miR-30-5p Functions as a Tumor Suppressor and Novel Therapeutic Tool by Targeting the Oncogenic Wnt/β-Catenin/BCL9 Pathway

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Abstract

Wnt/β-catenin signaling underlies the pathogenesis of a broad range of human cancers, including the deadly plasma cell cancer multiple myeloma. In this study, we report that downregulation of the tumor suppressor microRNA miR-30-5p is a frequent pathogenetic event in multiple myeloma. Evidence was developed that miR-30-5p downregulation occurs as a result of interaction between multiple myeloma cells and bone marrow stromal cells, which in turn enhances expression of BCL9, a transcriptional coactivator of the Wnt signaling pathway known to promote multiple myeloma cell proliferation, survival, migration, drug resistance, and formation of multiple myeloma cancer stem cells. The potential for clinical translation of strategies to re-express miR-30-5p as a therapeutic approach was further encouraged by the capacity of miR-30c and miR-30 mix to reduce tumor burden and metastatic potential in vivo in three murine xenograft models of human multiple myeloma without adversely affecting associated bone disease. Together, our findings offer a preclinical rationale to explore miR-30-5p delivery as an effective therapeutic strategy to eradicate multiple myeloma cells in vivo. Cancer Res; 74(6); 1801–13. ©2014 AACR.

Introduction

Multiple myeloma is a cancer of plasma cells that accumulate in the bone marrow. Despite recent advances in understanding the molecular pathogenesis of multiple myeloma and the unveiling of promising new therapies, it remains incurable, highlighting the need for continual efforts to develop novel and hopefully more effective therapies (1, 2).

The canonical Wnt/β-catenin pathway is implicated in the pathogenesis of a broad range of cancers, and has emerged as a promising target for therapy. Loss-of-function mutations in APC and Axin as well as activating mutations in β-catenin itself, enable β-catenin nuclear translocation, and drive oncogenic Wnt transcription (3). Coactivators for β-catenin transcription have been identified, including Pygo, B-cell lymphoma 9 (BCL9), and its homolog B-cell lymphoma 9-like (B9L), among others (4, 5). The formation of a quaternary complex consisting of TCF, β-catenin, BCL9 (or B9L), and Pygo enhances β-catenin-dependent transcriptional activity (6).

The canonical Wnt pathway is constitutively active in multiple myeloma and promotes tumor cell proliferation and disease progression (7–11); however, mutations in APC, Axin, or β-catenin have not been reported (12). Instead, the mechanism of pathologic Wnt signaling in multiple myeloma has been linked to posttranscriptional regulation of β-catenin (13) and/or increased levels of BCL9, implicating this β-catenin cofactor as a bona fide oncogene (9). The oncogenic role of BCL9 is further highlighted by the following observations: human BCL9 was first identified by cloning the (t(1;14)(q21;q32) translocation from a patient with B-cell acute lymphoblastic leukemia (14); chromosome 1q21 amplifications containing the BCL9 locus are observed in a broad range of human cancers (15), including multiple myeloma, and is associated with poor clinical outcome (8); shRNA-induced knockdown of BCL9 or treatment with stabilized α-helix of BCL9 (SAH-BCL9), which selectively suppress Wnt transcription, elicit mechanism-based antitumor responses in colorectal cancer cells and multiple myeloma (9, 10). Collectively, these data indicate that targeting the BCL9 component of aberrantly activated Wnt signaling in cancer may attenuate invasion, metastasis, and resistance to therapy, highlighting the importance of this pathway and BCL9 for target drug discovery.
In previous studies, we found that BCL9 is overexpressed in a large subset of patients with multiple myeloma (8, 9). However, only a weak correlation was observed between BCL9 DNA copy number gains and BCL9 mRNA expression levels in patient multiple myeloma cells (Supplementary Fig. S1A), indicating that mechanisms other than gene dosage because of chromosome1q21 amplification may be involved in regulating expression of BCL9 in multiple myeloma.

