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Thyroid-specific knockout of the tumor suppressor mitogen-inducible gene 6 activates epidermal growth factor receptor signaling pathways and suppresses nuclear factor- κ B activity

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Abstract

Background—Mitogen-inducible gene 6 (Mig-6) is a putative tumor suppressor gene and prognostic biomarker in papillary thyroid cancer. We hypothesized that Mig-6 knockout would activate pro-oncogenic signaling in mouse thyrocytes.

Methods—We performed a thyroid-specific knockout using the Cre/*loxP* recombinase system.

Results—Four knockout and 4 control mouse thyroids were harvested at 2 months of age. Immunoblotting confirmed Mig-6 ablation in knockout mice thyrocytes. Epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK) phosphorylation levels were increased in Mig-6 knockout compared to wild-type mice. Total EGFR levels were similar in knockout and wild-type mice. However, EGFR was absent in the caveolae-containing membrane fraction of knockout mice, indicating that Mig-6 depletion is associated with a change in the membrane distribution of EGFR. Although p65 localized to the nucleus in wild-type mice, it was distributed in both cytoplasm and nucleus in knockouts, suggesting that Mig-6 loss decreases p65 activity.

Conclusion—Our results confirm the feasibility of targeted, thyroid-specific gene knockout as a strategy for studying the relevance of specific genes in thyroid oncogenesis. We suggest that the loss of Mig-6 alters the membrane distribution of EGFR, which may limit receptor degradation and activate this oncogenic signaling pathway.

Mitogen-inducible gene 6 (*Mig-6*) is a multifunctional adaptor protein that inhibits epidermal growth factor receptor (EGFR) activity,¹ directly down-regulates extracellular signal-regulated kinase (ERK) signaling,² and up-regulates nuclear factor- κ B (NF- κ B) activity.³ *Mig-6* levels are consistently lower in well-differentiated papillary thyroid cancers

in comparison to normal matched thyroid tissue.⁴ In addition, *Mig-6* knockdown in thyroid cancer cell lines results in increased levels of phospho-EGFR, and independently, increased phospho-ERK.⁴ Low *Mig-6* expression is also associated with an increased risk of recurrence after thyroidectomy for papillary thyroid cancer.⁵ Altogether, the available body of evidence suggests that *Mig-6* is a relevant tumor suppressor in thyroid cancer of follicular origin. However, no previous studies have investigated the loss of *Mig-6* in thyrocytes in vivo.

Mig-6 germline null allele (*Mig-6*^{-/-}) mice develop many abnormalities of the skin and solid organs. A 50% reduction in litter size is observed in newborn *Mig-6*^{-/-} mice, and the remaining survivors typically die by the 6 months of age.⁶ *Mig-6*^{-/-} mice suffer from severe joint deformities and tumorigenesis in various organs including the lung, gallbladder, bile ducts, uterus, endometrium, and gastrointestinal tract.^{2,7,8} However, previous studies have not examined the effects of *Mig-6* knockout in the thyroid. In addition, embryonic lethality and multiorgan carcinogenesis in *Mig-6*^{-/-} mice precluded characterization of the effects of *Mig-6* loss in the thyroid.^{6,7,9} Therefore, thyroid-specific *Mig-6* ablation is required to study the role of *Mig-6* loss in vivo.

Homologous recombination of genes in embryonic stem cells is a useful tool for deletion of targeted genes in vivo. However, this system results in gene deletion in all tissues of the body. The Cre/*loxP* system is a powerful tool that allows gene deletion in a particular tissue or cell type. This system uses the site-specific recombinase Cre, which recognizes consensus *loxP* target sequences and excises any sequence placed between 2 *loxP* sites of the same relative orientation. By crossing a transgenic mouse expressing Cre under a cell-specific promoter with a mouse containing a gene flanked by *loxP* sites, one can obtain an animal with a cell-specific knockout of the particular gene of interest. This technique is useful tool to avoid embryonic lethality and multiorgan carcinogenesis.

We established thyroid-specific knockout transgenic mice (*Mig-6*^{flx/flx}; TPO-Cre⁺) by breeding *Mig-6*^{flx/flx} mice with mice expressing the Cre recombinase under the control of the human thyroid peroxidase (TPO) promoter. TPO is expressed exclusively in the thyroid when the mouse thyroid enters the final steps of differentiation.¹⁰ Target genes flanked by *loxP* sequences can be ablated in a thyroid-specific manner using a TPO-Cre transgenic mouse.¹¹ We hypothesized that *Mig-6* ablation in vivo would activate pro-oncogenic signaling in thyrocytes.

