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Cancer Biology and Signal Transduction

Molecular Cancer Therapeutics

IQGAP1 Scaffold–MAP Kinase Interactions Enhance Multiple Myeloma Clonogenic Growth and Self-Renewal

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Abstract

Despite improved outcomes in newly diagnosed multiple myeloma, virtually all patients relapse and ultimately develop drug-resistant disease. Aberrant RAS/MAPK signaling is activated in the majority of relapsed/refractory multiple myeloma patients, but its biological consequences are not fully understood. Self-renewal, as defined by the long-term maintenance of clonogenic growth, is essential for disease relapse, and we examined the role of RAS/MAPK activation on multiple myeloma self-renewal by targeting IQ motif–containing GTPase-activating protein 1 (IQGAP1), an intracellular scaffold protein required for mutant RAS signaling. We found that loss of IQGAP1 expression decreased MAPK signaling, cell-cycle progression, and tumor colony formation. Similarly, a peptide mimicking the WW domain of IQGAP1 that interacts with ERK inhibited the clonogenic growth and self-renewal of multiple myeloma cell lines and primary clinical specimens *in vitro* as well as tumor-initiating cell frequency in immunodeficient mice. During multiple myeloma progression, self-renewal may be enhanced by aberrant RAS/MAPK signaling and inhibited by targeting IQGAP1. *Mol Cancer Ther; 15(11); 2733–9. ©2016 AACR.*

Introduction

Multiple myeloma remains an incurable disease with a continual risk of relapse even in patients achieving complete remissions to initial therapy (1). Disease relapse is dependent on the maintenance of clonogenic growth over time (herein referred to as self-renewal) for tumor regrowth, and the frequency of clonogenic tumor cells may increase with disease progression (2). Therefore, the determinants of multiple myeloma self-renewal may be associated with factors that are increased or activated in advanced disease. The frequency of RAS/MAPK pathway mutations increases with disease progression to eventually become the most common genetic alterations in multiple myeloma (3–11). Moreover, intratumor subclones with RAS mutations often establish dominance despite their late acquisition in clonal evolution (10). Therefore, RAS/MAPK signaling may be involved in aberrant self-renewal in advanced multiple myeloma similar to other diseases (12–16).

The biological consequences of RAS/MAPK signaling in multiple myeloma are not fully understood, but the expression of mutant RAS in an IL6-dependent multiple myeloma cell line induces cytokine independence and chemoresistance (17–22). RAS mutations are also associated with extramedullary disease and bortezomib resistance that can be features of advanced disease (6, 11, 23). Thus, the inhibition of RAS/MAPK signaling may be most effective in patients with relapsed and refractory disease, but clinical approaches to target mutant RAS remain a challenge.

IQ motif–containing GTPase-activating protein 1 (IQGAP1) is a cytoplasmic scaffold for RAS/MAPK signaling that binds to MAPK through a small WW domain and is upregulated in various solid tumors (24–28). IQGAP1 is dispensable for normal skin homeostasis, but its loss decreases RAS/MAPK signaling and mitigates RAS-mediated tumorigenesis in solid tumor models (24). In this study, we examine a novel approach to disrupt the interaction between IQGAP1 and MAPK and determine the biological effects of RAS/MAPK signaling on multiple myeloma self-renewal. We found that IQGAP1 knockdown inhibited RAS/MAPK signaling, as has been previously been shown (29, 30). Moreover, targeting IQGAP1 with a small peptide mimetic that binds to ERK also effectively blocked MAPK signaling, decreased clonogenic growth and self-renewal *in vitro*, and inhibited multiple myeloma tumor-initiating cell frequency of NCI-H929 cells engrafted in immunodeficient mice. Therefore, RAS/MAPK signaling may regulate aberrant self-renewal during disease progression, and targeting IQGAP1 using an IQGAP1–ERK interaction blockade may represent a novel strategy for advanced multiple myeloma.

