

Published in final edited form as:

*Biochem Pharmacol.* 2010 December 15; 80(12): 2057–2065. doi:10.1016/j.bcp.2010.07.003.

## Resveratrol modulates the levels of microRNAs targeting genes encoding tumor-suppressors and effectors of TGF $\beta$ signaling pathway in SW480 cells

Esmerina Tili<sup>1</sup>, Jean-Jacques Michaille<sup>1,2</sup>, Hansjuerg Alder<sup>1</sup>, Stefano Volinia<sup>1</sup>, Dominique Delmas<sup>2</sup>, Norbert Latruffe<sup>2</sup>, and Carlo M. Croce<sup>1</sup>

<sup>1</sup>Ohio State University, Department of Molecular Virology, Immunology and Medical Genetics, Columbus, OH 43210, USA

<sup>2</sup>LBMN-INSERM U866, Université de Bourgogne, 21000 Dijon, France

### Abstract

Resveratrol (trans-3,4',5-trihydroxystilbene) is a natural antioxidant with cardiovascular and cancer preventive properties, that is currently at the stage of pre-clinical studies for human cancer prevention. Beside its known effects on protein coding genes, one possible mechanism for resveratrol protective activities is by modulating the levels of non-coding RNAs. Here, we analyzed the effects of resveratrol on microRNA populations in human SW480 colon cancer cells. We establish that resveratrol treatment decreases the levels of several oncogenic microRNAs targeting genes encoding Dicer1, a cytoplasmic RNase III producing mature microRNAs from their immediate precursors, tumor suppressor factors such as PDCD4 or PTEN, as well as key effectors of the TGF $\beta$  signaling pathway, while increasing the levels of *miR-663*, a tumor-suppressor microRNA targeting *TGF $\beta$ 1* transcripts. We also show that, while upregulating several components of the TGF $\beta$  signaling pathway such as TGF $\beta$  receptors type I (TGF $\beta$ R1) and type II (TGF $\beta$ R2), resveratrol decreases the transcriptional activity of SMADs, the main effectors of the canonical TGF $\beta$  pathway. Our results establish that protective properties of resveratrol may arise at least in part from its capability to modify the composition of microRNA populations in cells, and suggest that the manipulation of the levels of key microRNAs, such as *miR-663*, may help to potentiate the anti-cancer and anti-metastatic effects of resveratrol.

### Keywords

Colon cancer; microRNAs; *miR-663*; resveratrol; SW480 cells; TGF $\beta$

### 1. Introduction

Colorectal cancer (CRC) is the third most common malignancy and the fourth biggest cause of cancer mortality worldwide [1, 2]. Despite the increased use of screening strategies such as fetal occult blood testing, sigmoidoscopy and colonoscopy, more than one third of patients with colorectal cancer will ultimately develop metastatic disease [2]. It is now widely accepted that genetic factors play key roles in the predisposition to colorectal cancer development and progression. Thus, more than 11% of patients with colorectal cancer have at least one first degree relative with the same disease [3], however twin studies suggest that

---

Address correspondence to: Jean-Jacques Michaille, Ph.D., Université de Bourgogne, LBMN-INSERM U866, Faculté Gabriel, 6 Bd. Gabriel, 21000 DIJON, France. Phone: +33-380-39-62-11. Fax: +33-380-39-62-50. jean-jacques.michaille@u-bourgogne.fr.

**Conflict of Interest:** The authors declare no conflict of interests.

roughly 35% of CRCs are inherited [4]. Understanding the genes and pathways that cause CRC are thus of greatest interest, for they will allow to improve both the diagnoses and prognoses of the disease as well as to optimize its prevention and treatment. The genetic basis of familial CRC has been actively analyzed in the last decades, and the various CRC sub-types linked to several gene mutations responsible for initiating them [5]. For example, Lynch syndrome, formerly known as hereditary non-polyposis CRC, is characterized by rapid progression of colorectal tumors, related to germline mutation of one of the DNA mismatch repair (MMR) genes: *MLH1*, *MSH2*, *MSH6* or *PMS2* [6].

