hsa-miR29b, a critical downstream target of non-canonical Wnt signaling, plays an anti-proliferative role in non-small cell lung cancer cells via targeting MDM2 expression

Sreedevi Avasarala1, Michelle Van Scoyk1, Jianbin Wang2, Marybeth Sechler1, Katherine Vandervest1, Christine Brzezinski1, Colin Wekes3, Michael G. Edwards1, John Arcaroli3, Richard E. Davis2, Rama Kamesh Bikkavilli1 and Robert A. Winn1,4,∗

1Division of Pulmonary and Critical Care Sciences, School of Medicine, University of Colorado, Anschutz Medical Campus, Aurora, CO 80045, USA
2Department of Biochemistry and Molecular Genetics, School of Medicine, University of Colorado, Aurora, CO 80045, USA
3Division of Medical Oncology, School of Medicine, University of Colorado, Anschutz Medical Campus, Aurora, CO 80045, USA
4Veterans Affairs Medical Center, Denver, CO 80220, USA

*Author for correspondence (Robert.Winn@ucdenver.edu)

Biology Open 2, 675–685
doi: 10.1242/bio.20134507
Received 18th February 2013
Accepted 8th April 2013

Summary
In non-small cell lung cancer cell lines, activation of β-catenin independent signaling, via Wnt7a/Frizzled9 signaling, leads to reversal of cellular transformation, reduced anchorage-independent growth and induction of epithelial differentiation. miRNA expression profiling on a human lung adenocarcinoma cell line (A549) identified hsa-miR29b as an important downstream target of Wnt7a/Frizzled9 signaling. We show herein that hsa-miR29b expression is lost in non-small cell lung cancer (NSCLC) cell lines and stimulation of β-catenin independent signaling, via Wnt7a expression, in NSCLC cell lines results in increased expression of hsa-miR29b. Surprisingly, we also identify specific regulation of hsa-miR29b by Wnt7a but not by Wnt3, a ligand for β-catenin-dependent signaling. Interestingly, knockdown of hsa-miR29b was enough to abrogate the tumor suppressive effects of Wnt7a/Frizzled9 signaling in NSCLC cells, suggesting that hsa-miR29b is an important mediator of β-catenin independent signaling. Finally, we show for the first time that hsa-miR29b plays an important role as a tumor suppressor in lung cancer by targeting murine double mutant 2 (MDM2), revealing novel nodes for Wnt7a/Frizzled9-mediated regulation of NSCLC cell proliferation.

© 2013. Published by The Company of Biologists Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

Key words: Frizzled 9, MDM2, Non-small cell lung cancer, p53, Wnt7a, hsa-miR29b

Introduction
Lung cancer represents the leading cause for cancer-related deaths in the world (Jemal et al., 2011; Siegel et al., 2013). Lung cancers, especially, non-small cell lung cancers (NSCLC) display frequent loss of not only tumor suppressor genes like p53 (~50%), Rb (retinoblastoma protein, 15–30%) and p16INK4 (30–70%) but also Wnts, particularly Wnt7a (Sekido et al., 1998). Wnts are secreted glycoproteins that bind seven-transmembrane containing receptors Frizzleds, and stimulate diverse array of morphogenic and developmental-specific programs (Peifer and Polakis, 2000). Wnt binding to Frizzleds, mediated by G-proteins and Dishevelled, leads to post-transcriptional and post-translational mechanism/s-mediated stabilization of β-catenin (Bikkavilli and Malbon, 2010; Bikkavilli and Malbon, 2012; Malbon, 2005). Stabilization of β-catenin allows its nuclear translocation, where it functions as a transcriptional co-activator along with the T-cell factor (TCF) and lymphoid enhancer factors (LEF) (Behrens et al., 1996; Molenaar et al., 1996). Deregulated Wnt/β-catenin signaling leads to many cancers (Karim et al., 2004; Mazieres et al., 2005). In strong contrast, Wnt7a/Frizzled9 signaling was shown to play a protective role in lung cancer (Winn et al., 2005). Interestingly, Wnt7a expression is lost in majority of NSCLC cells (Winn et al., 2005), and restoration of Wnt7a expression in the same cells leads to reduced cell proliferation, reduced anchorage-independent growth, increased cell differentiation and reversal of transformed phenotype (Winn et al., 2005). Previously, we have shown that Wnt7a binding to Frizzled9 (Fzd9) receptor activates a potent tumor suppressor, the peroxisome proliferator activated receptor γ (PPARγ) (Winn et al., 2006). However, the nature of the downstream targets of Wnt7a/Fzd9-stimulated PPARγ and the mechanism/s of Wnt7a regulation of NSCLC cell growth remains largely unknown.

Among the non-coding RNAs, microRNAs (miRNAs) represent a major subset, which regulate target mRNAs either by site specific cleavage or by translation repression (Garzon et al., 2006). Although an estimate, miRNAs constitute nearly 1%
of all the predicted genes in nematodes, flies or mammals (Baskerville and Bartel, 2005; Lai et al., 2003; Lim et al., 2003a; Lim et al., 2003b). miRNAs not only play critical roles during development but also during cell proliferation, differentiation, and apoptosis (Calin et al., 2004). The role of miRNAs during oncogenesis is also evident, with altered expressions in several cancers (Esquela-Kerscher and Slack, 2006). Similar to that of regulators of gene expression, modulators of miRNA biogenesis are also coordinately regulated (Cai et al., 2009). In this context, the role of Wnt signaling in regulating miRNA biogenesis remains largely unknown, and of great interest.

