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Targeting M3 Muscarinic Receptors for Colon Cancer Therapy

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Abstract

Background: Expression and activation of subtype-3 muscarinic receptors (M3R) plays an important role in the progression of colorectal neoplasia.

Methods: Herein, we describe the role of muscarinic receptors in colon cancer, focusing specifically on M3R, illustrate how M3R over-expression and activation of post - receptor signaling pathways potentiates tumor progression, and explore the efficacy and safety of a variety of therapeutic approaches that can target the molecules involved.

Results: Colon cancers overexpress M3R mRNA (*CHRM3*) and protein, and post-M3R signaling stimulates cell proliferation. Post-M3R signal transduction is complex, involving interplay between epidermal growth factor receptors (EGFR)/ERK and protein kinase C (PKC)/p38 mitogen-activated protein (MAP) kinase signaling pathways. In particular, the development of an invasive and metastatic phenotype requires that these signaling interactions augment cellular release of a key collagenase, matrix metalloproteinase-1 (MMP1). Blocking either M3R activation or post-M3R signaling attenuates MMP1 release and colon cancer invasiveness.

Conclusions: Parsing the complexities of these signaling interactions is important, not only to understand these mechanisms of cancer initiation and progression, but also to develop novel treatment modalities. Since the vast majority of persons with colon cancer die from disseminated disease, preventing or reversing metastatic spread of cancer cells by targeting M3R, post-M3R signaling, or MMP1 has therapeutic potential.

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Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

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Keywords

Colon cancer; muscarinic receptor; M3R; matrix metalloproteinase; MMP1; colon cancer; EGFR; MMP inhibitor

1. INTRODUCTION

In the United States, colorectal cancer is the fourth most prevalent neoplasm and the second most frequent cause of cancer-related death [1]. While there have been recent advances in early detection and prevention of colon cancer, treatment options remain limited, especially for patients with advanced disease [2, 3]. In addition to identifying genetic mutations that initiate colon cancer, to develop new treatment modalities it is essential to have a better understanding of the mechanisms that govern cancer progression [2, 3]. Subtype-3 muscarinic receptor (M3R) expression and activation have been associated with the progression of colorectal neoplasia and, in particular, post-M3R signal transduction stimulates release of matrix metalloproteinase-1 (MMP1). Matrix metalloproteinases have long been considered important players in cancer [4], and MMP1 specifically is strongly associated with tumor metastasis and adverse outcomes [3]. Targeting the *M3R/MMP1 axis* may offer potential novel therapeutics for Stage II and III colon cancer, possibly retarding or preventing colon cancer metastasis, an intervention that may improve prognosis for the millions of colon cancer patients.

2. MUSCARINIC RECEPTORS

Five muscarinic receptor subtypes (M1R-M5R) were first described in 1987 [5]. These G protein-coupled receptors (GPCR), each composed of seven transmembrane domains, are expressed in various organ systems [5]. In the gastrointestinal system, the primary muscarinic receptor subtypes are M1R, M2R, and M3R [5]. When expressed in cells capable of proliferation, M1R, M3R, and M5R are conditional oncogenes [3]. Colon epithelial cells and many colon cancer cell lines specifically express M1R and M3R [5, 6]. In humans, acetylcholine (ACh) and secondary bile acids are the only known naturally occurring endogenous muscarinic receptor agonists [5].

The majority of cancers derived from epithelial and endothelial cells express muscarinic receptors and activation of M1R, M3R, and M5R increases cell proliferation [7]. For most of these cancers, acetylcholine synthesized and released by cancer cells stimulates cell growth, acting as an autocrine and paracrine growth factor [7].

