Chapter 1: Introduction

1. Introduction

1.1. Colorectal Cancer (CRC)

1.1.1. Epidemiology, Incidence, Mortality Rate

Colorectal cancer (CRC), a major cause of morbidity and mortality throughout the world, accounts for 9% of all cancer incidence and is the third most common cancer occurring in men and second most common cancer occurring in women. According to World Health Organization, there are 1.8 million new cases and 861,000 deaths due to CRC happening worldwide in 2018 (Macrae, 2018).

Regional incidence of CRC varies up to 10-fold, where historically the highest incidence rates are found in North America, Europe, Australia, and New Zealand, and the lowest rates are found in South-Central Asia and Africa (Fitzmaurice, 2017). These differences within geographical distribution are owed to the differences in diet and other environmental conditions imposed upon variant of backgrounds and genetically determined suceptibility (Macrae, 2018).

Unfortunately, however, the countries with historically low rates of CRC have recently experiencing increased risk, causing the greatest concern (Torre, 2016). This is due to low socioeconomic status (SES) countries transitioning into high-income, westernized lifestyle contributing to the increase risk of the disease, such as physical inactivity, unhealthy diet, smoking, and obesity (Arnold, 2016). Other factor, such as lower rates of CRC screening, also contribute substantively outside the lifestyle to SES differences in CRC risk (Klabunde, 2011). In addition to SES lifestyle and low rates of CRC screening, high blood levels of insulin, gastrointestinal inflammation, and certain meat cooking methods may contribute to the increase risk of colorectal carcinogenesis (Harris, 2016).

Current trends in mortality statistics from several developing countries are promising, despite the difficulty to interpret temporal changes in mortality as they are greatly influenced by trends in between incidence and survival. Incidence rate is more appropriate to indicate disease occurrence than survival rate. CRC incidence is unaffected by changes in treatment and survival, but it has been shown to be influenced by increase screening programs and improved diagnostic techniques (Haggar, 2009).

1.1.2. Causes of CRC

There are several risk factors associated to the incidence of CRC, categorized into environmental risk factors and nonmodifiable risk factors. Environmental risk factors covers a wide range of ill-defined cultural, social, and lifestyle factors such as nutritional practices, physical activity and obesity, tobacco smoking, heavy alcohol consumption. As of nonmodifiable risk factors, one of the established risks that accounts to 5-10% of CRC incidence is inherited genetic risk, with the most common inherited conditions are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) (Haggar, 2009). While it is generally understood that FAP is initiated by the inactivation of body's tumor suppressor gene called adenomatous polyposis coli (APC) (Bienz, 2000), the initiating event(s) for mixed hyperplastic/serrated adenomatous polyposis-carcinoma sequence for colorectal cancer still remains unclear.

Hereditary mixed polyposis syndrome (HMPS) is a condition characterized with the development of colonic polyps of mixed types (both FAP and HNPCC) that eventually develops into CRC, which is why it makes an ideal model to study the underlying genetic basis of mixed polyposis-carcinoma sequence for both hereditary and sporadic CRC (Cao, 2006). A polyp is a lump forming from the growth of normal tissue and people with HMPS typically have higher number of

precancerous polyps in their digestive tracts. The cause of HMPS is due to the inherited mutation of specific gene called *GREM1*, where it leads to overexpression of protein Grem1 (ASCO, 2017).

1.1.3. Colorectal (CRC) stages

CRC staging has had a long evolution dating back to 1926 when Lockhart-Mummery proposed a system where lymph node positivity detected in specimens removed during surgery and depth of invation were classified as important prognostic factors (Lockhart-Mummery, 1926).

Six years later, Dukes stated that in CRC's earliest stages, it begins as an epithelial proliferation that rises from the surface, whereas carcinoma develops from a previously existing adenoma. Cancer then metastasizes through the bowel wall to the lymphatics, and the following is where classes begins to appear. Cases where carcinoma is only limited to the wall of the rectum is classified as A. Those that has spread to the extrarectal tissue are classified as B. Cases which metastases are present in regional lymph nodes are called C (figure 1) (Dukes, 1932).

