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Report

miR-34 Cooperates with p53 in Suppression of Prostate Cancer by Joint Regulation of Stem Cell Compartment

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SUMMARY

The miR-34 family was originally found to be a direct target of p53 and is a group of putative tumor suppressors. Surprisingly, mice lacking all *mir-34* genes show no increase in cancer formation by 18 months of age, hence placing the physiological relevance of previous studies in doubt. Here, we report that mice with prostate epithelium-specific inactivation of *mir-34* and *p53* show expansion of the prostate stem cell compartment and develop early invasive adenocarcinomas and high-grade prostatic intraepithelial neoplasia, whereas no such lesions are observed after inactivation of either the *mir-34* or *p53* genes alone by 15 months of age. Consistently, combined deficiency of p53 and miR-34 leads to acceleration of MET-dependent growth, self-renewal, and motility of prostate stem/progenitor cells. Our study provides direct genetic evidence that *mir-34* genes are bona fide tumor suppressors and identifies joint control of MET expression by p53 and miR-34 as a key component of prostate stem cell compartment regulation, aberrations in which may lead to cancer.

INTRODUCTION

The microRNA-34 (miR-34) is highly evolutionarily conserved (Corney et al., 2007; He et al., 2007). In mammals, the miR-34 family is composed of three processed microRNAs (miRNAs) that are encoded by two different genes: miR-34a is encoded by its own transcript, whereas miR-34b and miR-34c share a common primary transcript as a cluster. Due to the high homology among these three members, they have many similar targets and may be functionally redundant (He et al., 2007). miR-34 was the first miRNA reported to be directly transactivated by tumor suppressor p53 (aka Trp53/TP53) and is considered to be an important component of the p53 network (Hermeking, 2012).

In addition to a transactivation-dependent decrease in expression levels in p53-deficient tumors, *mir-34* is also deleted or epigenetically downregulated in multiple cancer cell lines and human malignancies (Bader, 2012; Hermeking, 2012). Ectopic expression of miR-34 has been shown to counteract various oncogenic processes by regulating target genes that function in cell cycle, apoptosis, senescence, cell migration, and invasion (Hermeking, 2012). Furthermore, treatment with miR-34 mimics inhibits cancer formation in transplantation experiments (Bader, 2012; Liu et al., 2011).

Contrary to the expectations raised from experiments based on nonphysiological approaches, such as exogenous miR-34 introduction and miR-34 knockdown, only minor defects have been reported in studies of mice with targeted inactivating mutations of *mir-34* (Concepcion et al., 2012; Wei et al., 2012). Moreover, complete genetic inactivation of miR-34 did not impair the p53 response in a variety of ex vivo and in vivo assays (Concepcion et al., 2012). Most surprisingly, no increase in spontaneous or irradiation-induced carcinogenesis has been observed in mice lacking all *mir-34* genes by 18 month of age (Concepcion et al., 2012). The absence of all *mir-34* genes also did not accelerate B cell lymphomagenesis in mice overexpressing c-Myc under the control of the $E\mu$ promoter (Concepcion et al., 2012). These data question the native tumor-suppressive function of miR-34. Clarification of the miR-34 role as a tumor suppressor is of particular importance because reintroduction of this miRNA into cancer cells has already reached phase 1 clinical trials (Bouchie, 2013).

A number of recent studies have provided evidence of p53-independent expression of miR-34. For example, miR-34a can be upregulated to repress MYC during oncogene-induced senescence in human TIG3 fibroblasts (Christoffersen et al., 2010) and contributes to megakaryocytic differentiation of K562 cells (Navarro et al., 2009) in a p53-independent fashion. Consistent with these observations, levels of all miR-34 family members remain high in the brains, testes, and lungs of mice lacking p53 (Concepcion et al., 2012).

Methylation of *mir-34a* and *mir-34b/c* has been found in prostate cancers carrying mutant p53 (Fujita et al., 2008; Kojima et al., 2010; Liu et al., 2011; Lodygin et al., 2008). Furthermore,

frequent hypermethylation of *mir-34* in cancers with a high occurrence of *p53* mutations, such as ovarian and mammary carcinomas and soft tissue sarcomas (Corney et al., 2010; Lodygin et al., 2008; Vogt et al., 2011), suggests the coexistence of both alterations in the same neoplasms. These findings, together with reports of p53-independent regulation of miR-34, suggest that p53 and miR-34 may cooperate in cancer suppression. This possibility is also supported by our observation that p53 and miR-34 may jointly regulate MET receptor tyrosine kinase as part of a coherent feedforward loop in primary ovarian surface epithelium cells (Hwang et al., 2011). However, there is no direct experimental evidence for p53 and miR-34 cooperation in MET regulation in animal models. By using newly generated mice carrying conditional alleles of *mir-34a* and *mir-34b/c*, we show that miR-34 cooperates with p53 in suppression of prostate carcinogenesis by joint MET-mediated control of the stem cell compartment.

