-B) Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
Figure 33: Effect of butyric acid (BA) on stemness markers of DRP1 transduced HCT116 cells. (A) Effect of BA treatment on CD44\(^{\text{high}}\)/CD133\(^{\text{high}}\) population relative control. Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test. (B) Immunoblotting for LGR5 and Keratin-8 using lysates from transduced HCT116 cells treated with BA as detailed in Materials and Methods. Membranes were stripped and re-probed with anti-beta actin antibody to ensure equal protein loading. Numbers on top of the bands represent changes in protein levels as determined by densitometric analysis of the immunoreactive bands and corrected for beta-actin loading control.
Figure 34: Effect of butyric acid (BA) on angiogenesis markers of DRP1 transduced HCT116 cells. Cells were treated with vehicle (distilled water) or 1 mM concentrations of BA for 12 h. (A) Immunoblotting for VEGF and HIF1α using lysates from transduced HCT116 cells treated with BA as detailed in Materials and Methods. Membranes were stripped and re-probed with anti-beta actin antibody to ensure equal protein loading. Numbers on top of the bands represent changes in protein levels as determined by densitometric analysis of the immunoreactive bands and corrected for beta-actin loading control. (B) VEGF released in media of transduced HCT116 cell was measured after 12 h of treatment as described in Materials and Methods. Volume of medium loaded was normalized with equal number of live cells for each lane.
Figure 35: Effect of butyric acid (BA) treated DRP1 transduced HCT116 cell’s conditioned media (CM) on HUVEC growth and proliferation. DRP1 transduced HCT116 cells were treated with vehicle (distilled water) or 1 mM concentrations of BA for 12 h and conditioned media was collected in 0.2% serum media as detailed in Materials and Methods. 8000 HUVEC cells were plated in 96 well plate and next day, treated with different CM. Volume of CM mixed (maximum 50% of total volume) with HUVEC cell expansion media with 0.2% serum. CM volume was normalized with equal number of live DRP1 transduced HCT116 cells at the time of collection of conditioned media. Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
**Figure 36:** Effect of butyric acid (BA) treated DRP1 transduced HCT116 cell’s conditioned media (CM) on capillary tube formation by HUVEC on matrigel. DRP1 transduced HCT116 cells were treated with vehicle (distilled water) or 1 mM concentrations of BA for 12 h and conditioned media was collected in 0.2% serum media as detailed in Materials and Methods. 5x10^4 HUVEC cells were plated on matrigel coated 48 well plate and simultaneously treated with different CM. Volume of CM mixed (maximum 50% of total volume) with HUVEC cell expansion media with 0.2% serum. CM volume was normalized with equal number of live DRP1 transduced HCT116 cells at the time of collection of conditioned media. (A) Representative images depicting formation of capillary tubes on matrigel by HUVEC after 6 h treatment with CM. (B) Quantitative depiction of capillary tube formation after 6 h. Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
Figure 37: Effect of butyric acid (BA) treated DRP1 transduced HCT116 cell’s conditioned media (CM) on Migration of HUVEC cells. DRP1 transduced HCT116 cells were treated with vehicle (distilled water) or 1 mM concentrations of BA for 16 h and conditioned media was collected in 0.2% serum media as detailed in Materials and Methods. 5x10^4 HUVEC cells were seeded on upper Boyden chamber and in lover chamber different CM containing media was added. Cells were allow to migrate for 16 h. Volume of CM mixed (maximum 50% of total volume) with HUVEC cell expansion media with 0.2% serum. CM volume was normalized with equal number of live DRP1 transduced HCT116 cells at the time of collection of conditioned media. (A) Representative images effect of CM on HUVEC migration in Boyden chambers after 16 h treatment. (B) Quantitative depiction of migrated cells after 16 h. Data are shown as mean ± SD of triplicate samples. *, P<0.05, significantly different compared with respective controls by Student t-test.
**Figure 38:** Effect of butyric acid (BA) treated DRP1 transduced HCT116 cell’s conditioned media (CM) on PCNA and N-cadherin expiration in HUVEC cells. DRP1 transduced HCT116 cells were treated with vehicle (distilled water) or 1 mM concentrations of BA for 16 h and conditioned media was collected in 0.2% serum media as detailed in Materials and Methods. Immunoblotting for PCNA and N-cadherin using lysates from HUVEC cells treated with CM as detailed in Materials and Methods. Membranes were stripped and re-probed with anti-beta actin antibody to ensure equal protein loading. Numbers on top of the bands represent changes in protein levels as determined by densitometric analysis of the immunoreactive bands and corrected for beta-actin loading control. CM volume was normalized with equal number of live DRP1 transduced HCT116 cells at the time of collection of conditioned media.

Blank: 0.2% serum media without growth factors
Table 1: All Cancers (excluding non-melanoma skin cancer) incidence, mortality and 5-year prevalence worldwide in 2012, Summary.

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<td>537.5</td>
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<td></td>
<td></td>
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<td>of skin</td>
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<td>(EU-28)</td>
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<td>2205.9</td>
<td>Lung, Stomach, Liver, Colorectum, Oesophagus</td>
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</table>

Table shows the incidence and mortality of all cancers (excluding non-melanoma skin cancer) in different geographical regions of the world. It is clear that less developed countries like India have lower incidence rates than developed ones like United States; however, death rates are higher. Colorectal cancer is among top five most frequent cancers. Numbers indicated are in thousands. (Source: GLOBOCAN-2012, Ferlay et al., 2015)
<table>
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<th></th>
<th>5-year prevalence</th>
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<td>Female</td>
<td>Total</td>
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<td>Female</td>
<td>Total</td>
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<td>----</td>
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<tr>
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<td>356730</td>
<td>326100</td>
<td>682830</td>
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</table>

Table shows the incidence and mortality of all cancers (excluding non-melanoma skin cancer) in India.
(Source: GLOBOCAN-2012, Ferlay et al., 2015)
Table 3: Colorectal cancer incidence mortality and prevalence worldwide in 2012.

<table>
<thead>
<tr>
<th>Regions</th>
<th>Incidence</th>
<th></th>
<th>Mortality</th>
<th></th>
<th>5-year prevalence</th>
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<td></td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
<td>Male</td>
<td>Female</td>
</tr>
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<td>737</td>
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<td>107</td>
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<td>79</td>
<td>60</td>
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</tbody>
</table>

Table shows the incidence and mortality rates of colorectal cancer in various regions of the world based on economic developments. Five-year prevalence is also indicated against respective region. It is clear that India has lower incidence rates than developed ones like United States and European Union. Numbers indicated are in thousands. (Source: GLOBOCAN-2012, Ferlay et al., 2015)