Materials and Methods

Cell lines and clinical specimens

Human multiple myeloma cell lines NCI-H929 (year: 2012), RPMI-8226 (year: 2012), and MM1.S (year: 2013) were obtained from the ATCC and KMS12 cells from the Japanese Collection of Research Bioresources (Year: 2012; National Institutes of

Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Health Sciences, Japan). The KMS12 cell line is RAS and BRAF wild-type. All cell lines were independently authenticated using short tandem repeat profiling and tested for mycoplasma using PCR within 3 months of use. For peptide treatment, cells were cultured in complete media (CM) and incubated with the SCR or WW peptides as described previously (2, 24). For long-term treatment with peptide, cells were collected by centrifugation ($300 \times g$) and resuspended in CM containing peptide twice per week for 2 weeks.

Clinical bone marrow and peripheral blood samples were obtained from multiple myeloma patients or healthy donors providing informed consent in accordance with the Declaration of Helsinki, as approved by the Johns Hopkins Medical Institutes Institutional Review Board. Mononuclear cells were isolated by density centrifugation (Ficoll–Paque; Pharmacia). $CD138^+$ cells from normal bone marrow donors were isolated using magnetic microbeads (Miltenyi Biotec).

Lentivirus production and transduction

Lentiviral supernatants were produced by transfecting pLKO.Tet. On shRNA (Addgene) or pLVX-hIQGAP1-IRES-mCherry (Clontech) lentivectors into 293FT cells along with pMDG.2 and psPax2 packaging plasmids. Multiple myeloma cells were transduced with equal amounts of lentiviral supernatants. When appropriate, puromycin selection was used and maintained throughout each experiment. The targeting sequences for each shRNA were as follows: IQGAP1 5′-GCCCACATTGTGCCTTTATTTCTC-3′, SCR 5′-CCTAAGGTTAAGTCGCCCTCGCTC-3′. The IQGAP1 shRNA used here recognizes the 3′ UTR and, therefore, should not significantly impact the expression of the hIQGAP1 ORF expressed from the rescue plasmid.

Tumor cell colony formation

Tumor colony formation in methylcellulose was used to quantify *in vitro* clonogenic growth according to our previously published methods (2, 31). Multiple myeloma cell lines (1,000 cells/mL) were washed after treatment to remove drug or peptide and then plated in duplicate into 35-mm^2 tissue culture dishes containing 1.2% methylcellulose, 10% FBS, 1% BSA, 10^{-4} mol/L 2-mercaptoethanol, and 2 mmol/L L-glutamine in the absence of doxycycline, drug, or peptide. For clinical specimens, unfractionated mononuclear cells (without $CD34^+$ depletion) were isolated from bone marrow aspirates, treated with peptide, washed, and then plated (5×10^5/mL) in methylcellulose cultures containing 10% lymphocyte conditioned media as a source of growth factors. After 14 to 21 days of culture at 37°C and 5% CO_2, tumor colonies were quantified using an inverted microscope as described previously (2, 31).

Mouse studies

For limiting dilution assays, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice (8–12 weeks, female) were injected intravenously with NCI-H929 cells resuspended in 200 μL of RPMI1640 media without serum. Engraftment was assessed by detecting serum human (Ig) kappa light chain using an ELISA per the manufacturer's protocol (Bethyl Laboratories). Tumor-initiating cell frequency and *P* values were determined using extreme limiting dilution analysis software (http://bioinf.wehi.edu.au/software/elda/; ref. 32).

Immunoblot analysis

Western blot analysis was carried out on whole-cell lysates separated by 4% to 15% SDS-PAGE, transferred to a polyvinylidene fluoride membrane (Life Technologies), and then incubated with antibodies against pERK [1:2,000, Cell Signaling Technology (CST), #9101S], total ERK (1:1,000, CST #9192S), pAKT (1:1,000, T308, CST #2965S), total AKT (1:1,000, CST #4691S), and IQGAP1 (1:1,000, Abcam #ab86064). Detection was carried out using a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase and ECL chemiluminescence reagent (Millipore). Densitometry was performed with Image Lab software (Bio-Rad), and for relative quantitation, bands derived from the same immunoblot were used.

