REVIEW

# *MEG3* noncoding RNA: a tumor suppressor

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## Abstract

Maternally expressed gene 3 (*MEG3*) is an imprinted gene belonging to the imprinted *DLK1–MEG3* locus located at chromosome 14q32.3 in humans. Its mouse ortholog, *Meg3*, also known as gene trap locus 2 (*Gtl2*), is located at distal chromosome 12. The *MEG3* gene encodes a long noncoding RNA (lncRNA) and is expressed in many normal tissues. *MEG3* gene expression is lost in an expanding list of primary human tumors and tumor cell lines. Multiple mechanisms contribute to the loss of *MEG3* expression in tumors, including gene deletion, promoter hypermethylation, and hypermethylation of the intergenic differentially methylated region. Re-expression of *MEG3* inhibits tumor cell proliferation in culture and colony formation in soft agar. This growth inhibition is partly the result of apoptosis induced by *MEG3*. *MEG3* induces accumulation of p53 (TP53) protein, stimulates transcription from a p53-dependent promoter, and selectively regulates p53 target gene expression. Maternal deletion of the *Meg3* gene in mice results in skeletal muscle defects and perinatal death. Inactivation of *Meg3* leads to a significant increase in expression of angiogenesis-promoting genes and microvessel formation in the brain. These lines of evidence strongly suggest that *MEG3* functions as a novel lncRNA tumor suppressor.

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## Introduction

A tumor suppressor gene is broadly defined as a gene whose product normally inhibits tumor initiation and progression. Inactivation of tumor suppressors plays a major role in the molecular pathogenesis of human neoplasms. Therefore, the search for new tumor suppressors and investigation of their functions are challenging and exciting areas in cancer research. To date, the known tumor suppressors are predominantly protein-coding genes. Their tumor suppression functions are mediated by their gene products – proteins. However, recent studies reveal that another class of genes, whose products are long noncoding RNAs (lncRNAs) with sizes >200 nt, may also play an important role in tumor suppression (Gibb *et al.* 2011). One such lncRNA gene is maternally expressed gene 3 (*MEG3*), which is an imprinted gene, located on chromosome 14q32 (Miyoshi *et al.* 2000). *MEG3* was first identified as the ortholog of gene trap locus 2 (*Gtl2*) in mice (Schuster-Gossler *et al.* 1998), officially known as *Meg3* and located on mouse distal chromosome 12. The *MEG3* gene belongs to the *DLK1–MEG3* imprinting locus, containing multiple maternally and paternally imprinted genes (Fig. 1), including at least three paternally expressed protein-coding genes and numerous maternally expressed noncoding RNAs (see review da Rocha *et al.* (2008)). The gene expression in this locus is tightly controlled by at least two differentially methylated regions (DMRs): the intergenic DMR (IG-DMR) and the *MEG3*-DMR. The imprinted expression of these genes plays an important role in development and growth (see review da Rocha *et al.* (2008)). In humans, *MEG3* is expressed in many normal tissues. The loss of *MEG3* expression has been found in various types of human tumors and tumor cell lines. In addition, re-expression of *MEG3* inhibits *in vitro* tumor cell proliferation (Zhang *et al.* 2003, 2010*a*, Zhou *et al.* 2007, Braconi *et al.* 2011, Wang *et al.* 2012). Therefore, it has been hypothesized that *MEG3* is a tumor suppressor gene. In this review, we will discuss the evidence from studies by us and others to support this hypothesis.

## Identification of *MEG3* as a pituitary tumor-associated gene by representational difference analysis

A major focus of our research is the molecular pathogenesis of human pituitary adenomas. These tumors arise from the anterior pituitary, which contains


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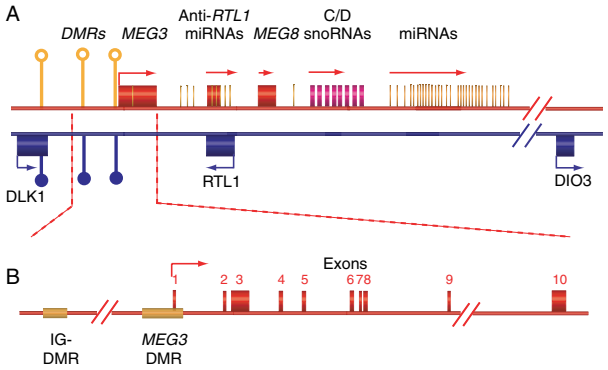