METHODS

Generation of transgenic mice and genotyping

Mice were housed and maintained at the animal facility of Brigham and Women's Hospital according to the institutional guidelines for the care and use of laboratory animals. *Mig-6* floxed (*Mig-6*^{flx}) transgenic mice, harboring 2 *loxP* sites flanking *Mig-6* exons 2 and 4 on a C57BL/6 background.¹² One *loxP* site was inserted downstream of exon 4. The other *loxP* site was inserted upstream of exon 2. The 5' ends of the targeting vector were constructed by cloning 2 kb of *Mig-6* homologous sequence upstream of *Mig-6* exon 2, whereas the 3' end contains a 2-kb homologous sequence downstream of *Mig-6* exon 4. The linearized targeting construct was transfected into R1 embryonic stem cells by electroporation. Correctly targeted clones were microinjected into blastocysts derived from C57BL/6 mice. Offspring mice were analyzed by polymerase chain reaction (PCR) genotyping to validate the germline transmission of the *Mig-6*-floxed allele. Genotyping was performed by PCR using primers: 5'-GGTCAGGGCTGTGCAG TCCGTAGA-3' (*Mig-6*-F), 5'-CGATACCCACCGA GACC-3' (Neo-R), 5'-CTTCCCAAATCTAACACCC GACAC-3' (*Mig-6*-R), yielding 460-bp and 212 bands for *Mig-6*^{flx} and wild-type mice, respectively. Transgenic mice in which the TPO promoter drives expression of Cre recombinase (TPO-

Cre⁺) on a nearly pure FVB/N background were previously described.¹¹ In brief, to establish the TPO-Cre transgenic construct, DNA 6.3 kb upstream of the human TPO gene was connected to the Cre recombinase gene and the construct was then flanked at both ends by 2 sets of insulator DNA. The linearized transgenic construct DNA was microinjected into pronuclei of mouse embryos, resulting in the production of 2 TPO-Cre transgenic founder lines. Northern Blots were performed to confirm the expression patterns for Cre recombinase, and therefore the line was chosen as TPO-Cre⁺ mouse line. Genotyping was performed by PCR using primers 5'-AGGTGTAGAGAAGGCACTTAGC-3' and 5'-CTAATCGCCATCTTCCAGCAGG-3', yielding a 412-bp band for TPO-Cre⁺ mice. Genotyping was performed by PCR as previously described.¹² Mice of the genotype *Mig-6^{Δd}* (*Mig-6^{Δf}*; TPO-Cre⁺) were obtained by breeding *Mig-6^{Δf}* and TPO-Cre⁺ mice. Mice with various genotypes were killed at 6 and 8 weeks of age. Thyroid tissue specimens were resected from mice, flash frozen, and then stored at -80°C. Tissue specimens were also fixed in 10% neutral-buffered formalin and embedded in paraffin. Contiguous sections were cut at 4 μm for hematoxylin–eosin staining and immunostaining with antibodies against human thyroid transcription factor-1 (TTF-1) and Ki-67. Hematoxylin–eosin and TTF-1 staining confirmed that the resected specimens were thyroid tissue.

Reagents

Anti-mouse *Mig-6* antibody was obtained from Sigma-Aldrich (St. Louis, MO). Anti-mouse EGFR, phospho-ERK, and clathrin antibodies were obtained from Cell Signaling (Beverly, MA). Anti-mouse ERK1/2 was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-mouse Caveolin-1, NF-κB subunit p65, Hsp90, and OCT-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin antibody pan Ab-5 was purchased from Neomarker (Fremont, CA). Anti-phospho-tyrosine antibody was obtained from Calbiochem (San Diego, CA). Secondary horseradish peroxidase-conjugated anti-rabbit and -goat antibodies were purchased from Vector Laboratories (Burlingame, CA).