Flow cytometry

Cells were stained in PBS supplemented in 0.5% BSA (staining buffer) along with FITC-conjugated mouse anti-human CD138 or relevant isotypic control antibodies (BD Pharmingen) for 20 minutes at 4°C. Cells were subsequently washed and resuspended in staining buffer containing 0.1 ng/μL propidium iodide (PI; Sigma) and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Cells were initially gated to exclude PI-positive cells and then analyzed for CD138 expression. Annexin V-FITC and bromodeoxyuridine (BrdUrd)/7AAD staining was performed per the manufacturer's protocol (BD Pharmingen). For BrdUrd labeling, cells were incubated for 40 minutes with 10 μmol/L BrdUrd and stained with 0.5 μL of anti-BrdUrd antibody. Flow cytometry was performed on a BD FACSCalibur, followed by analysis using FlowJo 8.7 software.

Statistical analysis

Student *t* test, one-way ANOVA with multiple comparisons, or Kaplan–Meier analyses were performed using GraphPad Prism 6. The log-rank test was used to test for differences between study groups. $P < 0.05$ was considered significant.

Results

IQGAP1 is expressed in advanced multiple myeloma

IQGAP1 is overexpressed in solid tumors (28), and we initially examined its expression in clinical multiple myeloma specimens. Within clinically annotated gene expression datasets (33–38), *IQGAP1* levels were similar between normal $CD138^+$-selected plasma cells and tumor cells isolated from MGUS or multiple myeloma patients (not shown). However, it was significantly overexpressed in $CD138^+$-selected cells from patients with plasma cell leukemia (PCL) compared with those with multiple myeloma (Supplementary Fig. S1A) and associated with increased mortality (Supplementary Fig. S1B). We also quantified IQGAP1 protein expression in freshly collected $CD138^+$-selected clinical specimens and similarly found that it was overexpressed in secondary PCL as well as multiple myeloma cell lines (Fig. 1A). Therefore, IQGAP1 expression may be associated with disease progression in multiple myeloma.

Targeting IQGAP1 in multiple myeloma decreases MAPK signaling and induces a cell-cycle arrest

Previous studies have shown that IQGAP1 expression is required for RAS/MAPK signaling and cell growth both in solid tumor models and in multiple myeloma (24, 29, 30). However, it

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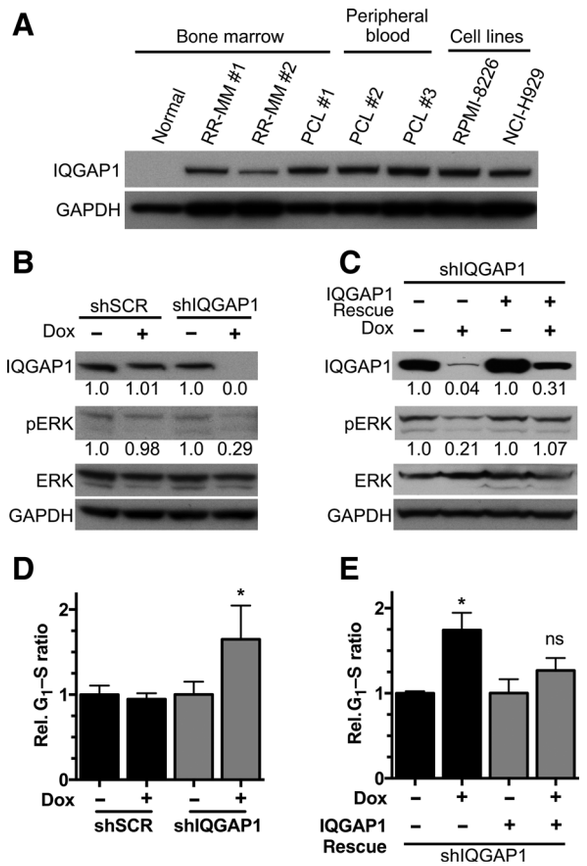

Figure 1.