2.1 Subtype-3 Muscarinic Receptor

M3Rs are widely expressed throughout the normal gut, and over-expressed in both colon adenomas and cancers [2, 3, 8]. Muscarinic signaling, most specifically through M3R, plays an important role in mediating intestinal epithelial fluid and electrolyte transport via cholinergic nerve endings [9]. Whereas normal colon epithelium expresses the genes encoding both M1R and M3R, *CHRM1* and *CHRM3*, respectively, only *CHRM3* is upregulated in colon cancer [10]. In comparison to normal colon, Cheng *et al.* detected

CHRM3 over-expression in 56% of colon cancers, and in eight samples, *CHRM3* expression was increased 2- to 128-fold [8]. Furthermore, they found a significant relationship between the level of *CHRM3* expression and the presence of colon cancer metastases, consistent with studies showing the key role of M3R for cell migration and invasion, two important features of tumor cells with metastatic capability [8]. They also detected increased M3R expression in both the cytoplasmic and plasma membranes of colonocytes compared to normal epithelium; mean M3R staining intensity in colon cancer was increased greater than two-fold [8]. Notably, in lymph node or liver metastases M3R staining intensity was not increased, suggesting the role of M3R is confined to early progression and invasion of colon cancer, but is not as important once tumors have spread [8].

In azoxymethane-treated mice, Raufman *et al.* found genetic ablation of M3R attenuated epithelial cell proliferation, as well as the number of adenomas and adenocarcinomas [11]. In contrast to the multiplicity of cancers in azoxymethane-treated wild-type mice, M3R-deficient mice had no more than one adenocarcinoma per colon, and the overall colon tumor number and volume were reduced by approximately 60% [11]. These findings, replicated in a second study [6], clearly suggest M3R plays an important role in promoting colon cancer.

3. M3R SIGNALING

Acetylcholine (ACh) and conjugated secondary bile acids are the only known endogenous muscarinic receptor ligands in humans [10]. Both *in vitro* studies using human colon cancer cell lines and *in vivo* studies using murine models of colonic neoplasia confirmed the importance of muscarinic signaling in colon cancer [10].

In human colon cancer cells, by stimulating interactions between post-M3R signaling pathways, muscarinic receptor agonists stimulate cell proliferation, survival, migration, and invasion [8, 12] (Fig. 1). Activation of M3R in colon cancer cells stimulates complex signaling pathways involving both epidermal growth factor receptor (EGFR)-dependent and -independent pathways that ultimately stimulate gene transcription leading to cell proliferation and other functional outcomes necessary to promote colon cancer. EGFR is a key member of the tyrosine kinase receptor family [12]. EGFR activation results in protein tyrosine phosphorylation and the formation of phosphotyrosine-dependent intracellular protein complexes. This pathway is important for colon cancer cell proliferation and has been a major therapeutic target [12]. M3R activation results in transactivation of EGFR, post-EGFR signaling cascade and colon cancer cell proliferation [12]. Cheng *et al.* showed that treating colon cancer cells with ACh stimulates EGFR-dependent phosphorylation of the p44/42 mitogen-activated protein kinase (MAPK), also referred to as ERK1/2, and of p90 ribosomal S6 kinase (p90 RSK) [12].

Selected bile acids also activate M3R and p90 RSK, and stimulate proliferation of colon cancer cells. In animal models, it was previously shown that increasing concentrations of secondary bile acids were associated with increased colon tumor growth [13]. Cheng *et al.* found taurine conjugates of lithocholic and deoxycholic acids stimulate colon cancer cell proliferation by muscarinic mechanisms [13], actions that are EGFR-dependent [14]. The pro-neoplastic effects of bile acids on colon cancer cells are distinguished from those of

traditional muscarinic agonists, like Ach, only in that bile acids tend to be somewhat less efficacious, achieving 30–40% of the maximal effect seen with Ach.