Immunoprecipitation and western blot analysis

Immunoprecipitation was performed as previously described.¹³ Immunoprecipitated products were prepared and processed for Western blot analysis using a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis–separating gel under reduced conditions as previously described.⁴

Caveolae-containing membrane isolation

Caveolae-containing membrane was isolated using detergent-free methods as previously described.¹⁴ In brief, mouse thyroid specimens were homogenized and sonicated in ice-cold buffer B (20 mM tricine pH 7.8, 1 mM ethylenediaminetetraacetic acid, and 250 mM sucrose) containing 1 mg/mL leupeptin, 1 mg/mL pepstatin A, 1 mg/mL soybean trypsin inhibitor, and 1 mg/mL benzamidine. A postnuclear supernatant fraction was prepared by spinning the lysate at 90 *g* for 10 minutes. The postnuclear supernatant was layered over 23 mL of ice-cold 30% Percoll in buffer B. After centrifugation at 85,000 *g* for 30 minutes, the cytosol and plasma membrane fractions were collected. The plasma membrane fraction was briefly sonicated, mixed with buffer C (50% w/v iodixanol in buffer B plus 40 mM sucrose) to a final iodixanol concentration of 23% and overlaid with 6 mL of linear (10–20%) gradient of iodixanol in buffer B. Samples were centrifuged at 53,000 *g* for 90 minutes. The bottom 4 mL of the gradient was pooled and designated noncaveolae membrane. The top 5 mL of the gradient was mixed with 4 mL of buffer C, overlaid with 1 mL of 15% w/v iodixanol in buffer B, and followed by 0.5 mL of 5% w/v iodixanol in buffer B. The gradients were centrifuged at 53,000 *g* for 90 minutes, and caveolae were collected from the 5%/15% interface (0.5 mL).

Isolation of nuclear and cytosolic cell fractions

Mouse thyroid tissue specimens were homogenized, sonicated, and subjected to nuclear and cytosolic cell fractionation as previously described.⁴ The total protein concentration was measured using the BCA assay kit (Sigma-Aldrich) with bovine serum albumin as a standard, according to the manufacturer's instructions. The protein extracts were stored in aliquots at -80°C until used for Western blot analysis. Films were scanned and band density was quantified using digital image densitometry analysis to determine the relative intensity of each band (ImageJ software; National Institutes of Health, Bethesda, MD).

RESULTS

Generation of mice with thyrocyte-specific *Mig-6* ablation

To generate a model of *Mig-6* loss in the thyroid, we crossed *Mig-6^{fl/f}* transgenic mice with transgenic mice expressing Cre recombinase under the control of the human TPO promoter. PCR genotyping analysis, using primers selectively amplifying the unrecombined or the recombined allele, showed that mice numbers 1, 9, 12, and 15 were *Mig-6^{d/d}* (*Mig-6^{fl/f}*; TPO-Cre⁺) and that mice numbers 5, 10, 14, and 17 were *Mig-6^{fl/f}* (*Mig-6^{fl/f}*; TPO-Cre⁻) mice, while others were wild-type *Mig-6* with or without the TPO-Cre gene (Fig 1, A) The level of *Mig-6* expression was specifically ablated in *Mig-6^{d/d}* but not *Mig-6^{fl/f}* or wild-type mice (Fig 1, B).

Mig-6^{d/d} mice were born at the expected Mendelian ratios and developed normally, without displaying obvious physical or behavioral abnormalities. No significant differences in body weight among *Mig-6^{d/d}*, *Mig-6^{fl/f}*, and wild-type mice were found in a 6- to 8-week follow-up (data not shown).

The histologic examination of these thyroid specimens did not reveal adenomatous changes or evidence of thyroid cancer in wild-type, *Mig-6^{fl/f}*, or *Mig-6^{d/d}* mice. Less than 1% of cells of thyroid tissue resected from each mouse were Ki-67 positive (data not shown). *Mig-6* loss alone is not sufficient to increase thyrocyte proliferation at 6 to 8 weeks of age.

Thyroid-specific *Mig-6* ablation causes EGFR and ERK hyperactivation

To determine the effect of *Mig-6* ablation on EGFR and ERK signaling in vivo, we compared the levels of phospho-EGFR and phospho-ERK in thyroid homogenates prepared from control and knockout mice. Our results showed that EGFR and ERK phosphorylation levels were undetectable in wild-type and *Mig-6^{fl/f}* mice. In comparison, EGFR and ERK phosphorylation levels were high in *Mig-6^{d/d}* mice (Fig 2). These results show that *Mig-6* is a negative inhibitor of EGFR and ERK signaling in thyrocytes in vivo.

