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Tumor Suppression by MEG3 lncRNA in a Human Pituitary Tumor Derived Cell Line

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Abstract

Human clinically non-functioning pituitary adenomas (NFAs) account for approximately 40% of diagnosed pituitary tumors. Epigenetic mutations in tumor suppressive genes play an important role in NFA development. *Maternally expressed gene 3 (MEG3)* is a long non-coding RNA (lncRNA) and we hypothesized that it is a candidate tumor suppressor whose epigenetic silencing is specifically linked to NFA development. In this study, we introduced MEG3 expression into PDFS cells, derived from a human NFA, using both inducible and constitutively active expression systems. MEG3 expression significantly suppressed xenograft tumor growth *in vivo* in nude mice. When induced in culture, MEG3 caused cell cycle arrest at the G1 phase. In addition, inactivation of p53 completely abolished tumor suppression by MEG3, indicating that MEG3 tumor suppression is mediated by p53. In conclusion, our data support the hypothesis that MEG3 is a lncRNA tumor suppressor in the pituitary and its inactivation contributes to NFA development.

Keywords

MEG3; long non-coding RNA; p53; pituitary tumor; tumor suppression

1. INTRODUCTION

Human pituitary adenomas are classified as clinically functioning or non-functioning. Functioning tumors secrete excess anterior pituitary hormones, causing hormone-specific clinical syndromes. For example, tumors secreting GH cause acromegaly and those secreting ACTH cause Cushing's disease. Clinically non-functioning adenomas (NFA) account for more than 40% of all diagnosed pituitary tumors (Chaidarun and Klibanski, 2002). NFAs do not produce excess functional hormones and their main manifestations are mass effect, leading to hypopituitarism and neurological symptoms. There are no approved medical

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therapies for these tumors and they typically require surgical intervention and repeat surgery and/or post-operative radiation therapy may be needed.

Early studies from our laboratory and others revealed that human NFAs are monoclonal in origin (Alexander et al., 1990; Herman et al., 1990; Jacoby et al., 1990). This finding indicates that a pituitary tumor is most likely initiated from an individual transformed cell, caused by genetic or epigenetic mutations. Data demonstrate that genetic mutations are very rare in NFAs. In contrast, epigenetic mechanisms play an important role in dysregulation of many genes in NFAs. At least twenty tumor suppressive genes have been identified whose expression is reduced or silenced in sporadic pituitary tumors including both functioning tumors and NFAs (see review) (Zhou et al., 2014). Among these genes, *maternally expressed gene 3 (MEG3)* is of particular interest because it is the only gene found to be completely silenced in NFAs (Cheunsuchon et al., 2011; Gejman et al., 2008; Zhang et al., 2003; Zhao et al., 2005). *MEG3*, an imprinted gene, belongs to the *DLK1-MEG3* locus located on chromosome 13q32 (Miyoshi et al., 2000), and is expressed only from the allele on the maternal chromosome. The *MEG3* gene has 10 exons and produces multiple transcript isoforms due to alternative RNA splicing (Miyoshi et al., 2000; Zhang et al., 2010; Zhou et al., 2007). In adult tissues and cell lines, the predominant mature MEG3 transcript consists of all exons except exons 5, 6 and 7 (Zhang et al., 2010). Sequence analysis and functional studies indicate that the MEG3 transcripts do not encode any proteins and function as long non-coding RNAs (lncRNA) (Miyoshi et al., 2000; Zhang et al., 2010; Zhou et al., 2007).

The association between MEG3 and NFAs was first discovered when gene expression profiles between NFAs and normal pituitaries were compared in our laboratory (Zhang et al., 2003). We found that MEG3 transcripts are highly expressed in normal human pituitaries but not detected in NFAs (Zhang et al., 2003). Our subsequent studies and those from other laboratories indicate that MEG3 expression is mostly lost in NFAs, but readily detected in functioning tumors (Binse et al., 2014; Cheunsuchon et al., 2011; Gejman et al., 2008; Mezzomo et al., 2012). MEG3 silencing in NFAs is in part due to promoter methylation (Zhao et al., 2005). These data suggest that loss of MEG3 expression is involved in the development of NFAs. However, whether MEG3 suppresses NFA growth has not been tested. In this study, we established stable MEG3 clones using an inducible expression system in PDFS cells, a cell line established from a human NFA in our laboratory (Danila et al., 2000). We found that MEG3 expression induced G1 arrest and suppressed xenograft tumor growth *in vivo* in nude mice. Importantly, tumor suppression by MEG3 requires functional p53. These results suggest that MEG3 is a lncRNA tumor suppressor in the pituitary and loss of its expression contributes to the development of NFAs.

2. MATERIALS AND METHODS

2.1. Primary culture of human NFA cells and histone deacetylase inhibitor treatment

Human clinically non-functioning pituitary tumor tissues were obtained from Massachusetts General Hospital Pathology Department as discarded tissues. The use of human tissue in the study was approved by the Partners Institutional Review Board. Primary tumor cells from

NFA tissues were isolated and cultured as described previously (Klibanski et al., 1988). For RNA extraction, cells (6×10^5) were seeded into P60 collagen coated dishes in duplicates. Twenty-four hours later, one dish was added with the pan histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) (SML0061, Sigma-Aldrich, St. Louis, MO) stock solution to a final concentration of 5 μM and the other dish with equal volume of DMSO as control. Forty-eight hours after the treatment, total RNAs were isolated from the cells using *TRI*zol reagent according to the manufacture's instruction (Life Technology). To facilitate RNA recovery, ten μg of glycogen (Roche, Indianapolis, IN) was added before RNA precipitation.