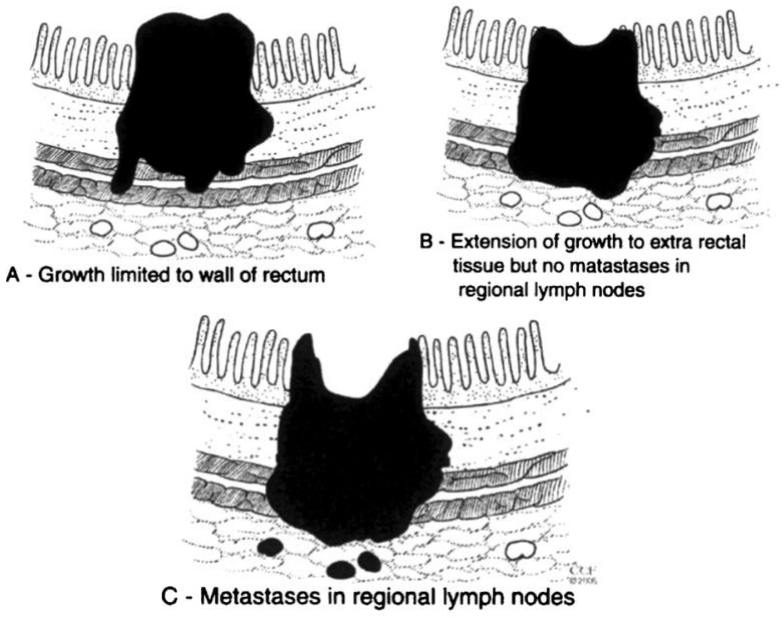

Fig 1. Rectal cancer staging system proposed by Dukes in 1932. From the Cleveland Clinic Foundation, Cleveland, Ohio.

In 1949, Kirklin, Dockerty, and Waugh proposed another modified system from Dukes' classification. Despite preserving the A, B, C framework, they added subscript designations to B (for lesions), where "1" is for lesions that have extended into, but not through the muscularis propria and "2" for tumors that have penetrated muscularis propria (Kopelson, 1983).

The next 5 years, Astler and Coller proposed the next modified system that is based on Dukes' classification as modified by Kirklin et al. The classification list is named as Astler-Coller (MAC) classification, based on reports of specimens from the rectum and colon removed post surgery (Wu, 2007).

Turnbull et al. (1967) would then assign stage D to identify tumors that metastasized to the lung, parietes, bones, liver, and adjacent organs.

In 1987, the three main stages established by the American Joint Committee on Cancer (AJCC) are the invasion (tumor), the presence and number of nodal metastases (node), and the presence of distant metastases (metastases) which in join is abbreviated as TNM and it is widely used today to predict the prognosis for CRC patients, guide adjuvant therapy after curative surgery, and classify patients for clinical trials (Li, 2016). Each stages has substages assigned for advanced prognosis on colorectal patients (figure 2).

Tumor, Node, Metastases (TNM) Classification of Colorectal Cancer

T-Primary tumor
Tx Primary tumor cannot be assessed
T0 No evidence of primary tumor
Tis Carcinoma in situ: intraepithelial or invasion of lamina propria
T1 Tumor invades submucosa
T2 Tumor invades muscularis propria
T3 Tumor invades through muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues
T4 Tumor directly invades other organs or structures and/or perforates visceral peritoneum
N-Regional lymph nodes
NX Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Metastasis in one to three regional lymph nodes
N2 Metastasis in four or more regional lymph nodes
M-Distant metastasis
MX Distant metastasis cannot be assessed
M0 No distant metastasis
M1 Distant metastasis

Fig 2. Tumor, Node, Metastases (TNM) classification of colorectal cancer along with its substages, proposed in 1987 by American Joint Committee on Cancer.