IQGAP1 loss of function impacts MAPK signaling and proliferation in multiple myeloma (MM). **A,** $CD138^+$ cells were isolated from normal, relapsed/refractory multiple myeloma (RR-MM), and secondary PCL patient specimens, followed by Western blotting for the indicated proteins. **B,** RPMI-8226 multiple myeloma cells carrying a doxycycline (Dox)-inducible shRNA against scramble control or IQGAP1. After treatment of cells for 2 days with doxycycline (100 ng/mL), the cells were subjected to Western blotting. Numbers, normalized values of IQGAP1 or phospho-ERK relative to total ERK. **C,** cells containing IQGAP1 shRNA and control-IRES-mCherry or hIQGAP1 (shRNA resistant)-IRES-mCherry were sorted for mCherry, followed by doxycycline treatment and Western blotting. **D** and **E,** cell-cycle analysis was done using BrdUrd/7AAD and displayed as a ratio of G_1–S-phase. ns, not significant. Error bars, three biological replicates. *P* values were determined by one-way ANOVA with multiple comparisons. *, $P < 0.05$; ns, not significant.

is not known whether IQGAP1 loss of function mimics the cell-cycle arrest observed with direct MAPK pathway inhibition in multiple myeloma (19, 21, 39). To determine whether the loss of IQGAP1 causes a similar phenotype and impacts multiple myeloma cell cycle, we generated stable cell lines expressing doxycycline-inducible shRNA against *IQGAP1*. In RAS-mutated RPMI-8226 cells, IQGAP1 knockdown significantly decreased pERK1/2 levels and cell-cycle progression compared with cells with a scrambled control shRNA construct (Fig. 1B–D). Moreover, the effects of the IQGAP1 shRNA were specific, as they were rescued by an *IQGAP1* expression vector resistant to the shRNA (Fig. 1C–E). Consistent with a role for IQGAP1 in RAS/MAPK signaling, we also found that clinical specimens with increased IQGAP1 expression had gene expression patterns indicative of RAS pathway activation using gene set enrichment analysis (Supplementary Fig. S2A and S2B). Therefore, targeting scaffold–MAPK interactions may be a feasible means of inhibiting RAS/MAPK signaling in multiple myeloma.

A peptide mimetic of IQGAP1 WW domain inhibits MAPK signaling in multiple myeloma

The WW domain of IQGAP1 binds to ERK, and a synthetic peptide that mimics this domain competitively inhibits MAPK signaling in RAS- or BRAF-mutated solid tumor cells (24). We examined the effects of the WW peptide in multiple myeloma cells and found that it significantly decreased the phosphorylation of ERK1/2 (Fig. 2A). This effect appeared to be specific for ERK as AKT phosphorylation was unaffected (Fig. 2A). Similar to IQGAP1 knockdown, the treatment of multiple myeloma cells with the WW peptide significantly inhibited cell cycling compared with a scrambled control peptide (SCR; Fig. 2B). Therefore, the WW peptide has anti-multiple myeloma activity *in vitro*.

IQGAP1 is required for multiple myeloma clonogenic growth and self-renewal

Tumor regrowth and disease relapse following remission induction is dependent on the long-term maintenance of clonogenic potential, and aberrant RAS/MAPK signaling has been associated with self-renewal in both solid tumors and AML (12–16). We studied the impact of IQGAP1 on the clonogenic growth of RAS-mutated multiple myeloma cells and found that IQGAP1 knockdown significantly inhibited tumor cell colony formation compared with cells with a control shRNA (Fig. 3A). The effects on tumor colony formation were specific, as the expression of an shRNA-resistant IQGAP1 construct rescued this

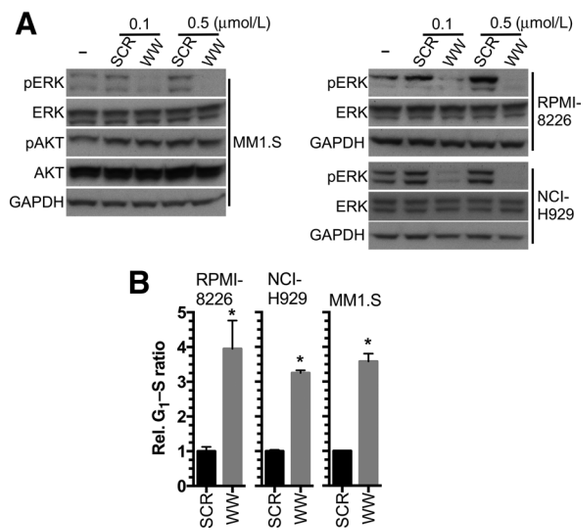

Figure 2.