In addition, germline mutations in *APC*, a gene encoding a gatekeeper that acts as a negative regulator of the WNT signaling pathway, were initially found in Familial Adenomatous Polyposis (FAP), a syndrome that accounts for less than 1% of CRC in the United States, but then somatic mutations of *APC* have been found in the great majority of sporadic CRCs [6]. Indeed, activating mutations of the canonical WNT signaling pathway, which signals through the nuclear relocalization of  $\beta$ -CATENIN, have been found in more than 90% of CRCs [7]. Once in the nucleus,  $\beta$ -CATENIN functions as a cofactor for transcription factors of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family, which regulate the transcription of genes mainly determining cell fate and regulating cell proliferation [7].

On the other hand, the TGF $\beta$  signaling pathway is involved in a number of biological processes, including cell proliferation, differentiation, migration and apoptosis [8]. It is one of the most commonly altered cellular signaling pathways in human cancers [9]. TGF $\beta$  signaling is initiated by the binding of TGF $\beta$  ligands to type II receptors (TGF $\beta$ R2). Three TGF $\beta$  isoforms (TGF $\beta$ 1, TGF $\beta$ 2 and TGF $\beta$ 3) are expressed in mammalian epitheliums, each being encoded by a unique gene and expressed in both a tissue-specific and developmentally regulated manner, with TGF $\beta$ 1 being the most abundant and ubiquitously expressed isoform. Once bound by TGF $\beta$ , TGF $\beta$ R2 recruits, phosphorylates and thus activates of the type I TGF $\beta$  receptor (TGF $\beta$ R1). TGF $\beta$ R1 then phosphorylates two transcriptional regulators, namely SMAD2 and SMAD3, which subsequently bind to SMAD4. This results in the nuclear translocation of SMAD complexes, allowing SMADs to interact with transcription factors controlling the expression of a multitude of TGF $\beta$  responsive genes [10]. TGF $\beta$ 1 is usually considered a tumor suppressor, due to its cytostatic activity in epitheliums. However, on advanced stages of tumors, TGF $\beta$ 1 behaves as a tumor promoter, due to its capability to enhance angiogenesis, epithelial-to-mesenchymal transition, cell motility and metastasis [11-13]. Not only the expression of *TGF $\beta$ 1* in both tumor and plasma was found to be significantly higher in patients with metastatic colorectal cancer, but increasing colorectal tumor stage was correlated with higher *TGF $\beta$ 1* expression in tumor tissues [14].

MicroRNAs (miRNAs) are short non-coding RNAs which regulate the translation and/or degradation of target messenger RNAs, and whose molecular malfunctions are associated with cancers [15, 16]. Depending on the effects of their downregulation or over-expression, miRNAs have been described either as oncogenic (onco-miRs) or tumor suppressors. For example, genomic amplification and over-expression of *miR-17-92* miRNAs is found in B-cell lymphomas as well as in breast and lung cancers [15, 16]. *MiR-21* is over-expressed in several cancers, including CRCs, gliomas, as well as breast, gastric, prostate, pancreas, lung, thyroid and cervical cancers [17]. On the other hand, *let-7* miRNAs, frequently downregulated in cancers like lung, colon or other solid tumors, are therefore considered as tumor suppressor miRNAs in these types of cancers [15]. Also, *miR-15* and *miR-16-1* suppress tumorigenicity by inhibiting cell proliferation and promoting apoptosis of cancer cells [18]. Beside *miR-21*, several miRNAs are overexpressed in CRCs, such as for example *miR-17*, *miR-25*, *miR-26a* or *miR-181a* [19, 20]. Furthermore, several miRNAs, including *miR-21*, have been shown to activate metastasis by acting on multiple signaling pathways and targeting various proteins that are key players in this process [19, 21].