1.1.4. Intestinal Stem Cell Biology: Colonic Crypt Organisation

One of the fastest self-regeneration process found in mammals happen in the intestinal epithelium, which can undergo self-renewal every 2-3 days in mice and 3-5 days in human. This mono layer of cells is responsible for both absorption of nutrients and water as well as a formation of protective sheet-like cells that prevents invasion of pathogens and other harmful substances to penetrate in and invade the lamina propria. These cells are not proliferating on their own, but

instead are progeny of highly proliferative immature cells that can be found in the small pits lining the intestinal tract, which often called the crypts of Leberkühn (figure 3).

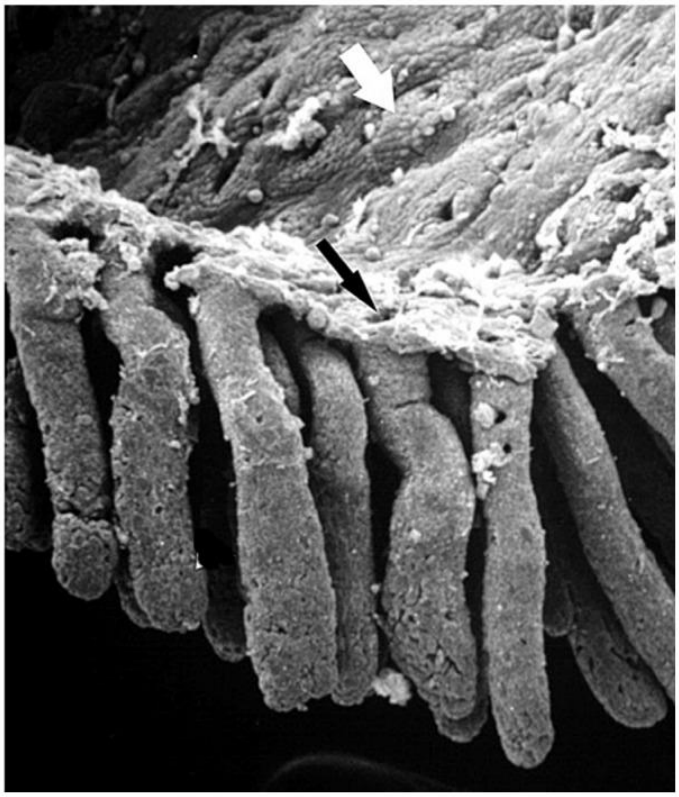

Fig 3. An electron micrograph scan of colonic crypt finger-like protrusions in the mouse (120x magnification). White arrow shows flat luminal surface of the colon with no villi and black arrow indicates the orifice of the crypt lumen. Picture taken from a paper by Magney et al. (1986).

They have to continuously replace old and damaged cells to sustain function. The renewal is driven by stem cells that upon activation can generate transit amplifying cells, the proliferating progeny. In a picture (figure 4) taken from a paper by Mittal et al. (2009), stem cells and transit amplifying cells reside in a region near the base of the crypt. When they migrate out from their niche cells, they stop proliferating and begin differentiating into different cell lineages, resulting into adult villus.