As a strategy to identify potential miRNAs involved in the Wnt7a-dependent regulation of NSCLC cell growth, we performed miRNA expression profiling on Wnt7a-stimulated human lung adenocarcinoma cell line (A549) and identified hsa-miR29b as an important downstream target of Wnt7a. We show herein that hsa-miR29b expression is lost in NSCLC cell lines and Wnt7a-stimulation of NSCLC cell lines results in increased expression of hsa-miR29b. In addition, ERK5 and PPARγ, key effectors of Wnt7a/Fzd9 pathway, were also observed to be strong inducers of hsa-miR29b expression. Interestingly, knockdown of hsa-miR29b was enough to abrogate the tumor suppressive effects of Wnt7a and Fzd9 expression in NSCLC cells, suggesting that Wnt7a and/or hsa-miR29b plays a critical during lung tumorigenesis. Finally, we also show that hsa-miR29b plays an important role as a tumor suppressor in lung cancer by targeting murine double mutant 2 (MDM2), revealing novel nodes for Wnt7a/Fzd9-mediated regulation of NSCLC cell proliferation.

Results
Identification of Wnt7a regulated miRNAs in NSCLC cell lines
To identify potential miRNAs involved in Wnt7a-dependent regulation of NSCLC cell growth, we performed miRNA expression profiling of human lung adenocarcinoma cell line A549 as described in Materials and Methods (Table 1; supplementary material Table S1). A549s were of particular interest as they express Fzd9 but not Wnt7a, the ligand for Fzd9. The strategy was to transiently express either Wnt3 or Wnt7a in A549 cells, isolate total RNA, and screen for miRNA expression by using a cancer-specific miRNA super-array (Table 1; supplementary material Table S1). Interestingly, our screening succeeded in identifying hsa-miR29b as a novel miRNA regulated by Wnt7a. The reason for selecting hsa-miR29b over other miRNAs is 2-fold: 1) hsa-miR29b was upregulated by more than 19-fold in A549 cells expressing Wnt7a in comparison to empty vector control (Table 1), and 2) several studies have shown either a direct or an indirect role for hsa-miR29b in human cancers (Fabbri et al., 2007; Kole et al., 2011; Rothschild et al., 2012; Ru et al., 2012).

The hsa-miR-29 family includes three members: hsa-miR29a, hsa-miR29b and hsa-miR29c (Fig. 1A). It was interesting to note that Wnt7a induced the expression of only hsa-miR29b but not hsa-miR29a or hsa-miR29c (supplementary material Table S1). To corroborate our PCR array data, we performed q-RT-PCR analyses on RNAs isolated from two NSCLC cell lines (H661 and H157) transiently transfected with either empty vector, Wnt3, or Wnt7a expression vectors and by using primers specific for the hsa-miR29 family members (Fig. 1B,C). H157 cells were additionally transfected with Fzd9 as they have no expression of endogenous Fzd9 (H157+Fzd9). Consistent with our PCR array data, Wnt7a induced the expression of hsa-miR29b, but not hsa-miR29a or hsa-miR29c (Fig. 1B,C). Additionally, we also performed Northern blot analysis using 32P-labelled hsa-miR29b or hsa-miR29a/c specific probes to confirm the Wnt7a-induced hsa-miR29b expression (Fig. 1D). Consistent with our PCR array and q-RT-PCR data, Northern analysis revealed robust Wnt7a-induced expression of hsa-miR29b, as detected by hsa-miR29b specific probes (Fig. 1D). On the other hand, probing the same blot with hsa-miR29a and hsa-miR29c specific probes (since there is only one base difference between the mature forms of hsa-miR29a and hsa-miR29c, we probed the blot with both the probes) showed no such increase in hsa-miR29a and hsa-miR29c expression (Fig. 1D). It was also interesting to note that Wnt7a stimulated the expression of only the mature form of hsa-miR29b but not its primary or precursor form (Fig. 1D). These data suggest that Wnt7a regulates hsa-miR29b, but not hsa-miR29a or hsa-miR29c.

We also interrogated Wnt7a-mediated regulation of hsa-miR29b by using an hsa-miR29b-specific luciferase reporter plasmid (Fig. 1E). In this reporter, the complimentary sequence of hsa-miR29b has been engineered downstream of luciferase gene (Fig. 1E). In cells expressing the reporter, mature hsa-miR29b targets the binding site downstream of luciferase gene resulting in repression in luciferase gene expression and as detected by reduced luciferase enzyme activity. Therefore, a decreased luciferase activity represents increased expression of hsa-miR29b and vice versa. Similar luciferase reporters have