**Figure 1** (A) Schematic representation of the *DLK1–MEG3* locus on human chromosome 14. The *DLK1–MEG3* locus is ~837 kb long and consists of three known protein-coding genes including *DLK1, RTL1*, and *DIO3*, noncoding RNAs including at least three lncRNAs, and numerous small nucleolar RNAs (snoRNAs) and micro-RNAs (miRNAs). The maternal chromosome is in red and the paternal chromosome in blue. Differentially methylated regions are shown as circles. Filled circle, methylated; open circle, unmethylated. (B) The *MEG3* gene is 35 kb long and consists of ten exons. The IG-DMR is ~13 kb upstream of the *MEG3* gene. The *MEG3*-DMR overlaps with the *MEG3* promoter.

somatotrophs (producing growth hormone), lactotrophs (prolactin), gonadotrophs (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)), thyrotrophs (thyroid-stimulating hormone), and corticotrophs (adrenocorticotrophic hormone). Pituitary tumors are classified as clinically nonfunctioning adenomas (NFAs), which are mostly derived from gonadotroph cells (Chaidarun & Klibanski 2002), and clinically functioning adenomas, which are derived from the other four types of hormone-secreting cells. Patients with NFAs do not have symptoms associated with excess hormone secretion. All types of human pituitary adenomas are monoclonal in origin (Alexander *et al.* 1990, Herman *et al.* 1990, Biller *et al.* 1992), indicating that these tumors are derived from somatic cells with genetic or epigenetic mutations. To search for new genes linked to NFAs, we compared gene expression profiles between normal pituitaries and NFAs by representational difference analysis (RDA) techniques (Zhang *et al.* 2002). RDA identifies genes that are over- or under-expressed in tumor tissues compared with normal tissues. RNAs from four normal anterior pituitaries and four NFAs were used in the analysis (Zhang *et al.* 2002). Four genes identified as the most under-expressed genes in tumors are *GADD45γ* (*GADD45G*), histone deacetylase 6, *MEG3*, and delta-like 1 homolog (*Drosophila*; *DLK1*; Zhang *et al.* 2002). *MEG3* and *DLK1* were particularly noteworthy because they are known imprinted genes and belong to the same imprinted locus. Importantly, these genes are located on chromosome 14q32, a region proposed to contain putative tumor suppressors (Mutirangura *et al.* 1998, Bando *et al.* 1999). *DLK1*, also known as *Pref-1*, is a protein-coding gene and functions as an adipocyte differentiation inhibitor (Smas & Sul 1993). At that time, very little was known about the *MEG3* gene and we chose to focus on this gene and its function.

## *MEG3* expression is lost in human clinically NFAs

To confirm the finding that *MEG3* is under-expressed in NFAs, we first examined *MEG3* expression in five normal anterior pituitaries and eight NFAs of gonadotroph origin by RT-PCR. *MEG3*s were detected in all five normal pituitaries. In contrast, *MEG3* was not detected in any NFA (Zhang *et al.* 2003). Using *in situ* hybridization techniques, we detected *MEG3* transcripts in gonadotroph cells in the normal human pituitary (Zhang *et al.* 2003). Therefore, the lack of *MEG3* transcripts in NFAs is due to the loss of *MEG3* expression. This observation was further validated in our follow-up studies including 16 more normal pituitaries and 50 additional NFAs of gonadotroph origin in which *MEG3* expression was examined by conventional or quantitative RT-PCR (qRT-PCR) techniques (Zhao *et al.* 2005, Gejman *et al.* 2008, Cheunsuchon *et al.* 2011). In one study using qRT-PCR, we found that the average expression level of *MEG3* in 19 NFAs was < 2% of that seen in normal pituitaries (Cheunsuchon *et al.* 2011). In an independent study, Mezzomo *et al.* (2012) reported that *MEG3* expression was lost in all five NFAs, which stained positive for LH or FSH. These data demonstrated that *MEG3* expression was lost in all human gonadotroph-derived NFAs. We also examined tumors that stained negative for LHβ and FSHβ, and *MEG3* expression in these tumors was similar to that in gonadotroph NFAs (Cheunsuchon *et al.* 2011). In Mezzomo *et al.*'s (2012) study, four out of nine LHβ- and FSHβ-negative NFAs had lost *MEG3* expression. Additional supportive data were obtained using PDFS, a folliculostellate cell line derived from a human NFA in our laboratory (Danila *et al.* 2000). Using RT-PCR, we did not detect any *MEG3* expression in this cell line (Zhang *et al.* 2010*a*). These data indicate that loss of *MEG3* expression is a common phenomenon in NFAs.