MicroRNAs (miRNA) function as negative regulators of gene expression (16) and have been implicated in the pathogenesis of multiple myeloma (17) and other cancers (18), offering the promise to create novel therapeutic approaches if they can be effectively applied in vivo. However, a functional link between miRNAs and the Wnt pathway and its clinic and pathologic significance has not been established. Here, we document for the first time that expression of BCL9 is regulated by the miR-30s family. miR-30s are expressed at very low levels in a large subset of the multiple myeloma samples compared with normal plasma cells, and there is a reverse relation between miR-30s and BCL9 mRNA expression levels. Bioinformatics analysis revealed that BCL9 mRNA has two different binding sites for miR-30s in the 3′-untranslated region (3′UTR). Enhanced expression of miR-30s in multiple myeloma cell lines leads to a reduction in cellular proliferation, survival, migration, and invasion as well as colony formation and number of side population cells. These changes were mediated through direct binding of miR-30s to the 3′UTR of BCL9 mRNA, thereby downregulating BCL9 and Wnt transcriptional activity. Overall our studies establish a functional link between miR-30s and BCL9, unveiling their role in multiple myeloma progression and providing a proof-of-concept for the potential translation of miR-30s as novel therapeutic agent to target oncogenic Wnt/β-catenin/BCL9 complex in multiple myeloma and other cancers with deregulated Wnt activity.

Materials and Methods

Patients’ tissue preparation and cell lines

Bone marrow specimens were obtained from patients with multiple myeloma and normal donors in accordance with Dana-Farber Cancer Institute Review Board approval, and informed consent performed in compliance with the Declaration of Helsinki. Multiple myeloma and normal plasma cells were purified from bone marrow aspirates using CD138 magnetic beads (Miltenyi Biotec) as described (19). CD138 negative mononuclear cells were used to establish long-term bone marrow stem cells (BMSC). Stable stroma cell line HS-5 was infected with V-ds-red and sorted to generate a stable cell line for coculture experiments. After a confluent layer of adherent cells was obtained, the cells were ready for coculture experiments and dexamethasone drug treatment experiments. Multiple myeloma cell lines: H929, MM1S, and RPMI8226 were obtained from American Type Culture Collection, OPM1, and MR20 kindly provided by Dr. T. Hideshima. All of the cells were routinely used and informed consent performed in compliance with the Dana-Farber Cancer Institute Review Board approval, and those of the primers of BCL9, Axin-2, and CD44 are listed in Supplementary Table S1. Western blot, miRNAs-LNA in situ hybridization, immunofluorescence, and immunohistochemistry (IHC) were carried out as previously described (21). Antibodies included: BCL9 (6109) antibody for Western blotting and immunofluorescence (10); Alexa Fluor 546 goat anti-rabbit immunoglobulin G (IgG; A-11010; Invitrogen) as secondary antibody for immunofluorescence; as well as actin-horseradish peroxidase (HRP; C-11; Santa Cruz) and anti-Rabbit IgG HRP conjugated secondary antibodies (W401b; Promega) for Western blotting as well as BCL9 (ab37305; Abcam), caspase-3 (*9662; Cell Signaling Technology), Ki-67 (NB110-90592; Novus), Axin-2 (*2151; Cell Signaling), CD44 (*5640s; Cell Signaling), and BCL6 (ab94797; Abcam) were used for IHC.

Lentiviral infection of miRNAs and transient transduction of mature miRNAs

Expression plasmids of miR-30s were purchased from SBI and referred to as V-miR-30s. The construct expresses the pre-miR-30s, which is processed into mature miRNAs in infected cells. Lentiviral packaging and infection of multiple myeloma cells were done according the protocol from manufacturer (SBI). Multiple myeloma cells were infected with viral supernatant containing polybrene, and GFP-expressing cells sorted by FACS. RPMI-8226, H929, and MM1S-Luc-neo cells were transiently transduced with either mature miR-30a/b/c/d/e, miR-30c only, or control cel-miR-67 (IDT) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cel-miR-67 has minimal sequence identity with miRNAs in human, mouse, and rat (sequence is listed in Supplementary Table S1). Following transduction of cells, BCL9 immunoblots were performed.

2′-O-methyl oligoribonucleotides for knockdown of miR-30s and Bcl9 3′UTR luciferase constructs

The knockdown of miR-30s family members in multiple myeloma cells was achieved by transfection with antisense 2′-O-methyl oligoribonucleotides (ASO) against miR-30s using Lipofectamine 2000. Transfection complexes were prepared according to manufacturer’s instructions and added directly to the cells at a final oligonucleotide concentration of 10 nmol/L. The sequences of ASO against miR-30s and scrambled miR-30s are listed in Supplementary Table S1. Following 48-hour incubation, cells were assayed for BCL9 mRNA and protein expression as described (9). pmir-Report-Bcl9 were created by cloning Bcl9 3′UTR 329–335 and 486–492 bp, which match the seed sequence of miR-30s into downstream luciferase gene of pmIR reporter plasmid (pmir-0), as we previously described (20).
Their mutants (e.g., pmiR-Report-BCL9-mut-1 and pmiR-Report-BCL9-mut-2) were obtained by deletion of the matching seed sequence to indicated nucleotides (Fig. 2A). The sequences used to create these plasmids are listed in Supplementary Table S1.