### Table 1. Wnt7a regulated miRNAs in A549 cells.

| miRNA ID     | Wnt7a (fold over empty vector control) | Wnt 3 (fold over empty vector control) |
|--------------|----------------------------------------|----------------------------------------|
| mir-29b      | 19.56                                  | 1.36                                   |
| mir-10a      | 12.21                                  | 1.2                                    |
| mir-100      | 10.34                                  | 0.83                                   |
| mir-21       | 9.58                                   | 0.93                                   |
| mir-10b      | 9.32                                   | 0.8                                    |
| mir-125b     | 8.4                                    | 1.66                                   |
| mir-128      | 7.84                                   | 1.05                                   |
| mir-335      | 7.11                                   | 1.88                                   |
| mir-183      | 7.06                                   | 0.51                                   |
| mir-125a-5p  | 6.73                                   | 1.01                                   |
| mir-20a      | 6.73                                   | 1.35                                   |
| mir-92a      | 6.11                                   | 1.26                                   |
| mir-25       | 5.98                                   | 1.05                                   |
| mir-16       | 5.5                                    | 1.09                                   |
| let-7c       | 5.56                                   | 1.03                                   |
| mir-15b      | 5.39                                   | 0.94                                   |
| let-7b       | 4.47                                   | 0.99                                   |
| mir-126      | 4.4                                    | 0.9                                    |
| mir-181c     | 4.44                                   | 0.72                                   |
| mir-27a      | 4                                     | 0.8                                    |
| mir-191      | 3.76                                   | 0.86                                   |
| mir-200c     | 3.63                                   | 0.94                                   |
| mir-215      | 2.99                                   | 0.5                                    |
| let-7g       | 2.91                                   | 1.53                                   |
| mir-7        | 2.89                                   | 1.33                                   |
| mir-19a      | 2.45                                   | 0.92                                   |
| mir-218      | 2.28                                   | 0.71                                   |
| let-7c       | 2.17                                   | 0.9                                    |
| mir-127-5p   | 2.13                                   | 0.62                                   |
| mir-214      | 1.11                                   | 2.35                                   |
| mir-15a      | 0.87                                   | 0.5                                    |
| mir-301a     | 0.71                                   | 0.45                                   |
| mir-155      | 0.62                                   | 0.4                                    |
been widely utilized for miRNA studies (Bao et al., 2012; Xin et al., 2012; Yang et al., 2012). Consistent with our q-RT-PCR and Northern analysis, Wnt7a stimulation of three different NSCLC cells (A549, H157+Fzd9, and H661) expressing hsa-miR29b-luciferase reporter plasmid displayed a similar reduction in luciferase activities, strongly indicating an increased hsa-miR29b expression upon Wnt7a stimulation (Fig. 1F). Of note, Wnt7a-mediated induction of hsa-miR29b is unidirectional. Expression of hsa-miR29b, on the contrary, failed to impact Wnt7a expression (data not shown). In total, by using several distinct and powerful analyses we establish that activation of a β-catenin-independent pathway by Wnt7a stimulates the expression of hsa-miR29b, but not hsa-miR29a or hsa-miR29c, in NSCLC cells.

hsa-miR29b regulates NSCLC cell proliferation

Since, Wnt7a induce hsa-miR29b expression in NSCLC cell lines (Table 1; Fig. 1) and Wnt7a expression is lost in a majority of NSCLC cell lines (Winn et al., 2005), we probed next for the expression levels of hsa-miR29b in a panel of NSCLC cell lines using quantitative RT-PCR (qPCR, Fig. 2A). For these experiments, total RNA was extracted from normal lung bronchial epithelial cells (Beas2B), lung adenocarcinoma (A549, H2122), squamous cell carcinoma (H157+Fzd9) and large cell carcinoma cell lines (H661), reverse transcribed and the cDNAs were later used to measure the levels of hsa-miR29b expression (Fig. 2A). Q-PCR established the relative expression of hsa-miR29b in normal and NSCLC cell lines (Fig. 2A). Interestingly, hsa-miR29b expression was severely attenuated in all the NSCLC cell lines tested when compared to non-transformed bronchial epithelial cell line (Beas2B, Fig. 2A). Earlier, we have shown a loss in Wnt7a expression in a similar panel of NSCLC cell lines in comparison to short-term bronchial epithelial cell line [STBE (Winn et al., 2005)]. Since, hsa-miR29b expression was attenuated in all the NSCLC cell lines tested; we probed next if hsa-miR29b could...
affect NSCLC cell proliferation, either by using gene specific knockdowns (Fig. 2B,C) or re-expression of hsa-miR29b (Fig. 2D,E) in NSCLC cells. Since, normal bronchial epithelial cells (Beas2B) express high levels of hsa-miR29b (Fig. 2A), we first probed the effects of chemically synthesized double stranded miR29b precursor molecules (Ambion, anti-miR29b1 and anti-miR29b2) on Beas2B cell proliferation. Treatment of Beas2B cells with miR29b precursors showed a significant decrease in hsa-miR29b levels (\( \text{\%}\)) (Fig. 2B). Interestingly, depletion of hsa-miR29b in Beas2B cells revealed a significant increase in cell proliferation (by 3-fold), as detected by clonogenic assays (Fig. 2C). Consistent with the effects of hsa-miR29b knockdown on Beas2B cell proliferation, re-expression of hsa-miR29b was inhibitory to the cell growth of A549 or H157 cells as determined by clonogenic (Fig. 2D) or MTS cell proliferation assays (Fig. 2E).

Stimulation of NSCLC cells with Wnt7a not only induced the expression of hsa-miR29b but also attenuated NSCLC cell proliferation (Tennis et al., 2010; Winn et al., 2005). To test if the anti-proliferative effects of Wnt7a in NSCLC cells are mediated...
via the induction of hsa-miR29b, we first stimulated A549 cells with Wnt7a (to induce hsa-miR29b expression), followed by treatment with miR29b precursors. Interestingly, the expression of Wnt7a in A549 cells (that lack endogenous Wnt7a) severely attenuated A549 cell proliferation (Fig. 2F), whereas depletion of hsa-miR29b in A549 cells re-expressing Wnt7a blocked the inhibitory effects of Wnt7a expression on A549 cell growth (Fig. 2F). These data suggest that hsa-miR29b is a novel downstream target of Wnt7a/Fzd9 signaling and the anti-tumorigenic effects of Wnt7a in NSCLC cells.