Resveratrol (trans-3,4',5-trihydroxystilbene) is a dietary polyphenolic, non-flavonoid antioxidant derived from grapes, berries, peanuts, and other plant sources. Resveratrol has various health benefits, such as cardiovascular and cancer preventive properties [22-24], and it is currently at the stage of pre-clinical studies for human cancer prevention [24, 25]. Resveratrol induces apoptosis by up-regulating pro-apoptotic genes while simultaneously down-regulating anti-apoptotic genes. Resveratrol induces the redistribution of CD95 and other death receptors in lipid rafts, thus contributing to their sensitization to death receptor agonists [26]. In addition, resveratrol causes growth arrest at G1 and G1/S phases by inducing the expression of CDK inhibitors p21/CDKN1A and p27/CDKN1B [23]. However, the molecular bases for resveratrol effects remain unclear.

Here, we address the effects of resveratrol treatment on miRNA populations from human SW480 cells. We show that resveratrol modulates the levels of miRNAs targeting both oncogenes and tumor suppressor genes, which provides a rationale for manipulating the levels of miRNAs depending on the nature and the stage of tumors to optimize the anti-tumors effects of resveratrol.

## 2 Materials and methods

### 2.1 Micro-array analyses

RNAs extracted with TRIzol (Invitrogen, Carlsbad, California, USA) were subsequently subjected to DNase digestion (Turbo-DNase from Ambion, Invitrogen, Carlsbad, California, USA). MiRNA micro-array analyses were done at the Ohio State University micro-array facility. Data were submitted to MIAME database with the accession numbers to be received after confirmation.

### 2.2 Cell culture, transfection and treatments

SW480 cells were maintained in culture following standard procedures. Transfections were done using Lipofectamine 2000 (Invitrogen). Cells were treated for 14 hours with either the vehicle (ethanol) or resveratrol 50  $\mu$ M (Sigma-Aldrich, Saint Louis, Missouri, USA). Whenever needed, cells were either mock-treated or stimulated for 14 hours with 100 ng/ml TGF $\beta$ 1 (Invitrogen).

### 2.3 Preparation of clones and subclones

The 307 nt-long 3'-UTR (untranslated region) of the *TGF $\beta$ 1* gene was cloned by PCR from genomic DNA extracted from HEK-293 cells. *Luc-TGF $\beta$*  constructs were prepared by inserting the *TGF $\beta$ 1* 3'-UTR downstream of the *luciferase* gene in the *Xba*I site of the *pGL3-Control* vector (Promega, Madison, Wisconsin, USA). The sequences of the oligonucleotides used for cloning are available upon request.

### 2.4 Mutagenesis

The two sets of overlapping *miR-663* target sites in *TGF $\beta$ 1* 3'-UTRs, respectively containing three and two *miR-663* sites, (site *663-1*: CCCC GCCCGCCCCGCC, starting at nt 8, and site *663-2*: CCCC GCCCGGCC, starting at nt 43) were respectively mutated to *M663-1* (CGGCGCGCGGCCGGGCC) and *M663-2* (GCCGCGCCGCGG) using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies, Inc., Santa Clara, California, USA). *M663-1,2* mutant carry both mutations simultaneously.

### 2.5 Luciferase assays

SW480 ( $1 \times 10^6$ ) in 6-well plates were transfected with 0.4  $\mu$ g of DNA (Promega *pGL3-Control* vector or derived constructs), or with RNA 50 nM final, i.e., pre-miR Precursor

Molecule-Negative Control #1 (Ambion) or either *pre-miR-663* precursor RNA (Ambion) or a *miR-663* antisense inhibitory RNA (*663-I*, Ambion), along with 20 ng of *Renilla* luciferase control vector (*pRL-TK* from Promega). Assays were performed 48 hours after transfection using the Dual Luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity. TGF $\beta$  signaling was tested in sixplicates in 12-wells plates using the Cignal SMAD Reporter Assay Kit from Super Array Bioscience Corporation (SABioscience, Qiagen, Valencia, California, USA). This kit is designed to monitor the activity of the canonical TGF $\beta$ -induced signal transduction pathway, i.e., to give a measure of the rate of SMAD2 and/or SMAD3 nuclear transcriptional activity in conjunction with SMAD4 on a reporter construct containing a functional SMAD binding site.

