THE ROLE OF MCC IN MATURE B-CELL PROLIFERATION AND CARCINOGENESIS

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Abstract

Non-Hodgkin’s lymphoma is the leading cause of hematologic cancer in adults and the 7th leading cause of death in the United States.\textsuperscript{1} Importantly, B-cell lymphomas account for 85% of Non-Hodgkin’s Lymphoma and 90% of lymphomas altogether.\textsuperscript{2} Therefore, it is necessary to further delineate the molecular mechanisms of B-cell lymphomagenesis in order to develop novel therapeutic treatments for the disease. This study sought to investigate a novel candidate oncogene, Mutated in Colorectal Cancer (MCC), identified in a new mouse B-cell lymphoma model with the tumor suppressor gene TRAF3 specifically deleted in B-cells (B-TRAF3\textsuperscript{-/-} mice). Significantly, a striking upregulation of MCC mRNA and protein levels was observed in TRAF3\textsuperscript{-/-} mouse B lymphomas as compared to splenic B-cells of littermate control mice (LMC). A robust increase of MCC mRNA and protein expression levels was also observed in a variety of human patient-derived multiple myeloma cell lines with TRAF3 deletions or relevant mutations. Therefore, the aberrant upregulation of MCC was verified at both the mRNA and protein levels in TRAF3\textsuperscript{-/-} mouse B lymphomas and human multiple myeloma cells. In order to further investigate the role of MCC in B-cell lymphomagenesis, a series of lentiviral shRNA constructs were generated and used to transduce human multiple myeloma cells. Interestingly, lentiviral shRNA-mediated knockdown of MCC protein expression in multiple myeloma cells resulted in decreased cell viability, induction of apoptosis, inhibition of cell cycle progression, and reduced cell proliferation. Taken together, our results indicate that MCC plays oncogenic roles in B-cell lymphomagenesis, which is in sharp contrast to its tumor suppressive function in colorectal cancer. Our findings suggest that aberrant upregulation of MCC may serve as a diagnostic marker and therapeutic target of B lymphoma and multiple myeloma.
Introduction

Cancer is the second leading cause of death in the United States and has contributed significantly to rising incidence rates of disease nationwide over the past decade.\(^1\) Non-Hodgkin’s Lymphoma is the seventh most predominant cancer in the United States and the sixth leading cause of cancer among women. Crucially, 70,000 new cases of Non-Hodgkin’s Lymphoma were diagnosed in 2012.\(^1\) In fact, the number of new-onset diagnoses for Non-Hodgkin’s Lymphoma has doubled over the past three decades\(^3\). Specifically, B-cell lymphomas account for 85% of all cases of Non-Hodgkin’s Lymphoma annually and 90% of lymphomas altogether.\(^4,5\) Despite recent advances in detection and chemotherapeutic treatments for patients with this disease, Non-Hodgkin’s Lymphoma still accounted for 19,000 deaths in 2012.\(^1\) Thus, it is paramount that new methods for detection and treatment of Non-Hodgkin’s Lymphoma be developed in order to optimize patient outcomes and improve upon current diagnostic capabilities and limitations in order to ameliorate the burden of this disease. However, novel therapeutic alternatives for treating Non-Hodgkin’s Lymphoma necessitate a greater molecular understanding of the mechanisms of B-cell lymphomagenesis.

Increasingly, genetic alterations and epigenetic modifications have been linked to deregulated signaling pathways, which promote the growth and survival of lymphoma cells.\(^6-10\) More specifically, step-wise mutations and alterations in cancer-related genes have been implicated in the development of carcinogenesis.\(^11\) Therefore, it is crucial that the specific oncogenic alterations required for tumor progression in B-cell lymphomagenesis be further investigated to identify biomarkers of disease progression and to classify genetic risk factors for Non-Hodgkin’s Lymphoma. Importantly, the recommended chemotherapeutic approach typically taken to treat patients with the same variation of B-cell lymphoma may not take into
account key histological and genetic differences unique to the tumors of each patient. Therefore, targeted biomarkers possess significant prognostic value and can be used to guide the development of patient-specific gene therapies for individuals with Non-Hodgkin’s Lymphoma.

A search for new genetic risk factors of B-cell malignancies has recently identified TRAF3, a cytoplasmic adaptor protein, as a new tumor suppressor gene in B lymphocytes. Both homozygous deletions and inactivating mutations of the TRAF3 gene frequently occur in human patients with a variety of non-Hodgkin lymphomas and multiple myeloma. Specifically, deletions and inactivating mutations of TRAF3 have been observed in clinical presentations of splenic marginal zone lymphoma (SMZL), B-cell chronic lymphocytic leukemia (B-CLL), mantle cell lymphoma (MCL), as well as multiple myeloma (MM) and Waldenström’s macroglobulinemia (WM). 12-15 To explore the in vivo functions of TRAF3 in B lymphocytes, our laboratory generated B cell-specific TRAF3-deficient (B-TRAF3−/−) mice. 16 Interestingly, TRAF3 deletion leads to remarkably prolonged survival of mature B-cells independent of the B-cell survival factor BAFF, due to constitutive activation of the non-canonical NF-κB (NF-κB2) pathway. 16 These studies identify TRAF3 as a key inhibitor of the NF-κB2 pathway and B-cell survival (Fig. 1).
Figure 1. TRAF3 Inhibits the Non-Canonical NF-κB Pathway in B-cells.