**ERK5 and PPARγ modulate hsa-miR29b expression in NSCLC cells**

Wnt7a/Fzd9 signaling leads to activation of ERK5 and PPARγ and their associated effects on the inhibition of NSCLC growth (Winn et al., 2006). We therefore probed if ERK5 could also modulate hsa-miR29b expression (Fig. 3A,B). For these experiments, A549 or H157 cells were transfected with either empty vector or pcDNA3.2-ERK5 (Fig. 3A,B). Interestingly, Q-RT-PCR analyses of RNA isolated from ERK5 overexpressing NSCLC cells revealed a 4-fold (A549) and a 3-fold (H157) increase in hsa-miR29b expression, but not hsa-miR29a or hsa-miR29c expression (Fig. 3A,B). We also confirmed that ERK5-induced hsa-miR29b levels in A549 cells via Northern analyses (Fig. 3C). In order to test the specificity of ERK5-mediated induction of hsa-miR29b, we made use of hsa-miR29b-specific luciferase reporter and MEK inhibitors, PD98059 and U0126 (Fig. 3D). For these studies, A549 or H157 cells expressing either hsa-miR29b luciferase reporter alone or together with Wnt7a-HA plasmid were treated without or with MEK inhibitors (Fig. 3D). H157 cells were additionally transfected with Fzd9 as they do not express endogenous Fzd9. Expression of Wnt7a, as expected induced hsa-miR29b expression as revealed by the reduced luciferase activities (Fig. 3D) in both the cell lines. Interestingly, treatment of Wnt7a expressing A549 or H157+Fzd9 cells with PD98059 [that blocks, MEK 1, 2 and 5, (Cameron et al., 2003; Kamakura et al., 1999; Winn et al., 2006)], blocked Wnt7a-induced hsa-miR29b expression, as revealed by an increase in luciferase activities (Fig. 3D). In strong contrast, treatment of Wnt7a expressing A549 or H157 cells with U0126 [that blocks only MEK 1 and 2 (Cameron et al., 2003; Kamakura et al., 1999; Winn et al., 2006)] has no impact on Wnt7a-induced hsa-miR29b expression (Fig. 3D), strongly suggesting that ERK5 (but not ERK 1 and 2) mediates Wnt7a-induced hsa-miR29b expression.

We next probed if the distal downstream effector of Wnt7a/Fzd9 pathway viz., PPARγ (Winn et al., 2006) could also regulate the expression of hsa-miR29b (Fig. 3E,F). Similar to the effects of ERK5 expression, overexpression of PPARγ in A549 or H157 cells also induced a 28-fold (A549, Fig. 3E) and 54-fold (H157, Fig. 3F) increase in hsa-miR29b expression, but not hsa-miR29a or hsa-miR29c expression, as determined by Q-RT-PCR (Fig. 3E,F). Consistent with our Q-RT-PCR analyses, PPARγ expression also induced hsa-miR29b expression in NSCLC cells (A549, H157 and H661), as revealed by reduced hsa-miR29b-luciferase activities (Fig. 3G). Furthermore, PPARγ-induced anti-proliferative effects on A549 cell growth was abrogated by the knockdown of hsa-miR29b, as determined by clonogenic assays (Fig. 3H) and MTS cell proliferation assay (Fig. 3I). These data strongly suggest that the anti-proliferative effects of PPARγ are also mediated via the induction of hsa-miR29b expression.

**hsa-miR29b regulates MDM2 expression**

In order to identify potential hsa-miR29b targets, which are specific to lung cancer and tumor suppressor pathway, we scanned for hsa-miR29b targets in silico (http://www.microrna.org; Table 2). Among the several targets identified is the human homologue of murine double mutant 2, MDM2 (Fig. 4A). MDM2 is an important negative regulator of p53 tumor suppressor pathway (Oliver et al., 2011; Zhan et al., 2012). Since, hsa-miR29b expression in NSCLC cells is anti proliferative, we hypothesize that expression of hsa-miR29b might downregulate MDM2 expression. We tested our hypothesis by measuring MDM2 transcript levels by Q-PCR in A549 and H157 cells upon re-expression of hsa-miR29b (Fig. 4B). In the presence of increased hsa-miR29b expression (Fig. 4B), we observed a corresponding decrease in MDM2 mRNA expression (by more than 50%) in both the cell lines tested (Fig. 4C). To further validate our findings, we also tested the effects of hsa-miR29b re-expression on MDM2 protein levels. Consistent to their effects on MDM2 mRNA, re-expression of hsa-miR29b in A549 or H157 cells resulted in reduced MDM2 expression (Fig. 4D). To ascertain that the effects of hsa-miR29b expression on MDM2 were specific and that there were no off-target effects, we also tested the effects of hsa-miR29b re-expression on other proteins identified in silico, viz., phosphatase and tensin homologue deleted on chromosome Ten, PTEN (Fig. 4E) and Cdk2 (Fig. 4F). Although PTEN and Cdk2 display hsa-miR29b target sites (Fig. 4A), expression of hsa-miR29b in A549 or H157 cells had no impact either on PTEN (Fig. 4E) or Cdk2 (Fig. 4F) expression, suggesting that the observed effects of hsa-miR29b on MDM2 expression are indeed specific. Taken together, these data suggest that hsa-miR29b regulates NSCLC cell proliferation via repressing MDM2 expression.