Secondary bile acids stimulate colon cancer cell proliferation through post-EGFR signaling. The effects of bile acids are dependent on activation of both M3R and EGFR signaling with post-receptor ERK1/2 activation [10]. *In vitro* evidence suggests that in addition to post-EGFR signaling mediated by ERK1/2, mutations in genes that regulate phosphatidylinositol-3'-OH kinase (PI3K)/Akt signaling are present in approximately 40% of colorectal cancers [15]. Previous studies showed conjugated bile acids activate PI3K/Akt signaling in liver cells by EGFR-dependent and -independent mechanisms. Raufman *et al.* showed exposing colon cancer cells to deoxycholytaurine (DCT) stimulated EGFR-dependent activation of downstream PI3K/Akt signaling [15]. This subsequently results in phosphorylation of GSK, BAD, NF- κ B, and other anti-apoptotic molecules targets that govern colon cancer cell survival [10, 15]. When activated by bile acids through the EGFR-dependent pathway, NF- κ B plays a key role in rescuing colon cancer cells from apoptosis [16]. Bile acids also increase resistance to both TNF- α - and UV-induced apoptosis in colon cancer cells, an action mediated by the activation of Akt [9, 10]. A more recent study further confirmed that treating cancer stem cells in normal human colonic epithelial cells with unconjugated secondary bile acids leads to a greatly elevated M3R expression [17]. This was also accompanied by a marked increase in EGFR expression and activation. Furthermore, induction of Wnt/ β -catenin signaling (which plays a role in regulation proliferation of colon cancer stem cells) by bile acids in M3R-downregulated colonic epithelial cells resulted in greatly diminished expression of M3R, which is essential for colon carcinogenesis [17]. Thus, downstream effects of bile-acid induced activation of EGFR regulate colon cancer cell apoptosis and survival.

In a study of gastric tumorigenesis, Hayakawa *et al.* reported M3R activation modulates Wnt pathway signaling [18]. In the stomach, cholinergic stimulation induced nerve growth factor (NGF) expression, which in turn promoted carcinogenesis. Hayakawa *et al.*'s findings also suggest M3R-mediated Wnt activation requires the involvement of YAP (yes-associated protein), a downstream target of M3R [18]. YAP plays a role in activation of β -catenin-dependent cancer growth and tumorigenesis [18]. Although these findings pertain to gastric cancer, they warrant mention in this review since the mechanism is similar to that observed in colon cancer, involving interaction between acetylcholine, M3R, and Wnt.

TACE/ADAM-17 (tumor necrosis factor- α converting enzyme/a disintegrin and metalloproteinase-17) plays a role in the EGFR pathway as well in colon cancer cell migration and invasion [19–21]. TACE/ADAM-17 is part of the EGFRPI3K-Akt pathway and is upregulated and overexpressed in primary and metastatic colon cancer tumors [20, 21]. Its co-expression with EGFR in neoplastic cells suggests it plays a significant role in tumor growth [21]. In fact, superoxide-generating NADPH oxidase 1 (Nox1) was found to be an important regulator of TACE/ADAM-17 expression. Nox1 stabilizes TACE/ADAM-17 from degradation and thus leads to activation of the EGFR pathway and promotes colorectal cancer metastasis [19].

In addition to release of Ach from enteric neurons, there is evidence normal and neoplastic non-neuronal cells can produce and release Ach. Cheng *et al.* showed several human colon cancer cell lines express choline acetyltransferase (ChAT), and Ach synthesis and release by colon cancer cells mediates autocrine and paracrine stimulation of proliferation [9]. This finding further supports the notion that both neuronal and non-neuronal Ach release may stimulate M3R-mediated colon cancer cell proliferation [9].

4. MATRIX METALLOPROTEINASES

Before linking M3R with MMP1 and contemplating the role of the *M3R/MMP1* axis in colon cancer, it is necessary to understand the nature of matrix metalloproteinases. MMPs were discovered initially in 1962 when they were found to degrade the extracellular matrix (ECM) in tadpole tails [22]. Until about 20 years ago, MMPs were primarily considered to promote cancer progression. Since then, our understanding of MMPs has greatly evolved [22]. MMPs are part of the metzincin superfamily of metalloproteinases, comprised of MMPs, a disintegrin and metalloproteinases (ADAMs), and ADAM proteases with thrombospondin motifs (ADAMTSs) [22, 23]. The family of matrix metalloproteinases (MMPs) comprises at least 24 zinc-containing proteases that cleave one or more components of the extracellular matrix (ECM) [24, 25]. Commonly produced by cells as secreted or membrane-bound pro-enzymes, MMPs are activated by proteolytic cleavage [25, 26]. Although major classes of MMPs, include collagenases, stromelysins, and gelatinases, each play a different role and some do not readily fit these traditional classifications [22].