RESULTS

miR-34 Cooperates with p53 in Suppression of Prostate Carcinogenesis

By using gene targeting of *mir-34a* and *mir-34b/c* loci (Figure S1) and subsequent crosses of mice, we prepared mice with conventional (*mir-34a*$^{-/-}$*mir-34b/c*$^{-/-}$) and conditional (floxed, *mir-34a*$^{loxP/loxP}$*mir-34b/c*$^{loxP/loxP}$) triple knockout alleles and designated them as *mir-34*$^{-/-}$ and *mir-34*$^{L/L}$, respectively. Consistent with a previous report by Concepcion et al. (2012), our findings indicate that germline genetic inactivation of *mir-34* has only a minor effect on normal development (Supplemental Results, Supplemental Discussion, and Figure S2). We also have not observed any significant pathological phenotypes, including cancers, in *mir-34*$^{-/-}$ mice (n = 19) between 15 and 18 months of age.

To rule out the possibility that mice somehow physiologically compensate for germline *mir-34* deficiency, we performed prostate epithelium-specific *mir-34* deletion. This was accomplished by using a *PB-Cre4* transgene, in which a modified *probasin* promoter drives postnatal expression of Cre recombinase in the prostate epithelium (Chen et al., 2005; Zhou et al., 2006). Consistent with previous reports and our findings in *mir-34*$^{-/-}$ mice, mice lacking all *mir-34* genes in the prostate epithelium cells (*mir-34*$^{PE-/-}$ mice) did not show any atypical lesions by 15 months of age (Figures 1A, 1B, and S3A; Table S1).

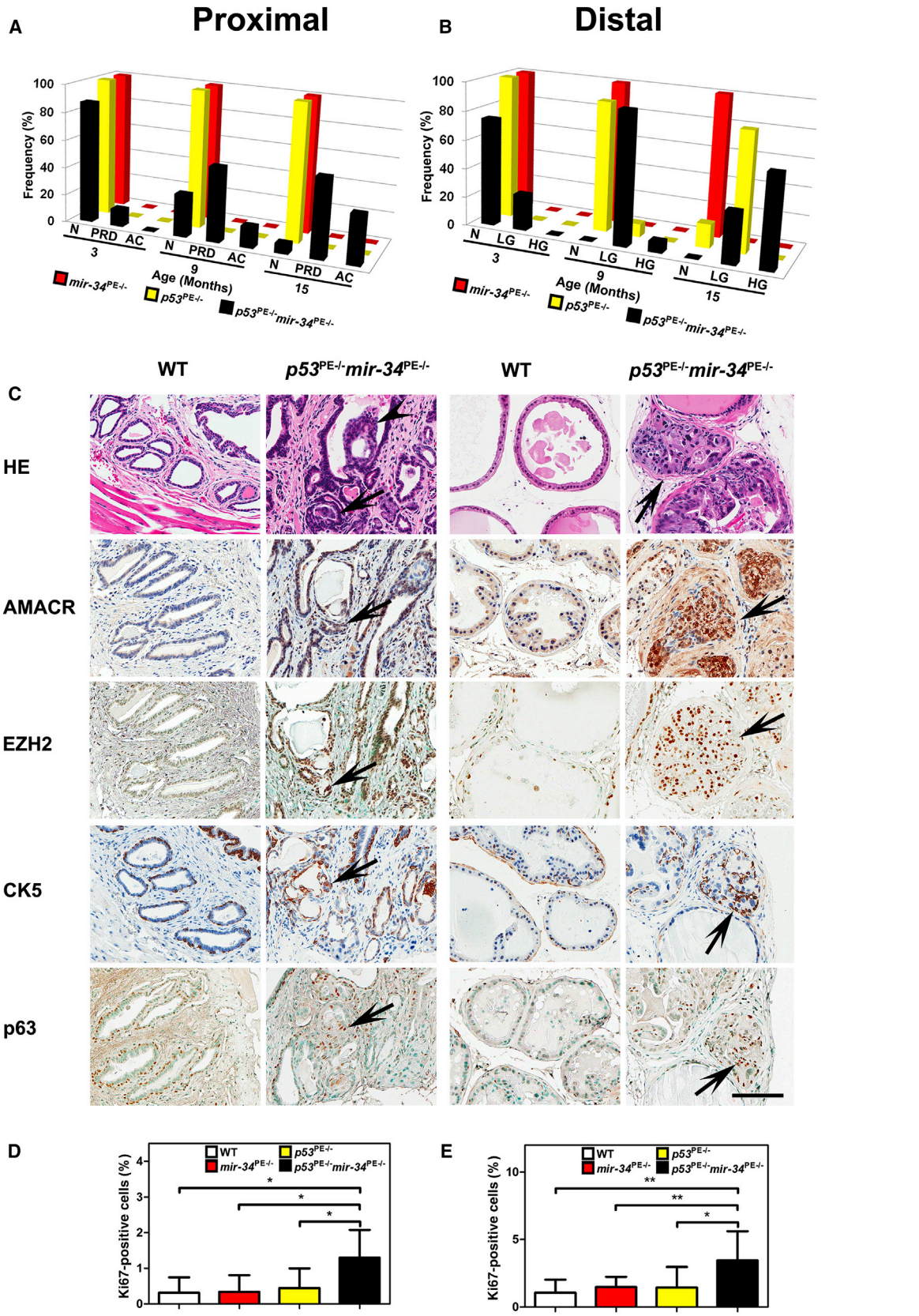
To test if miR-34 may have p53-independent function, we determined the expression levels of the miR-34 family after *p53* deletion in FACS-purified *p53*$^{L/L}$ prostate epithelium cells exposed to Ad-*Cre*. Significant levels of miR-34 expression were still detected after *p53* inactivation (Figures S3B and S3C).