Finally, since MDM2 is a known regulator of p53 tumor suppressor, we went further to probe the effects of hsa-miR29b expression on p53 expression (Fig. 4G,H). Consistent with the effects on MDM2 expression, expression of hsa-miR29b in A549 cells increased p53 expression as determined by western blots with anti-p53 antibodies (Fig. 4G). Since, p53 is a transcription factor that binds DNA and induce gene transcription, increased p53 expression (Fig. 4G) should result in increased p53-mediated gene transcription. To test this possibility, we made use of a p53-luciferase (Stratagene) construct, with luciferase under the control of 14 repeats of p53-binding sequence [TGCCTG-GACTTGG] (Wang et al., 2001). Co-expression of hsa-miR29b and p53-luciferase reporter in A549 cells resulted in an 8-fold induction in p53-dependent gene transcription in comparison to A549 cells transfected with p53-luciferase reporter alone (Fig. 4H). Consistent with the induction of p53-dependent gene transcription upon hsa-miR29b expression, knockdown of hsa-miR29b expression in non-transformed cells (Beas2B) resulted in reduced p53-dependent gene transcription (Fig. 4I), an effect similar to that of PPARγ inhibition (Fig. 4I) (Winn et al., 2006). In total, the results from several distinct and powerful analyses reveal a consistent story: Wnt7a stimulates hsa-miR29b expression and hsa-miR29b modulates NSCLC cell proliferation via repressing MDM2 expression.

**Discussion**

Wnt7a is frequently lost in a subset of NSCLC (Winn et al., 2005), and restoration of Wnt7a expression in the context of Fzd9
results in increased differentiation and decreased transformed phenotype in cancer cell lines (Winn et al., 2005). In the current study, we identify hsa-miR29b as a novel tumor suppressor, which is regulated by Wnt7a in NSCLC cells. We show herein that the activation of a β-catenin-independent pathway, mediated by Wnt7a/Fzd9, strongly induce hsa-miR29b expression in
Table 2. List of lung cancer-specific tumor-suppressor genes with potential hsa-miR29b targets.

| Gene ID   | Synonyms                          | hsa-miR29b site |
|-----------|-----------------------------------|----------------|
| TP53      | Tumor protein p53                  | No             |
| TP53N1P1  | Tumor protein p53 inducible nuclear protein 1 | Yes            |
| RB1       | Retinoblastoma 1                   | No             |
| CDKN2A    | p16                               | No             |
| FHT       | Fragile histidine triad            | No             |
| RBL1      | Retinoblastoma like protein-1      | No             |
| RBL2      | Retinoblastoma like protein-2      | No             |
| CDK2      | Cyclin dependent kinase-2          | Yes            |
| CDK4      | Cyclin dependent kinase-4          | No             |
| CDKN2B    | p15                               | No             |
| CDKN2C    | p18                               | No             |
| PTEN      | Phosphatase and tensin homolog     | Yes            |
| DNMT3B    | DNA (cytosine-5-)methyltransferase 3 beta | Yes            |
| MDM2      | p53 E3 ubiquitin protein ligase homolog | Yes            |
| CDKN1B    | P27                               | No             |
| SMAD2     | SMAD family member 2               | No             |
| SMAD4     | SMAD family member 4               | No             |
| TGFBI     | transforming growth factor, beta 1 | No             |
| TGFBI2    | transforming growth factor, beta 2 | No             |
| CDC42     | cell division cycle 42 (GTP binding protein) | Yes            |
| ATR       | ataxia telangiectasia and Rad3 related | No             |
| ATM       | ataxia telangiectasia mutated      | No             |

miRNAs offer precise gene regulation through post-transcriptional regulation of gene expression. However, the mechanism/s of regulation of miRNA expression is just beginning to emerge. We show herein for the first time that Wnt7a expression leads to increased expression of hsa-miR29b (Fig. 1). We have shown previously that activation of Wnt7a/Fzd9 signaling pathway leads to activation of the mitogen activated protein kinase, ERK5 and subsequent activation of PPARγ (Winn et al., 2006). Interestingly, ERK5, obligate for Wnt7a-stimulated PPARγ activation, was also observed to be indispensable for hsa-miR29b expression. The ability of PPARγ to induce hsa-miR29b expression suggests that induction of hsa-miR29b expression is the most distal event of Wnt7a signaling. In prostate cancer, hsa-miR-143 was shown to interfere with ERK5 signaling (Clapé et al., 2009). Although miRNA-mediated regulation of ERK5 is evident, ERK5-mediated regulation of miRNAs has not been identified thus far. Our study provides the first evidence for an important role of a MAPK in regulating miRNA. In a similar manner, the role of PPARγ in regulating miRNAs has been extensively studied (Shah et al., 2007), but the role/s of PPARγ on miRNA regulation remains largely unknown. Although a speculation, PPARγ might impose an indirect control on hsa-miR29b expression and regulate the biogenesis of mature form of hsa-miR29b, since Wnt7a or ERK5 could stimulate only the expression of mature form of hsa-miR29b (Figs 1, 3).