**BCL9 3′ UTR luciferase reporter assay and TOP/FOP luciferase assay**

Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega). 293T cells were cotransfected with miRNAs virus (V-miR-30s or V-GFP), reporter plasmids, and Renilla luciferase using lipofectamine 2000 (Invitrogen). Twenty-four-hour posttransfection, cells were subjected to luciferase reporter assay using Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were analyzed as activity of firefly relative to Renilla. To measure Wnt reporter activity, H929 and MM1S cells were transfected with TOP-FLASH, FOP-FLASH plasmid (Millipore Corporation), along with an internal Renilla control plasmid (hRL-null), according to the manufacturer’s protocol. The results were normalized to control Renilla activity. The reported data represent the average of three independent transfection experiments performed in triplicate.

**The MM1S-Luc-neo tumor dissemination mouse model**

NOD/SCID mice were injected intravenously with 5 × 10⁶ MM1S-Luc-neo cells, and 1 week after injection, groups of eight mice were randomly separated and treated by intraperitoneal injection once a week with RNA-LANCErII (BioScience; vehicle) or 100 pmol total of an echymolar mixture of miR-30a/b/c/d/e (miR-30 mix) premixed in RNA-LANCErII. Tumor development was monitored by whole body imaging using Xenogen software. Mice were evaluated every week after initiation of treatment, and the tumor ratio of fluorescence intensity was compared among the two groups. Fluorescent tissues were harvested for histologic and IHC evaluation. To exclude toxic effects of miR-30s therapy, nonfluorescent tissue were also evaluated by histopathology. At day 21 of treatment, two mice from control and treated groups were euthanized and their spines subjected to microcomputed tomographic analysis.

**Results**

**miR-30s is the only predicted miR binding to the 3′ UTR of BCL9 mRNA**

We investigated whether BCL9 mRNA expression is regulated by miRNAs. By searching databases TargetScan (22), PicTar (23), miRDB, and microCosm, we found that the 3′UTR of BCL9 mRNA contains 2 sequence motifs designated wt-1 (9129–9135 bp) and wt-2 (9880–9886 bp; Supplementary Fig. S1B), which perfectly match with the “seed” sequence of the miR-30s family members (Supplementary Fig. S1C and Fig. 2A).

**miR-30s are downregulated in multiple myeloma cells, and their expression is inversely related with BCL9 expression**

Our expression profiling data from 78 patients with multiple myeloma samples showed that the levels of each miR-30 family member (miR-30a/b/c/d/e) were variable, and that 60% (45/78) of samples expressed low levels of miR-30s compared with normal plasma cells (Fig. 1A). We next asked whether low levels of miR-30s were associated with high BCL9 mRNA expression levels, and vice versa using qRT-PCR analysis. A total of six normal plasma cells (N1–N6) and six patient multiple myeloma cells (T1–T6) were examined in parallel for expression of miR-30s and BCL9 mRNA (Fig. 1B). We observed that normal plasma cells with undetectable levels of BCL9 mRNA display high levels of miR-30s expression, whereas patient multiple myeloma cells with variable levels of BCL9 mRNA were almost devoid of miR-30s expression. This inverse relation was also detected in multiple myeloma cell lines (Fig. 1C). For example, the H929 cell line that expresses relatively low levels of miR-30s showed high levels of BCL9 mRNA expression, whereas the MM1S cell line that expresses high levels of miR-30s showed relatively low levels of BCL9 mRNA expression. Although miR-30s members are located at 3 different chromosomal regions: 1p34.2 (miR-30e and miR-30c-1), 6q13 (miR-30c-2 and miR-30a), and 8q24.22 (miR-30b and miR-30d) they all have similar expression patterns among different multiple myeloma patient samples (Fig. 1A), suggesting that they share a similar regulatory network that is independent of chromosomal copy number alterations. Indeed miR-30c, the only member with two copies in the human genome, does have the most abundant expression levels in normal plasma cells, and show the lowest levels of expression in multiple myeloma patient samples, which frequently have chromosomal 1p34 and 6q13 deletions (24).