IQGAP1 WW peptide impacts MAPK signaling and cell-cycle proliferation in multiple myeloma. **A,** the indicated RAS-mutated multiple myeloma cell lines were treated with the WW peptide or SCR (500 nmol/L) for 24 hours and subjected to Western blotting. **B,** the indicated RAS-mutated multiple myeloma cell lines were subjected to BrdUrd/7-AAD cell-cycle analysis after exposure to the peptide and expressed as a ratio of G_1–S-phase. Error bars, biological replicates. *P* values were determined by one-way ANOVA with multiple comparisons. *, $P < 0.05$.

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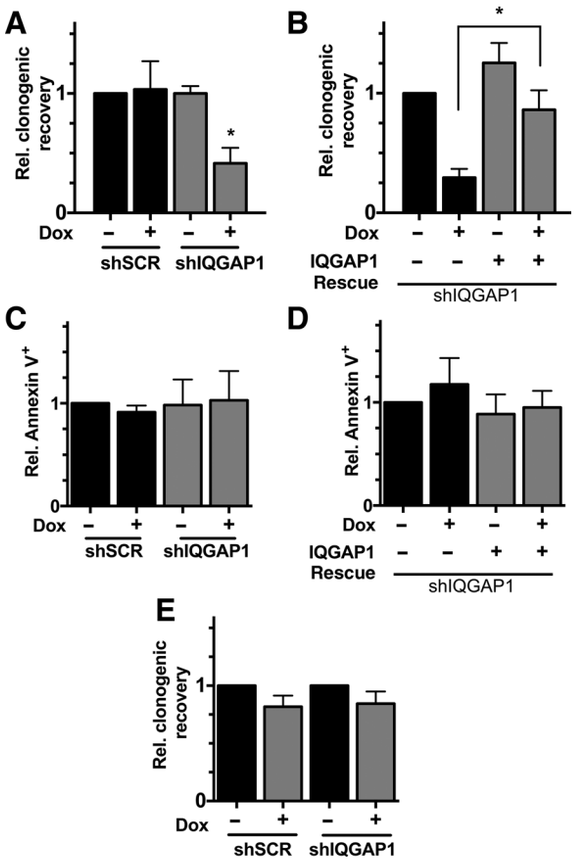

Figure 3.

IQGAP1 is required for clonogenic growth in multiple myeloma. **A,** RPMI-8226 cells (RAS mutant) containing doxycycline (Dox)-inducible shRNA targeting scramble (shSCR) or IQGAP1 were treated with doxycycline for 6 days. Phenotypically unselected cells were then plated in methylcellulose without doxycycline. Quantification of colonies occurred 7 to 10 days later. Results are the average of three biological replicates. **B,** RPMI-8226 cells containing doxycycline-inducible shRNA targeting IQGAP1 and ectopic control or hIQGAP1 rescue were subjected to doxycycline treatment for 9 days, followed by colony formation as in **A**. Results are the average of three biological replicates. *, $P < 0.05$. **C** and **D,** the cells from **A** and **B** were subjected to Annexin V staining, followed by flow cytometric analysis. Results are the average of two biological replicates. **E,** KMS12 cells (RAS/BRAF wild-type) containing the indicated doxycycline-inducible shRNA were treated with doxycycline and subjected to colony-forming cell assays as in **A**. Results are the average of two biological replicates.