The current study also reveals a novel role for hsa-miR29b in MDM2 regulation. In silico analysis for hsa-miR29b complimentary sites identified MDM2 as a potential target (Fig. 4A). We confirmed our observation experimentally through hsa-miR29b expression, wherein expression of hsa-miR29b could block the expression of MDM2 both at the transcript level and protein level (Fig. 4). Similar effects for hsa-miR143/145 in regulating MDM2 have been reported (Zhang et al., 2013). These data suggest that loss of hsa-miR29b in cancers might lead to MDM2 upregulation and corresponding downregulation of p53 tumor suppressor. Indeed, re-expression of hsa-miR29b in NSCLC cells restored p53 expression and attenuated NSCLC cell proliferation (Fig. 4). A subset of NSCLC characteristically displays loss in Wnt7a (Winn et al., 2005), hsa-miR29b (current study) and p53 (Rom and Tchou-Wong, 2003), indicating that proper activation of Wnt7a signaling might be critical for p53 regulation and NSCLC cell proliferation.

In summary, we propose herein a novel role for Wnt7a/Fzd9 signaling in inducing hsa-miR29b expression. Absence of Wnt7a in NSCLC fails to activate the Wnt7a/Fzd9 pathway, which in turn fails to induce hsa-miR29b expression. Furthermore, the loss of hsa-miR29b expression results in increased levels of MDM2, reduced p53 expression, and increased cell proliferation (Fig. 5). On the contrary, activation of Wnt7a/Fzd9 signaling by Wnt7a, and
mediated by ERK5 and PPARγ, leads to the induction of hsa-miR29b. hsa-miR29b induction later promotes downregulation of MDM2, increased p53 expression, and reduced cell proliferation (Fig. 5). Thus, Wnt7a mediated regulation of hsa-miR29b represents a novel mechanism for Wnt7a/Fzd9-mediated regulation of NSCLC cell proliferation.

Fig. 4. See next page for legend.
miRNA extraction and miRNA expression profiling

Total RNA was isolated from cell lines using TRIzol reagent (15956, Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. cDNA were made using iScript II RT kit (218161) and for qPCR, miScript SYBR Green PCR kit (218023, Qiagen, Valencia, CA). The hsa-miR29b pcDNA plasmid was a kind gift from Dr. Gregory Gores (Mayo Clinic). The hsa-miR29b luciferase reporter plasmid was purchased from Sigma (Cat. no. LR-0062), in which the mature hsa-miR29b complementary sequence (TGCCTGGACTTGCCTGG) was obtained from Stratagene. The luciferase reporter, with luciferase under the control of 14 repeats of p53-binding sequence (TGCCTGGACTTGCCTGG), was purchased from Promega Corporation, Madison, WI.

Materials and Methods

Cell culture and inhibitors

NSCLC cell lines A549, H157 and H661 and a human non-transformed lung epithelial cell line (Beas2B) were cultured in RPMI 1640 medium (10-040-CV, Cellgro, Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO2 incubator at 37 °C. The cell lines were cultured bi-weekly and stocks of cell lines were passaged no more than ten times for use in experiments. The inhibitors used in our studies include, MEK inhibitors, PD98059 (Sigma), U0126 (Calbiochem) and PPARγ antagonist (T0070907, Calbiochem/EMD Biosciences). For miRNA expression studies, total RNA was isolated from NSCLC cells using TRIzol reagent (15956, Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. To make cDNAs, miScript II RT kit (218161) and for qPCR, miScript SYBR Green PCR kit (218023) (Qiagen, Valencia, CA) were used. The qPCR primer for mouse miR29b (MPM00629A) was purchased from Qiagen (Qiagen, Valencia, CA).

Clonogenic and MTS assays

Clonogenic assays were performed in triplicates by seeding 1×10^3 cells in a well of 12-well culture plate followed by incubation at 37 °C in 5% CO2 incubator. After 72 h, cell colonies were stained using a staining solution (0.5% of Crystal Violet, 12% Glutaraldehyde, 87.5% of deionized HO) for 1 h at room temperature. After destaining in water and drying, colonies were quantified using Biorad Chemidoc Imaging System. Cloning efficiency represents the mean number of colonies formed per well.

MTS assays were performed in duplicates by seeding 500 cells in a well of 96-well culture plate, followed by incubation at 37 °C in a 5% CO2 incubator. Cell proliferation was measured after 24, 48 and 72 h by adding 20 μl of MTS reagent (Cell Titer 96 Aqueous One Solution, G3582, Promega Corporation, Madison, WI) to each well, followed by incubation at 37 °C. After 1 h, the absorbance of the formazan product was measured at 490 nm using a plate reader. Normalized absorbance values (sample readings-readings of media only blank) were represented in the graphs.

Transfections and luciferase reporter assays

The reporter plasmids (hsa-miR29b-luciferase reporter and p53 luciferase-reporter), expression plasmids (pLNCX-Wnt7a-HA, pCDNA3.1-PPARγ and Fzd9) and CMV-β-galactosidase control plasmids were transiently transfected into NSCLC cells using LipofectAmine reagent (18324-012, Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s recommendations. The hsa-miR29b pcDNA plasmid was a kind gift from Dr. Gregory Gores (Mayo Clinic). The luciferase reporter plasmid was purchased from Signosis (Cat. no. LR-0062), in which the mature hsa-miR29b complementary sequence was used as a luciferase gene. Mature hsa-miR29b was then transfected into its complementary sequence in the reporter plasmid. The luciferase reporter was co-transfected with a downstream of luciferase gene. Mature hsa-miR29b was then transfected into its complementary sequence in the reporter plasmid. Therefore, a decrease in luciferase activity represents increased expression of hsa-miR29b and vice versa. All of the luciferase activities were normalized to CMV-β-gal activities. Wild-type pCDNA3.1-PPARγ plasmid was generously provided by Dr. Raphael Nemenoff (University of Colorado, Anschutz Medical Campus). p53-luciferase reporter, with luciferase under the control of 14 repeats of p53-binding sequence (TGCGTGGACCTGCGTGG) was obtained from Stratagene. The expression plasmid for pLNCX-Wnt7a-HA was a gift from Dr Jan Kitajewski (Columbia University) and pLNCX-Wnt3 was a gift from Dr Randall Moon

hsa-miR29b regulation of MDM2 in NSCLC 683
The authors have no competing interests to declare.