To further investigate the relation between BCL9 mRNA and miR-30s levels, we analyzed the published dataset GSE17306 (25) in which information for both mRNA and miR expression was available. We found that BCL9 mRNA is highly expressed in late stage multiple myeloma patient samples, and that its expression inversely associated with expression of miR-30a (also known as miR-30a-5p), miR-30b, miR-30c, miR-30d, and miR-30e (also known as miR-30e-5p; P < 0.05; Fig. 1D), but not with miR-30a-3p and miR-30e-3p. 2 miRNAs functionally unrelated to the miR-30s family (P > 0.05, data not shown). In addition, we examined the relation between miR-30s and BCL9 protein levels on bone marrow biopsies. Because miR-30c showed the most significant changes above (Fig. 1D), we selected this family member for this and further studies. miR-LNA in situ hybridization of miR-30c and IHC staining of BCL9 protein on 6 bone marrow biopsies from patients with multiple myeloma also showed an inverse association between miR-30s expression and BCL9 protein levels (Fig. 1E).

**BCL9 mRNA is a direct target of miR-30s**

To establish a functional relationship between miR-30s and BCL9 mRNA regulation, we first transduced individual premiR-30s into HEK293T cells using lentiviral vectors expressing GFP as a marker for flow sorting of stably transduction of cells. Cells transduced with vector alone (V-GFP) were used as a control. Ectopic expression of each miR-30s member (Fig. 2B) was associated with downregulated expression of BCL9 mRNA and protein levels, as evaluated by qRT-PCR (Fig. 2C) and immunoblot analysis (Fig. 2D) respectively. To further
demonstrate that miR-30s directly regulates expression of BCL9 mRNA through binding to the 3'UTR, 2 wild type (pmiR-BCL9-30-wt-1 and pmiR-BCL9-30-wt-2) and 2 mutant (pmiR-BCL9-30-Mut-1 and pmiR-BCL9-30-Mut-2) BCL9-3'UTR reporter vectors were cotransfected into HEK293T cells, together with each individual V-miR-30s member or V-GFP.
Luciferase activity of wild type, but not mutant, was significantly decreased with each V-miR-30s compared with V-GFP (Fig. 2E), confirming the specificity of the interaction between miR-30s and BCL9-3′ UTR mRNA.

miR-30s regulates BCL9 mRNA expression in multiple myeloma cells

We next investigated whether BCL9 mRNA expression is regulated by miR-30s in multiple myeloma cells using both gain- and loss-of-function studies. For gain-of-function studies, we used H929 cells that express the lowest levels of miR-30s among the multiple myeloma cell lines examined (Fig. 1C). We first induced ectopic expression of individual V-miR-30s family members in H929 cells using lentiviral infection. After flow sorting of GFP positive cells, levels of miR-30s were verified by qRT-PCR (Fig. 3A) and then used in all of the following experiments. As shown in HEK293T cells (Fig. 2B and C), ectopic expression of miR-30c in multiple myeloma cells was also associated with a significant reduction in the expression of BCL9 mRNA (Fig. 3B). Ectopic expression of miR-30c was also associated with a reduction in the expression of BCL9 protein, as evaluated by immunoblot (Fig. 3C) and immunofluorescence (Fig. 3D) studies. Consistent with the role of BCL9 as a transcriptional coactivator of the Wnt signaling pathway, ectopic expression of miR-30c was also associated with reduced expression of "bona fide" Wnt pathway downstream targets CD44 and Axin-2 (Fig. 3B; refs. 9 and 10) as well as Wnt reporter FOP/TOP activity (Fig. 3E). We next focused on evaluating the effect of the miR-30s family member miR-30c and validated its effect on BCL9 downregulation in other multiple myeloma cell lines using immunoblot (Supplementary Fig. S1D). Wild-type TOP reporter activity was inhibited in V-miR-30c stable H929 cells compared with V-GFP stable H929 cells, whereas the mutant FOP activity was not changed (Fig. 3E). To exclude the possibility that the observed changes in BCL9 expression may be because of nonspecific and/or secondary effects of stable transfection of V-miR-30c, we also performed transient transfection with increasing amounts of mature miR-30c into RPMI8226 cells, using cel-miR-67 as a control (Fig. 3F). To confirm transfection efficiency, cells were collected 72 hours after transfection, and miR-30c expression levels were checked by qRT-PCR (Fig. 3F, top). We found an inverse association between downregulation of BCL9 protein expression and increasing amounts of transfected mature miR-30c (Fig. 3F bottom). To verify miR-30's potential for therapy in patients with multiple myeloma, CD138+ primary cells (n = 3) were transfected with 40 pmol of mature miR-30c or same amount of cel-miR-67 as control. Expression of BCL9, CD44, and Axin-2 were downregulated as evaluated by immunofluorescence in all 3 patient samples. One representative case is shown in Fig. 3G (top). Furthermore, multiple myeloma cell proliferation was dramatically inhibited by miR-30c (Fig. 3G, bottom).
For loss-of-function studies we used the MM1S cell line, which expresses relatively high levels of miR-30s and relatively low levels of BCL9 mRNA (Fig. 1C). MM1S cells were transfected with a pool of 2'-O-me anti–miR-30a/b/c/d/e cocktail (anti–miR-30 mix) or scrambled oligonucleotides as a control. qRT-PCR analysis revealed a significant reduction of all members of miR-30s in cells treated with individual anti–miR-30s compared with cells transfected with scrambled oligonucleotides (Supplementary Fig. S2A). Levels of BCL9 mRNA (Supplementary Fig. S2B) and protein (Supplementary Fig. S2C) were increased in 2'-O-me anti–miR-30 treated cells compared with cells transfected with scrambled nucleotides. Furthermore, expression of the Wnt downstream targets Axin-2 and CD44 was upregulated (Supplementary Fig. S2B), as it was Wnt reporter activity (Supplementary Fig. S2D), in MM1S cells treated with anti–miR-30 mix, but not with scrambled, oligonucleotides.