To test if p53 and miR-34 may cooperate in suppressing prostate carcinogenesis we generated *p53*$^{PE-/-}$ and *p53*$^{PE-/-}$ *mir-34*$^{PE-/-}$ mice by crossing *p53*$^{L/L}$ mice with *mir-34*$^{L/L}$ and *PB-Cre4* mice. Consistent with previous reports on lack or low frequency of neoplastic lesions in mice with prostate epithelium-specific *p53* inactivation (Chen et al., 2005; Zhou et al., 2006), only 1 out of 11 *p53*$^{PE-/-}$ mice (9%) showed prostatic intraepithelial neoplasia 1 (PIN1) by 9 months of age in the distal regions of prostatic ducts. By 15 months of age more of *p53*$^{PE-/-}$ mice developed PINs. However, all of them were of low-grade (PIN1 or PIN2; Figures 1A, 1B, and S3A; Table S1). No significant changes were observed in the proximal regions of prostatic ducts, which are known to encompass a prostate epithelium stem cell compartment (Leong et al., 2008; Tsujimura et al., 2002).

In contrast, beginning at 3 months of age *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice showed dysplastic lesions characterized by varying degree of nuclear atypia and loss of normal cellular arrangement in the proximal regions of prostatic ducts (Figures 1A and S3A; Table S1). From 9 months of age majority of mice had advanced dysplastic lesions which frequently filled up expanded ducts, and 15% and 36% of mice developed early invasive adenocarcinomas at 9 and 15 months, respectively (Figures 1A and S3A; Table S1). In the distal regions of prostatic ducts, the first PIN1 lesions were detected already by 3 months of age (Figures 1B and S3A; Table S1). High-grade PIN lesions (PIN3 and 4) have been observed by 9 months of age and 64% (9 out of 14) of mice had such lesions at 15 months of age. Consistent with these findings, adenocarcinomas and high-grade PIN lesions of the proximal and distal regions of prostatic ducts, respectively, characterized by elevated expression of markers of early prostate cancer, such as AMACR and EZH2, and increased number of CK5 and p63-positive cells (Figure 1C). Similarly, higher proliferative activity has been observed in both proximal and distal regions of prostatic ducts of *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice (Figures 1D and 1E). In summary, these results show that miR-34 and p53 cooperate in suppression of prostate carcinogenesis.

p53 and miR-34 Cooperate in the Control of Prostate Stem/Progenitor Cell Activity

According to our pathological evaluation, the stem/progenitor cell-enriched proximal regions of prostatic ducts were specifically affected in *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice. To test if combined p53 and miR-34 deficiency affects functional properties of prostate stem/progenitor cells, we isolated such cells by fluorescence-activated cell sorting (FACS) as a CD49f^{hi}/Sca-1^{+} fraction. Mice with prostate-specific deletions of either *mir-34* or *p53* had slightly more stem/progenitor cells than background-matched wild-type (WT) mice (Figure 2A). However, the pool of CD49f^{hi}/Sca-1^{+} cells deficient for both miR-34 and p53 increased by 39% and constituted 7.1% of the prostate epithelium versus 5.1% in WT. Notably, the CD49f^{hi}/Sca-1^{+} fraction isolated from the prostates of *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice formed prostaspheres far more efficiently and of larger size (Figures 2B and 2C). Both higher frequency and the size of spheres formed by p53 and miR-34-deficient CD49f^{hi}/Sca-1^{+} stem/progenitor cells were maintained over multiple passages (dissociation and clonal formation), suggesting a role for these genes in the control of self-renewal. At the same time, no difference among genotypes was observed in CD49f^{lo}/Sca-1^{-} luminal cells (Figure 2D). These cells formed very few spheres after the first plating, and no spheres were observed after the first passage. Thus, miR-34 and/or p53 deficiency is unlikely to reprogram differentiated cells toward the stem cell state.