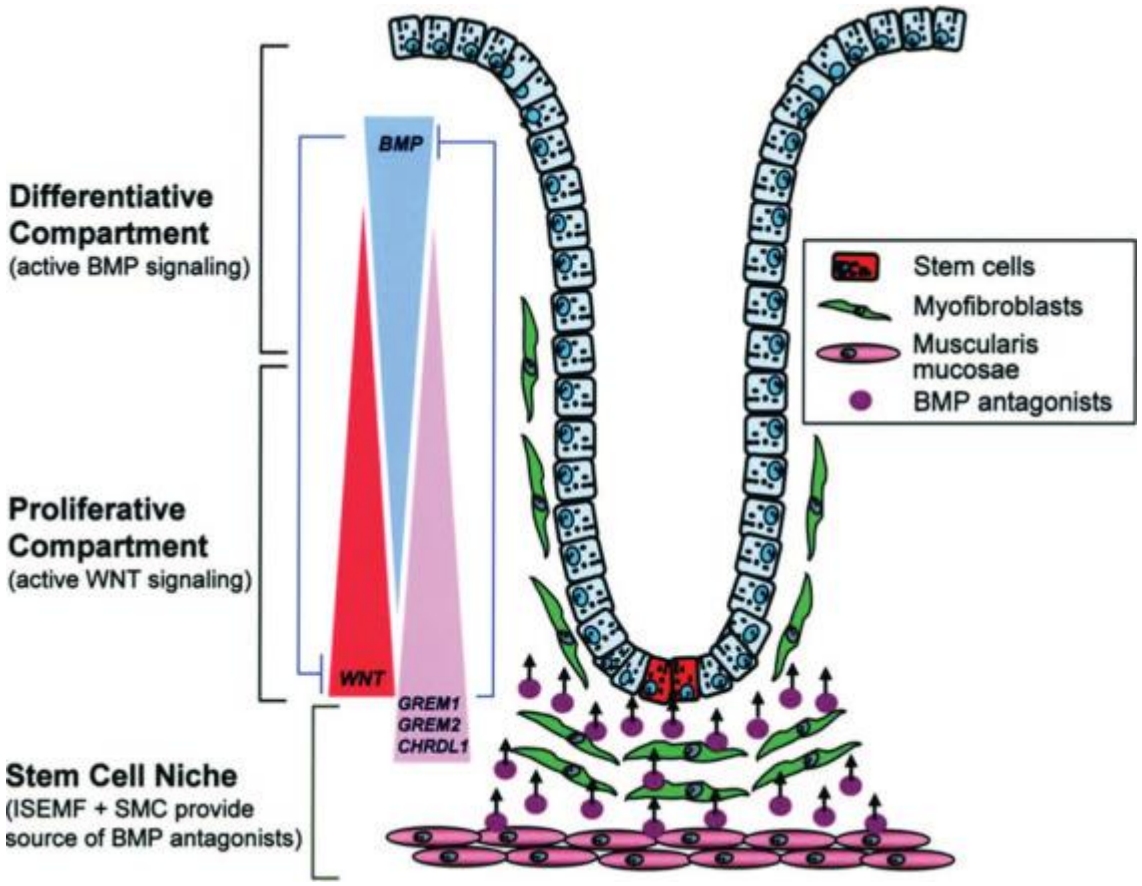

Fig 4. Stem cell niche of human colon. Bone Morphogenetic Protein signaling becomes more active as cell moves up the colonic crypt whereas Grem1 signaling is more active at the base of the crypt to promote proliferation and inhibit differentiation. Taken from a paper by Mittal et al. (2009)

Regeneration and remodeling of intestinal epithelium depends on epithelial-mesenchymal crosstalk. Wnt, bone morphogenetic protein (BMP), and hedgehog (Hh) signaling regulates the information flow between epithelium and mesenchymal cells of the niche and the niche in the small intestine and colon is formed by subepithelial sheath of activated fibroblasts known as myofibroblasts. BMP signaling works antagonistically with crypt proliferation as it has a function of a negative regulator for that area. When Gremlin-1 (Grem1) is overexpressed, proliferation occurs and expression of BMP in the subepithelium is regulated by hedgehog signaling from the epithelium to the mesenchyme to ensure proper spacing between the crypts.

The master switch between proliferation and differentiation in the epithelial cells is constituted by Wnt pathway, and opposing gradients of Wnt and BMP signals are present along the crypt axis with Wnt signal being the strongest at the base of the crypt. All in all, these signals work in tandem to generate distinct features of stem cells including self-regeneration and proliferation. Deregulation of the signals can result to a diseased state, such as malignancy (Mittal, 2009).