miR-30c inhibits multiple myeloma cell proliferation, invasion, and migration and induces apoptosis

Then we examined if miR-30s can mimic the functional consequences of BCL9 deregulation in multiple myeloma (9,
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10). Based on results shown in Fig. 1D and E, we focused on miR-30c. A consistent pattern emerged whereby H929 and OPM1 cells overexpressing miR-30c (V-miR-30c), but not control cells (V-GFP), showed significantly reduced proliferation (Fig. 4A), colony formation (Fig. 4B), invasion, and migration (Fig. 4C). In addition, we found that miR-30c induces a modest increase in apoptosis, from 10.1% ± 1.1% in H929 cells expressing GFP (V-GFP) to 14.2% ± 1.9% in H929 cells expressing miR-30c (V-miR-30c; n = 3, P < 0.05; Fig. 4D). Taken together, these data demonstrate that miR-30c specifically disrupts a series of physiologic processes regulated by the Wnt/BCL9/b-catenin transcriptional complex, and highlighting the potential therapeutic role of miR-30c in multiple myeloma.

miR-30c inhibits multiple myeloma cancer stem cell formation

We next investigated whether miR-30c is involved in regulating behavior of cancer stem cells (CSC) in multiple myeloma (Fig. 5). Functional Hoechst 33342 staining assay was used to define the side population in V-miR-30c stably infected cells, and V-GFP cells were used as a control (Fig. 5A). The stem cell side population was significantly reduced from 0.698% ± 0.04% in H929 V-GFP cells to 0.068% ± 0.05% in H929 cells expressing V-miR-30c (n = 3, P < 0.05; Fig. 5B). Moreover, in experiments using stem cell medium to culture sorted side population cells (Fig. 5C), the sphere numbers (Fig. 5D) and size of spheres as evaluated by cell number per sphere (Fig. 5E), were significantly decreased in H929 V-miR-30c side population compared with H929 V-GFP control CSCs. These results highlight the potential role of miR-30c in blocking the Wnt signaling pathway in CSC, further confirming the relevance of this pathway for target drug discovery in multiple myeloma.

miR-30c restores drug sensitivity in bone marrow stroma cell-induced drug resistance of multiple myeloma

BMSCs promote migration, homing, proliferation, survival, and drug resistance in multiple myeloma (26). The relatively high levels of miR-30s in multiple myeloma cell lines compared with patient multiple myeloma cells (Fig. 1B and C) prompted us to next investigate the possible role of BMSCs in regulating miR-30s expression in multiple myeloma cells. GFP-labeled H929 cells were cocultured with HS-5 dsRed stable BMSCs. After 48 hours of coculture, GFP-positive H929 cells were flow sorted and total RNA was isolated for qRT-PCR analysis. As shown in Supplementary Fig. S3A, coculture with HS-5 dsRed downregulates expression of miR-30s in H929 cells, associated with enhanced expression of BCL9 mRNA and the Wnt downstream targets Axin-2 and CD44, but not of GAPDH, a non-Wnt target gene used as a control (Supplementary Fig. S3B).