To detect MEG3 expression, reverse transcription reactions were carried out using the ProtoScript® First Strand cDNA Synthesis Kit according to the manufacturer's instruction (New England Biolabs, Ipswich, MA). MEG3 levels were determined by quantitative PCR. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the internal control. Primer pairs are 5'-CCTGCTGCCCATCTACACCTC-3' (forward) and 5'-CCTCTTCATCCTTTGCCATCCTGG-3' (reverse) for detection of MEG3, and 5'-CTGGGCTACACTGAGCACC-3' (forward) and 5'-AAGTGGTCGTTGAGGGCAATG-3' (reverse) for detection of GAPDH. The sequences for GAPDH primers were obtained from PrimerBank (ID 378404907c3) (Spandidos et al., 2010; Wang and Seed, 2003). The qPCR was performed using the SYBR Select Master Mix from Life Technology on an Applied Biosystems 7500 fast thermocycler according to the manufacturers' instruction. The MEG3 expression levels in tumor cells after SAHA treatment were represented as fold changes compared to the corresponding controls. The fold changes were calculated using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{SAHA} - \Delta\ Ct_{control}$.

2.2. Plasmid constructs

Plasmids pPur, pTet-On and pTRE-Tight were obtained from ClonTech (Mountain View, CA). Plasmid pcDNA3.1 was obtained from Life Technologies (Grand Island, NY). pSuper.Retro.puro and pSuper.p53 were obtained from OligoEngine (Seattle, WA). To construct inducible MEG3 expression system, the MEG3 cDNA from pCI-MEG3 (Zhou et al., 2007) was cloned into pTRE-Tight between restriction enzyme sites of EcoRI and NotI to generate pTRE-MEG3. The neomycin resistant gene in pTet-On was replaced with the puromycin resistant gene to generate pCMV-rtTA-puro. To make a construct constitutively expressing MEG3, the MEG3 cDNA was cloned into pcDNA3.1-puro, derived from pcDNA3.1 and pPur, to generate pCMV-MEG3-puro. The negative control pCMV-MEG3-del5-puro was similarly constructed by cloning MEG3-del5 cDNA from pCI-MEG3-del5 (Zhou et al., 2007) into pcDNA3.1-puro. To generate the retroviral vector containing the p53 RNA interference (p53i) hairpin, The p53i hairpin cassette from pSuper.p53 (Brummelkamp et al., 2002) was cloned into pSuper.Retro.hyg to generate pSR-p53i-hyg. The pSuper.Retro.hyg was generated by replacing the puromycin resistant gene with the hygromycin gene in pSuper.Retro.puro. The negative control, pSR-NSi-hyg, containing a non-specific RNAi hairpin (NSi), was similarly constructed. To construct retrovirus vector expressing HPV E6 oncoprotein, a retroviral vector pRCA-hyg was constructed by replacing H1 RNA promoter in pSuper.Retro.hyg with a DNA fragment containing CMV promoter,

multiple cloning sites and poly adenylation signal from pcDNA3.1. The E6 gene coding region was cloned into pRCA-hyg to generate pRCA-E6-hyg.

2.3. Cell culture and stable transfection

PDFS cells were maintained in DMEM (11965-118, LifeTechnology) supplemented with 10% fetal bovine serum, 1% non essential amino acids and 1% insulin-transferrin-selenium at 37% with 10% CO_2. To establish stable clones with inducible MEG3 expression, PDFS cells were co-transfected with pTRE-MEG3 and pCMV-rtTA-puro at a ratio of 3 to 1 using Mirus *Trans*IT-LT1 according to the manufacture's instruction (Madison, WI). Cells were re-plated and treated with puromycin 48 hour after transfection. Individual clones were isolated and propagated. MEG3 expression was induced by addition of doxycycline (Dox) with a final concentration of 1 μg/ml in culture media. Total RNAs were extracted from cells treated with or without Dox. MEG3 transcripts were detected by RT-PCR. Clones with good MEG3 induction were saved and further evaluated by Northern blotting. Finally two clones with a minimal background in the absence of Dox and a significant induction of MEG3 in the presence of Dox were isolated. They were designated as PDFS-TetO-MEG3 clone 1 and clone 2, respectively.

To establish stable clones with constitutive expression of MEG3, PDFS cells were transfected with pCMV-MEG3-puro. Drug resistant clones were similarly isolated as above. MEG3 expressions were evaluated by Northern blotting. Clones with MEG3 expression were designated as PDFS-CMV-MEG3. Clones expressing mutant MEG3-del5, PDFS-CMV-MEG3-del5, were similarly established. Cells containing blank vector, PDFS-vect, were also established by pooling puromycin resistant clones.

To knockdown p53, cells from MEG3-TetO-MEG3 clone 1 were transduced with retrovirus expressing p53 RNAi. Hygromycin resistant clones were isolated. P53 expression as well as MEG3 expression was evaluated by Northern blotting. Clones with a significant reduction in p53 levels and maintaining MEG3 expression in the presence of Dox were kept. The obtained cells were designated as PDFS-TetO-MEG3-p53kd. Control cells containing NSi were similarly established and designated as PDFS-TetO-MEG3-NSi. PDFS-TetO-MEG3-E6 and PDFS-TetO-MEG3-vect were similarly generated by transduction of PDFS-TetO-MEG3 clone 1 cells with retroviruses expressing E6 and blank viruses.

To produce retroviral vectors for transduction of PDFS-TetO-MEG3 cells as described above, retrovirus packaging cells 90.74 (CR-11654, ATCC, Manassas, VA) were seeded into P60 dishes and transfected with 4 μg/dish of plasmid pSR-p53i-hyg, pSR-NSi-hyg, pRCA-E6-hyg or pRCA-hyg using Mirus TransIT-LT1 according to the manufacture's instruction. Approximately forty-eight hours post-tansfection, the supernatants containing viruses were filtered through 0.45 micro filter and added polybrene at the final concentration of 4 μg/ml. The medium in target cells in P60 dishes was removed and 2 ml of virus mixes were added. After incubation at 37 C for 30 min, additional 3 ml of fresh medium were added and the cell continued incubation for 24 hours. After virus mixes were removed, fresh media containing hygromcyin (50 μg/ml) was added. The cells were continuously treated with hygromycin until the drug-resistant clones were ready to be isolated.

2.4. Flow cytometry

Cell cycle was analyzed by flow cytometry as described previously (Zhou et al., 2000). Cells (4×10^5) were treated with Dox (1 μg/ml) or without for 48 hours. Cells were trypsinized, washed with cold PBS and fixed with ice-code ethanol. Before cell sorting, the fixed cells were stained with a staining solution containing 10 mg/ml propidium iodide, 0.1% Triton X-100, 0.1 mM EDTA, 10 mg/ml RNase A in PBS for at least 30 min at room temperature. The cell samples were sorted using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Approximately 2×10^4 cells were analyzed for each sample.