In both normal and pathological processes, MMPs degrade ECM [27]. Under normal conditions, MMP activity is tightly controlled on several levels, including activation of secreted latent MMPs as well as inhibition by tissue inhibitors of matrix metalloproteinases (TIMPs) [27]. However, in certain pathological processes, like cancer, MMPs are no longer normally regulated and MMP-TIMP expression may be unbalanced, favoring proteolysis [27]. MMPs enhance tumor angiogenesis, disrupt local tissue architecture, and break down basement membranes, thereby contributing to cancer spread [28].

Several studies implicated MMPs in tumor invasion and metastasis [24]. Giambernardi *et al.* found inappropriate expression of several MMP mRNAs in breast, prostate, bone, colon, and oral tumor-derived cell lines [24]. Evidence suggests several proto-oncogenes control MMP gene expression, and the inappropriate expression of one or more MMPs plays a role in the progression of cells towards malignancy [24]. In tumors, both cancer cells and the surrounding stromal cells produce MMPs, and they may trigger infiltration by inflammatory and immune cells [29]. Further, the role of MMPs may differ according to cancer stage [29].

In addition to playing a role in immunity by degrading ECM, MMPs regulate several cellular actions, including reproduction, embryogenesis, angiogenesis, and tissue remodeling, as well as cell migration and release of bound signaling molecules [22]. MMPs contribute to several aspects of immunity by directing activation of signaling molecules like TNF and other cytokines [22].

4.1. Matrix Metalloproteinase-1 (MMP1)

By degrading ECM, MMP1, a member of the collagenase family, plays a role in colon cancer cell migration and meta-static invasion [24, 25]. Thus, it is not surprising that MMP1 expression is strongly correlated with advanced colon cancer and hematogenous metastasis [25]. In colorectal cancer, MMP1 expression is associated with a poor prognosis [30]. Murray *et al.* found patients with MMP1-positive colorectal tumors had significantly reduced survival compared to those whose tumors did not express MMP1 [30].

Based on the findings of Murray *et al.*, Shiozawa *et al.* aimed to elucidate the clinicopathologic role of MMP1 expression in colon cancer [31]. They compared the immunohistochemistry of colorectal adenomas and colorectal adenocarcinomas. Whereas MMP1 was not expressed in colorectal adenomas, it was present in 76.1% of adenocarcinomas [31]. Furthermore, they found MMP1 expression enhanced in invasive compared to intramucosal carcinomas, suggesting MMP1 was over-expressed at an early stage of tumor invasion. MMP1 expression was correlated with depth of tumor invasion and was greater in infiltrative tumors with expansive patterns [31]. Previously, MMP1 mRNA was only found in stromal cells surrounding tumors, but not in carcinomas; this study established the presence of MMP1 mRNA in carcinoma cells [31]. Finally, MMP1 expression in primary cancers correlated significantly with lymphatic and venous invasion, as well as lymph node and liver metastasis [31]. Clearly, MMP1 plays an important role in colon cancer invasion and metastasis.

MMP1 expression is also associated with poor cancer-specific survival. Langenskiold *et al.* showed that in patients with colorectal cancer, high levels of MMP1 and MMP9 expression in tumor-free mucosa were significantly associated with T stage and lymph node involvement [26]. In colonic enterocytes, MMP1 protein was expressed at the surface layer of tumor-free mucosa while in tumor tissue it was evenly distributed in tumor cells. The authors postulated that MMP1 may reflect a reaction to tumor development in normal tissue and may identify patients needing more aggressive therapy [26].