The B-TRAf3−/− mice spontaneously developed SMZL and B1 lymphomas (a mouse mimic of B-CLL) by the age of 18 months. The spontaneous, highly penetrant development of B lymphomas in the B-TRAf3−/− mice provides conclusive evidence that TRAF3 is a tumor suppressor in B-cells.17 However, TRAF3−/− B-cells do not exhibit autonomous proliferation despite of their prolonged survival. In accordance with this, B lymphoma development in B-TRAf3−/− mice exhibits a long latency of 9 months, indicating that additional oncogenic alterations are required for the development of malignancies in B-cells. To identify the secondary oncogenic alterations required for B-cell malignant transformation, our laboratory performed microarray analysis to comparatively assess the differential expression of genes in TRAF3−/− B lymphomas versus tumor-free littermate control splenic B-cells. Interestingly, Mutated in
Colorectal Cancer (MCC) was identified as one of the most strikingly upregulated genes in TRAF3−/− B lymphomas.

MCC is emerging as a multifunctional protein with a demonstrated role in a variety of cellular processes and pathways, including UV damage response, cellular proliferation, and cellular migration.6-8,10-13 The MCC gene was initially isolated from the human chromosome 5q21 by positional cloning in order to identify tumor suppressor genes implicated in familial adenomatous polyposis (FAP)18. Subsequently, MCC has been identified as a putative tumor suppressor gene in colorectal cancer cells (CRC). Kinzler et al. have reported the frequent early promoter methylation of MCC in CRC cells19. MCC gene promoter hypermethylation and gene silencing are associated with high tumor grade and metastasis in colorectal cancer. However, the observed promoter hypermethylation in CRC stands in notable contrast to studies conducted in non-small cell lung cancer cell lines.20 Upon further analysis of the molecular pathways of MCC-mediated carcinogenesis in CRC cells, it was found that MCC acts by suppressing Wnt signaling and β-catenin/T-cell factor/lymphoid-enhancer factor dependent transcription and proliferation21. It was recently demonstrated that MCC is highly expressed in murine colonic epithelial cells and other well-differentiated cells, indicating a potential physiologic role for MCC in cell differentiation and cell signaling22-24. Notably, the overexpression of the MCC protein inhibited the cell cycle transition from the G1 to S phase in NIH3T3 fibroblasts.22 These results suggest that MCC plays a negative role in cell cycle progression in fibroblasts. Further studies have identified MCC as a novel target in the DNA damage repair pathway. MCC knockdown impaired the G2/M checkpoint arrest following UV damage, suggesting that MCC is needed for full arrest at the G2/M checkpoint in response to DNA damage in colon epithelial cells25.
Collectively, recent evidence indicates that MCC functions as a tumor suppressor gene in colorectal cancer cells.

However, the functional roles of MCC in B-cell lymphomas have not yet been investigated. The aim of this research project is therefore to test the central hypothesis that aberrant up-regulation of MCC is implicated in TRAF3 inactivation-initiated B lymphomagenesis.

**Objectives**

Specifically, my thesis research aims to: (1) verify that MCC is upregulated in TRAF3\(^{-/-}\) mouse B lymphomas and human multiple myelomas; (2) further delineate the functional roles of MCC in TRAF3\(^{-/-}\) tumor B-cells through lentiviral shRNA-mediated knockdown studies.

In order to address the aforementioned objectives, the MCC mRNA levels in TRAF3\(^{-/-}\) mouse B lymphomas were quantified using reverse-transcription PCR (RT-PCR). Subsequently, the upregulation of MCC was verified in TRAF3\(^{-/-}\) mouse B lymphomas at the protein level using Western Blot Analysis. To extend the clinical relevance of the study, several human patient-derived multiple myeloma cell lines with TRAF3 deletions or relevant mutations were also assessed for MCC expression levels. To further elucidate the function of MCC in TRAF3\(^{-/-}\) tumor B-cells, lentiviral shRNA constructs were generated and transduced into the TRAF3\(^{-/-}\) human patient-derived multiple myeloma cells. The effects of MCC knockdown on cell survival, apoptosis, and cell cycle progression were subsequently examined and quantified using immunofluorescence staining and flow cytometry.
Materials and Methods

RNA Extraction:

Total cellular RNA was prepared from splenocytes of LMC mice, or splenic B lymphomas and ascites of B-TRAF3\(^{-/-}\) mice. Total cellular RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s protocol. Briefly, cells were first lysed with 1 mL of TRIzol reagent in an Eppendorf tube, and chloroform was added. After centrifugation, RNA in aqueous phase was collected, and then precipitated using isopropanol. RNA pellets were subsequently washed using 75% ethanol, and dissolved in RNase-free water. The RNA concentration was determined using a Nanodrop spectrophotometer (ND-1000.V.3.0 software).

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR):

High capacity cDNA was prepared from the extracted RNA using the cDNA Reverse Transcription Kit (Applied Biosystems). Real time PCR analyses of MCC were performed using the TaqMan gene assay kits specific for mouse and human MCC genes, respectively (Applied Biosystems). Fifty cycles of quantitative real-time PCR reactions were performed using a 7500 Fast Real-Time PCR system. Taqman primers and probes specific for mouse or human MCC were used. Each reaction also included the probe (VIC-labeled) and primers for mouse or human \(\beta\)-actin mRNA, which served as an endogenous control. Relative mRNA expression levels of MCC were analyzed using the 7500 Sequencing Detection Software (Applied Biosystems) and the comparative Ct (\(\Delta\Delta\text{Ct}\)) method following the manufacturer’s procedures. For each biological sample, duplicate PCR reactions were performed.