## 2.6 RNase-protection assays

Assays were performed using the *mirVana* miRNA Probe Construction Kit and the *mirVana* miRNA Detection Kit from Ambion. RNAs were incubated overnight at 46 °C in the presence of  $8 \times 10^4$  cpm of [ $^{32}$ P]-labelled antisense RNA probe and then digested for 40 minutes at 37 °C using a 1/50 dilution of the provided RNase A/RNase T1 solution. The relative intensities of the bands were determined using the Adobe Photoshop software (Adobe Systems Incorporated, San Jose, California, USA).

## 2.7 Western blots

Anti-TGF $\beta$ 1 and anti-SMAD3 antibodies were from Cell Signaling Technology (Danvers, Massachusetts, USA). Anti-TGF $\beta$ R1, anti-TGF $\beta$ R2, anti-SMAD7, anti-PDCD4 and anti-E-CADHERIN antibodies were from Abcam (Abcam Inc., Cambridge, Massachusetts, USA). Anti-PTEN and anti-GAPDH antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA). The relative intensities of the bands on Western blots were determined using the Adobe Photoshop software.

## 2.8 Statistical analysis

Results are expressed as mean  $\pm$  S.D. The statistical significance of the results was determined by Student *t* tests using the Microsoft Excel software (Microsoft Campus, Redmond, Washington, USA).

## 3. Results

### 3.1 Resveratrol modulates the levels of miRNAs targeting known tumor suppressors as well as components of the TGF $\beta$ signaling pathway

MiRNA micro-arrays (MIAME accession number) showed that a 14 hours resveratrol treatment of SW480 cells significantly ( $P < 0.05$ ) increased the levels of 22 miRNAs while decreasing those of 26 other miRNAs (Table 1). The changes in miRNA levels were generally limited, with a few exceptions such as a 17 and 11 fold upregulation of *miR-146b-5p* and *miR-1*, respectively. This suggests that resveratrol effects on miRNA populations are not global, but rather miRNA-specific. Several miRNAs downregulated by resveratrol, as for example *miR-17*, *miR-21*, *miR-25*, or *miR-92a-2*, are generally considered as onco-miRs [15, 16], and are known to be overexpressed in CRCs [19, 20]. Surprisingly, *miR-16-1* was also downregulated by resveratrol, while usually working along *miR-15* to suppress tumorigenicity by inhibiting cell proliferation and promoting apoptosis of cancer cells [18].

In silico analysis using TargetScan (<http://www.targetscan.org/>) showed that several of miRNAs under resveratrol control potentially target known tumor suppressor genes, such as components of the mismatch repair machinery (MLH3, MSH2 and MSH3), PDCD4 and

PTEN, as well as several effectors and regulators of the TGF $\beta$  signaling pathway (Table 2). Of note, the same miRNAs may also potentially target *DICER1* transcripts, which encode the RNase III producing mature miRNAs from their immediate precursors in the cytoplasm. A majority of the miRNAs potentially targeting the above targets were downregulated by resveratrol (Table 1). Altogether, these results indicate that some of resveratrol antitumor properties may arise from its effects on the levels of miRNAs targeting transcripts encoding key regulators of cell homeostasis.

### 3.2 The levels of tumor suppressor factors encoded by putative target transcripts of oncogenic miRNAs downregulated by resveratrol increase following resveratrol treatment