Elevated MMP1 expression in colon cancer is associated with aberrant activation of the transcription factor STAT3. STAT3 hyperactivation is associated with various types of cancer and promotes tumorigenesis and tumor development via different pathways [32]. In colorectal carcinoma, STAT3 is a determinant of malignancy and is persistently activated (phosphorylated; pY-STAT3) in about 90% of biopsies. pYSTAT3 drives CRC cell line proliferation and increases cancer invasiveness [32]. Using colorectal cancer cells, Zugowski *et al.* discovered expression of MMP1 and activation of STAT3 are connected in a complex manner. STAT3 contributed to the activity of the MMP1 promoter with a high affinity of the STAT binding site upstream of the transcriptional start site [32]. AP-1, another transcription factor, also has affinity for a binding site close to the site of transcription initiation. Both STAT3 and AP-1 are essential for STAT3-mediated transcription of the MMP1 promoter; combined inappropriate activation of STAT3 and AP-1 signaling drives increased MMP1 expression and, consequently, CRC invasion [32].

As noted above, M3R signaling is important in colon cancer cell proliferation and survival – MMP1 may play key role in this pathway. Raufman *et al.* found treating several human

colon cancer cell lines with Ach selectively increased MMP1 expression [25]. Pre-incubating cells with atropine or anti-MMP1 neutralizing antibody completely blocked the actions of Ach. These findings indicate muscarinic receptor agonists induce MMP1 gene expression and therefore, colon cancer invasion; conversely, blocking muscarinic receptor and MMP1 activation prevents human colon cancer cell invasion [25].

Xie *et al.* reported that in addition to stimulating MMP1 expression in colon cancer cells, M3R activation also increases the levels of other selected MMPs, specifically MMP7 and MMP10 [33]. *In vitro*, Ach-induced colon cancer cell proliferation requires MMP7 activation and release of heparin-binding EGF-like growth factor (HBEGF), an EGFR ligand [33]. The critical role of MMP7, another metalloproteinase overexpressed in the majority of colon cancers, in communication between M3R and EGFR signaling, mediates a variety of pro-neoplastic effects [33]. Currently, the role of MMP10 in colon cancer is unknown. Regardless, treatment of H508 colon cancer cells with Ach stimulated dose- and time-dependent 50-fold increase in mRNA levels of *MMP1*, *MMP7*, and *MMP10* [33]. When pre-treated with atropine and inhibitors of EGFR activation and ERK signaling, these actions are attenuated. This effect primarily results from upregulation of *MMP* gene transcription, thus further indicating that there may be a connection between M3R activation and increasing levels of MMPs in colon cancer cells [33].

Similarly, Farhana *et al.* found a relationship between M3R and MMP expression. In their study using secondary bile acids to promote stemness in colonic epithelial cells and leading to the generation of cancer stem cells, they found increased expression and activation of M3R and EGFR [17]. The increase in activation of EGFR was also marked by a significant rise in MMP mRNAs, *MMP1*, *MMP3*, and *MMP10* in normal human colonic epithelial cells [17]. While the exact pathway connecting M3R and MMP1 has not yet been clearly elucidated, there is a definite connection between M3R activation and MMP expression in colon cancer that promotes proliferation and progression.