The anterior pituitary contains five types of hormone-secreting cells, all of which express *MEG3*, as demonstrated by *in situ* hybridization (Gejman *et al.* 2008). We examined *MEG3* expression in functioning pituitary tumors derived from somatotrophs, lactotrophs, and corticotrophs. We found that *MEG3* transcripts were readily detected in all those tumor types assessed by RT-PCR (Gejman *et al.* 2008). Although *MEG3* expression levels appeared lower in functioning tumors than in normal pituitaries, this difference was not statistically significant (Cheunsuchon *et al.* 2011). Similar results were also reported by Mezzomo *et al.*

(2012). These data indicate that *MEG3* expression is exclusively lost in NFAs but not in functioning tumors and suggests that inactivation of the *MEG3* gene may play an important role in the development of NFAs.

## *MEG3* expression is lost in other human cancers

In addition to the normal pituitary, *MEG3* is highly expressed in the brain, adrenal gland, and placenta. Its transcripts are also detected in the testes, ovary, pancreas, spleen, mammary gland, and liver (Zhang *et al.* 2003). Multiple human cancer cell lines of various tissue origins have been examined for *MEG3* expression. Loss of *MEG3* expression has been found in all cancer cell lines examined including those derived from brain, bladder, bone marrow, breast, cervix, colon, liver, lung, meninges, and prostate (Table 1; Zhang *et al.* 2003, 2010*a*, Astuti *et al.* 2005, Zhou *et al.* 2007, Braconi *et al.* 2011, Wang *et al.* 2012). Loss of *MEG3* expression is also found in primary tumors. *MEG3* expression is lost or significantly reduced in 25% of neuroblastomas (Astuti *et al.* 2005), 81% of hepatocellular cancers (Braconi *et al.* 2011), and 82% of gliomas (Wang *et al.* 2012). Recently, we examined *MEG3* expression in meningiomas and found that loss of *MEG3* expression is strongly associated with tumor grade; MEG3 was detected in four of nine grade I, one of 11 grade II, and none of seven grade III meningiomas (Zhang *et al.* 2010*a*). These data suggest that loss of *MEG3* expression contributes to tumor development in a wide range of tissues.

## DNA methylation plays a major role in silencing the *MEG3* gene in tumors

One of the possible mechanisms whereby *MEG3* expression is lost in NFAs is the allelic deletion of the gene. To investigate this possibility, two microsatellite markers (D14S985 and WI-16835) flanking exons 4–9 of the *MEG3* gene were quantified by qPCR in NFAs and normal pituitaries (Zhao *et al.* 2005). No significant differences in their respective levels were detected between NFAs and normal pituitaries (Zhao *et al.* 2005). For example, the relative levels for D14S985 are $1{\cdot}15 \pm 0{\cdot}17$ in NFAs and $1{\cdot}02 \pm 0{\cdot}25$ in normal pituitaries (Student's *t*-test, $P = 0{\cdot}447$). The relative levels for WI-16835 are $1{\cdot}17 \pm 0{\cdot}23$ in NFAs and $1{\cdot}04 \pm 0{\cdot}34$ in normal pituitaries ($P = 0{\cdot}574$). These data indicate that there are no genomic deletions in the *MEG3* gene in NFAs. Individual exons of the *MEG3* gene were also amplified by PCR and the sequences were confirmed. None of the ten exons carried deletions or point mutations in NFAs (Zhao *et al.* 2005). These findings suggest that genetic alterations do not play a role in *MEG3* silencing in NFAs. In meningiomas, allelic deletion was not found in relatively benign, grade I tumors (Zhang *et al.* 2010*a*). However, three of 11 grade II and four of seven grade III tumors had *MEG3* gene allelic loss (Zhang *et al.* 2010*a*). These data indicate that genomic DNA deletion is associated with aggressive tumors.