inhibition (Fig. 3B). Notably, apoptosis was not significantly increased by IQGAP1 knockdown (Fig. 3C–D). In distinction to the above results, IQGAP1 knockdown had no effect on clonogenic growth of a RAS wild-type multiple myeloma cell line (Fig. 3E). We also found that treatment with the WW peptide significantly inhibited colony formation by 3 of 4 multiple myeloma cell lines compared with the SCR control peptide. Notably, the one unaffected cell line was also RAS wild-type (Fig. 4A). The inhibition of colony formation was maintained during secondary plating, suggesting that the disruption of IQGAP1–MAPK interactions limits multiple myeloma self-renewal (Fig. 4B). The effects of the WW peptide on colony formation were not directly due to its effects on proliferation, as cells were not exposed to additional WW peptide over the course of serial plating and removal of the WW peptide results in correction of cell-cycle defects (Supplementary Fig. S3). These effects were also not due to cell death, as apoptosis was not significantly induced following treatment (Fig. 4C). MAPK signaling was also critical for multiple myeloma clonogenic growth, as the MEK inhibitor selumetinib (AZD6244) similarly inhibited pERK1/2 levels (data not shown) and colony formation (Fig. 4D). Therefore, IQGAP1 enhances multiple myeloma self-renewal through MAPK signaling.

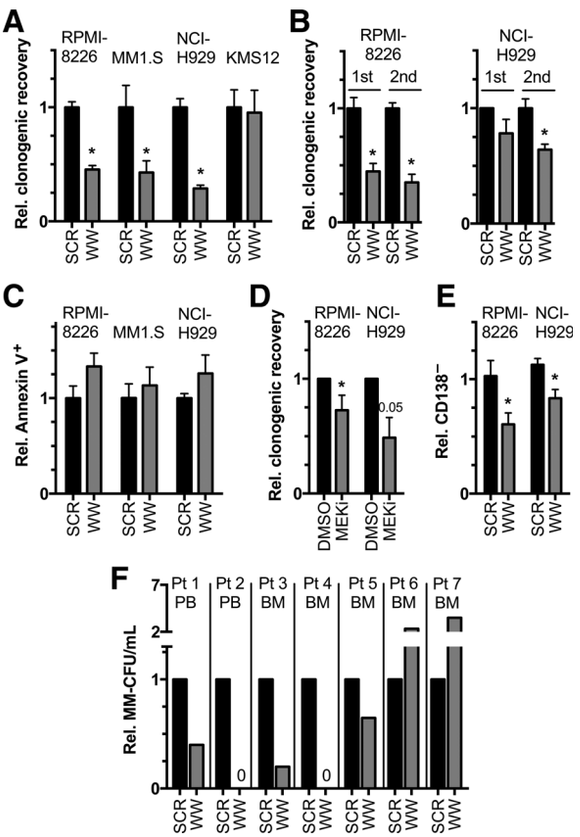

Figure 4.

IQGAP1 WW peptide impacts multiple myeloma clonogenic growth and self-renewal. **A,** the indicated multiple myeloma cell lines were treated with 500 nmol/L of scrambled (SCR) or WW peptide for 4 days (RPMI-8226, NCI-H929, and MM1.S are RAS mutated, whereas KMS12 is RAS/BRAF wild-type). Unselected cells were then plated in methylcellulose in the absence of peptide. Colonies were scored 7 to 10 days later. Results are the average of three biological replicates. *P* values were determined by one-way ANOVA followed by multiple comparison testing. *, $P < 0.05$. **B,** multiple myeloma cells were treated with peptide and then plated onto methylcellulose as in **A**. Colonies were scored and then harvested for replating using equal volumes of samples after washing. **C,** cells from **A** were subjected to cell staining using Annexin V followed by flow cytometric analysis. **D,** multiple myeloma cells were treated with 100 nmol/L of MEK inhibitor selumetinib or DMSO control for 4 days, followed by quantification of colony formation as in **A**. **E,** multiple myeloma cells were treated with peptide (100 nmol/L) and subjected to staining with CD138-FITC and PI. The percentage of $CD138^-$, PI^- cells was then calculated after flow cytometric analysis (absolute values ranged from 0.5%–1%) and normalized to a control sample. *P* values were determined by Student *t* test. **F,** unsorted bone marrow (BM) or peripheral blood (PB) mononuclear cells from multiple myeloma (MM) patient (Pt) samples were treated with SCR or WW peptide (500 nmol/L) for 4 days, followed by plating in methylcellulose. CFU, colony-forming unit.