Because CD44 is a downstream target of Wnt/b-catenin/BCL9 transcriptional complex (9, 10), miR-30c downregulates expression of CD44 (Fig. 3B) in multiple myeloma cells, and CD44 is a functional component of cell adhesion–mediated drug resistance (CAM-DR; ref. 26), we investigated the possible role of miR-30s in multiple myeloma drug resistance in the context of bone marrow microenvironment. After ectopic overexpression of miR-30c or miR-30a/b/c/d/e cocktail (miR-30 mix) or a negative control (cel-miR-67) in H929 cells (Supplementary Fig. S3C), we cocultured these cells alone or in the presence of HS-5 cells for 48 hours and treated them with 200 nmol/L dexamethasone. Interestingly, we found that both miR-30c and miR-30 mix can sensitize the H929 cells to dexamethasone treatment with miR-30 mix apparently being more effective than miR-30c (Supplementary Fig. S3D). Overall these findings indicate that expression of miR-30s in multiple myeloma cells can be modulated by the bone marrow microenvironment and further support its therapeutic potential to overcome CAM-DR.
miR-30c inhibits tumor progression in murine xenograft models of human multiple myeloma

To further explore the therapeutic potential of miR-30c, we next examined its capacity to suppress tumor growth and metastatic potential in vivo using 2 established murine xenograft models of human multiple myeloma (9). In the first model (i.e., subcutaneous), H929 V-GFP control and H929 V-miR-30c stably transduced cells were injected subcutaneously into opposite flanks of SCID mice; tumor volume was evaluated over time up to day 25, when mice were sacrificed and whole body imaging was performed. As shown in Fig. 6A (top and bottom), tumor growth was significantly decreased in mice injected with H929 V-miR-30c (green arrows) as compared with H929 V-GFP control cells (red arrows). In the second model (i.e., intravascular), H929 V-GFP control or H929 V-miR-30c stably transduced cells were injected by tail vein into SCID mice; survival, tumor burden, and spreading were assessed (Fig. 6B). Tumor involvement was observed in the intestine, spine, and skull, which was similar in V-miR-30c and V-GFP control groups (122.5 ± 33.0 days vs. 162.2 ± 21.7 days, n = 6, P = 0.03; Fig. 6B, top). However, tumor burden was decreased and survival was significantly increased in mice injected with H929 V-miR-30c compared with H929 V-GFP control cells (Fig. 6B, bottom). In agreement with our in vitro studies, tumors developing in mice injected with stable V-miR-30c H929 cells showed decreased expression levels of BCL9, Ki-67, CD44, and Axin-2 proteins, as well as increased levels of caspase-3 expression compared with V-GFP control tumors evidenced by IHC analysis (Fig. 6C). BCL6 that is a target of miR-30 in diffuse large B-cell lymphomas (27) was not identified in multiple myeloma (Supplementary Fig. S1E). The miR-30c overexpression and downregulation of BCL9 in GFP-positive harvested tumors was further verified by qRT-PCR (Fig. 6D, top) and immunoblot (Fig. 6D, bottom).

To further evaluate the possibility of translating this biological proof-of-concept into a pharmacologic strategy for inhibiting oncogenic Wnt/β-catenin/BCL9 complex using miR-30s, we next determined whether intraperitoneal delivery of miR-30s could inhibit multiple myeloma tumor growth in vivo. To achieve therapeutic benefits in vivo, miRNA-based drugs require trans-membrane delivery systems to protect encapsulated miRNAs from degradation in the circulation, promote accumulation in target tissue, facilitate intracellular delivery into target cells and must also be relatively nontoxic. Lipid nanoparticles are currently the most favored delivery system for satisfying these needs;