There are two DMRs located upstream of the *MEG3* gene, IG-DMR and *MEG3*-DMR (Fig. 1B). A recent study by Kagami *et al.* (2010) analyzing patients carrying microdeletions at these DMRs indicates that the

**Table 1** *MEG3* expression is lost in human cancer cell lines

| Cancer | Cell lines | *MEG3* status | References |
|---|---|---|---|
| Brain | H4 | Not detected | Astuti *et al.* (2005) |
| | Kelly | | Zhang *et al.* (2003) |
| | SK-N-AS | | Wang *et al.* (2012) |
| | SK-N-DZ | | |
| | SK-N-F1 | | |
| | U251 | | |
| | U-87MG | | |
| Bladder | T24 | Not detected | Zhang *et al.* (2003) |
| | 5637 | | |
| Bone marrow | K562 | Not detected | Zhang *et al.* (2003) |
| Breast | MCF7 | Not detected | Zhang *et al.* (2003) |
| | T47D | | |
| Cervix | HeLa | Not detected | Zhang *et al.* (2003) |
| Colon | HCT116 | Not detected | Zhang *et al.* (2003) |
| | H29 | | Zhou *et al.* (2007) |
| Liver | HepG2 | Not detected | Braconi *et al.* (2011) |
| | Huh-7 | | |
| | PLC/PRF/5 | | |
| Lung | H1299 | Not detected | Zhang *et al.* (2003) |
| Meninges | CH157-MN | Not detected | Zhang *et al.* (2010*a*) |
| | IOMM-Lee | | |
| Prostate | Du145 | Not detected | Zhang *et al.* (2003) |

IG-DMR and *MEG3*-DMR are imprinting control centers for the *DLK1–MEG3* locus in the placenta and the body respectively. In the body, however, the methylation status of the *MEG3*-DMR is governed by the IG-DMR, suggesting a hierarchical relationship between these two DMRs (Kagami *et al.* 2010). *MEG3*-DMR covers a DNA segment that starts ~1·5 kb upstream of the first *MEG3* exon and extends to the first intron (Paulsen *et al.* 2001, Takada *et al.* 2002). Kagami *et al.* (2010) reported that *MEG3* transcripts were not detected in cells obtained from a patient with a microdeletion at the *MEG3*-DMR, indicating that this region is required for expression of the *MEG3* gene. The *MEG3* promoter overlaps with the *MEG3*-DMR and is GC rich (Zhao *et al.* 2005). Bisulfite sequencing revealed that the methylation in regions 1 and 4 of the *MEG3* promoter is significantly higher in NFAs than in normal pituitary (Zhao *et al.* 2005; Fig. 2). Interestingly, this promoter hypermethylation is also observed in meningiomas (Zhang *et al.* 2010*a*). Astuti *et al.* (2005) reported that the *MEG3*-DMR is completely methylated in neuroblastoma cell lines. These studies indicate that inactivation of the *MEG3* gene in these tumors is in part attributed to promoter silencing by hypermethylation. We have identified a transcription factor-binding site within the *MEG3* promoter, which is a cAMP response element (CRE). Deletion of the CRE significantly reduces promoter transcription activity (Zhao *et al.* 2006). In addition, the CRE sequences contain a CpG dinucleotide, suggesting that methylation of this CpG may prevent transcription factors from binding to the site. This likely plays a role in promoter silencing of the *MEG3* gene in tumors.

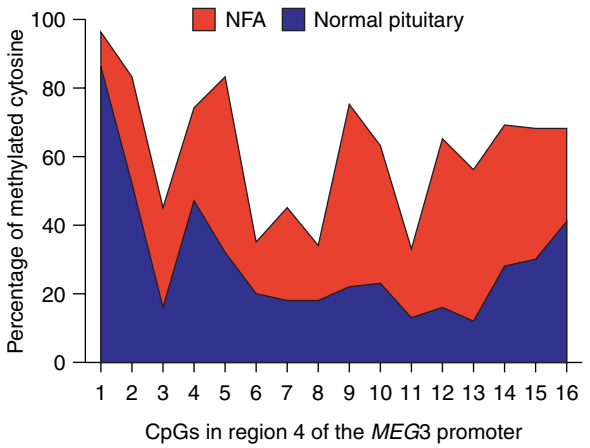


**Figure 2** Hypermethylation of the *MEG3* promoter in NFAs. Region 4 of the *MEG3* promoter contains 33 CpG dinucleotides. The methylation status of the first 16 CpGs are shown (Zhao *et al.* 2005). The percentage of methylated CpGs in tumors is in red and that in normal pituitaries is in blue.