This study was supported by a Merit Award from the U.S. Department of Veterans Affairs, NIH grants R01CA138528-252271 and 5R21CA153268-02 to R.A.W. The study was supported by a Merit Award from the U.S. Department of Veterans Affairs, NIH grants R01CA138528-252271 and 5R21CA153268-02 to R.A.W. The authors have no competing interests to declare.

Biology Open

The authors have no competing interests to declare.

252271 and 5R21CA153268-02 to R.A.W. This study was supported by a Merit Award from the U.S. Department of Veterans Affairs, NIH grants R01CA138528-252271 and 5R21CA153268-02 to R.A.W. The authors have no competing interests to declare.

References

Bao, B., Ahmad, A., Kong, D., Ali, S., Azmi, A. S., Li, Y., Banerjee, S., Padhye, S. and Sarkar, F. H. (2012). Hypoxia induced aggressiveness of prostate cancer cells is linked with deregulated expression of VEGF, IL-6 and miRNAs that are attenuated by CDK4. PLoS ONE 7, e43726.

Baskerville, S. and Bartel, D. P. (2005). Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA 11, 241-247.

Behrens, J., van Krieis, J. P., Kühl, M., Bruhn, L., Wiedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of beta-catennin with the transcription factor LEF-1. Nature 382, 638-642.

Bikavalli, R. K., Malbon, C. C. and Malbon, C. C. (2010). Dishevelled-KSRP complex regulates Wnt signaling through post-transcriptional stabilization of beta-catennin mRNA. J. Cell Sci. 123, 1352-1362.

Bikavalli, R. K. and Malbon, C. C. (2012). Wnt3a-stimulated LRP6 phosphorylation is dependent on neighboring myosin of GJBP2. J. Cell Sci. 125, 2446-2456.

Cai, Y., Yu, X., Hu, S. and Yu, J. (2009). A brief review on the mechanisms of miRNA regulation. Genomics Proteomics Bioinformatics 7, 147-154.

Carlin, G. A., Garzon, R., Cimmino, A., Fabbri, M. and Croce, C. M. (2006). MicroRNA and leukemias: how strong is the connection? Leuk. Res. 30, 653-655.

Cameron, S. J., Malik, S., Akaile, M., Lerner-Marmarosh, N., Yan, C., Lee, J. D., Abe, Y. and Yang, J. (2003). Regulation of epidermal growth factor-induced connexin 43 gap junction communication by big mitogen-activated protein kinase/ ERK5 but not ERK1/2 kinase activation. J. Biol. Chem. 278, 18682-18688.

Clapé, C., Fritz, V., Henriquet, C., Apparailly, F., Fernandez, P. L., Iborra, F., Avancés, C., Villalba, M., Culine, S. and Fajas, L. (2009). miR-143 interferes with ERK5 signaling, and abrogates prostate cancer progression in mice. PLoS ONE 4, e6900.

Esquela-Kerscher, A. and Slack, F. J. (2006). Oncomirs – microRNAs with a role in cancer. Nat. Rev. Cancer 6, 259-269.

Fabbri, M., Garzon, R., Cimmino, A., Liu, Z., Zanesi, N., Callegari, E., Liu, S., Adamovich, C., Costinean, S. and Calin, G. A. (2007). MicroRNA-29 family reverts aberrant differentiation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc. Natl. Acad. Sci. USA 104, 15805-15810.

Garzon, R., Fabbri, M., Cimmino, A., Carlin, G. A. and Croce, C. M. (2006). MicroRNA expression and function in cancer. Trends Mol. Med. 12, 580-587.

Jenal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E. and Forman, D. (2011). Global cancer statistics. CA Cancer J. Clin. 61, 69-90.

Kamakura, S., Moriguchi, T. and Nishida, E. (1999). Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases: Identification and characterization of a signaling pathway to the nucleus. J. Biol. Chem. 274, 26563-26571.

Karim, R., Tse, G., Putti, T., Scudier, R. and Lee, S. (2004). The significance of the Wnt pathway in the pathogenesis of human cancers. Pathology 36, 120-128.

Kolter, L., Swahari, V., Hammond, S. M. and Deshmukh, M. (2011). miR-29b is activated during neuronal maturation and targets BH1-only genes to restrict apoptosis. Genes Dev. 25, 120-130.

Lai, E. C., Tomancak, P., Williams, R. W. and Rubin, G. M. (2005). Computational identification of Drosophila microRNA genes. Genome Biol. 4, R42.

Lim, L. P., Glasner, M. E., Yekta, S., Burge, C. B. and Bartel, D. P. (2003a). Vertebrate microRNA genes. Science 299, 1540.