2.5. Northern and Western Blotting

For Northern blotting, total RNAs were isolated using *TRI*zol reagent according to the manufacture's instruction (Life Technology). Northern blotting was performed to detect MEG3 transcripts using the NorthernMax kit from Life Technology. Ten μg total RNA for each sample was loaded with dye containing ethidium bromide on 1.5% agarose gel. After electrophoresis, resolved RNAs were transferred to a Nytran membrane using a TurboBlotter from GE Healthcare (Pittsburgh, PA). The membrane was hybridized with MEG3 cDNA probe labeled with [α-^{32}P]dCTP using the Ready-To-Go DNA Labeling Beads from GE Healthcare. After washing, the membrane was exposed to a storage phosphor screen and analyzed by GE Storm 860 phosphor imager. The membrane was then stripped and re-probed to detect GAPDH as the internal control.

For Western blotting, total protein was isolated by lysis of cells with RIPA buffer containing protease inhibitor cocktail from Sigma Aldrich (P8340). Ten μg of total protein was resolved by 10% SDS-PAGE. After transfer to a PVDF membrane, the blot was probed with anti-p53 (FL393, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-β-actin antibody (C4, Santa Cruz Biotechnology). P53 and β-actin were detected using Pierce ECL Plus Western blotting substrate (LifeTechnology) on a C-DiGit blot scanner (LI-COR Biotechnology, Lincoln, NE).

2.6. Xenograft tumor growth

Use of mice and procedures involved in the xenograft tumor study were approved by the Institutional Animal Care and Use Committee (IACUC) at MGH. Female immunodeficient nude mice (NU/NU) of 6 weeks old were purchased from Charles River Laboratories (Wilmington, MA). For each experiment, ten mice were used. Five million cells suspended in 0.1 ml PBS were inoculated subcutaneously bilaterally in the flanks of the mice. After injection, mice were equally divided into two groups. One group was fed with regular acidified water and the other with water containing 1 mg/ml doxycycline and 5% sucrose. Tumor dimensions were measured every other day by a caliber. Tumor sizes were calculated as $\pi \times (\text{length} \times \text{width} \times \text{height}) \div 6$ in mm. The tumor size was considered as zero if no tumor grew at the injection site.

3. RESULTS

3.1. MEG3 suppresses PDFS xenograft tumor growth in nude mice

Previously, we demonstrated that DNA methylation is significantly higher in two regions of the MEG3 promoter in NFAs compared to that in normal pituitaries (Zhao et al., 2005), indicating that methylation plays a role in silencing the *MEG3* gene in these tumors. However, we also observed that the methylation in these regions is not observed in all tumors (Zhao et al., 2005), suggesting that other epigenetic modifications are involved in MEG3 silencing. Histone deacetylation is commonly associated with gene silencing in human tumors caused by dysregulation of histone deacetylases (HDACs) (Barneda-Zahonero and Parra, 2012; Glozak and Seto, 2007). To investigate whether this is the case in MEG3 silencing in NFAs, we treated primary pituitary NFA cells with a pan HDAC inhibitor, SAHA (Bradner et al., 2010). We found that the treatment increases MEG3 expression by more than 4-fold measured by quantitative RT-PCR (Fig. 1A), indicating that histone deacetylation is one of the epigenetic mechanisms inactivating MEG3 in NFAs.

To investigate the effects of MEG3 on pituitary tumor growth, we chose the PDFS cell line as an *in vitro* model system. PDFS is a cell line spontaneously established from a human NFA by multiple passages (Danila et al., 2000). We established two stable clones in PDFS cells in which MEG3 expression is controlled by the Tet-On system, designated as PDFS-TetO-MEG3. In the presence of doxycycline (Dox), MEG3 RNA is significantly induced in both clones (Fig 1B). To test MEG3 tumor suppression function, we inoculated PDFS-TetO-MEG3 cells subcutaneously into the flanks of nude mice. After inoculation, mice were divided equally into two groups. One group was fed with Dox to induce MEG3 expression. The size of the tumors, if any, was measured every other day. We found that the average tumor size at a given day was significantly smaller in mice fed with Dox water compared to that in mice fed with regular water (Fig. 1C and 1D), indicating that tumor growth in mice with MEG3 induction is significantly slower than that in mice without MEG3 induction.

We examined MEG3 expression in tumors from mice fed with Dox water. The MEG3 transcript was readily detected in those tumor cells (Fig. 1E), indicating that MEG3 is indeed induced in PDFS-MEG3 cells by Dox. The detection of MEG3 in tumors raises two possibilities. One is that those tumor cells have developed resistance to MEG3. The other is that MEG3 expression slows down proliferation of those cells, resulting in smaller tumors. To explore this further, we established cells with constitutive expression of MEG3 by stable transfection of PDFS cells with plasmids in which the MEG3 cDNA was under the control of a CMV promoter. Multiple drug resistant clones were selected and MEG3 expression was determined by Northern blotting (Fig. 2A). These clones were pooled and designated as PDFS-CMV-MEG3. As a control, we also established cells expressing MEG3-del5, a non-functional mutant MEG3 (Fig. 2B) (Zhou et al., 2007) and tested their tumor growth in nude mice. We found that cells carrying empty vectors (PDFS-CMV) or MEG3del5 (PDFS-CMV-MEG3del5) rapidly grew tumors in nude mice. In contrast, cells with wt MEG3 (PDFS-CMV-MEG3) grew much smaller tumors (Fig. 2C and 2D). These data further indicate that MEG3 suppresses tumor growth in PDFS cells. Because MEG3 expressing cells still grew tumors, albeit at a slower rate than cells expressing no MEG3, these data

indicate that MEG3 significantly reduces, but does not completely abolish, the tumorigenic potential of PDFS cells.