Although the absolute levels of miRNAs upregulated or downregulated by resveratrol as well as their relative activities on their target transcripts in SW480 cells cannot be definitely ascertained, the above results suggested that resveratrol treatment may change the levels of several of the factors encoded by transcripts targeted by these miRNAs (Table 2). We thus investigated the effects of resveratrol on several factors known to behave as tumor suppressors, such as PTEN and PDCD4, as well as on critical effectors of the TGF $\beta$  signaling pathway. Indeed, the levels of TGF $\beta$  receptors type II and type I (TGF $\beta$ R2 and TGF $\beta$ R1, respectively) increased by roughly 60% and 70%, respectively (Figures 1A and 1C, respectively). The levels of both PTEN, a phosphatase targeting PI3K (Phosphoinositide 3-kinase), and PDCD4, a factor upregulated during apoptosis, also increased by roughly 60% (Figures 1A and 1B, respectively). The levels of E-CADHERIN, a component of adherens junctions implicated in the maintenance of epithelial phenotype, and of SMAD7, a negative regulator of TGF $\beta$  signaling, respectively increased by nearly 50% and 35% (Figures 1B and 1D, respectively). In contrast, the upregulation of SMAD3 following resveratrol treatment was almost neglectable (Figure 1C), which may possibly arise from the fact that *SMAD3* transcripts are also potentially targeted by five miRNAs upregulated by resveratrol, including *miR-1* and *miR-146b-5p*, the two miRNAs which showed the greatest upregulation by resveratrol (Table 1). Nevertheless, resveratrol treatment of SW480 cells globally lead to the increase of the levels of effectors of TGF $\beta$  signaling pathway as well as of several tumor suppressor factors.

### 3.3 Resveratrol upregulates *miR-663*, a miRNA targeting *TGF $\beta$ 1* transcripts

The 3'-UTR of *TGF $\beta$ 1* is short (307 nt in length) and is potentially targeted by no more than 28 miRNAs, which probably results from the existence of multiple levels of regulation of the rate of TGF $\beta$ 1 signaling. Remarkably, however, resveratrol upregulated one and only one of these miRNAs in SW480 cells, namely *miR-663*, a miRNA previously shown to be downregulated in hormone refractory prostate cancer cells [27]. (Table 1). Of note, TGF $\beta$ 1 behaves as a tumor promoter on advanced stages of tumorigenesis, due to its capability to enhance angiogenesis, epithelial-to-mesenchymal transition, cell motility and metastasis [11-13]. Especially, it has been shown that increasing colorectal tumor stage correlates with higher TGF $\beta$ 1 expression in tumor tissues [14]. We therefore double-checked the upregulation of *miR-663* by resveratrol using RNase-protection assays. This experiment suggested that the levels of mature *miR-663* in SW480 cells may increase by roughly 100% following resveratrol treatment (Figure 2), a result in good agreement with those of miRNA micro-array experiments (Table 1). Of note, the levels of *miR-663* primary transcripts, namely *pri-miR-663*, increased 50% further, suggesting that resveratrol may also regulate the processing of *miR-663* from its primary transcripts (Figure 2).

The effects of *miR-663* on *TGF $\beta$ 1* transcripts were subsequently analyzed in SW480 cells transfected with either a control RNA (pre-miR-Control) or *pre-miR-663* along with a construct containing the whole 307 nt-long 3'-UTR of *TGF $\beta$ 1* inserted downstream the *luciferase* gene in the *pGL3-Control* vector in sense or in antisense orientation. *TGF $\beta$ 1* 3'-



UTR namely contains five *miR-663* target sites (TS) (Figure 3A) arranged in two sets of three and two overlapping sites, respectively (sites TS1 to TS3, starting at nt 8, and sites TS4 and TS5, starting at nt 43). *Pre-miR-663* reduced the luciferase activity produced from sense *Luc-TGFβ1* construct by about 60%, while remaining without effects on antisense construct (Figure 3B). The lack of the first 41 nt of *TGFβ1* 3'-UTR, which contain the three first *miR-663* sites (i.e., TS1 to TS3), in *Luc-TGFβ-Del-663-1*, or the mutation of the three same sites by replacing a few nt by the complementary nt in *Luc-TGFβ-M663-1* (Figure 3A), still allowed a residual down-regulation of the luciferase activity of roughly 20%, while mutating the two last *miR-663* sites (TS4 and TS5) in *Luc-TGFβ-M663-2* still lead to about 50% decrease of the luciferase activity (Figure 3B). However, the luciferase activity produced from a construct bearing both sets of mutations (*Luc-TGFβ-M663-1,2*) remained essentially unaffected by resveratrol. Collectively, these results suggest that *miR-663* may target *TGFβ1* transcripts in vivo.