Figure 5. miR-30c decreases population of multiple myeloma cancer stem cells. A and B, side population fraction of H929 cells transduced with V-GFP or V-miR-30c, as detected by functional Hoechst 33342 stem cell staining assay. Verapamil is used as an inhibitor of side population cells. C, representative phase contrast (left) and fluorescence microscopy images (right) of cell spheres formed after culture of side population cells isolated from H929 cells transduced with either V-GFP or V-miR-30c in stem cell medium. Sphere numbers per 1,000 sorted side population cells (D) and numbers of cells per sphere (E) in cells transduced with V-GFP or V–miR-30c.
therefore, we used miR-30s premixed with RNA-LANCErII, consisting of a mixture of neutral lipid, nonionic detergent, and oil (28) to perform this \textit{in vivo} experiment. We first evaluated if lipid nanoparticles could deliver miR-30s into H929 multiple myeloma cells and if there was preferential delivery and inhibition of BCL9 expression among individual members of the miR-30s family. qRT-PCR analysis revealed that all miR-30 members were taken up by the cells, although at different extent when added to the medium individually or as a miR-30a/b/c/d/e echymolar cocktail (miR-30 mix; Fig. 7A). In addition, all individual miR-30 members or the echymolar cocktail decreased expression of BCL9 to similar extent, evaluated by immunoblot analysis (Fig. 7B). Reasoning that treatment with a cocktail mixture containing lower amounts of each individual member could be better tolerated by the mice we performed this \textit{in vivo} experiment with miR-30 mix. Therefore, we first investigated the specificity of miR-30 mix treatment in inhibiting expression of Wnt/\beta-catenin/BCL9 transcriptional targets by performing comparative genome-wide expression analyses (Fig. 7C). We generated triplicate gene expression profiling datasets from H929 cells transduced with V-GFP or V-miR-30c and isolated from mice injected subcutaneously (#1 and #2) or intravenously (#3 to #6).
or scrambled sequences (control; ref. 9) using Affimetrix oligonucleotides microarrays. Gene set enrichment analysis revealed a statistically significant correlation between the genes downregulated by miR-30 mix and by sh-BCL9 [family-wise error P-value < 0.001; false discovery rate q-value < 0.001], documenting the specificity of miR-30 mix in blocking expression of Wnt/β-catenin/BCL9 transcriptional targets (Fig. 7C).

Then, we performed an in vivo miR-30 mix delivery experiment to determine whether tumor growth is antagonized in a well-established MM1S-Luc-neo murine xenograft model of human multiple myeloma (Fig. 7D–F) after intraperitoneal delivery using lipid nanoparticles. As shown in Fig. 7D, survival was increased in mice treated with miR-30 mix (vehicle group, 31.9 ± 2.3 days vs. miR-30 mix group, 35.7 ± 4.4 days, n = 8, P < 0.05), and associated with decreased tumor burden (Fig. 7E). metastasis to the kidney (Fig. 7F, top), as well as decreased expression of BCL9 and CD44 proteins (Fig. 7F, bottom). In vivo delivery of miR-30s to target cells was confirmed using miRNA LNA-ISH (Fig. 7F second row, bottom).

Because of the documented role of Wnt activity in bone metabolism (29), and the potential side effect of worsening osteolytic bone disease in patients with multiple myeloma treated with Wnt inhibitors (30), we evaluated the effect of miR-30s therapy in our murine xenograft model using micro-computed tomography (µCT) of bones. We first evaluated the effect of miR-30c in SCID mice no transplanted with myeloma cells. No apparent development of bone osteolytic lesions was observed in mice treated with miR-30c as compared with mice treated with vehicle (Supplementary Fig. S4A and S4B). We also evaluated by µCT analysis the spines of 2 vehicle-treated and 2 miR-30 mix-treated SCID mice transplanted with MM1S-Luc-Neo cells at day 21 of treatment (Fig. 7D). As shown in Supplementary Fig. S4C and S4D, no major differences in trabecular bone volume and cortical void fraction was observed between mice treated with vehicle or miR-30 mix. In addition, the cortical void fraction of mice transplanted with multiple myeloma cells and treated with miR-30 mix was similar to mice not transplanted with multiple myeloma cells (Supplementary Fig. S4B and Supplementary S4D). Furthermore, no evidence of bone lytic lesions as evaluated histologically was observed in long bones, which were not involved by multiple myeloma cells (data not shown). Overall these results suggest that miR-30s treatment does not have a negative impact on bone, an issue that need to be further explored. Thus, miR-30s treatment effectively inhibited BCL9-driven Wnt transcriptional activity in vivo, thereby suppressing tumor growth, invasion, and enhancing survival, highlighting the potential role of miR-30s as a novel therapeutic approach in multiple myeloma. The lack of improvement in multiple myeloma-associated bone disease in miR-30s treatment suggest that this approach should be implemented in association with therapies that reduce osteoclast-mediated bone resorption, such as bisphosphonates (29).