3.2. MEG3 induces G1 arrest in PDFS cells

To determine mechanisms whereby MEG3 suppresses xenograft tumor growth, we examined the effects of MEG3 on cell cycle. PDFS-TetO-MEG3 cells were treated with Dox for 48 hours to induce MEG3 expression. Cell cycle profiles were analyzed by flow cytometry. We found that the G1 population is significantly increased in Dox treated cells compared to cells without Dox treatment (Fig. 3A). In agreement, proliferation was reduced in cells treated with Dox compared to that in cells without Dox treatment (Fig. 3B). These data suggest that MEG3 suppresses tumor growth by causing cell cycle G1 arrest.

3.3. MEG3 tumor suppression requires p53

We have shown that MEG3 activates p53 (Zhou et al., 2007). To determine if p53 plays a role in mediating MEG3 tumor suppression, we knocked down p53 expression in PDFS-TetO-MEG3 cells by RNA interference. We isolated six clones in which p53 levels were significantly reduced (Fig. 4A). We pooled cells from all six clones, designated as PDFS-TetO-MEG3-p53kd and tested their tumor growth in nude mice. As expected, we found that MEG3 induction suppresses tumor growth in PDFS-TetO-MEG3 cells expressing non-specific RNAi (PDFS-TetO-MEG3-NSi), which express p53 normally (Fig. 4B). Surprisingly, PDFS-TetO-MEG3-p53kd cells rapidly grew tumors even larger than parental PDFS cells (Fig. 4B). In addition, there was no significant difference in tumor growth between cells with and without MEG3 induction (Fig. 4B), indicating that the tumor suppression function of MEG3 is obliterated by p53 inactivation.

We also inactivated p53 in PDFS-TetO-MEG3 cells by expression of HPV E6 oncoprotein. E6 binds to p53, blocks its function and promotes its degradation (Howie et al., 2009). E6 was introduced into PDFS-TetO-MEG3 cells by transduction of an E6 expressing retrovirus vector. After drug selection, the surviving cells were pooled and designated as PDFS-TetO-MEG3-E6 (Fig. 4C). The control was PDFS-TetO-MEG3, cells transduced with a blank retrovirus vector (PDFS-TetO-MEG3-vect). We found that PDFS-TetO-MEG3-E6 behaved similarly as PDFS-TetO-MEG3-p53kd. At day 18 after cell inoculation, tumors growing from PDFS-TetO-MEG3-E6 cells are significantly larger than those from PDFS-TetO-MEG3-vect regardless of Dox treatment (Fig. 4D). Furthermore, MEG3 induction did not significantly affect tumor growth from PDFS-TetO-MEG3-E6 cells (Fig. 4D). Taken together, these data strongly indicate that p53 is required for tumor suppression by MEG3.

4. DISCUSSION

The molecular mechanism for NFA development has not been elucidated. Although the expression of many genes have been reported to be down regulated in human NFAs, to date, most have not been shown to play a role in suppression of NFA tumor growth. MEG3 is a lncRNA gene and its expression is lost in the vast majority of human NFAs. In this study, we demonstrated that re-expression of MEG3 suppresses *in vivo* tumor growth from a human NFA cell line and this tumor suppression requires functional p53. This is the first

demonstration of tumor suppression from a human NFA-derived cell line *in vivo* by MEG3. These data indicate that MEG3 is a long non-coding RNA tumor suppressor in the pituitary and it functions through activation of p53. The data also suggest that human NFA development is at least in part attributed to the epigenetic inactivation of the MEG3 gene.

Human pituitary tumors are typically benign and slow growing, making it very difficult to establish cell lines for long term studies. The most frequently used human pituitary tumor cell line is HP75, which was established by expression of SV40 large T antigen (Jin et al., 1998). Its limitation is that the T antigen is an oncogene, known to transform cells by inactivation of p53 (Pipas and Levine, 2001). We have shown that p53 is a target of MEG3 (Zhou et al., 2007). Therefore, HP75 is not an appropriate cell line to assess tumor suppression by MEG3. PDFS is a human pituitary tumor derived cell line developed in our laboratory. It was established by spontaneous transformation during the culture of primary cells from an NFA of gonadotroph origin (Danila et al., 2000). Western blotting detected high levels of expression of three major tumors suppressors, RB, $p16^{Ink4a}$ and p53 in PDFS cells (Danila et al., 2000). In our original report we suspected that the p53 was mutant, judged by its high expression and positive staining with antibody pAb240 (Danila et al., 2000), which was thought to only recognize mutant p53 (Gannon et al., 1990; Said et al., 1992). To investigate further, we examined p53 mRNA in PDFS cells by sequencing its cDNA and found no mutations (data not shown), indicating that PDFS cells contain wt p53. Therefore, we used the PDFS cell line as the *in vitro* model to test the hypothesis that MEG3 lncRNA plays a role in tumor suppression in NFA cells. Xenograft tumor growth in immunodeficient mice is a proven and widely accepted *in vivo* method to evaluate tumorigenecity of human cells. Therefore, we used tumor growth in nude mice to test our hypothesis. Two expression systems were used to control MEG3 expression in PDFS cells, inducible and constitutive, respectively. Data using both systems consistently showed that PDFS cells expressing MEG3 grew much smaller tumors in nude mice compared to cells not expressing MEG3 or expressing the non-functional mutant MEG3-del5 (Fig. 1 and 2). These data strongly indicate that MEG3 suppresses tumor growth in this human NFA derived cell line.