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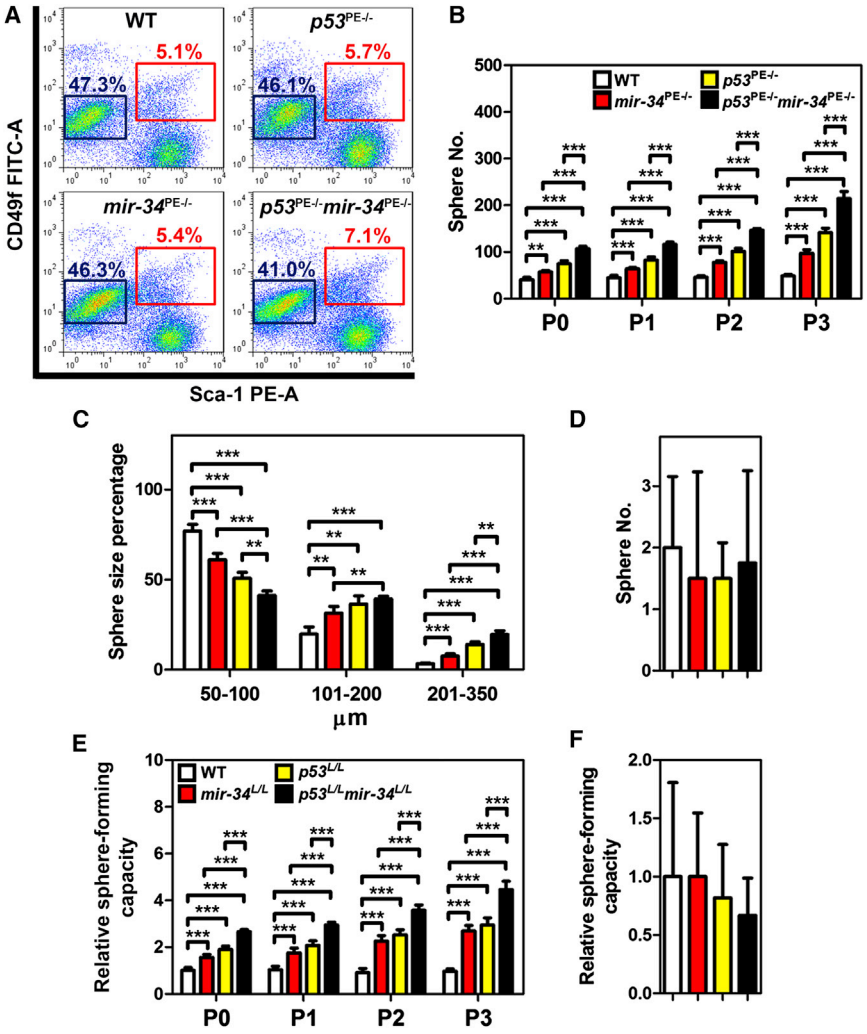

Figure 2. Deletions of Both *p53* and *mir-34* Promote Prostate Stem/Progenitor Cell Expansion and Sphere-Forming Capacity

(A) A quantitative analysis of distribution of CD49f^{hi}/Sca-1^{+} stem/progenitor cells and CD49f^{lo}/Sca-1^{-} luminal cells from 3-month-old WT, *mir-34*$^{PE-/-}$, *p53*$^{PE-/-}$, and *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice (n = 3). Red and blue frames represent stem/progenitor cell and luminal cell populations, respectively.

(B–D) The frequency (B and D) and size (C) of spheres formed by CD49f^{hi}/Sca-1^{+} stem/progenitor cells (B and C) and CD49f^{lo}/Sca-1^{-} (D) luminal cells from 3-month-old WT, *mir-34*$^{PE-/-}$, *p53*$^{PE-/-}$, and *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice (n = 3). P0–P3, passages 0–3.

(E and F) The relative frequency of sphere formation by CD49f^{hi}/Sca-1^{+} stem/progenitor cells (E) and CD49f^{lo}/Sca-1^{-} luminal cells (F) isolated from WT, *mir-34*$^{L/L}$, *p53*$^{L/L}$, and *p53*$^{L/L}$*mir-34*$^{L/L}$ mice followed by Ad-*Cre* infection (n = 3). Sphere counts were normalized to the Ad-*blank*-infected spheres of each passage.

**p < 0.01; ***p < 0.001. Error bars denote SD.

p53 and miR-34 Regulation of Stem/Progenitor Cells Depends on MET

In addition to the invasive growth of cells in the prostate stem cell compartment of *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice, we have noted that some of the cells from the p53 and miR-34-deficient prostaspheres were spreading into the surrounding matrix (Figure 3A). Because MET plays a crucial role in regulation of cell motility and invasion (Trusolino et al., 2010) and is a known target of p53 (Hwang et al., 2011) and miR-34 (Corney et al., 2010; He et al., 2007; Hwang et al., 2011), we have tested its expression in FACS-isolated populations of the prostate epithelium. CD49f^{hi}/Sca-1^{+} prostate stem/progenitor cells had far higher levels of expression as compared to CD49f^{lo}/Sca-1^{-}luminal cells (Figure 3B). Deficiency for either miR-34 or p53 slightly increased MET levels in stem/progenitor cells, whereas such cells from *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ showed the highest MET expression (Figures 3B and 3C). Consistently, CD49f^{hi}/Sca-1^{+} prostate stem/progenitor cells deficient for both miR-34 and p53 had the highest motility in the migration assay (Figure 3D)