Western blot analysis:

Cell pellets were resuspended in 2x SDS lysis buffer and were sonicated to prepare total cellular proteins. Protein samples were boiled at 98\(^\circ\) for 10 minutes and chilled on ice. Proteins
were then separated by SDS-PAGE, electroblotted onto nitrocellulose membranes (ProTran, Schleicher & Schuell BioScience, Keene, NH) and incubated with specific antibodies. Blocking and secondary Ab incubations were performed at room temperature for 1h. Primary Ab incubation was carried out at 4°C overnight. A chemiluminescent substrate (Pierce, Rockford, IL) was used to detect HRP-labeled Abs on the Western blots. Images of immunoblots were then acquired using a low-light imaging system (MiniLAS-4000, FUJIFILM Medical Systems). Typically, the same protein blot was stripped and re-immunoblotted with 3 or 4 different antibodies sequentially. Actin blot was used as a loading control.

Splenic B-Cell Purification:

Splenic B-cells were purified using anti-mouse CD43-coated magnetic beads (Miltenyi) and a MACS separator (Miltenyi Biotec Inc.). During the procedure of splenocyte and B-cell purification, spleens were kept on ice. A hypotonic ACK buffer was used to lyse red blood cells. Cells of each genotype were kept separate in appropriate tubes throughout the protocol. The purity of the splenic B-cells was then monitored through the use of FACS analysis. Cell preparations of at least 95% purity were used for the protein and RNA extractions. RNA and protein levels were analyzed using RT-PCR and Western blot analysis, respectively.

Lentiviral shRNA vector-mediated knockdown of human MCC:

Lentiviruses of shRNA vectors for human MCC or a scrambled shRNA vector were packaged and titered according to the manufacturer’s protocol (Sigma). Human multiple myeloma (MM) cells lines KMS11 and LP1 were transduced with the packaged lentiviruses at a MOI of 1:5 (cell:virus) in the presence of 8 µg/ml polybrene. For puromycin-resistant lentiviral vectors, at 48 hr post transduction, puromycin (0.5 µg/ml) was added to the culture to select successfully transduced cells. Three days after puromycin selection, proteins were extracted for
immunoblot analysis. For GFP-expressing lentiviral vectors, GFP+ve transduced cells were sorted using a FACS cell sorter (Beckman Coulter Moflo XDP) on day 4 post transduction. Sorted cells were plated in 6-well plates for growth curve determination, or analyzed by Annexin V (Invitrogen) and PI staining (eBioscience).

Survival assay and cell cycle analysis:

For PI staining, cells were fixed with an equal volume of ice-cold 70% ethanol. PI staining (eBioscience) was performed as previously described\textsuperscript{26}, and DNA content was quantified by a benchtop FACScan (Becton Dickinson). Labeling of MM cells with eFluor 670 (eBioscience) for proliferation analysis was performed following the manufacturer’s instructions. Cells were then fixed, and the decline in eFluor670 fluorescence as a measure of proliferation was determined by FACS analysis. The results were analyzed using FlowJo software (TreeStar, San Carlos, CA).

Statistics:

Statistical analyses were performed using the Student’s t-test. P values less than 0.05 are considered significant, and P values less than 0.01 are considered very significant.

Results

\textit{Up-regulation of MCC in TRAF3\textsuperscript{-/-} Mouse B Lymphomas}

Our laboratory recently reported that B lymphoma development was not observed in any B-TRAF3\textsuperscript{-/-} mice younger than 9 months old.\textsuperscript{27} These findings indicate that TRAF3 inactivation and its downstream signaling pathways are not sufficient and that additional oncogenic pathways are required for B lymphoma development in B-TRAF3\textsuperscript{-/-} mice. To identify the secondary alterations involved in TRAF3 inactivation-initiated B lymphomagenesis, our laboratory performed microarray analysis using TRAF3\textsuperscript{-/-} splenic B lymphomas in comparison to littermate
control mice (LMC) spleens. Results of the microarray analysis identified MCC as one of the most strikingly up-regulated genes in TRAF3⁻/⁻ splenic B lymphomas. In order to verify if MCC mRNA transcripts are up-regulated in large quantities in TRAF3⁻/⁻ B lymphomas as compared to LMC splenic B-cells, we performed real-time PCR (RT-PCR) analysis. Results from the RT-PCR indicate that mRNA expression of MCC was barely detectable in the splenocytes of LMC mice. In sharp contrast, mRNA levels of MCC in splenic B lymphomas and ascites of diseased B-TRAF3⁻/⁻ mice were strikingly elevated (Fig. 2). These results confirmed the aberrant upregulation of MCC mRNA transcripts in TRAF3⁻/⁻ mouse B lymphomas.

![MCC](image)

**Fig. 2. Real time PCR analyses of MCC in mouse splenocytes and B lymphomas.** Total cellular RNA was prepared from splenocytes of LMC mice, or splenic B lymphomas and ascites of B-TRAF3⁻/⁻ mice. Real-time PCR was performed using TaqMan primers and the probe (FAM-labeled) specific for mouse MCC. Each reaction also included the probe (VIC-labeled) and primers for mouse β-actin mRNA, which served as endogenous control. Relative mRNA expression levels of MCC were analyzed using the Sequencing Detection Software (Applied
Biosystems) and the comparative Ct method. Graphs depict the results of two independent experiments with duplicate reactions in each experiment (mean ± SD).