The PDFS cell line is folliculostellate in origin. Folliculostellate cells are believed to play an important role in the intracellular communications between various pituitary cells (Fauquier et al., 2002). They may regulate growth of endocrine cells by producing cytokins and growth factors (Ooi et al., 2004). Multiple cases have been reported showing that some pituitary tumors could arise from folliculostellate cells. For example, Roncaroli et al. (Roncaroli et al., 2002) examined five pituitary spindle cell oncocytomas and found those tumors mainly contain cells that stained positive for S-100, vimentin, galectin-3 and epithelial membrane antigen. They suggested that those tumors were derived from folliculostellate cells. Additional cases have been reported by several independent researchers subsequently (Coire et al., 2009; Hori et al., 2009; Min et al., 2007). Therefore, although most tumors are not derived from these cells, it has been reported. Our data, at the least, indicate that MEG3 inhibits growth of pituitary tumors of folliculostellate origin. This cell line was derived from a clinically non-functioning human pituitary tumor of gonadotrope origin. Because folliculostellate cells play an important role in supporting endocrine cells, our data also

suggest that MEG3 could suppress growth of pituitary tumors of endocrine cell origin by inhibition of folliculostellate cells. We demonstrated that MEG3 inhibits tumor growth via the p53 pathway, which is a ubiquitous tumor suppressor. It is likely that MEG3 plays a direct tumor suppression role in endocrine cells. Our previous data demonstrated that MEG3 expression is primarily lost in human NFAs, not in most functioning pituitary tumors (Cheunsuchon et al., 2011; Gejman et al., 2008). The PDFS cell line used in the study does not express MEG3. Therefore, our data suggest that loss of MEG3 expression plays a role in the development of human NFA and a subset of functioning pituitary adenomas.

To investigate mechanisms whereby MEG3 suppresses tumor growth, we analyzed the effects of MEG3 on cell cycle progression. We found that MEG3 induction results in an accumulation of G1 phase cells (Fig. 3), which is consistent with previous findings that MEG3 induces cell cycle arrest in a number of human cancer cell lines (Jia et al., 2013; Lu et al., 2013; Wang et al., 2012), indicating that induction of cell cycle arrest plays a role in mediating MEG3 suppression of xenograft tumor growth. We also observed that the G1 arrest caused by MEG3 expression is moderate, suggesting that additional mechanisms may be involved in MEG3 tumor suppression. Indeed, that MEG3 expression causes apoptosis has been reported in several cancer cell lines, including tongue squamous cell carcinoma lines SCC-15 and CAL-27 [Jia et al., 2013], non-small cell lung cancer lines A549 and SPC-A1 [Lu et al., 2013], glioma line U251(Wang et al., 2012), gastric cancer cell lines AGS and MGC-803 (Sun et al., 2014), and hepatocellular cancer line PRC/PRF/5 (Braconi et al., 2011). However, we did not observe apoptosis in either PDFS-TetO-MEG3 cells after MEG3 induction, or PDFS-CMV-MEG3 cells with constitutive MEG3 expression. This difference may be due to differences in the expression of genes regulating cell cycle and apoptosis. For example, PDFS cells are derived from a human NFA and contain wild type $p16^{Ink4a}$, RB and p53. In contrast, cancer cell lines usually contain mutations in at least one of these tumor suppressor genes. For example, genetic mutations or epigenetic silencing of the $p16^{Ink4a}$ gene are found in A549, AGS, MGC-803, SCC-15 and U251 cells (Albanese et al., 2013; Cody et al., 1999; Mack et al., 1999; Meng et al., 2007; Qin et al., 2014). P53 mutations are found in CAL-27, PRC/PRF/5, SSC-15 and U251 cells (Brazdova et al., 2009; Kaino, 1997; Kraljevic Pavelic et al., 2009; Min et al., 1994). The role of these tumor suppressors in MEG3 induced apoptosis remains to be investigated. It is also possible that this difference is due to different techniques used to introduce MEG3 expression. The above studies usually involve transient transfection of cancer cells with MEG3 expression constructs. Using this method, MEG3 expression levels can be very high in some cells if they took in multiple MEG3 plasmids. MEG3 at very high levels may be toxic, inducing apoptotic response in those cells. In our studies, we established stable clones in which MEG3 is expressed constitutively or inducible by Dox. The cloning process involving prolonged drug selection is likely to have eliminated cells with high MEG3 expressions. In addition, MEG3 should be expressed at a similar level in all cells in a clone. These cells are likely to have a more homogenous response to MEG3 than a population consisting cells with various levels of MEG3 expression. Therefore, further investigations are needed to determine additional mechanisms mediating MEG3 tumor suppression in PDFS cells.

Over 20 tumor suppressive genes have been identified to be silenced or down regulated in human pituitary tumors. We found that all these genes can be functionally linked to RB

and /or p53 pathways (Zhou et al., 2014). Numerous studies indicate that the RB pathways are epigenetically silenced in most NFAs. However, no p53 mutations have been identified in these tumors. Because p53 function is activated by its upstream signals, p53 is as "functionally effective" as its regulators. Therefore, we hypothesized that the p53 function is compromised in NFAs due to inactivation of its upstream regulators. In agreement, several such genes have been identified to be silenced or down regulated in NFAs. Examples of these genes include *Pleiomorphic adenoma gene-like 1 (PLAGL1)* (Michaelis et al., 2011; Pagotto et al., 2000; Vieria Neto et al., 2013), *Suppressor of cytokine signaling 1 (SOCS1)* (Buslei et al., 2006) and MEG3 (Zhou et al., 2012). *PLAGL1* is a transcription cofactor which binds to p53 and activates its target genes including $p21^{Cip1}$ and PPARγ (Zhou et al., 2014). *SOCS1* is a negative regulator of cytokine signaling (Zhang et al., 2012). It induces cellular senescence by activation of p53 (Calabrese et al., 2009). Normally, the p53 protein is rapidly degraded by the MDM2 and MDM4 mediated-ubiquitin proteolysis pathway (Wade et al., 2010). Functional inhibition of MDM2/4 leads to p53 stabilization and activation (Kruse and Gu, 2009; Wade et al., 2010). We previously showed that ectopic expression of MEG3 reduces MDM2 protein levels (Zhou et al., 2007), suggesting that MEG3 lncRNA may activates p53 by downregulation of MDM2.

When p53 expression was detected by Western blotting, we observed a high level of p53 protein in PDFS cells (Fig. 4C). Because PDFS cells rapidly grow tumors in nude mice, it suggests that the p53 protein expressed in these cells is not functional concerning tumor suppression. It is known that the function of p53 is controlled by site-specific modifications and molecular interactions with its regulators (Kruse and Gu, 2009). It is possible that the p53 protein has not been properly activated in PDFS cells although it is accumulated at a high level. Previously we have shown that MEG3 activates p53 and selectively activates p53 target genes (Zhou et al., 2007). Therefore, we speculate that induction of MEG3 in our PDFS-MEG3 clones results in a specific functional activation of p53 protein. It is worth pointing out that no apparent increase in p53 protein levels was observed in cells with MEG3 induction compared to the control cells (Fig. 4C). This could be due to the high basal level of p53 protein exists in PDFS cells and Western blotting fails to distinguish an additional increase in p53 protein. Another possibility is that MEG3 may functionally activate p53 protein by novel mechanisms, such as a direct molecular interaction.