To test whether the observed properties represent the direct effects of p53 and/or miR-34 on prostate stem/progenitor cells, we have isolated CD49f^{hi}/Sca-1^{+} stem/progenitor cells and CD49f^{lo}/Sca-1^{-} luminal cells from the prostates of WT, *mir-34*$^{L/L}$, *p53*$^{L/L}$, and *p53*$^{L/L}$*mir-34*$^{L/L}$ mice and, followed by infection with Ad-*Cre* or Ad-*blank*, subjected them to the prostasphere-formation experiments (Figures 2E and 2F). Consistently, the lack of both p53 and miR-34 had the most pronounced effect on the frequency of stem/progenitor cells in consecutive passages.

Figure 1. miR-34 and p53 Cooperate in Suppression of Prostate Carcinogenesis

(A and B) A quantitative analysis of the frequency of neoplastic lesions in proximal (A) and distal (B) regions of prostatic ducts. N, normal; PRD, proximal dysplastic lesions; AC, adenocarcinoma; LG, low-grade PIN; HG, high-grade PIN.

(C) Proximal (left two columns) and distal (right two columns) regions of prostatic ducts in 15-month-old WT and *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice. Adenocarcinomas invading the surrounding stroma (arrows) and filling up the lumen (arrowheads) in the proximal regions of the prostatic ducts of *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice are shown. PIN4 (arrows) in the distal regions of the prostatic ducts of *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice is shown. As compared to the prostate epithelium of WT mice, both adenocarcinomas and PIN4 (arrows) show higher expression levels of AMACR and EZH2 and an increased number of CK5 and p63-positive cells. HE, hematoxylin and eosin staining. The ABC Elite method with hematoxylin (AMACR, CK5) or methyl green (EZH2, and p63) counterstaining was performed. Scale bar, 100 μm for all images.

(D and E) A quantitative analysis of the proliferation rate in proximal (D) and distal (E) regions of prostatic ducts. *p < 0.05; **p < 0.01. Error bars denote SD. See also Figure S3A.