1.1.5. CRC screening and treatment

Beck stated in his paper, The Importance of Colorectal Cancer Screening (2015), that CRC is preventable by early screening. Screening is seen as a preventable step, which in many ways better than treating the cancer itself through surgery if compliance rates are high and the cost of screening is reasonable. At the moment, the screening rate is not adequate and there are several factors that may explain this. The Centres for Disease Control recommends colonoscopy as the gold standard for CRC screening method because it allows physician to view entire colon and treat polyps and this requires bowel preparation, which includes cleaning the colon prior to colonoscopy. Some ways to do this are traditional lavage preparation and low-volume lavage preparations. Other factor is economic issue, as in some areas, health plans are still under developed and access to healthcare may still be limited. Another method would be computed tomography (CT) colonography, which is targeted mostly for patients with coagulation issues. It is accurate in detecting significant lesions but the hindering factors are still the same as mentioned above.

Based on a medical journal by Campos (2014), prophylactic surgery is the main FAP management. Several factors should be considered, however, before proceeding with the process. Timing of surgery is needed to be planned based on patient's condition, as well as their preferences. Adenoma-associated symptoms is also a crucial factor that encourages patient to prompty take the surgery as soon as possible, along with the identification of specific endoscopic

and histological features (like the presence of adenomas) during screening. Next, age is also a significant factor as it is observed previously that the mean age of CRC diagnosis and mortality have been reported to be 39 and 42 years, respectively (Bussey, 1979).

Aside of the preventable method by screening and surgerical treatment approach, another treatment would be chemotherapy. Campos stated that in inherited cancer syndrome, management can be accomplished by layer treatments including genetic counseling and screening, chemoprevention, prophylactic surgery and lifetime surveillance, whereas Kim Junghan (2015) elaborated in his paper on how to administer CRC patients, especially elderly ones, with the correct chemotherapy administration. As for now, adjuvant therapy seems to be the best choice within younger and eldery patient. The standard drug used is leucovori(LV)-modulated 5-fluorouracil (5-FU/LV). Its benefit is in terms of progression-free survival (PFS), disease-free survival (DFS), and overal survival (OS) and study shows that benefit of adjuvant chemotherapy with 5-FU/LV did not diminish with chronologic age. Despite its benefits, there is the risk of toxicity especially to elderly patient. To combat toxicity due to combinational therapy, monotherapy and stop-and-go strategy can be implemented instead. In conclusion, treatments for colon cancer are either taken from preventable angle by early screening or direct removal approach by surgery. Chemotherapy is an alternative treatment that requires no surgery but may pose risk such as toxicity. A proposed treatment would be target therapy, which is an efficient chemotherapy that targets the source of problem to inhibit the colon cancer progression with minimal posed risk. However, understanding pathophysiology of colon cancer, especially inherited cancer syndrome, and the role of gremlin1 along with bone morphogenetic protein in colon cancer progression is still under huge study, which is why working out on how gremlin1 is driving colon cancer cell growth will give new insight on how to target grem1 to reduce its growth in future chemotherapy strategies for patients.

1.2. Bone Morphogenetic Proteins (BMP)

1.2.1. Overview of BMP

First Bone Morphogenetic Protein (BMP) was discovered by an orthopaedic surgeon named Marshall Urist from University of California, Los Angeles, in 1960. These proteins were shown triggering the formation of bone and cartilage from mesenchymal stem cells in culture. Ever since, more than 22 members of BMP family have been identified, along with smaller set of plasma membrane receptors and Smad 1/5/8 proteins. Today, BMP signalling is known more than just regulating bone and cartilage formation. BMPs are secreted extracellular matrix (ECM)-associated proteins that regulates a broad range of developmental processes. It is involved in diverse biological processes combined with its antagonist in stem cell and organ formation, even cancer. Its known antagonists, such as Noggin, Chordin, Gremlin (Grem1), and twisted gastrulation-1 (Twsg1) have been shown to inhibit BMP action in several developmental stage-specific contexts (figure 5) (Brazil, 2014).