In this study, we used a human NFA derived cell line, PDFS, as a model system to investigate the role of MEG3 and its target gene p53 in tumor suppression in the pituitary. We observed that p53 inactivation results in a more aggressive phenotype in PDFS cells and abolishes tumor suppression function of MEG3 (Fig. 4). These data provide the first direct evidence suggesting that p53 mediates functions of multiple tumor suppressive genes, including MEG3, in the pituitary and plays a critical role in suppression of NFA development.

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Abbreviations

NFA	clinically non-functioning pituitary adenoma
MEG3	maternally expressed gene 3
lncRNA	long non-coding RNA
HPV	human papillomavirus
Dox	doxycycline

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Highlights

- We re-expressed MEG3 lncRNA in PDFS, a human pituitary tumor derived cell line.
- MEG3 expression suppressed PDFS xenograft tumor growth in nude mice.
- MEG3 expression induced G1 arrest in PDFS cells.
- Tumor suppression by MEG3 lncRNA is mediated by tumor suppressor p53.

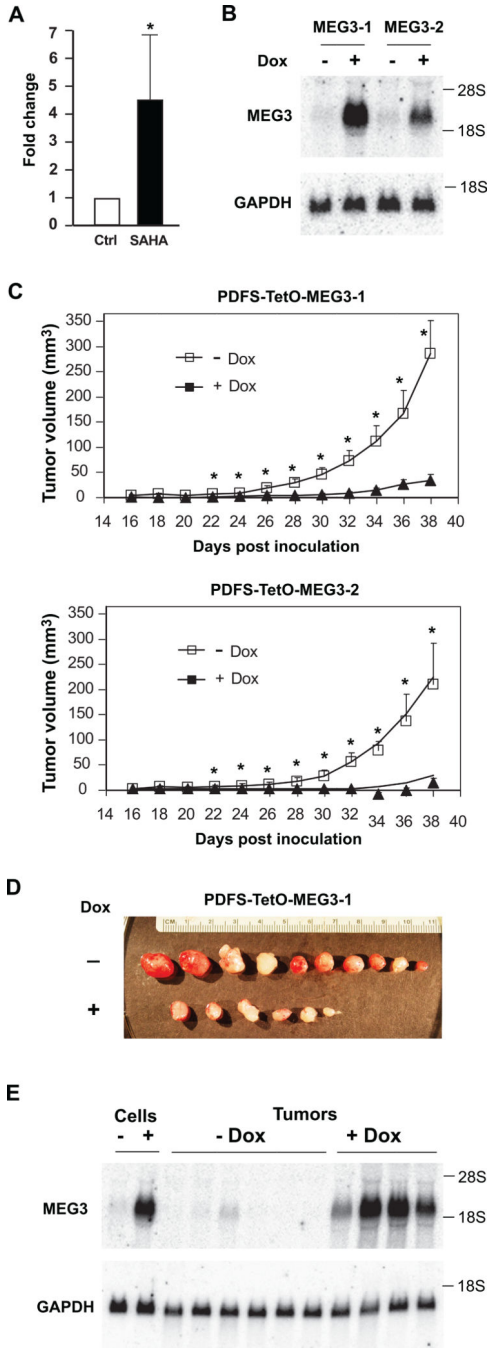

Figure 1.

Inducible expression of MEG3 suppresses xenograft tumor growth in nude mice. (A) SAHA treatment induces MEG3 expression human NFA cells. Cells dispersed from NFA tissues (n = 5) were seeded into 24 well plates and treated with 5 mM SAHA or with equal volume of DMSO for 24 hours. MEG3 expression was determined by qRT-PCR. The MEG3 fold change in SAHA treated cells was normalized against that in control cells as described in Methods and Materials. * $p < 0.05$, student t-test. (B) MEG3 expression is induced by Dox in PDFS-TetO-MEG3 clones. Two clones were established as described in Materials and

Methods. Cells were treated with or without Dox for 24 hours. MEG3 RNA was detected by Northern blotting. GAPDH was probed as the internal control. Ribosome RNAs 18S and 28S were used as markers. (C) Xenograft tumor growth curves of PDFS-TetO-MEG3 clones. Cells (5×10^6) from each PDFS-TetO-MEG3 clone were subcutaneously injected in two flanks of nude mice (n = 10). One group of mice (n = 5) was fed with regular water (−Dox) and the other (n = 5) with Dox containing water (+Dox). Tumor dimensions were measured every other day as indicated. Tumor sizes were calculated as $\pi \times (\text{length} \times \text{width} \times \text{height}) \div 6$ in mm. Values are means of 10 tumors (the size is considered as 0 if tumors fail to grow at an injection site) and the error bars are standard error of the mean. *, $p < 0.05$, student t-test. (D) Pictures of tumors from nude mice injected with cells from PDFS-TetO-MEG3 clone 1. Tumors were removed from mice at day 38 after cell injection. Upper row are tumors from mice fed with regular water (−Dox) and lower row are tumors from mice fed with Dox water (+Dox). (E) MEG3 expression in xenograft tumors described above. MEG3 RNAs are detected by Northern blotting.