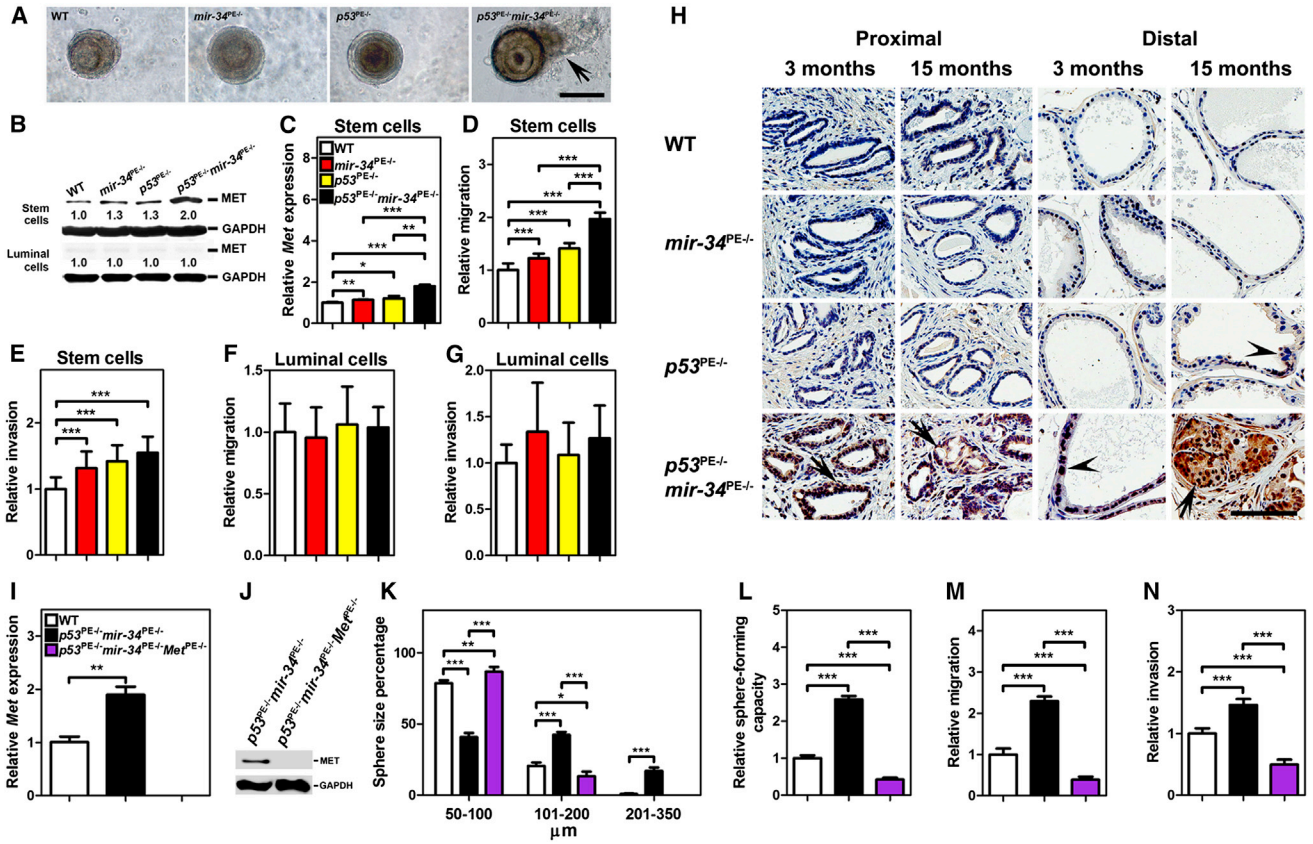

Figure 3. MET Expression Is Essential for the Increased Growth, Sphere-Forming Capacity, Motility, and Invasion of p53 and miR-34-Deficient Prostate Stem/Progenitor Cells

(A–H) Prostasphere formation (A), western blot (B), and qRT-PCR (C) of *Met* expression, migration (D and F), and invasion (E and G) by CD49f^{hi}/Sca-1^{+} stem/progenitor cells (A–E) and CD49f^{lo}/Sca-1^{-} luminal cells (B, F, and G) of 3-month-old WT, *mir-34*$^{PE-/-}$, *p53*$^{PE-/-}$, and *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice (n = 3). (A) Note the outgrowth of cells from prostaspheres prepared from *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice (arrow). (H) MET expression in the cells of proximal and distal regions of prostatic ducts of 3- and 15-month-old WT, *mir-34*$^{PE-/-}$, *p53*$^{PE-/-}$, and *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice is shown. MET expression (arrows) is detected in the proximal regions of the prostatic ducts of 3- and 15-month-old *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice and in the PIN4 of the distal region in 15-month-old *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice. PIN1 (arrowheads) in the distal regions of prostatic ducts lacks MET expression in both *p53*$^{PE-/-}$ and *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice. The ABC Elite method with hematoxylin counterstaining was performed. Scale bars, 100 μm.

(I–N) qRT-PCR (I) and western blot (J) of *Met* expression, prostasphere size (K), sphere-forming capacity (L), migration (M), and invasion (N) of CD49f^{hi}/Sca-1^{+} stem/progenitor cells isolated from 3-month-old WT, *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$, and *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$*Met*$^{PE-/-}$ mice (n = 3).

*p < 0.05; **p < 0.01; ***p < 0.001. Error bars denote SD.

and some trend, albeit not statistically significant, toward increased invasive activity (Figure 3E). To the contrary, luminal cells deficient for p53 and/or miR-34 had no significant differences in their motility (Figure 3F) or invasion (Figure 3G).

Consistent with ex vivo results, we also observed elevated levels of MET expression in cells of the proximal regions of prostatic ducts of *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice, as compared to WT mice and mice with inactivation of either *mir-34* or *p53* (Figure 3H). MET expression was below detectable levels in the epithelium of the distal regions of the prostatic ducts in all strains. The only exception was elevated MET expression in high-grade PINs in *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice, suggesting a possible increase in the number of stem cell-like cells in such lesions.

To test if MET overexpression is essential for the observed phenotypes, *Met* was inactivated using a conditional *Met*$^{L/L}$ allele. *Met* inactivation abrogated growth, sphere-forming capacity, cell motility, and invasion of p53 and miR-34-deficient CD49f^{hi}/Sca-1^{+} prostate stem/progenitor cells (Figures 3I–3N). Effects of MET downregulation on growth, sphere-forming capacity, cell motility, and invasion of WT prostate stem/progenitor cells were less prominent (Figures 4A–4G), consistent with the lower levels of MET expression in such cells. However, 2-fold induction of MET expression by hypoxia resulted in a comparable increase of all the above parameters (Figures 4A–4G). Similar to *p53*/*mir-34*-inactivation experiments, this phenotype was reversed after MET knockdown, indicating a critical role for MET in prostate stem/progenitor cell regulation.

Previously, it has been shown that p53 may negatively regulate MET expression by the miR-34-mediated targeting of MET (Corney et al., 2010; He et al., 2007; Hwang et al., 2011). Supporting these observations, we have detected the preserved 3′ UTR