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Multiple myeloma is characterized by the expansion of malignant plasma cells expressing high surface levels of CD138, a marker of plasma cell differentiation. However, prior studies have demonstrated that CD138$^-$ multiple myeloma cells are enhanced for self-renewal and tumor-initiating potential both *in vitro* and *in vivo* (2, 31, 40). We found that the WW peptide also decreased the relative proportion of CD138$^-$ cells in multiple myeloma cell lines (Fig. 4E). Furthermore, treatment with the WW peptide decreased multiple myeloma colony formation in unsorted whole bone marrow or peripheral blood mononuclear cells from 5 of 7 primary clinical specimens with relapsed and refractory myeloma or secondary PCL (Fig. 4F). Although colony formation was inhibited, the limited viability of primary colonies did not allow us to carry out serial plating and evaluate effects on self-renewal.

IQGAP1 WW peptide impacts multiple myeloma tumor-initiating cell frequency

The development of therapies inhibiting the propagation of multiple myeloma may decrease the likelihood of relapse. To determine whether disrupting IQGAP1–MAPK interactions can affect tumor-initiating potential *in vivo*, we treated multiple myeloma cells with the WW peptide and then examined engraftment following intravenous injection into NSG immunodeficient mice. Notably, cells were washed of the peptide prior to injection, and no further peptide treatment was given after injection so as to not conflate the effects on proliferation with tumor-initiating potential. Compared with treatment with the SCR control peptide, the tumor-initiating cell frequency of WW peptide–treated cells was significantly decreased by approximately 10-fold (Fig. 5A). Furthermore, mice engrafted with the WW peptide–treated cells survived significantly longer than those receiving control SCR peptide cells (Fig. 5B). Therefore, the WW peptide inhibits *in vivo* clonogenic growth.

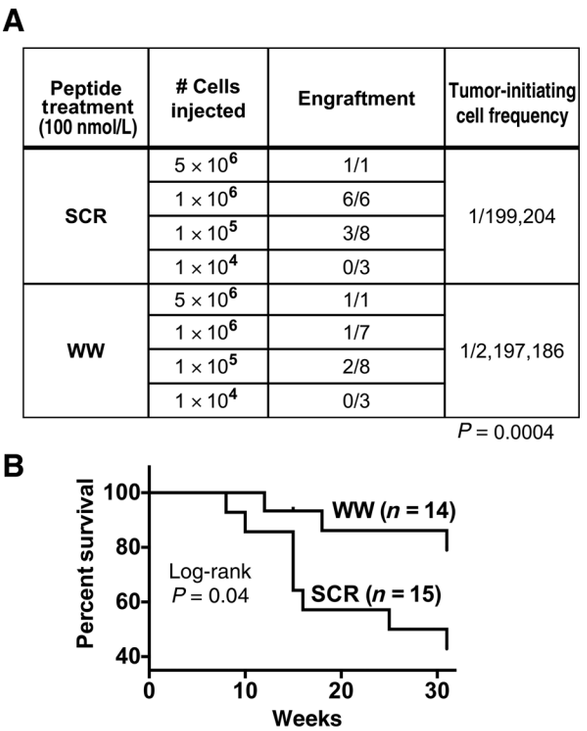

Figure 5.
IQGAP1 WW peptide impacts multiple myeloma tumor-initiating cell frequency and survival. **A,** NCI-H929 human multiple myeloma cells were treated *ex vivo* for 2 weeks with SCR or WW peptide (100 nmol/L), then washed to remove peptide, and then injected into NSG mice at the indicated cell doses with no further peptide treatment. Engraftment was detected from serum using a Human Kappa Light Chain ELISA Kit. Tumor-initiating cell frequencies and a *P* value for statistical comparison between the SCR- and WW-treated groups were then determined by applying a χ^2 analysis using all of the engraftment results listed in this figure (see Materials and Methods section). **B,** Kaplan–Meier survival curve of mice injected with 10^5 or 10^6 cells.