After confirming the upregulation of MCC mRNA levels in TRAF3−/− mouse B lymphomas, subsequent experiments aimed to assess if the observed increase in MCC at the mRNA level correlated to an increase of MCC at the protein level. In order to comparatively assess the protein levels in TRAF3−/− mice versus LMC mice, Western blot analysis was performed using SDS-PAGE electrophoresis. Importantly, a substantial elevation in MCC protein expression was noted in splenic B lymphomas and ascites of diseased B-TRAF3−/− mice. However, LMC mice displayed undetectable levels of MCC protein expression under the same experimental conditions (Fig. 3). Thus, the results from the RT-PCR indicating a robust increase in MCC mRNA levels were consistent with Western Blot analysis indicating that MCC was also aberrantly upregulated at the protein level.

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**Fig. 3. Western blot analysis of MCC in mouse splenic B-cells and B lymphomas.** Total cellular proteins were prepared from splenic B-cells purified from LMC mice, or B lymphomas of the spleens or ascites of diseased B-TRAF3−/− mice. Proteins were immunoblotted for MCC, followed by Actin. The Actin blot serves as loading control. Results are representative of 3 experiments.
Up-regulation of MCC in TRAF3−/− Human Multiple Myelomas

To strengthen the clinical relevance of the study, I next examined mRNA expression of MCC in normal human B-cells in comparison to several human patient-derived multiple myeloma cell lines (HMCLs) with TRAF3 deletions or relevant mutations. These include 6 HMCLs with TRAF3 bi-allelic deletions (KMS11 and 8226), TRAF3 frameshift mutations (U266 and LP1), or cIAP1/2 bi-allelic deletions (KMS20 and KMS28PE). Similar to TRAF3 inactivation-mediated upregulation of the NF-κB pathway, bi-allelic deletions of cIAP1/2 also lead to constitutive NF-κB2 activation. To answer the question of whether MCC expression was elevated in human patient-derived multiple myeloma cell lines, RT-PCR analysis was performed in the human patient-derived cell lines as well as in control B-cells. Interestingly, mRNA expression of MCC was strikingly elevated in human patient-derived multiple myeloma cell lines while normal B lymphocytes exhibited minimal expression levels of MCC at the mRNA level (Fig. 4). These results suggest that MCC mRNA expression is significantly elevated in human multiple myeloma cells.
**Fig. 4.** Real time PCR analyses of MCC in human patient-derived multiple myeloma cell lines. Total cellular RNA was prepared from purified normal human blood B lymphocytes (CTL), the 6 HMCLs or C3688. The 6 HMCLs include KMS11, 8226, U266, LP1, KMS28 and KMS20. C3688 is an EBV-transformed B lymphoblastoid cell line. Real-time PCR was performed using TaqMan primers and the probe (FAM-labeled) specific for MCC. Each reaction also included the probe (VIC-labeled) and primers for β-actin mRNA, which served as endogenous control. Relative mRNA expression levels of MCC were analyzed using the Sequencing Detection Software (Applied Biosystems) and the comparative Ct method. Graphs depict the results of two independent experiments with duplicate reactions in each experiment (mean ± SD).

In order to further investigate whether the increased mRNA levels of MCC in TRAF3\(^{-/-}\) multiple myeloma cells would also correlate to increased expression levels of the MCC protein, Western blot analysis was subsequently conducted. Specifically, MCC protein levels in normal B blood lymphocytes were measured in comparison to MCC protein levels in human patient-derived multiple myeloma cell lines. Importantly, MCC protein expression was robustly elevated in human multiple myeloma cell lines as compared to the undetectable MCC protein levels observed in the control B-cells. Interestingly, MCC expression was upregulated significantly in cell lines C3688, KMS11, LP1, 8226, and KMS20 (Fig. 5). Thus, a large increase in MCC protein expression was observed in cases of TRAF3 bi-allelic deletions, TRAF3 frameshift mutations, and cIAP1/2 bi-allelic deletions as well as the EBV-transformed B lymphoblastoid cell line C3688. Taken together, the results from the protein analysis have extended the significant upregulation of MCC demonstrated at the mRNA level to the protein level in human MM cells. Further, the upregulation of MCC has also been demonstrated and verified both in TRAF3\(^{-/-}\) B mouse lymphoma and in a variety of human-patient derived TRAF3\(^{-/-}\) MM cell lines.
Fig. 5. Western blot analysis of MCC in human patient-derived multiple myeloma cell lines. Total cellular proteins were prepared from purified normal human blood B lymphocytes (CTL), C3688 or the 6 HMCLs. C3688 is an EBV-transformed B lymphoblastoid cell line. The 6 HMCLs include KMS11, 8226, U266, LP1, KMS28 and KMS20. Proteins were immunoblotted for MCC, followed by TRAF3 and Actin. The Actin blot serves as loading control. Results are representative of 2 experiments.

**Generation of lentiviral shRNA constructs**

In order to further elucidate the role of MCC in TRAF3⁻/⁻ B lymphomas, genetic manipulation *in vitro* was used to knockdown MCC expression. A series of shRNA constructs were generated using molecular cloning applications based on cloning strategies devised to efficiently decrease protein levels of MCC in TRAF3⁻/⁻ cells. All the MCC lentiviral shRNA constructs were originally generated in a puromycin-resistant vector, which allows selection of successfully transduced cells using treatment with puromycin. A GFP tag was engineered into the lentiviral constructs to facilitate subsequent identification and sorting of successfully transduced cells by flow cytometry. DNA mini-prep, restriction enzyme digestion, and ligation and transformation experiments were carried out to obtain each of the shRNA constructs.
Constructs corresponding to the first nucleotide in the mRNA transcript of human MCC were named numerically as 1332, 1388, 2284, and 2689, respectively. Additionally, the Torc 2. scrambled shRNA vector served as a control in the subsequent lentiviral-mediated shRNA experiments. Clone Manager Software was used to devise the cloning strategy for each of the shRNA constructs developed specific for MCC knockdown. Each construct was engineered to contain an antibiotic (Amp) selectivity for bacteria culture, U6promoter, multiple cloning sites, an origin of replication, and a puromycin-resistance or GFP expression cassette. Molecular maps, such as the construct for lentiviral MCC shRNA vector phMCC 1332.GFP (Fig. 6), were engineered for each of the shRNA constructs used in subsequent analyses.