The IG-DMR is ~13 kb upstream of the *MEG3* gene (Paulsen *et al.* 2001, Takada *et al.* 2002). Hypomethylation of this region in the maternal allele is required for activation of all MEGs, including *MEG3*, and correct imprinting of the *DLK1–MEG3* locus. In mice, maternal deletion of the IG-DMR silences MEGs and activates paternally expressed genes, which are normally silenced on the maternal chromosome (Lin *et al.* 2007). In humans, *MEG3* expression was lost in cells obtained from a patient carrying a small DNA deletion containing the IG-DMR (Kagami *et al.* 2010). Therefore, it is believed that the IG-DMR is required for activation of its downstream MEG (see review da Rocha *et al.* (2008)). An increase in IG-DMR methylation has been found in human NFAs (Gejman *et al.* 2008). Interestingly, the degree of the methylation increase correlates with meningioma tumor grade (Zhang *et al.* 2010*a*). For example, the percentages of methylated CpG in IG-DMR are 50·1, 56·4, 61·0 and 68·8% in normal meninges, grade I, grade II, and grade III meningiomas respectively. Hypermethylation of the IG-DMR is also reported in neuroblastoma cell lines, a Wilms' tumor, two neuroblastomas, and a pheochromocytoma (Astuti *et al.* 2005). More importantly, treatment with a demethylating agent, 5-aza-2-deoxycytidine, resulted in re-expression of *MEG3* in multiple human breast cancer tumor cell lines (Zhao *et al.* 2005), meningiomas (Zhang *et al.* 2010*a*), neuroblastomas (Astuti *et al.* 2005), and hepatocellular carcinomas (Braconi *et al.* 2011). Taken together, these findings strongly indicate that epigenetic modification of the IG-DMR and *MEG3*-DMR plays a significant role in inactivation of the *MEG3* gene in human tumors.

## *MEG3* inhibits tumor cell proliferation *in vitro*

One characteristic of a tumor suppressor is inhibition of tumor cell proliferation. Whether *MEG3* negatively regulates cell proliferation was tested in a number of human cancer cell lines in several independent studies using four experimental assays: colony-formation, growth curves, BrdU incorporation, and anchorage-independent growth. In the colony formation assay experiments, MCF7, HeLa, H4, and CH157-MN cells were transfected with a plasmid expressing *MEG3* or a control vector. The transfected cells were treated with the selection drug and the number of drug-resistant colonies was counted. The colony number in cells transfected with *MEG3* was significantly lower than in cells transfected with controls (Zhang *et al.* 2003, 2010*a*). Secondly, in assessing cell proliferation using a growth curve assay, cells transfected with the *MEG3* plasmid grew significantly slower than cells transfected with control vectors. Cell lines used for this assay were

the cervical cancer HeLa (Zhang *et al.* 2003), hepatocellular cancer PRC/PRF/5 (Braconi *et al.* 2011), and gliomas U251 and U87 MG cell lines (Wang *et al.* 2012). Thirdly, using the BrdU incorporation assay, cells were transfected with a vector expressing both *MEG3* and *GFP*. DNA synthesis in cells expressing *MEG3* (GFP positive) was determined by BrdU incorporation. Re-expression of *MEG3* significantly inhibited BrdU incorporation in cell lines HCT116, IOMM-Lee, and CH157-MN (Zhou *et al.* 2007, Zhang *et al.* 2010*a*). Finally, using the anchorage-independent growth assay, the cell line PRC/PRF/5 was transfected with a *MEG3* expression construct and seeded into soft agar. *MEG3* significantly suppressed colony-formation efficiency in the PRC/PRF/5 cell line (Braconi *et al.* 2011). These studies demonstrated that *MEG3* inhibits proliferation of tumor cells. Braconi *et al.* (2011) reported that ectopic expression of *MEG3* causes apoptosis in PRC/PRF/5 cells. Apoptosis induced by *MEG3* has also been observed in U251 and U87 MG cells (Wang *et al.* 2012). These data suggest that *MEG3* inhibition of tumor cell proliferation is due in part to induction of apoptosis. We are currently investigating whether *MEG3* inhibits tumor growth *in vivo* in our laboratory. Our studies to date indicate that re-expression of *MEG3* in PDFS cells suppresses tumor growth in nude mice (Zhou *et al.*, manuscript in preparation).