Lim, L. P., Lau, N. C., Weinstein, E. G., Abdelhakim, A., Yekta, S., Rhoades, M. W., Burge, C. B. and Bartel, D. P. (2003b). The microRNAs of Caenorhabditis elegans. Curr. Biol. 13, 1484-1489.

Lim, L. P., Lau, N. C., Weinstein, E. G., Abdelhakim, A., Yekta, S., Rhoades, M. W., Burge, C. B. and Bartel, D. P. (2003a). The microRNAs of Caenorhabditis elegans. Curr. Biol. 13, 1484-1489.

Mazieres, J., He, B., You, L., Xu, Z. and Jaublons, D. M. (2005). Wnt signaling in lung cancer. Cancer Lett. 222, 1-10.

Molenaar, M. van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destrie, O. and Clevers, H. (1996). Wnt signaling through stabilization of beta-catenin induced axis formation in Xenopus embryos. Cell 86, 391-399.

Oliver, T. G., Meylan, E., Chang, G. P., Xue, W., Burke, J. R., Humpton, T. J., Cameron, S. J., Malik, S., Akaike, M., Lerner-Marmarosh, N., Yan, C., Lee, J. D., Abramson, M. J., Wu, N. and Tabin, C. J. (2012). A brief review on the mechanisms of miRNA regulation. Mol. Cancer Res. 10, 1866-1878.

Peifer, M. and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis – a look outside the nucleus. Science 287, 1600-1609.

Rom, W. N. and Tchou-Wong, K. M. (2003). Molecular and genetic aspects of lung cancer. Methods Mol. Med. 75, 3-26.

Rothschild, S. I., Tschan, M. P., Federoni, E. A., Jaggii, R., Fey, M. F., Gugger, M. and Gautschi, O. (2012). MicroRNA-29b is involved in the Src/ID1 signaling pathway and is dysregulated in human lung adenocarcinoma. Oncogene 31, 4221-4232.

Ru, P., Steele, R., Newhall, P., Phillips, N. J., Toth, K. and Ray, R. B. (2012). miRNA-29b suppresses prostate cancer metastasis by regulating epithelial-mesenchymal transition signaling. Mol. Cancer Ther. 11, 1166-1173.

Samakoglu, S., Devi, D. S., Li, H., Wang, S., Murphy, M., Bao, C., Bassi, R., Prewitt, M. and Tonra, J. R. (2012). Preclinical rationale for combining an EGFR antibody with ciplatin/gemcitabine for the treatment of NSCLC. Cancer Genomics Proteomics 9, 77-92.

Sekido, Y., Fong, K. M. and Minna, J. D. (1998). Progress in understanding the molecular pathogenesis of human lung cancer. Biochim. Biophys. Acta 1378, F21-F39.

Shah, Y. M., Morimura, K., Yang, Q., Tanabe, T., Takagi, M. and Gonzalez, F. J. (2007). Peroxisome proliferator-activated receptor alpha regulates a microRNA-mediated signaling cascade responsible for hepatocellular proliferation. Mol. Cell. Biol. 27, 4238-4247.

Siegel, R., Naishadhram, D. and Jemal, A. (2013). Cancer statistics, 2013. CA Cancer J. Clin. 63, 11-30.

Tennis, R. A., Van Scoy, M., Heasley, L. E., Vandervest, K., Weiser-Evens, M., Freeman, S., Keith, R. L., Simpson, F., Nemenoff, R. A. and Winn, R. A. (2010).
Prostacyclin inhibits non-small cell lung cancer growth by a frizzled 9-dependent pathway that is blocked by secreted frizzled-related protein 1. *Neoplasia* **12**, 244-253.

Wang, Y., Debatin, K. M. and Hug, H. (2001). HIPK2 overexpression leads to stabilization of p53 protein and increased p53 transcriptional activity by decreasing Mdm2 protein levels. *BMC Mol. Biol.* **2**, 8.

Wang, J., Czech, B., Crunk, A., Wallace, A., Mitreva, M., Hannon, G. J. and Davis, R. E. (2011). Deep small RNA sequencing from the nematode Ascaris reveals conservation, functional diversification, and novel developmental roles. *Genome Res.* **21**, 1462-1477.

Winn, R. A. and Heasley, L. E. (2004). Gamma-catenin expression is reduced or absent in a subset of human non-small cell lung cancers, and its re-expression inhibits cell growth. *Chest* **125 Suppl.**, 22S-123S.

Winn, R. A., Marek, L., Han, S. Y., Rodriguez, K., Rodriguez, N., Hammond, M., Van Scoyk, M., Acosta, H., Mirus, J., Barry, N. et al. (2005). Restoration of Wnt-7a expression reverses non-small cell lung cancer cellular transformation through frizzled-9-mediated growth inhibition and promotion of cell differentiation. *J. Biol. Chem.* **280**, 19625-19634.

Yang, Y. M., Seo, S. Y., Kim, T. H. and Kim, S. G. (2012). Decrease of microRNA-122 causes hepatic insulin resistance by inducing protein tyrosine phosphatase 1B, which is reversed by licorice flavonoid. *Hepatology* **56**, 2209-2220.

Zhang, J., Sun, Q., Zhang, Z., Ge, S., Han, Z. G. and Chen, W. T. (2013). Loss of microRNA-143/145 disturbs cellular growth and apoptosis of human epithelial cancers by impairing the MDM2-p53 feedback loop. *Oncogene* **32**, 61-69.