Figure 6. Map of the lentiviral MCC shRNA vector phMCC1332.GFP.
**Lentiviral shRNA-mediated knockdown of MCC in human MM TRAF3-/- cells**

To decrease MCC protein expression, lentiviral shRNA-mediated knockdown was employed. Human multiple myeloma (MM) cell line LP1 or KMS11 cells were transduced with lentiviruses expressing several human MCC shRNAs or a scrambled shRNA control construct (SCR), separately. Total cellular proteins were prepared from the transduced cells and assessed for MCC protein expression levels using Western Blot analysis. Significantly, transduced cells with two shRNA constructs exhibited a potent knockdown of MCC protein expression as compared to LP1 cells transduced with the scrambled shRNA. Specifically, MCC protein levels were markedly reduced by MCC shRNA constructs 1332 and 2689 in LP1 cells. The additional shRNA constructs 1388 and 2284 were also able to reduce MCC protein expression in LP1 cells, albeit to a much lesser extent (Fig. 7). Similar results were also obtained in KMS11 cells (data not shown). Importantly, the results from the Western Blot analysis demonstrated that the lentiviral shRNA vectors of MCC were able to knockdown MCC protein levels in TRAF3-/- human multiple myeloma cells.

![Western Blot Image](image-url)
proteins were prepared on day 5 after transduction, and levels of MCC protein were determined by immunoblot analysis. MCC shRNAs examined include 1332, 1388, 2284, and 2689. Immunoblot of actin was used as loading control. Results are representative of 3 experiments.

**MCC shRNA knockdown decreased cell viability**

The transduced cells were used to assess the effects of MCC knockdown on cell viability and apoptosis. Trypan blue cell staining was used to differentiate live versus dead cells. Trypan blue, a diazo dye, is able to selectively color dead cells while live cells with intact cell membranes remain uncolored. Thus, application of the Trypan blue dye allowed for the direct visualization and differentiation of colored, dead cells versus live, uncolored cells. Subsequently, careful analyses of live and dead cells for each transduced shRNA construct were carried out using a hemocytometer to maintain an accurate record of cell counts. The results from three independent experiments were tabulated and analyzed thereafter. In cells transduced with the scrambled shRNA control vector, 90% of cells were viable whereas only a 10% level of cell death was recorded. In sharp contrast, there was a 50% decrease in cell viability in LP1 cells transduced with the hMCC 1332 shRNA vector (Fig. 8). Similarly, cells transduced with hMCC shRNA 2689 also exhibited a marked decrease in cell viability. Remarkably, the decrease in MCC protein level expression observed through Western Blot analysis (Fig. 7) in turn correlated with a decrease in the proportion of live cells observed in the LP1 transduced cells (Fig. 8). These results suggest that downregulation of MCC protein levels was able to induce apoptosis in TRAF3−/− human multiple myeloma cells. Notably, the level of cell death observed in the Trypan blue cell viability assessment reflected each individual construct’s efficiency in reducing MCC protein levels.
Figure 8. Tryphan blue staining demonstrates decreased cell survival in human MM cells transduced with lentiviral shRNA constructs of human MCC. LP1 cells were transduced with lentiviruses expressing human MCC shRNA or a scrambled shRNA (SCR). Percentages of live versus dead cells were determined by trypan blue-stained cell counting on day 7 post transduction. The graphs depict the results of 3 independent experiments (mean ± SD). P values were analyzed using Student’s t test, and are indicated above bar graphs by asterisks. *, significantly different from pTorc.SCR control (t test, p < 0.05); **, very significantly different from pTorc.SCR control (t test, p < 0.01). Similar results are obtained in another human multiple myeloma cell line KMS11 cells.

**MCC shRNA knockdown increased apoptosis and inhibited cell cycle progression**

In preparation for the cellular proliferation analysis, growth curves for the transduced LP1 cells were analyzed on days 0, 1, 2, 3, and 4 after sorting. Trypan blue staining was applied as an indicator of cell viability to construct the growth curves. In comparison to the normal growth observed in cells transduced with the scrambled shRNA control, LP1 cells transduced with the hMCC shRNA 1332 construct were almost completely arrested in growth, and cells transduced with the hMCC shRNA 2689 also exhibited a significant decrease in growth (Fig. 9).
Figure 9. Growth Curves for live transduced MM human cells. Growth curves of live cells determined by trypan blue-stained cell counting. The graphs depict the results of 3 independent experiments (mean ± SD). P values were analyzed using Student’s t test, and are indicated above bar graphs by asterisks. *, significantly different from pTorc.SCR control (t test, p < 0.05); **, very significantly different from pTorc.SCR control (t test, p < 0.01).

Similar results are obtained in another human multiple myeloma cell line KMS11 cells.