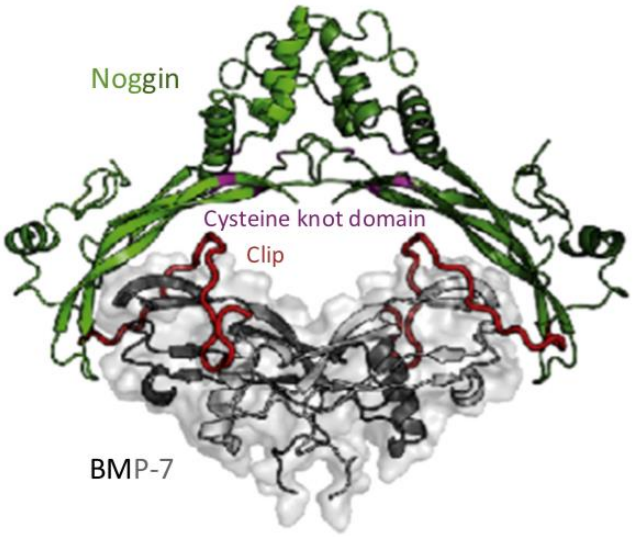

Fig 5. Structure of bone morphogenetic protein (BMP) and BMP antagonist Noggin. Taken from paper by Brazil et al. (2014)

1.2.2. Regulation of BMP signaling

BMPs are secreted members of the TGFβ family of signaling molecules and with their antagonists, they are associated with ECM as their glycosylation is likely to affect their interaction with the ECM and their function. BMP signaling is mediated through type I and type II serine/threonine kinase receptor (Karagiannis, 2015). Some BMP ligands bind to BMP type I receptors, which will then dimerize to BMP type II receptor and phosphorylate the type I receptor in the GS glycine-serine repeat domain. Activated type I receptor will then phosphorylate a set of Smad proteins called receptor Smads (R-Smad 1/5/8), that binds to a nuclear Smad called Smad4. The complex translocates to the nucleus, where it is recruited to transcriptional complexes and mediate BMP-dependent gene transcription (figure 6). Each level of BMP pathway is tightly regulated, hence it is critical to maintain strict control of BMP signaling in cells and tissues (Brazil, 2014).

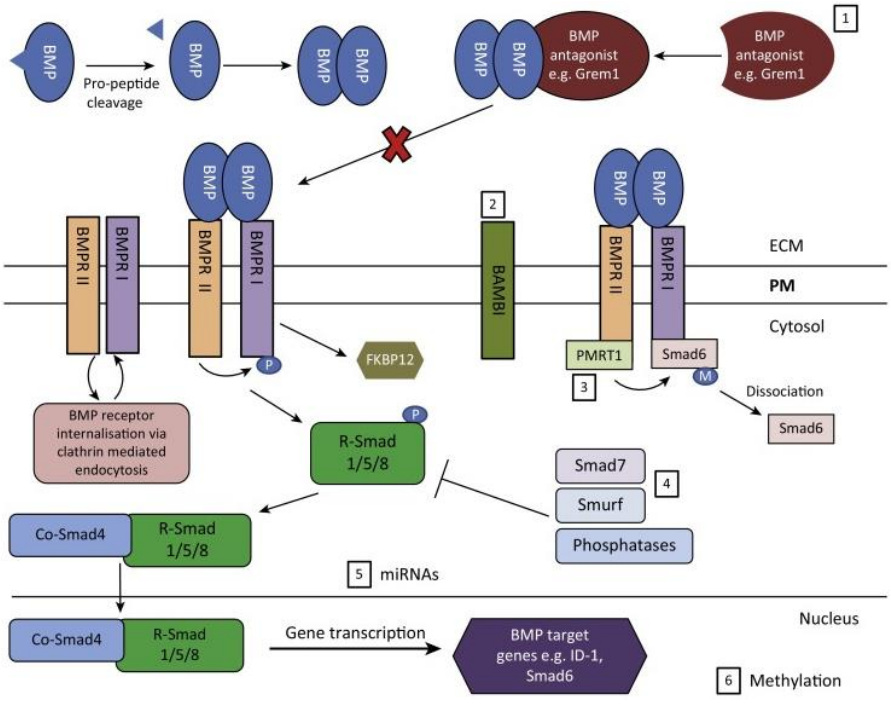

Fig 6. BMP signaling pathway with R-Smads induces formation of heterodimeric complex between R-Smad 1/5/8 and co-Smad that will then translocate to nucleus and regulate transcription of target genes. Brazil et al. (2015).