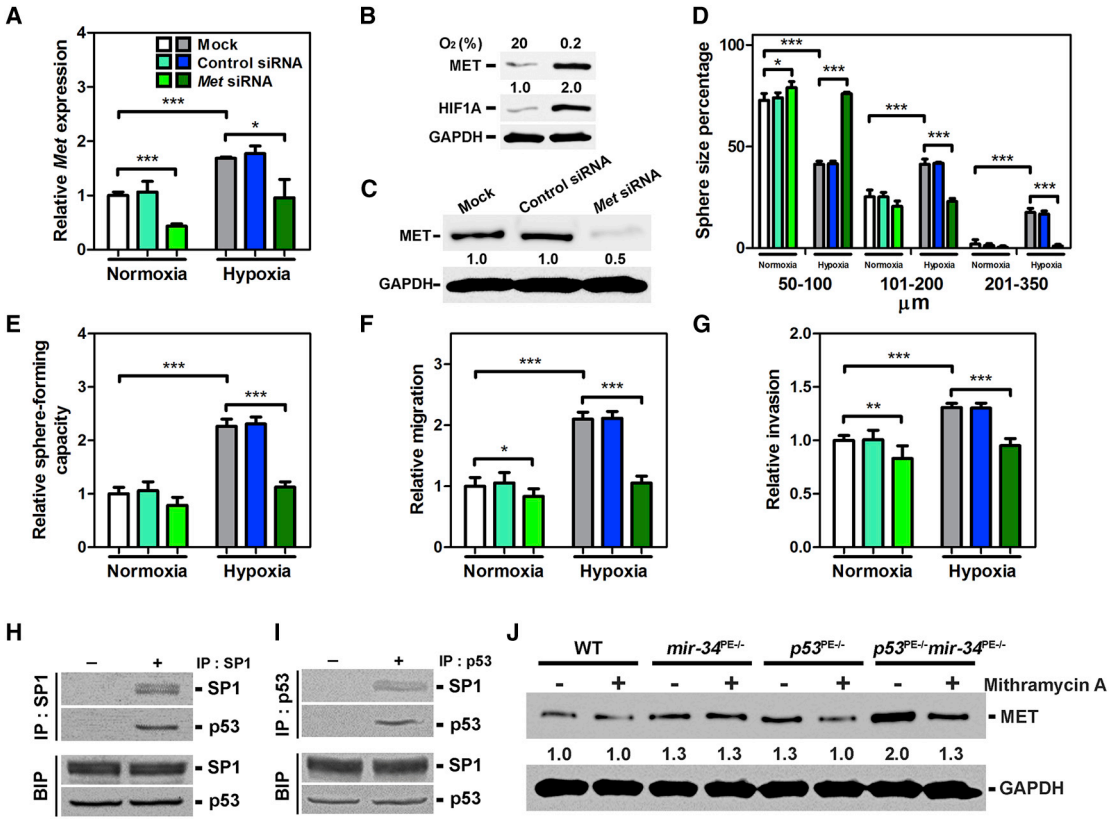

Figure 4. MET Is Essential for the Growth, Sphere-Forming Capacity, Motility, and Invasion of WT Prostate Stem/Progenitor Cells and Is Partially Regulated by SP1 Interacting with p53

(A–G) qRT-PCR (A) and western blot (B and C) of *Met* expression, prostasphere size (D), sphere-forming capacity (E), migration (F), and invasion (G) of CD49f^{hi}/Sca-1^{+} stem/progenitor cells isolated from 3-month-old WT mice (n = 3) and cultured under normoxic (20% O_2, A, B, and D–G) and hypoxic (0.2% O_2, A–G) conditions. *p < 0.05; **p < 0.01; ***p < 0.001. Error bars denote SD. Very similar results were obtained in separate experiments with two different *Met* small interfering RNAs (siRNAs).

(H and I) Coimmunoprecipitation of cell lysates with SP1 (H) or p53 (I) antibodies followed by western blot with p53 or SP1 antibodies, respectively (upper panels). Samples of the same lysates were used for western blot with p53 or SP1 antibodies before immunoprecipitation (lower panels). CD49f^{hi}/Sca-1^{+} stem/progenitor cells isolated from 3-month-old WT mice (n = 3) were used. IP, immunoprecipitation. BIP, before immunoprecipitation.

(J) The effect of mithramycin A (100 nM) on MET expression of CD49f^{hi}/Sca-1^{+} stem/progenitor cells isolated from 3-month-old WT, *mir-34*$^{PE-/-}$, *p53*$^{PE-/-}$, and *p53*$^{PE-/-}$*mir-34*$^{PE-/-}$ mice (n = 3).

carrying two binding sites for miR-34 in prostate stem/progenitor cells (Figure S4). We have also reported that p53 also represses MET expression by miR-34-independent inhibition of SP1 binding to the *Met* promoter in the ovarian surface epithelium cells (Hwang et al., 2011). Consistent with this mechanism, MET reciprocal coimmunoprecipitation experiments have shown that p53 physically interacts with endogenous SP1 in the prostate stem/progenitor cells (Figures 4H and 4I). Furthermore, SP1 inhibition results in reduction of MET expression in the prostate stem/progenitor cells deficient for either p53 or both miR-34 and p53, but not for miR-34 alone (Figure 4J).