To further examine the effects of shRNA knockdown on cell survival and apoptosis, FACS analysis was performed using the LP1 transduced cells. First, Annexin V and Propidium Iodide (PI) staining was applied to the cells to detect apoptotic and dead cells in transduced cells. Due to their intact cellular membranes, propidium iodide is not able to stain living cells and is only able to stain dead cells.32,33 Cells undergoing apoptosis, however, can be stained with Annexin V but not propidium iodide. Annexin V preferentially binds phosphatidyl serine in a calcium dependent manner.34 In normal, viable cells, phosphatidylserine is located in the inner leaflet of the plasma membrane. However, initiation of apoptosis translocates the phosphatidylserine to the outer leaflet of the plasma membrane as a signal for phagocytosis.35 Thus, Annexin V can bind to the phosphatidylserine once located on the outer leaflet of the plasma membrane in both apoptotic and dead cells.32,36 Therefore, live cells are annexin V-PI-,
apoptotic cells are annexin V+PI-, and dead cells are annexin V+PI+ populations. This differential staining pattern allows us to distinguish apoptotic and dead cells from live cells in transduced cell populations. Our results demonstrated that LP1 cells transduced with the hMCC shRNA 1332 construct exhibited a large increase (30.81%) in the apoptotic cell population in addition to a significant proportion of dead cells (13.27%). Similarly, LP1 cells transduced with the hMCC shRNA 2689 construct also demonstrated an increase in apoptosis and a decrease in cell viability (Fig. 10). Further, cells transduced with hMCC 1388 or hMCC 2284 shRNA constructs did not exhibit dramatic increases in apoptosis, which correlated to the efficiency of the knockdown of each construct. Conversely, the large majority of cells transduced with the scrambled shRNA control remained viable and only minimal levels of apoptosis were observed. Taken together, these results indicate that MCC knockdown induced apoptosis in TRAF3−/− human multiple myeloma cells.

![Image of FACS profiles](image)

**Figure 10. Lentiviral shRNA-mediated knockdown of MCC induced apoptosis in human MM cells.** Human MM cell line LP1 cells were transduced with lentiviruses expressing human MCC shRNA or a scrambled shRNA (SCR). Representative FACS profiles of transduced cells after annexin V and PI staining. Apoptotic cells were identified as annexin V+PI-, dead cells were annexin V+PI+, and live cells were annexin V-PI-. Similar results are obtained in another human multiple myeloma cell line KMS11 cells.

Subsequently, the transduced cells were sorted using a FACS sorter, and the DNA content of ethanol-fixed cells was assessed for cell cycle progression and apoptosis. The
fluorochrome propidium iodide was used to stoichiometrically stain the DNA to obtain accurate quantitative measurements. After sorting, cells were cultured for 24 hours and then were fixed prior to staining. Thereafter, the results from three independent experiments were tabulated. Markedly, cells transduced with the hMCC 1332 shRNA construct demonstrated a large increase in apoptosis concomitant with a substantial decrease in proliferation. Similarly, cells transduced with the hMCC 2689 shRNA construct exhibited an increase in apoptosis and a decrease in proliferation. However, cells transduced with the scrambled control demonstrated a large proportion of proliferating cells and a much smaller proportion of apoptotic cells (Fig. 11). Importantly, these analyses indicate that when MCC expression is knocked down, there is a large increase in apoptosis and an inhibition of cell cycle progression.

Fig. 11. Lentiviral shRNA-mediated knockdown of MCC induced apoptosis and inhibited cell cycle progression in human MM cells. Human MM cell line LP1 cells were transduced with lentiviruses expressing human MCC shRNA or a scrambled shRNA (SCR). Representative FACS histograms of cell cycle analysis. Transduced GFP+ve LP1 cells were sorted by a FACS sorter, cultured for 24 hr, and then fixed. Fixed cells were stained with PI and analyzed by FACS. Representative histograms of PI staining are shown, and percentage of apoptotic cells (DNA content < 2n) and proliferating cells (2n < DNA content ≤ 4n) are indicated. Data are
representative of 3 independent experiments. Similar results are obtained in another human multiple myeloma cell line KMS11 cells.

To further delineate the effects of MCC knockdown on cellular proliferation, transduced cells were analyzed by Alexa-eFluor labeling. The transduced LP1 cells were analyzed using a two-parameter plot with the proliferation dye eFluor 670 and Green Fluorescent Protein (GFP). The red fluorochrome eFluor 670 is able to bind primary amines in the cell and can be detected with a 660/20 band pass filter, making the use of this dye compatible with the application of GFP. As the cell divides, the eFlour 670 dye is distributed equally between daughter cells that can be measured as successive halving of the fluorescence intensity of the dye. Thus, as cell division progresses, there will be a reduction in the amount of red fluorescence measured by flow cytometry. GFP expression served as an indicator of successfully transduced cells in these experiments. In LP1 cells transduced with the scrambled shRNA control, transduced (GFP+) cell population proliferated at an equivalent rate as untransduced (GFP-) cell population, as evidenced by the equivalent decrease of eFluor 670 dye on day 2 and day 4 post labeling. In contrast, in LP1 cells transduced with the hMCC shRNA 1332 construct, transduced (GFP+) cell population proliferated at a much slower rate as untransduced (GFP-) cell population or cells transduced with the scrambled shRNA control, as evidenced by the much smaller decrease of eFluor 670 dye on day 2 and day 4 post labeling (Fig. 12). Taken together, these results indicate that the lentiviral shRNA vector-mediated knockdown of MCC efficiently inhibited cell cycle progression and decreased cellular proliferation in TRAF3−/− human multiple myeloma cells.
Fig. 12. Lentiviral shRNA-mediated knockdown of MCC inhibited proliferation in human MM cells. Human MM cell line LP1 cells were transduced with lentiviruses expressing human MCC shRNA or a scrambled shRNA (SCR). Representative FACS profiles of proliferation assay by a proliferation dye Alexa-eFluor 670. On day 4 post-transduction with human MCC shRNA 1332-eGFP or SCR-eGFP, cells were labeled with a proliferation dye, and cultured for additional 4 days. Cells were fixed on day 0, day 2 and day 4 after labeling to analyze the dilution of the proliferation dye and GFP+ve transduced cells. The data showed that the proliferation of MCC shRNA 1332 transduced cells was markedly slower as compared to untransduced GFP-ve cells or cells transduced with the scrambled shRNA. Results are representative of 3 independent experiments.