Thyroid-specific *Mig-6* ablation causes EGFR disassociation from caveolae

Upon ligand binding, EGFR can be internalized by either clathrin-mediated endocytosis (CME) or nonclathrin endocytosis (NCE). CME is necessary for activation of EGFR signaling pathways, whereas NCE is thought to be initiated from caveolin-containing sterol-rich cell membrane domains and primarily leads to receptor degradation.^{13,15,16} Therefore, regulating the distribution of EGFR to caveolae may regulate its internalization and signaling. Because *Mig-6* ablation activates EGFR, we next determined the impact of *Mig-6* ablation on EGFR disassociation from caveolae. To verify if *Mig-6* loss triggers EGFR trafficking in a NCE-dependent mechanism and leads to receptor degradation, we purified the caveolae-containing membrane fraction from mice thyroid, then determined EGFR levels by Western blot analysis to examine the association between EGFR and caveolae. We also collected total cell lysates then immunoblotted EGFR to examine if *Mig-6* loss impacts EGFR protein degradation. EGFR levels were undetectable in caveolae-containing

membranes purified from *Mig-6^{d/d}* mouse thyroids. Abundant EGFR levels were detected in *Mig-6^{f/f}* mice thyroid cell caveolae-containing membranes (Fig 3, A). We also observed that EGFR is not degraded in *Mig-6^{d/d}* mouse thyroid (Fig 3, B). Therefore, *Mig-6* may normally regulate EGFR activation by altering its distribution in the plasma membrane in a NCE-independent pathway and thereby control its pathway of internalization. To verify if *Mig-6* loss triggers EGFR trafficking in a CME-dependent mechanism, we immunoprecipitated clathrin then immunoblotted EGFR to examine the association between EGFR and clathrin. Interaction of EGFR and clathrin was found in *Mig-6^{d/d}* but not *Mig-6^{f/f}* mouse thyroid (Fig 3, C). These findings suggested that *Mig-6* loss in mouse thyroid triggers EGFR internalization in a CME-dependent mechanism.

Thyroid-specific *Mig-6* ablation attenuates NF- κ B activation

Mig-6 promotes NF- κ B activation by displacing p65 NF- κ B from its inhibitor, I κ B.³ To determine the impact of *Mig-6* loss in the thyroid on NF- κ B activity, we characterized the cell nuclear localization ratio of the p65, which is an indicator of NF- κ B activity.¹⁷ p65 was evenly localized in the nucleus and cytoplasm in *Mig-6^{f/f}* mouse thyroid tissue. In contrast, p65 preferentially localized to the cytoplasmic component in *Mig-6^{d/d}* mice (Fig 4). These results suggest that *Mig-6* promotes NF- κ B activity in the thyroid.

DISCUSSION

Mig-6 ablation in the thyroid gland in vivo increases EGFR and ERK activity but decreases NF- κ B activity. These effects on EGFR/ERK and NF- κ B are consistent with our previous findings in thyroid cancer cell lines.⁴ However, we previously observed increased proliferation in *Mig-6* knockdown cells in vitro. In contrast, the histologic examination of *Mig-6^{d/d}* thyroids did not reveal adenomatous changes or evidence of increased proliferation by Ki-67 immunohistochemical staining, at least up to 8 weeks of age. This may be because of the young age of the mice at analysis, and more time may be necessary to observe the histologic evidence of hyperproliferation in *Mig-6^{d/d}* mice.

A previous study revealed that the thyroid-specific loss of phosphatase and tensin homolog (PTEN), a known tumor suppressor, attenuated Akt signaling activation in 10-week-old mice. However, goiters and adenomas were observed only later in 10-month-old mice.¹⁸ PTEN knockdown, in addition to overexpression of PAX8/peroxisome proliferator-activated receptor γ fusion protein (PPFP), synergistically caused thyroid hyperplasia by 12 months of age.¹⁹ Perhaps *Mig-6^{d/d}* mice may also develop hyperplastic changes in later stages of development.

Alternatively, it is possible that *Mig-6* loss alone is not sufficient for tumorigenesis. Others have observed that the *Mig-6* and PTEN loss together cause endometrial tumors in mice as early as 2 to 4 weeks of age.² Skin-specific p53 knockout in addition to oncogene K-RAS activation synergistically causes squamous cell carcinoma in mice as early as by 2 weeks.²⁰ Therefore, *Mig-6* ablation alone may not be sufficient for the development of thyroid cancer; oncogenesis in *Mig-6^{d/d}* mice may require the acquisition of other genetic defects, such as the loss of an additional tumor suppressor or oncogenic activation.