Discussion

We demonstrate that targeting IQGAP1–MAPK signaling using a peptide mimicking the WW domain of IQGAP1 effectively inhibits MAPK signaling and leads to a loss of cell-cycle proliferation. More importantly, we provide novel evidence that blocking RAS/MAPK signaling in multiple myeloma reduces tumor initiation and self-renewal. In multiple myeloma, MAPK signaling is a major effector of oncogenic RAS mutations. In addition, other genetic alterations, such as BRAF mutations or chromosomal rearrangements that drive overexpression of MMSET/FGFR3, can lead to aberrant activation of MAPK pathway (41–43). The activity of the WW peptide appears to be dependent on aberrant MAPK signaling, and it is possible that mutations in RAS or BRAF can serve as predictive biomarkers of response. We also found that IQGAP1 expression levels correlate with disease progression and poor outcomes, but note that these studies were limited by analysis of CD138$^+$-selected cells only and, therefore, may not reflect the IQGAP1 levels in the entire tumor population. Nonetheless, these data suggest that IQGAP1 may play a role in multiple myeloma disease progression.

MEK inhibitors are active against multiple myeloma cell lines (19, 21, 22). However, the activity of these agents has been variable in the clinical setting. A recent retrospective study reported significant anti-multiple myeloma activity of a MEK inhibitor in patients with RAS- and BRAF-mutated tumors (44), whereas in a more recent prospective study, there was little activity in relapsed/refractory patients (45). This latter trial also included wild-type as well as mutant RAS and BRAF tumors, and it is possible that RAS mutations were limited to minor subclones that would limit efficacy against the entire tumor. Differences between the particular MEK inhibitors used in these studies may also be an explanation for any lack of efficacy seen, given that some particular MEK inhibitors also have suboptimal activity in solid tumor models (46). The disruption of scaffold–MAPK interactions may be advantageous over MEK inhibition, as the direct inhibition of ERK can prevent feedback activation of MAPK signaling that may arise in MEK and BRAF inhibitor–resistant solid tumors (24). Furthermore, IQGAP1 is required for RAS-mediated tumorigenesis but not MAPK signaling in normal cells (24), and the WW peptide may represent an active and safe strategy to inhibit MAPK signaling in multiple myeloma. Although the potential clinical use of peptides as anticancer agents may be limited, our studies demonstrate that targeting scaffold–MAPK interactions represents an effective means of inhibiting RAS/MAPK signaling in multiple myeloma.

The increasing proportion of both patients with RAS-mutant multiple myeloma and intratumoral clonal dominance suggests that aberrant RAS/MAPK signaling enhances self-renewal (4, 8,

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10, 23). In addition to decreased cell proliferation, we found that blocking IQGAP1–MAPK interactions decreased clonogenic multiple myeloma growth and self-renewal as well as the frequency of $CD138^-$ precursors. One limitation of our study is that most of our functional assays were done on multiple myeloma cell lines. However, we did find that the WW peptide impacts clonogenic growth in primary patient samples. An additional limitation to our studies is that we did not determine the impact of the WW peptide on isolated subsets of multiple myeloma cells (e.g., $CD138^-$ and $CD138^+$). However, the precise phenotype of clonogenic myeloma cells is controversial, and our studies indicate that the WW peptide inhibits cancer stem cell function, that is, colony and tumor initiation, without bias toward or against a specific cell-surface phenotype. Given these findings, it is possible that RAS/MAPK signaling drives self-renewal in relatively differentiated myeloma progenitors similar to findings in CML (47). Therefore, inhibition of RAS/MAPK signaling may be useful in both debulking tumors as well as preventing tumor regrowth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C.B. Gocke, W. Matsui
Development of methodology: C.B. Gocke, Q. Wang, V. Penchev
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.B. Gocke, R. McMillan, Q. Wang, A. Begum, C.A. Huff
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.B. Gocke, C.A. Huff, W. Matsui
Writing, review, and/or revision of the manuscript: C.B. Gocke, S.A. Ali, I. Borrello, C.A. Huff, W. Matsui
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.B. Gocke, Q. Wang, V. Penchev
Study supervision:, W. Matsui

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