Discussion

The aberrant upregulation of MCC mRNA transcripts in TRAF3⁻/⁻ mouse B lymphomas has been demonstrated as compared to the undetectable levels of MCC expressed in littermate control non-malignant splenic B-cells. Further, Western Blot analysis has confirmed the robust
expression of MCC protein levels in TRAF3\(^{-/-}\) mouse B lymphomas. To further extend the clinical relevance of the study, MCC mRNA and protein levels were quantified as compared to normal B-cells. Results indicate that MCC expression is markedly upregulated at both the mRNA and protein levels in a variety of human patient-derived multiple myeloma cell lines with TRAF3 bi-allelic deletions, TRAF3 frameshift mutations, or cIAP1/2 bi-allelic deletions. How MCC is upregulated in TRAF3\(^{-/-}\) malignant B-cells remains to be determined. The aberrant upregulation of MCC may be attributed to the development of abnormal genetic alterations such as chromosomal translocations, gene amplification, or mutations in the promoter region of MCC\(^{38-43}\). Genetic alterations in B-cells may play a distinct role in B-cell-lymphomagenesis development. In fact, V (D) J recombination, somatic hypermutations, and class switch recombination of the immunoglobulin genes (Ig) strongly increase the risk of genomic instability in B-cells.\(^{44-48}\) Aberrations in non-homologous recombination processes may result in double-stranded breaks, leading to chromosomal translocations, deletions, or amplifications mediated by the cellular repair machinery. Alternatively, aberrant epigenetic mechanisms such as DNA methylation and histone modifications influence chromatin architecture and may also result in altered gene expression of MCC.\(^{49}\)

To further investigate the role of MCC in TRAF3\(^{-/-}\) malignant B-cells, a series of lentiviral shRNA constructs were generated using molecular cloning techniques. Subsequently, the shRNA constructs specific for MCC knockdown were transduced into TRAF3\(^{-/-}\) human multiple myeloma cells. The efficacy of each shRNA construct was assessed by examining the protein levels of MCC in the transduced cells through Western Blot analysis. Remarkably, MCC protein expression levels were significantly reduced in human multiple myeloma cells transduced with hMCC shRNA constructs 1332 and 2689. The lentiviral shRNA-mediated
knockdown of MCC was able to markedly decrease cell viability, induce apoptosis, inhibit cell cycle progression and decrease proliferation in TRAF3<sup>−/−</sup> human multiple myeloma cells. Taken together, these results provide strong evidence that MCC functions as an oncogene in TRAF3<sup>−/−</sup> malignant B-cells.

In notable contrast, previous research in colorectal carcinogenesis has indicated that MCC functions as a tumor suppressor in colorectal cancer cells (CRC).<sup>19,22,24</sup> Importantly, MCC is reported to be localized in the nucleus and cytoplasm in CRC cells and functions through the Wnt/β-catenin signal transduction pathway.<sup>50</sup> MCC expression in CRC was able to suppress cellular proliferation by acting through a distinct mechanism in the Wnt/β-catenin/TCF/LEF-dependent promoter pathway. Specifically, MCC is able to suppress this pathway by interfering with β-catenin binding activity through the relocalization of β-catenin to the cytoplasm, which indirectly increases its degradation.<sup>21</sup> The reported interaction between MCC and the Wnt/β-catenin/TCF/LEF-dependent promoter pathway is conceivable in colorectal carcinogenesis due to the presence of Nuclear Exclusion Sequences (NES) and arginine-rich Nuclear Localization Sequences (NLS) within the MCC protein. The NES and NLS domains of MCC are needed for the proposed importin-exportin-dependent nuclear-cytoplasmic shuttling mechanism to interact with the Wnt/β-catenin dependent pathway in CRC cells.<sup>50,51</sup>

Although MCC is known as a tumor suppressor in colorectal carcinoma, there has been no prior research delineating the role of MCC in B-cell lymphomagenesis. In the present study, we found that MCC is strikingly up-regulated in TRAF3<sup>−/−</sup> mouse B lymphomas and human multiple myeloma cells. TRAF3<sup>−/−</sup> pre-malignant B-cells demonstrate increased survival yet lack the ability to proliferate, which is needed for malignancy to develop. Initially, TRAF3 inactivation in B-TRAF3<sup>−/−</sup> mice is characterized by aberrant, premalignant B-cells with longer
life-span as compared to normal B-cells from littermate control mice. Therefore, during the 9 month latency observed in B-TRAF3⁻/⁻ mice, additional oncogenic signaling events are needed in order to transform the nonproliferating, premalignant B-cells into proliferating, tumorigenic cells. Here, we have demonstrated that MCC knockdown in TRAF3⁻/⁻ human multiple myeloma cells markedly decreased cell viability, induced apoptosis, inhibited cell cycle progression, and suppressed cellular proliferation. Thus, results obtained from this study strongly suggest that aberrant MCC upregulation is one of the secondary oncogenic hits in TRAF3⁻/⁻ B-cell malignancies (Fig. 13).

![Diagram](image.png)

**Figure 13.** Additional secondary oncogenic alterations are needed for malignant transformation in B-cells. This schematic illustrates the pre-malignant B-cell phenotype observed in B-TRAF3⁻/⁻ mice. Further oncogenic modifications lead to the transformation of pre-malignant B-cells to proliferating, malignant B-cells.