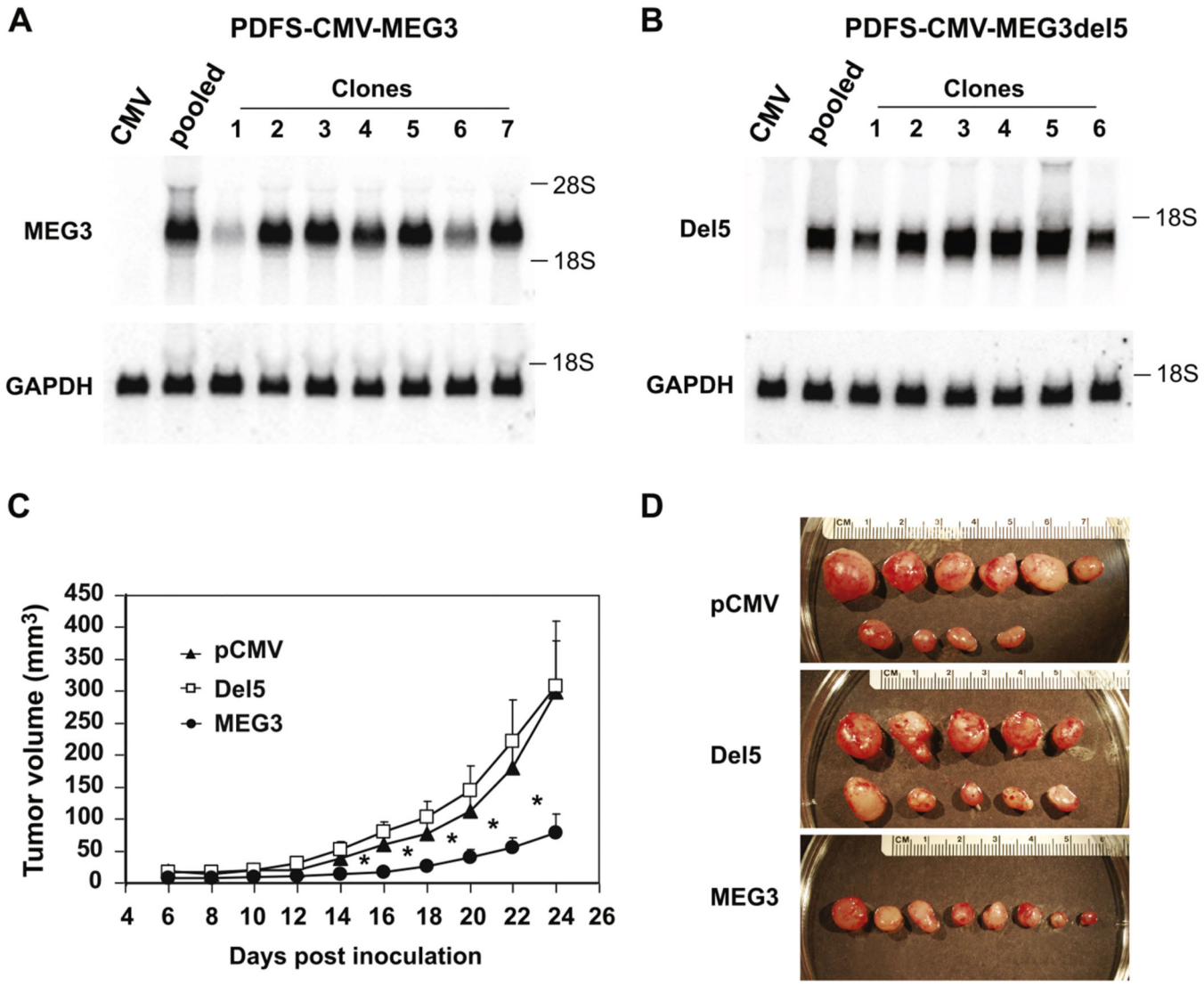

Figure 2.

Constitutively expressed MEG3 suppresses xenograft tumor growth in nude mice. PDFS cells were transfected with pcDNA3.1-puro vector, pCMV-MEG3-puro or pCMV-MEG3del5-puro constructs. Puromycin resistant clones were isolated. MEG3 (A) or MEG3del5 (B) expression in individual clones or pooled clones was detected by Northern blotting. (C) Xenograft tumor growth curves PDSF cells containing the blank vector (pCMV), expressing wt MEG3 (MEG3) or mutant MEG3 (del5). Cells (5×10^6) from pooled PDFS-CMV-MEG3, PDFS-CMV-MEG3del5 and pCMV clones were inoculated in bilateral flanks of nude mice (n = 5 for each type of cells). Tumor dimensions were measured every other day as indicated. Tumor sizes were calculated as $\pi \times (\text{length} \times \text{width} \times \text{height}) \div 6$ in mm. Values are means of 10 tumors and the error bars are standard error of the mean. *, $p < 0.05$, student t-test. (D) Pictures of tumors removed from nude mice injected with cells described in (C) at day 24 post injection.

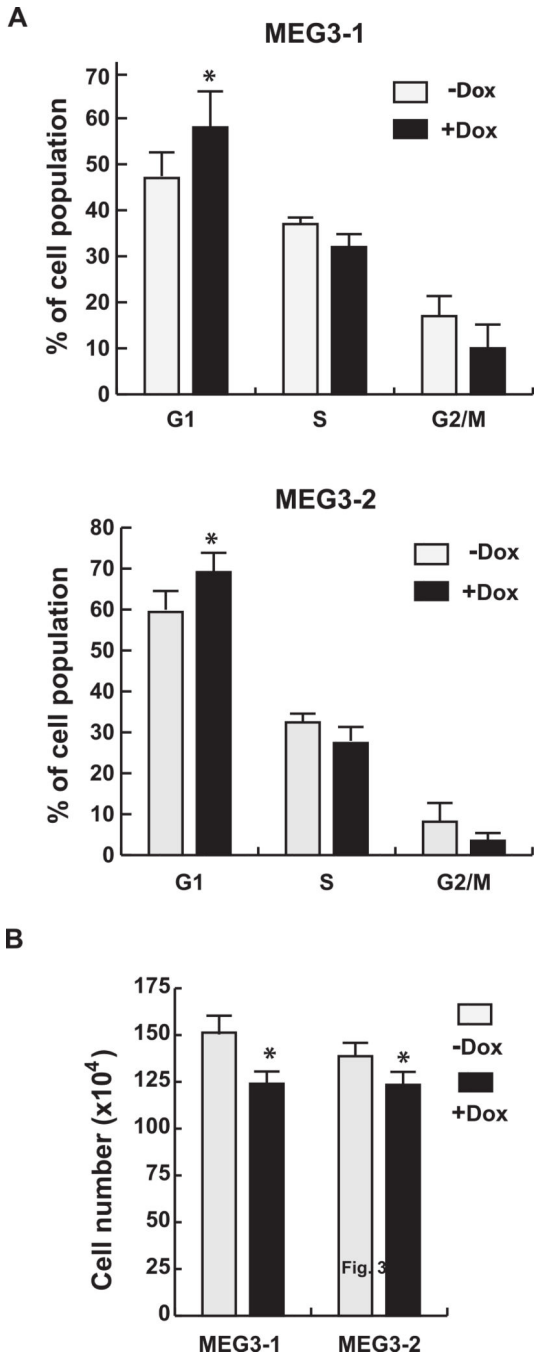

Figure 3.