Discussion

Here we provide evidence for a novel functional link between miR-30s and oncogenic Wnt/β-catenin/BCL9 transcriptional activity. Using multiple myeloma as a model cancer system with deregulated Wnt activity (7–11), we proved that miR-30s is a tumor suppressor and novel therapeutic tool by targeting BCL9, a critical coactivator of the canonical Wnt/β-catenin signaling pathway. Deregulated Wnt/β-catenin transcriptional activity underlies the pathogenesis of a wide variety of human carcinomas (31, 32), and hematologic malignancies, including multiple myeloma (7–11). Although the Wnt/β-catenin transcriptional complex is a promising target for cancer therapy (33), a major limitation to therapies targeting this pathway is the importance of β-catenin activity in normal adult tissue homeostasis (34). Thus, further investigation of novel and more selective Wnt signaling components are needed.

The BCL9 is an essential coactivator of Wnt/β-catenin transcriptional activity, and studies from our lab and others have unveiled its role as novel therapeutic target (9, 10). The lack of detectable phenotypic alterations in the intestinal tract of mice with conditional deletion of BCL9 and B9L (35) suggests that BCL9/BL9 proteins do not play an essential homeostatic role in mammalian Wnt signaling, although BCL9/B9L regulates Wnt target genes that control epithelial-to-mesenchymal transition and stem cell-like behavior (35). These data indicate that targeting the BCL9/B9L component of aberrantly activated Wnt signaling in cancer may attenuate tumor spread, and resistance to therapy, while leaving normal tissues relatively undisturbed. Indeed, treatment with SAH-BCL9 peptide elicits mechanism-based antitumor responses in vitro as well as in mouse xenograft models of colorectal cancer and multiple myeloma without detectable alterations in host tissues (10).

A link between miRNA-30c* and BCL9 was recently documented in ovarian carcinoma (36). miR-30c* (also called miR-30c-3p) and miR-30c (also called miR-30c-5p) are related miRNAs processed from the same precursor but from different regions denoted 3p and 5p, respectively, with almost complementary sequences, indicating they have different target genes (37). Using 4 different target prediction software algorithms we found that the 5p miR-30 family, but...
not the 3p complementary family, matched with the 3’UTR of BCL9 mRNA. Furthermore, our analysis of miRNAs and mRNA expression arrays in multiple myeloma patient datasets showed that expression of the miR-30s-5p family, but not the miR-30s-3p family, is inversely associated with BCL9 mRNA levels.

miR-30s also function as tumor suppressor genes in other cancers: miR-30s are downregulated in diffuse large B-cell lymphoma where BCL6 gene was identified as a target (27); ectopic expression of miR-30s inhibits the self-renewal capacity of breast tumor–initiating cells by reducing Ubc9, and induces apoptosis by silencing integrin-β3 (38); miR-30s regulate B-Myb expression, highlighting the pivotal role of miR-30s in Rb-driven cellular senescence (39), Interestingly, we did not find changes in the expression of BCL6, Ubc9, and B-Myb in multiple myeloma cells overexpressing miR-30c or miR-30 mix, in accordance with the notion that the target of miRNAs are tumor cell specific (40).

The small size of miRNAs makes them very attractive for drug development in multiple myeloma (41–43). miRNAs are natural antisense interactors and will not induce immune response (44), and the expression levels of specific miRs respond to physiologic stimuli (45) compare with other Wnt inhibitors (33). However, the success of miRNAs therapy has hold upon the development of suitable in vivo delivery systems. Because miR-30s plays its role as a tumor suppressor, and is expressed in all normal tissues, it is expected that replacement therapy will not affect normal cell function. In agreement with this scenario, we have observed that intraperitoneal delivery of miR-30 mix using lipid nanoparticles increased survival in a murine xenograft model of human multiple myeloma with lack of negative impact on normal tissues as it has been observed with other Wnt inhibitors (19, 33).

In conclusion, we have documented that downregulation of the tumor suppressor miR-30s is a frequent pathogenetic event in multiple myeloma, and provide evidence for a model in which the interaction between multiple myeloma cells and BMSCs decreases miR-30s levels in multiple myeloma cells, which in turn enhances expression of BCL9, as a transcriptional coactivator of the Wnt signaling pathway, promotes expression of downstream target genes involved in multiple myeloma tumor cell proliferation, survival, migration, drug resistance, and multiple myeloma CSC (Fig. 7G). The potential for clinical translation of strategies using miR-30s as a novel therapeutic tool is further confirmed by the capacity of miR-30c and miR-30 mix to reduce tumor burden and metastatic potential in vivo in 3 murine xenograft models of human multiple myeloma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
miR-30 as a Novel Therapeutic Tool for Multiple Myeloma

miR-30-5p Functions as a Tumor Suppressor and Novel Therapeutic Tool by Targeting the Oncogenic Wnt/β-Catenin/BCL9 Pathway

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