1.3. Gremlin1

1.3.1. Overview of Gremlin1

Grem1 is a bone morphogenetic protein (BMP) antagonist, a secreted extracellular matrix (ECM)-associated protein (Brazil, 2015). The human gremlin gene (*GREM1*) has been mapped to chromosome 15q13-q15 (Topol, 2000). Grem1 especially antagonizes with BMP2, BMP4, and BMP7 (Sato, 2015) and is a 20.7kDa, 184 amino acid, cysteine knot, transforming growth factor (TGF)-β superfamily protein (Wordinger, 2018) that functions by neutralizing ligands of bone morphogenetic protein (BMP) and inhibiting its signaling (Kisonaite, 2016). Grem1 forms heterodimers with BMP (Kim, 2012), thus it exerts antagonistic effect by preventing BMP to interact with BMP receptors, blocking BMP signaling (shown in figure 6) (Church, 2015). It also plays an important role in regulation of broad range of developmental processes such as stem cell homeostasis, cell lineage determination, normal formation of limb and kidney, neural crest cell differentiation along with dysregulation results in developmental diseases (Sato, 2015). Grem1 is a secreted protein and there are three isoforms have been reported from previous studies: isoform 1 as the most common isoform and isoform 2 and 3 have deletions of amino acids 39-79 and 10-79, respectively (Kim, 2012). Recent studies have demonstrated that Grem1 is overexpressed in various types of human cancers, name cervical cancer cells, endometrium, lung, ovary, kidney, breast, pancreas, and colorectal cancer cells (Karagiannis, 2015). A study done by Kim et al. (2012) stated that Grem1 binds to cancer cell lines A549, A172, A431, and HeLa. While some work of Grem1's role in several aspects are beginning to shed light on its importance, its role in carcinogenesis is yet to be studied in detail.

1.3.2. Gremlin1 in Colorectal Cancer (CRC)

Hereditary mixed polyposis syndrome (HMPS) is a condition characterized with the development of colonic polyps of mixed types that eventually develops into CRC, which is why it

makes an ideal model to study the underlying genetic basis of mixed polyposis-carcinoma sequence for both hereditary and sporadic CRC (Cao, 2006). Based on a paper written by Lieberman et al. (2017), it originally came from Ashkenazi Jewish (AJ) family whose members had multiple polyps of more than one histological type and/or individual polyps with overlapping histological features. The cause to this was a 40kb duplication upstream of GREM1 (Grem1 gene), resulting to increased and ectopic expression of GREM1 in colonic mucosa. Excess Grem1 suppresses Bone Morphogenetic Protein (BMP), which in mouse models showed epithelial cells to retain stem-cell like properties, form ectopic crypts, leading to neoplastic and ultimately cancerous state. Another report stated in the paper shows a patient with early onset colorectal cancer has a large duplication encompassing the entire GREM1. A targeted genotyping of the 40kb duplication was undertaken in 184 AJ patients to asses contribution of GREM1 duplication in CRC, and the result showed 142 had familial CRC and the remaining 42 patients had colonic polyps. GREM1 duplication was identified in a patient that was diagnosed with metachronous CRC.

2. Aim & Hypothesis

2.1. Aim

To identify Gremlin1's implication in colorectal cancer cells by understanding its expression in cancer cells and localization in intestinal tissue.

2.2. Hypothesis

Grem1 is able to bind and expressed by HeLa cells.

Grem1 is capable to induce cell proliferation on HeLa cells, which results to increase of cell viability.

Areas in the mouse intestine high with Grem1 will have low level of pSmad 1/5 and *vice versa*.