## *MEG3* functionally interacts with tumor suppressor p53

The p53 (*TP53*) gene is mutated in most human cancers and has been subject to intensive investigation and review (see reviews Olivier *et al.* (2010) and Soussi (2011)). It functions as a transcription factor capable of regulating expression of many target genes leading to suppression of tumor development and growth (Vousden & Prives 2009). p53 has also been shown to mediate functions of other tumor suppressors, such as ARF (CDKN2A; Sherr & Weber 2000), BRCA1 (Ouchi *et al.* 1998), and PTEN (Mayo & Donner 2002). Experimental data from our studies demonstrated that p53 is a target of *MEG3*. Transfection of *MEG3* expression constructs induces a significant increase in p53 protein levels in human cancer cell lines (Zhou *et al.* 2007). Normally, p53 protein levels are very low due to its rapid degradation via the ubiquitin-proteasome pathway (see review Brooks & Gu (2011)). The ubiquitination of p53 is mainly mediated by MDM2, an E3 ubiquitin ligase (Haupt *et al.* 1997, Kubbutat *et al.* 1997). Inhibition of *MDM2* function plays a major role in p53 stabilization (see review Harris & Levine (2005)). We found that *MEG3* downregulates *MDM2* expression (Zhou *et al.* 2007), suggesting that *MDM2* downregulation is one of the mechanisms whereby *MEG3* activates p53. *MEG3* stimulates p53-dependent transcription (Zhou *et al.* 2007, Zhang *et al.* 2010*a*, Wang *et al.* 2012). Interestingly, *MEG3* does not stimulate expression of $p21^{Cip1}$, a well-known p53 target gene (el-Deiry *et al.* 1993). Instead, MEG3 enhances p53-dependent expression of GDF15, an inhibitor of cell proliferation. Further investigation revealed that *MEG3* enhances binding of p53 to the GDF15 promoter but not to the $p21^{Cip1}$ promoter (Zhou *et al.* 2007). These findings indicate that *MEG3* activates p53 and selectively activates expression of p53 target genes.

## The product of the *MEG3* gene is a noncoding RNA

The *MEG3* gene consists of ten exons (Fig. 1B; Miyoshi *et al.* 2000). Transcription from the *MEG3* gene yields multiple *MEG3* transcript isoforms due to alternative RNA splicing (Zhou *et al.* 2007, Zhang *et al.* 2010*b*). A total of 12 MEG3 RNA isoforms have been identified, named MEG3, MEG3a to MEG3k (Fig. 3). The predominantly expressed isoform is MEG3, containing exons 1–4 and 8–10 (GenBank accession number, NR_002766.2; Zhang *et al.* 2010*b*). The mature *MEG3* RNA is ~1600 nt in length.

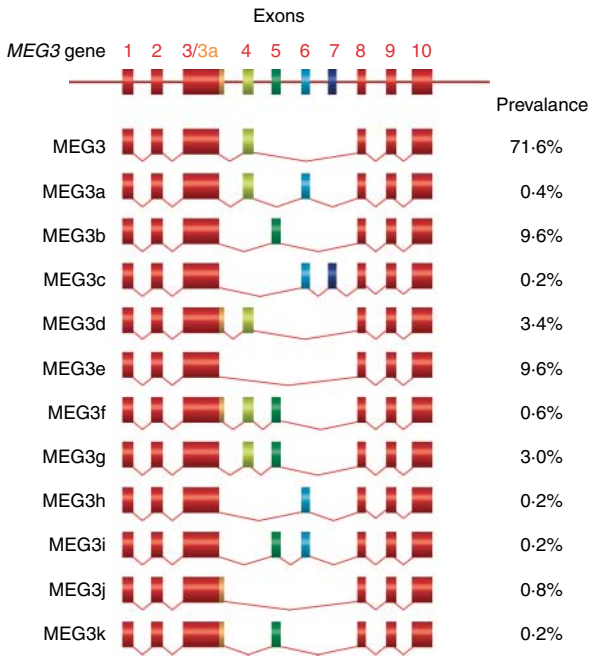


**Figure 3** Schematic representation of *MEG3* RNA isoforms generated by alternative splicing. The common exons are in red. Exon 3a contains 40 extra nucleotides at the 3′-end of the exon 3, which is in gold color. The prevalence of each *MEG3* RNA isoform is the average of values from five human tissues and cell lines (Zhang *et al.* 2010*b*).