While MCC interacts with the Wnt/β-catenin dependent pathway in CRC cells, it is likely that MCC regulates the canonical NF-κB (NF-κB1) pathway, which cooperates with the non-canonical NF-κB pathway (NF-κB2) in B-cell malignant transformation. The non-canonical NF-κB pathway regulates B-cell survival, maturation, and lymphoid organogenesis. Importantly, the deregulated NF-κB2 non-canonical pathway is associated with lymphoid malignancies. Both IKKα inactivation and NIK knockout mice display defective NF-κB2
activation and decreased numbers of mature B cells.\textsuperscript{54-56} Importantly, TRAF3 is a crucial negative regulator of the NF-κB2 pathway in B-cells and is needed to modulate the steady level of NIK in the cells by forming the complex of TRAF3-TRAF2-cIAP1/2-NIK. Therefore, disruption of any of the proteins of this complex will lead to dysregulation of the NF-κB2 pathway and altered B-cell survival.\textsuperscript{16,57-59} These findings are consistent with reported results indicating that specific ablation of TRAF3 in B-cells led to prolonged B-cell survival, expanded B-cell compartments in secondary lymphoid organs, splenomegaly, and lymphadenopathy.\textsuperscript{16} Furthermore, genetic deficiencies in TRAF3, TRAF2 and cIAP1/2, and amplification of NIK have been identified in human multiple myeloma, and are associated with aberrant non-canonical NF-κB2 activation. Furthermore, it has been demonstrated that constitutive NF-κB2 activation is a primary oncogenic pathway in TRAF3\textsuperscript{-/-} B-cells.\textsuperscript{17}

The canonical NF-κB (NF-κB1) pathway mediates both B-cell survival and proliferation, and is oncogenic in a variety of human cancers.\textsuperscript{60,61} In light of the evidence that MCC can directly interact with Valosin-Containing Protein (VCP), a regulator of the NF-κB1 pathway, I propose that MCC functions through the NF-κB1 pathway to promote the survival and proliferation of B-cells. MCC co-immunoprecipitated with VCP in HEK 293 human embryonic kidney cells, suggesting that VCP is a binding partner of MCC.\textsuperscript{52} The interaction between VCP and the inhibitor of the canonical NF-κB pathway, IκBα, has been well characterized. VCP contains two copies of the conserved ATP binding domain and an N-terminal polyubiquitin binding domain, which allows VCP to associate with ubiquitinated proteins for transport to the proteasome.\textsuperscript{60,62,63} Interestingly, the association of VCP with ubiquitinated IκBα has been demonstrated in a variety of cell lines, including human B-cell lines. In the canonical NF-κB1 pathway, VCP acts as a molecular chaperone to transport IκBα to the proteasome for ubiquitin-
mediated degradation and also functions as part of the 26S proteasomal complex. It is likely that MCC may function to stimulate the VCP-dependent ubiquitin-degradation and processing of IkBα, leading to constitutive activation of the NF-κB1 pathway. Active NF-κB1 can upregulate transcription of target genes needed for both B-cell survival and proliferation. Therefore, the constitutive activation of the NF-κB1 pathway may act cooperatively and synergistically with the TRAF3-inactivation initiated upregulation of the NF-κB2 non-canonical pathway to promote B lymphomagenesis (Fig. 14).

Figure 14. Potential mechanism for MCC-mediated oncogenic regulation in malignant B-cells. MCC-VCP protein-protein interactions may constitutively activate the NF-κB1 pathway. The synergistic and cooperative effects
of NK-κB1 and NF-κB2 constitutive activation will then induce the expression of the target genes needed for malignant B-cell proliferation and transformation.

Future Direction

It is necessary to investigate further the interaction between MCC and VCP in B-cells. I have generated FLAG-tagged MCC and MCC-SBP-6xHis lentiviral expression constructs, which can be used to transduce and express tagged MCC in human myeloma cells. These tools will be allow subsequent co-immunoprecipitation experiments of MCC and VCP in tumor B-cells. Furthermore, tandem affinity purification followed by mass-spectrometry based sequencing will be used to identify MCC- binding partners in tumor B-cells. Importantly, these experiments will identify novel MCC-interacting proteins, and are thus crucial for delineating the signaling mechanisms of MCC in B-cell malignant transformation. Additionally, the MCC knockdown studies were performed in transduced TRAF3−/- human multiple myeloma cells in vitro. Further in vivo tumorigenicity tests will be performed by injecting immunodeficient NSG recipient mice with transduced human multiple myeloma cells to assess the role of MCC in whole animals.

Significance

A greater understanding of the molecular mechanisms of lymphatic carcinogenesis is necessary to facilitate new treatment avenues for B-cell neoplasms. This project has further delineated the molecular mechanisms of MCC-mediated cancer pathogenesis and will eventually contribute to optimization of treatment courses for B-cell lymphomas. Specifically, it has been demonstrated that MCC is necessary for the maintenance of the malignant state of B-cell lymphomas and may serve as a therapeutic target. Interestingly, by surveying the public gene expression database of cancers (http://www.oncomine.org) identified by microarray, we found
that MCC is also vastly up-regulated in human primary effusion lymphoma and multiple myelomas. This evidence together with our findings suggests that MCC may also be used as a diagnostic marker for B-cell malignancies. Ultimately, improved molecular diagnostics and novel therapeutic approaches offer the potential for accurate prognostic and personalized treatment roadmaps for patients with B-cell lymphomas and multiple myelomas.
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