DISCUSSION

Our study provides direct genetic proof that miRNAs of the miR-34 family may act as tumor suppressors in concert with other genes, such as *p53*. These findings offer a solid physiological basis for the rational design of diagnostic and therapeutic approaches. Because the lack of *mir-34* genes alone is insufficient for cancer initiation, their downregulation is likely to occur at some point during tumor progression. However, the preexistence of *mir-34* methylation in some normal cells cannot be excluded. Further genomic studies in conjunction with animal modeling should be able to address this question. Although our current studies have been focused on prostate cancer, tissue-specific inactivation of *mir-34* and *p53* in other tissues will address likely interactions of these genes in other cell lineages.

Our observations confirm the earlier findings that p53 may negatively regulate MET expression by miR-34-mediated targeting of MET and by miR-34-independent inhibition of SP1 binding to the MET promoter. Notably, according to our previous ex vivo studies, inactivation of both mechanisms is required to achieve the highest MET overexpression, cell motility, and invasion (Hwang et al., 2011). Our present study supports this possibility in an autochthonous model of cancer. Our findings also

show that miR-34 effects on MET regulation occur both in a p53-dependent and -independent manner. Specific mechanisms for p53-independent miR-34 regulation remain to be determined.

Previous studies have shown that p53 and miR-34 affect induced pluripotent stem cell reprogramming (Choi et al., 2011; Krizhanovsky and Lowe, 2009). p53 mediates the onset of the senescence of endothelial progenitor cells (Rosso et al., 2006) and negatively regulates the proliferation and survival of neural stem cells (Meletis et al., 2006). Constitutive p53 activation results in depletion of adult stem cells in bone marrow, brain, and testes (Liu et al., 2010). It has been reported that ectopic expression of miR-34a may inhibit prostate cancer-propagating cells (also known as cancer stem cells or cancer-initiating cells) and metastasis by directly repressing CD44 (Liu et al., 2011). However, the role for miR-34 in regulation of normal adult stem cells has been unclear. Our study fills this gap by showing that miR-34 regulates prostate stem/progenitor cells in cooperation with p53. It will be of interest to see if a similar cooperation of miR-34 and p53 may play a role in the stem cell compartments of other cell lineages.

It has been previously reported that prostate cancer-propagating cells express MET, and depletion of MET results in a decrease in prostasphere formation (Rajasekhar et al., 2011). However, the direct role of MET in regulation of normal prostate stem/progenitor cells and mechanisms controlling its expression have been uncertain. Our studies on prostate cells collected either in the early stages of carcinogenesis or immediately after *mir-34* and *p53* inactivation provide a missing link between normal biological functions of MET and promotion of aberrant expansion of the stem/progenitor cell pool, which may eventually lead to cancer. Considering that MET is particularly overexpressed in stem/progenitor cells lacking both p53 and miR-34, its therapeutic targeting may be especially effective in p53 and miR-34-deficient cancer cases.

Some cancers arise from stem/progenitor cells (Flesken-Nikitin et al., 2013; Schepers et al., 2012), whereas others may originate from more differentiated cells (Friedmann-Morvinski et al., 2012). In our study, we have observed that neoplastic lesions in the distal regions of prostatic ducts, which are mainly populated by transit-amplifying and differentiated cells, never progress to frank invasive adenocarcinomas. These findings support observations in other models that cancers arising from stem cell compartments are more aggressive (Flesken-Nikitin et al., 2013). Our autochthonous mouse model of prostate cancer based on prostate epithelium-specific inactivation of *p53* and *mir-34* should provide a valuable tool for further elucidation of the role of individual cell subpopulations in prostate cancer pathogenesis.

EXPERIMENTAL PROCEDURES

Generation of Mice with *mir-34* Conventional and Conditional Targeted Mutations

All animal experiments were carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Laboratory Animal Use and Care Committee at Cornell University. *mir-34a* and *mir-34b/c* gene-targeting vectors were introduced into embryonic stem cells, and homologous recombinants were identified by positive/negative selection and by a quantitative approach, respectively. After germline transmission of the targeted allele, a *FRT*-flanked *Neo* cassette was excised by crosses with FLPeR transgenic mice. The resulting mice carrying conditional (floxed) alleles were crossed to *EIIa-Cre* mice to obtain conventional null alleles (Figures S1A and S1B). The lack of individual miR-34 family members was confirmed by qRT-PCR of the brain and the prostate of *mir-34*$^{-/-}$ mice (Figure S1C).

Animal Phenotyping, Cell Culture, and Molecular Biological Experiments

Pathological assessment, immunohistochemistry, and quantitative image analysis were performed as described earlier (Hwang et al., 2011; Zhou et al., 2006) and in the Supplemental Information. miRNA in situ hybridization, cell culture, qRT-PCR, western blot analysis, and coimmunoprecipitation were performed according to earlier established protocols (Corney et al., 2010; Hwang et al., 2011) and are described in detail in the Supplemental Information.

Statistical Analysis

Statistical analyses were performed with InStat 3.10 and Prism 6 software (GraphPad). A two-tailed unpaired t test, direct Fisher's tests, and a log rank Mantel-Haenszel test were used as appropriate.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results and Discussion, Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.02.023.

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