No single large open reading frame (ORF) has been found in any of the MEG3 isoforms. There are three small major ORFs in *MEG3* RNA with lengths ranging from 150 to 250 nt. No apparent consensus Kozak sequences are found around AUG in those ORFs, suggesting that these ORFs are not constructed for optimal translation. When transfected, all MEG3 isoforms stimulate transcription from a p53-reporter construct and inhibit BrdU incorporation in HCT116 cells, although the strength of the activity varies among MEG3 isoforms (Zhou *et al.* 2007, Zhang *et al.* 2010*b*). The major isoform MEG3 was chosen for further investigation. A series of *MEG3* expression constructs were generated to express individual ORFs, two ORFs in combination, or full-length molecules carrying point mutations that cause a frameshift in targeted ORFs leading to peptide premature termination if translated. These constructs were then tested for their ability to activate p53. We found that expression of individual ORFs failed to activate p53. In contrast, expression of full-length *MEG3*s in which the ORFs were destroyed by point mutations activated p53 comparable to wild-type *MEG3* (Zhou *et al.* 2007). These results indicate that *MEG3* function is not mediated by any individual ORFs. Instead, the full-length *MEG3* transcript is essential for MEG3 function. These data strongly support the concept that *MEG3* is an ncRNA.

More direct evidence supporting the concept that MEG3 is an ncRNA came from structural studies. The computer program *Mfold* is widely used to predict the secondary structures of nucleic acids based on their thermostability (Mathews *et al.* 1999, Zuker 2003). *MEG3* and its isoforms have similar secondary structures as predicted by *Mfold* (Fig. 4; Zhang *et al.* 2010*b*). For example, *MEG3* RNA folds into three major motifs, M1, M2, and M3 (Fig. 4). A *MEG3* RNA carrying a small deletion within M2 that does not significantly change the overall secondary folding activates p53. In contrast, a deletion of similar size within M2 that significantly alters its folding abolishes the p53-activating function of *MEG3* (Zhou *et al.* 2007). Furthermore, when a half sequence of M2 was replaced with nucleotides, which differ dramatically from the original sequences but do not significantly alter the M2 folding, the resultant hybrid *MEG3* functions as wild-type *MEG3* in both p53-dependent transcription activation and inhibition of BrdU incorporation (Zhang *et al.* 2010*b*). These data demonstrate that *MEG3* indeed functions as an ncRNA.

## *Meg3* gene deletion results in perinatal death in mice

Two *Meg3* knockout (KO) mouse models have been reported. We created a *Meg3* KO mouse model carrying

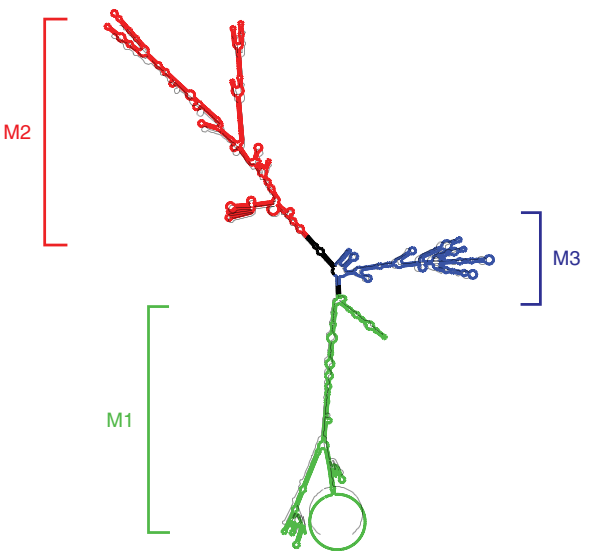


**Figure 4** *MEG3* RNA folding is predicted by *Mfold*. The stems and loops of folding are categorized into three groups designated as M1, M2, and M3.

a deletion of a 5 kb DNA, which contains the first five exons and a small portion of the *Meg3* promoter (Zhou *et al.* 2010). As expected, mice carrying the paternal deletion are alive and normal. In these mice, there are no changes in expression of either *Meg3* and its downstream MEGs or paternally expressed genes compared with wild-type controls. However, mice carrying the maternal deletion died perinatally and had major skeletal muscle defects. Expression of both *Meg3* and its downstream MEGs was completely abolished. In contrast, the paternally expressed genes within the *Dlk1–Meg3* locus were activated. These observations are very similar to those found in mice carrying the IG-DMR deletion (Lin *et al.* 2003). The other *Meg3* KO mouse model was created by Takahashi *et al.* (2009) and carries a 10 kb deletion including the Meg3-DMR and the first five exons of the *Meg3* gene. The phenotype of this mouse model is significantly different from our *Meg3* KO mice as well as the IG-DMR KO mice. For example, Takahashi's mice with the maternal deletion died 4 weeks after birth, while those with the paternal deletion died perinatally. Surprisingly, mice with the homozygous deletion survived and grew to fertile adults (Takahashi *et al.* 2009). The expression pattern of the imprinted genes in Takahashi's mice is also different. All the MEGs except *Meg3* were still moderately expressed in mice carrying the maternal deletion and their expression levels were significantly increased in mice carrying the paternal or homozygous deletion compared with those in wild-type mice (Takahashi *et al.* 2009). This dysregulated gene expression is likely due to the activity of the promoter controlling the *neo* expression cassette, which was used