Recent studies indicate post-transcriptional regulator microRNAs (miRNAs) play important roles in modulating MMP1 gene expression in colon and other cancers. Liu *et al.* demonstrated direct targeting of miR-222 to 3' untranslated regions (3'UTR) of MMP1 mRNA in squamous cell carcinoma of the tongue suppresses MMP1 gene expression [34]. Interestingly, miR-222 also controls MMP1 expression indirectly by targeting manganese superoxide dismutase 2 (SOD2) mRNA and lowering SOD2 protein levels [34]. Various indirect mechanisms were identified whereby miRNAs regulate MMP1. Wu *et al.* decreased MMP1 levels by transfecting exogenous miR-34a into HCT116 colon cancer cells, an effect that could be rescued by Fra-1 overexpression [35]. In gastric cancer cells, Zheng *et al.* found miR-145 directly targets the 3'UTR of v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1) mRNA. Overexpressing or knocking down miR-145 altered both the mRNA and protein levels of Ets1 and downstream MMP1 [36]. Multiple studies focus on MMP inhibitors, particularly the reversion-inducing-cysteine-rich protein with kazal motifs (RECK) and metalloproteinase inhibitor 3 (TIMP3), as major targets of miRNA that alter downstream MMP1 expression in cancer. It was confirmed that miR-220b/c, miR-221/222, miR-21 and miR-7 directly target RECK and miR-181b, miR-191 and miR21 target TIMP3 mRNA to inhibit gene expression [37–43]. These studies imply a complex network of

interactions involving MMP1 expression and multiple miRNAs. The effects of post-M3R signaling on changes in miRNAs remain largely uncharted territory – this is clearly an area that warrants further investigation.

5. PHARMACOLOGY

The current mainstay of treatment for metastatic colon cancer remains cytotoxic chemotherapy with irinotecan or oxaliplatin plus 5-FU and leucovorin or capecitabine [44]. These combinations provide overall survival of about 18 months. The recent addition of more targeted therapies has improved overall survival in patients with metastatic colon cancer to 20–29 months [44]. These targeted therapies include monoclonal antibodies directed against EGFR, vascular endothelial growth factor (VEGF), and VEGF receptor 2, recombinant fusion proteins, and multi-kinase inhibitors [44]. Unfortunately, the efficacy of EGFR inhibitors tends to be transient and drug resistance results in tumor recurrence, metastasis, and death [3]. Other therapeutic agents currently under investigation target cancer stem cells, mutant *BRAF* and *KRAS*, and overexpressed *HER2* [44].

Targeting MMPs for cancer treatment seems logical, but phase III trials in the 1990s and early 2000s failed to show benefit for several small molecule MMP inhibitors [4]. Early in the 1990s, there was a lack of information regarding MMP structure, therefore, pharmaceutical companies started to develop inhibitors targeting zinc at the center of the catalytic domain of MMPs [45]. While at least 50 agents showed promise in animal models, they failed in clinical trials. Failures may be attributable to the lack of inhibitor specificity and poorly understood, dose-limiting side-effects [4].

Since that time, a better understanding of MMPs has emerged. It is now known that 23 human MMPs function in ways other than ECM degradation, such as regulating the actions of cytokines, chemokines, antimicrobial peptides, and cell-surface proteins [45]. Proteolytic activity of MMPs also plays a role in governing immunity, inflammation, and cell transformation. Previously, it was unappreciated that the active site of MMPs shares structural similarity with other metzincin family members [23, 45]. Consequently, MMP inhibitors were insufficiently specific and the multitude of off-target effects was unanticipated. Indeed, some MMPs are considered “anti-targets” or molecules that play protective roles in normal cell or tissue function [29]. These MMPs perform beneficial actions, and down-modulating their activity can result in unacceptable side effects or even worsen the course of disease [29]. Ten of 24 MMPs have beneficial roles in several diseases; it is therefore essential to avoid agents that negatively affect these MMP anti-targets [29].

Recently, several groups developed highly selective small-molecule MMP inhibitors to a single MMP, generally in the form of highly selective and potent antibodies [22, 29]. Several were developed to target membrane-anchored MMPs [22]. For example, a monoclonal antibody targeted against MMP14 has been tested successfully *in vitro* and *in vivo* [22]. In a murine colitis model that leads to colorectal cancer, inhibitors of MMP2 and MMP9 show promise in treating inflammatory bowel disease [29].