MEG3 inhibits proliferation in PDFS cells. (A) MEG3 induction increases G1 population in PDFS cells. Cells from PDFS-TetO-MEG3 clone 1 (MEG3-1) and clone 2 (MEG3-2) were treated with Dox (+Dox) or without (−Dox) for 48 hours. After fixed with ethanol, cells were analyzed with a cytometer as described in Materials and Methods. Cells at each phase of the cell cycle were represented as percentages of total cell population. The data are derived from at least three independent experiments and presented as mean ± SD. *, $p < 0.05$, student t-test, compared to –Dox. (B) Cells (1×10^4) from PDFS-TetO-MEG3 clones

were seeded in 12-well plates in triplicates in the absence (−Dox) or presence of Dox (+Dox). Cell numbers were counted from each well at day 6. *, $p < 0.05$, student t-test, compared to –Dox.

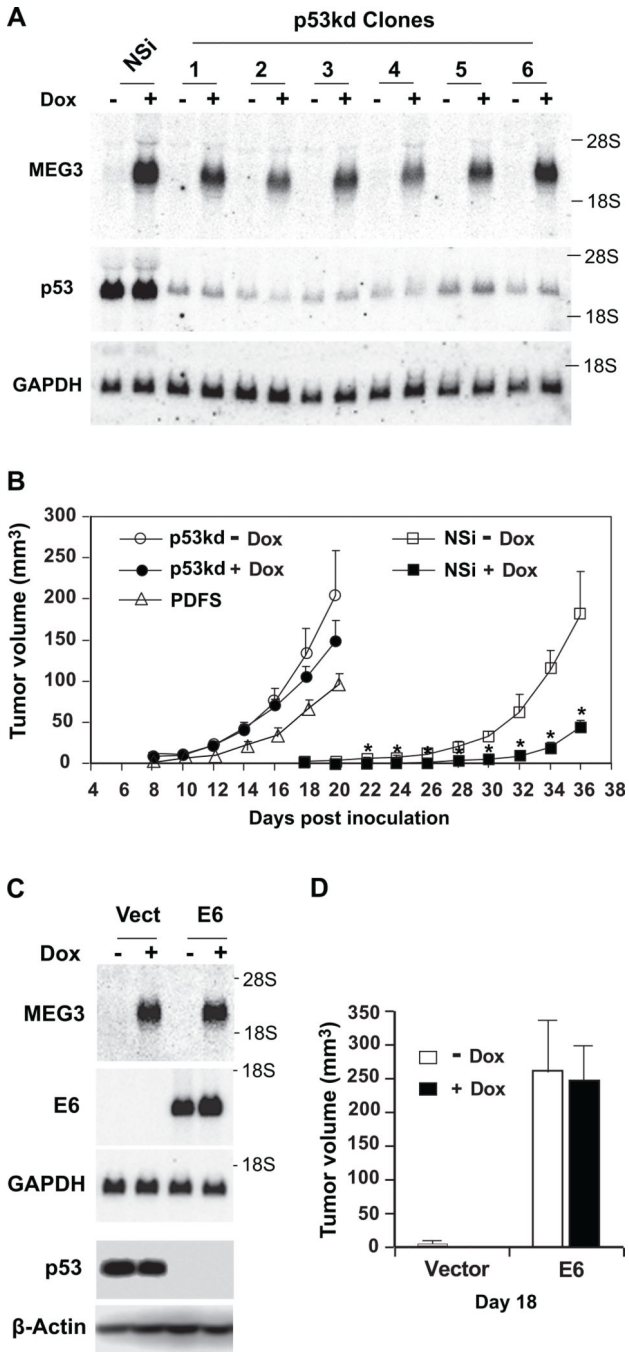

Figure 4.

MEG3 tumor suppression requires functional p53. (A) P53 in cells of PDFS-TetO-MEG3 clone 1 was knocked down by expressing p53 RNAi. Six clones with good p53 knockdown were selected. Cells expressing a non-specific RNAi (NSi) are used as a negative control. MEG3 and p53 expressions in the presence (+) or absence (−) of Dox (+) were detected by Northern blotting. (B) Xenograft tumor growth curves. Cells (5×10^6) from pooled PDFS-TetO-MEG3-p53kd clones (p53kd) and PDFS-TetO-MEG3-NSi (NSi) as well as parental PDFS cells were subcutaneously injected in two flanks of nude mice, ten for p53kd and NSi

cells respectively and five for PDFS. For mice injected with p53kd and NSi, they were divided into two groups, one group (n = 5) was fed with regular water (−Dox) and the other (n = 5) with Dox containing water (+Dox). Tumor dimensions were measured every other day as indicated. Tumor sizes were calculated as π × (length × width × height) ÷ 6 in mm. Values are means of 10 tumors and the error bars are standard error of the mean. *, $p < 0.05$, student t-test. (C) Cells from PDFS-TetO-MEG3 clone 1 were transduced with blank retrovirus (vect) or viruses expressing oncogene E6 (E6). E6 and MEG3 expressions were detected by Northern blotting. P53 and β-actin were detected by Western blotting. (D) Cells from PDFS-TetO-MEG3-E6 or -vect were inoculated subcutaneously in bilateral flanks of nude mice (n=10). After injection, mice were divided into two groups, one fed with Dox water (+Dox) and the other with regular water (− Dox). The tumor dimensions were measured at day 18 after injection. The tumor sizes were calculated as described above.