EGFR internalization and trafficking toward the cell cytoplasm and nucleus is a key mechanism for amplifying downstream signaling pathways, such as mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK).²¹ However, EGFR internalization also promotes trafficking toward the endosome and finally results in EGFR degradation.²² EGFR signaling can either be terminated or amplified by EGFR internalization, and these events are critical for oncogenesis.²³ Many tumor suppressors modulate EGFR downstream signaling through regulating EGFR internalization. Merlin has

been shown to inhibit EGFR internalization, and it therefore attenuates downstream signaling pathway including MAPK in renal carcinoma cells.²⁴ Our data also show that, in murine thyroid, *Mig-6* blocks EGFR activation and attenuates ERK, a subunit of the MAPK signaling pathway. *Mig-6* also appears to promote the distribution of EGFR to caveolin-containing membrane fractions and may thereby promote NCE leading to receptor degradation rather than activation of signaling pathways in response to ligand binding. In agreement with such a model, *Mig-6* promotes EGFR internalization, results in receptor degradation, leading to attenuation of ERK activity.²⁵ Unlike these previous studies, however, we do not observe an effect of *Mig-6* on total levels of EGFR in mouse thyroid. This may reflect differences in the basal levels of EGFR receptor-mediated endocytosis in these distinct cell types or may suggest that *Mig-6* attenuates EGFR signaling instead of affecting EGFR degradation in the thyroid.

In conclusion, our results show that *Mig-6* loss in vivo increases EGFR and ERK activity and inactivates NF- κ B in the mouse thyroid. We did not find histologic evidence of hyperproliferation in *Mig-6* knockout mice; this may be related to the young age of the mice studied, or it may indicate that loss of *Mig-6* alone is insufficient to cause tumorigenesis. Future studies should include more mature *Mig-6^{dd}* mice and should investigate the effects of adding other genetic defects in combination with *Mig-6* loss on the thyroid.

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Fig 1. Conditional ablation of *Mig-6* in the murine thyroid. Polymerase chain reaction (PCR) analysis of mouse tail DNA. (A) PCR analysis results showing the appearance of mice with various genotypes. (B) Cell lysates collected from mouse thyroids were subjected to Western blot analysis of *Mig-6*. Reprobing actin acted as a loading control. (C) Hematoxylin–eosin and thyroid transcription factor-1 staining representative microscopic images of thyroid tissue resected from mouse no. 1.

Fig 2.

Impact of *Mig-6* conditional ablation on epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK) phosphorylation in the murine thyroid. Cell lysates collected from mouse thyroids were subjected to immunoprecipitation of EGFR and ERK followed by immunoblotting of phosphotyrosine and phospho-ERK, respectively. Reprobing of EGFR and ERK was performed to ensure the equal loading. *Mig-6* and actin blotting results are the same as those used in Fig 1.

Fig 3.

Impact of *Mig-6* conditional ablation on epidermal growth factor receptor (EGFR) internalization from caveolae in the murine thyroid. (A) Caveolae fractions purified from *Mig-6^{fl/f}* and *Mig-6^{d/d}* mouse thyroids were prepared and then immunoblotted with anti-EGFR antibody. Reprobing with anti-caveolin-1 antibody was performed to ensure equal loading. (B) Cell lysates collected from *Mig-6^{fl/f}* and *Mig-6^{d/d}* mouse thyroids were subjected to Western blot analysis of EGFR and *Mig-6* expression level. Reprobing of actin was performed to ensure equal loading. (C) Cell lysates collected from *Mig-6^{fl/f}* and *Mig-6^{d/d}* mouse thyroids were subjected to immunoprecipitation of EGFR followed by immunoblotting of clathrin. Reprobing of EGFR was performed to ensure equal loading.

Fig 4.

Impact of *Mig-6* conditional ablation on nuclear factor- κ B activity in the murine thyroid. Western blot analysis of p65 cytoplasmic and nuclear localization in *Mig-6^{f/f}* and *Mig-6^{d/d}*. Cytosolic (C) and nuclear (N) extracts (100 μ g protein) of mouse thyroids were prepared as described in the Materials and Methods section and then subjected to Western blot analysis with p65 antibody. Reblotting of OCT-1 and Hsp90 were used as internal controls for nuclear and cytosolic extracts to ensure equal loading. Relative density of p65 bands (normalized to density of OCT-1 or Hsp90 bands) are shown.