Finally, a recent publication reports a novel approach to targeting MMPs. Investigators identified a highly selectively compound that inhibits activation of MMP9 [46]. This compound, designated JNJ0966, did not alter MMP1, MMP2, MMP3, MMP9, or MMP14 catalytic activity, nor did it inhibit the related MMP2 zymogen. JNJ0966 interacts with a pocket near the MMP9 zymogen cleavage site, distinct from the catalytic domain [46]. JNJ0966 attenuated the severity of disease in a mouse model of autoimmune encephalomyelitis [46]. This novel approach to MMP inhibition is likely to open the door to developing safe and effective inhibitors of other MMPs, including MMP1.

Other potential therapeutic targets include M3R and post-M3R signaling pathways. Compared to control mice, ApcMin mice treated with scopolamine butylbromide, a nonsubtype selective muscarinic inhibitor, had statistically significant reductions in tumor number and volume [33, 47]. Scopolamine butylbromide does not cross the blood-brain barrier and therefore does not cause central nervous system effects. It also most likely does not cause complete blockade of muscarinic receptors, and it is not specific to intestinal cells. However, it does prove that inhibition of muscarinic receptor activation can attenuate intestinal tumor formation and growth [47]. Although less impressive than the effects of M3R gene ablation [11], these findings are promising – suggesting more potent and selective M3R antagonists could be highly effective. Of course, the possibility of intolerable anticholinergic side effects is a concern.

Scopolamine (hyoscine) butylbromide, an anti-spasmodic, has been proposed to improve mucosal detection of polyps and adenomas during colonoscopy [48]. In a recent meta-analysis of randomized clinical trials, the use of scopolamine butylbromide during colonoscopy was shown to provide marginal improvements in adenoma and polyp detection rate. However, these conclusions are not definite, given the heterogeneity of the data [48]. Nonetheless, this speaks to the potential clinical use of such muscarinic inhibitors.

Recently, forkhead box D3 (FOXD3), a transcriptional repressor, was found to regulate the EGFR-Ras-Raf-MEK-ERK signaling pathway [49]. Li *et al.* showed knocking down *FOXD3* expression significantly promoted colon cancer cell proliferation and tumor formation in nude mice. Furthermore, FOXD3 knockdown activated EGFR-Ras-Raf-MEK-ERK signaling, a key player in human colon cancer cells [49]. While Li *et al.* could not clearly relate FOXD3 with the EGFR pathway, FOXD3 appears to be a tumor suppressor, thus enhancing its expression or activity may provide a novel therapeutic approach [49].

Said *et al.* showed that a combinatorial approach to blocking EGFR activity along with an agent that blocks M3R or EGFR-independent post-M3R signaling may have therapeutic promise [3]. Such a combination is likely to block induction of *MMP1* gene expression in colon cancer. At this time, no therapies under development target MMP1 specifically; *in vitro* findings suggest a selective antibody against MMP1 may be useful in decreasing advanced colon cancer, preventing metastasis, and improving prognosis.

6. CONCLUSION

Pathways underlying initiation and promotion of colon cancer are complicated and remain incompletely understood. Over the past 10–15 years, it has become apparent the *M3R/MMP1 axis* plays an important role in promoting colon cancer primarily by stimulating cell, proliferation, invasion, and dissemination. Either alone or in combination with current therapeutic modalities, targeting these complex interacting pathways is likely to identify novel therapies for colon cancer. Whereas inhibiting the *M3R/MMP1 axis* may be a useful adjunct to current chemotherapeutic regimens [4], it is necessary to develop inhibitors that selectively target disease-specific muscarinic receptors and MMPs, while protecting so-called “anti-targets” that actually prevent or inhibit progression of neoplasia [45].

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Fig. (1).

M3R-related signaling pathways with sites of action for inhibitors. PKC, protein kinase C; MAPK, mitogen-activated protein kinase; P13K phosphatidylinositol-3'-OH kinase; EGFR, epidermal growth factor receptor; RSK, ribosomal S6 kinase; MMP1, matrix metalloproteinase-1; FOXD3, forkhead box D3.