for selection of recombinant embryonic stem cells and is transcribed in the same direction as the *Meg3* gene. Another phenotypic difference between Takahashi's *Meg3* KO mice and our *Meg3* KO mice is that in Takahashi's mice, the maternal *Meg3* deletion did not affect the methylation status of the IG-DMR (Takahashi *et al.* 2009) but resulted in IG-DMR hypermethylation in our mice (Zhou *et al.* 2010). Taken together, data from these KO mice suggest that a function of *MEG3* is to maintain an active (unmethylated) status at the IG-DMR by positively regulating expression of its downstream MEGs, which may act as inhibitors of IG-DMR methylation.

## Deletion of the *Meg3* gene promotes angiogenesis *in vivo*

To identify the target genes of *Meg3 in vivo*, we compared gene profiles in embryonic brains between *Meg3* KO mice and wild-type mice using microarray techniques (Gordon *et al.* 2010). Among the genes that are up regulated in *Meg3* KO mice are several genes in the angiogenesis pathway. Using qRT-PCR and immunohistochemical staining, we confirmed that expression of vascular endothelial growth factor alpha (VEGFA) and its type I receptor (VEFGR1) is significantly increased in *Meg3* KO brain compared with their expression in the wild-type brain. Both VEGFA and VEFGR1 are primary regulators of angiogenesis (see review Veikkola & Alitalo (1999)). In agreement, the formation of cortical microvessels, identified by positive staining of *Pecam1* and *Vegfr2* (*Kdr*) markers, is significantly increased in the brains of *Meg3* KO mice (Gordon *et al.* 2010). These data indicate that deletion of the *Meg3* gene promotes angiogenesis. It is well known that novel blood vessel formation is required for tumor growth (see review Folkman (2002)). Therefore, enhanced angiogenesis may be one of the mechanisms by which inactivation of MEG3 contributes to tumor development.

## Conclusions

A bona fide tumor suppressor has to meet a minimum of three criteria: 1) it is functionally inactivated in tumors; 2) re-expression of the gene inhibits tumor growth *in vitro* and *in vivo*, and 3) knockout of the gene leads to tumor formation and/or developmental defects in an animal model (see reviews Weinberg (1991, 1993) and Hakem & Mak (2001)). Loss of *MEG3* expression in various human tumors has been well documented. Re-expression of *MEG3* inhibits proliferation, induces apoptosis, and inhibits anchorage-independent growth of human tumor cells. Its function, at least in part, is mediated by the tumor suppressor p53. Although *Meg3* KO mice died prematurely, rendering it impossible to test whether inactivation of *Meg3* leads to tumor formation, the requirement of *Meg3* for embryonic development is consistent with the function of many known tumor suppressors. For example, $Rb^{-/-}$ embryos die *in utero* between E13·5 and E15·5 due to nervous system and hematopoietic defects (Clarke *et al.* 1992, Jacks *et al.* 1992). $Pten^{-/-}$ embryos die due to defective chorioallantoic development between E6·5 and E9·5 (Suzuki *et al.* 1998). In addition, *in vivo* angiogenesis activity is enhanced in the absence of *MEG3*, indicating that one of the functions of *MEG3* is to inhibit angiogenesis. Taken together therefore, strong evidence exists to support the concept that *MEG3* is a novel lncRNA tumor suppressor. Currently, we are generating a *Meg3* conditional KO mouse model in which the *Meg3* gene will be specifically deleted in targeted tissues. We will be able to determine whether inactivation of *MEG3* leads to development of tumors *in vivo* and provides definite evidence demonstrating that *MEG3* is an ncRNA tumor suppressor. These studies will reveal novel functions and mechanisms of large noncoding RNAs in the development and pathogenesis of human tumors, which is a new field of investigation for the future.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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