### 3.4 Resveratrol decreases *TGFβ1* expression by both *miR-663*-dependent and *miR-663*-independent mechanisms

We then checked if the downregulation of *TGFβ1* by resveratrol in SW480 cells was *miR-663*-dependent. As shown by luciferase assays, resveratrol as expected decreased the expression of wild type *Luc-TGFβ-WT* as well as of both *Luc-TGFβ-M663-1* and *Luc-TGFβ-M663-2* mutant constructs, an effect impaired by the mutation of the two sets of overlapping *miR-663* target sites in *Luc-TGFβ-M663-1,2* (Figure 4). On the other hand, resveratrol effects on wild-type *Luc-TGFβ-WT* construct were blocked by cotransfecting the cells with a *miR-663* antisense inhibitory oligonucleotide (*663-I*). These results provide a further evidence that resveratrol should target the 3'-UTR of *TGFβ1* transcripts through the upregulation of *miR-663* (Figure 4).

Taking into account that *SMAD3* 3'-UTR also contains three putative *miR-663* target sites, we then investigated the combined effects of resveratrol, *pre-miR-663* and *663-I* on both endogenous *TGFβ1* and *SMAD3*. Without resveratrol treatment, both *pre-miR-663* and *663-I* worked as expected on *TGFβ1*, although with various efficiencies, i.e., *pre-miR-663* and *663-I* respectively decreased and increased the levels of *TGFβ1* (Figure 5). Of note, in the absence of resveratrol, *TGFβ1* levels were more affected by *pre-miR-663* than by *663-I*, suggesting that *miR-663* may normally not exert too tight a control on *TGFβ1* levels in SW480 cells. In agreement with the above results, resveratrol downregulated sharply *TGFβ1* levels. This was probably due to its upregulation of *miR-663*, as *pre-miR-663* proved unable to lower further *TGFβ1* levels (Figure 5). Still, *663-I* effects on *TGFβ1* surprisingly remained neglectable in the presence of resveratrol. By comparison, in the absence of resveratrol, *SMAD3* levels, while being only marginally affected by *pre-miR-663*, increased sharply following transfection with *663-I*, indicating that endogenous *miR-663* usually works to keep *SMAD3* levels below a certain threshold (Figure 5). In contrast, resveratrol effects on *SMAD3* were rather limited, as previously established in section 3.2 (compare Figure 5 and Figure 1C). Strikingly, however, *pre-miR-663* and *663-I* kept working as expected on *SMAD3* in the presence of resveratrol. This suggests that, at least in SW480 cells, resveratrol can also control the expression of *TGFβ1*, but not of *SMAD3*, through genetic circuitries independent of *miR-663*, possibly by decreasing the levels of *TGFβ1* positive regulators targeted by some of the resveratrol-upregulated miRNAs.

### 3.5 Resveratrol decreases the activation of SMADs through the canonical *TGFβ1* signaling pathway

The activation of the *TGFβ1* canonical pathway results in the C-terminal phosphorylation and activation of *SMAD2* and/or *SMAD3*, which then interact with *SMAD4*, that leads to their nuclear import. In the nucleus, *SMADs* collaborate with transcriptional activators and

repressors to control the expression of a number of TGF $\beta$  target genes. Given the above results, we then checked the effects of resveratrol on the expression of a *SMAD2/SMAD3/SMAD4 luciferase* reporter construct in order to measure the degree of activation of the above SMADS (Figure 6). Without TGF $\beta$ 1 treatment, resveratrol slightly but significantly decreased the basal level of expression of the reporter construct, which was possibly due to its effects on endogenous TGF $\beta$ 1 (see section 3.4). As expected, a treatment with TGF $\beta$ 1 robustly increased the expression of the reporter construct in presence of the vehicle. Quite surprisingly, the activation of the reporter construct was nearly completely abolished by resveratrol treatment (Figure 6). Of note, transfecting SW480 cells with *663-I* before resveratrol treatment remained without measurable consequence. This suggests that resveratrol, while restoring the levels of key components of the TGF $\beta$  signaling pathway, such as TGF $\beta$ R1 and TGF $\beta$ R2 (see section 3.2), nevertheless worked to put a cap to the overall activity of this SMAD-dependent pathway in a *miR-663* independent manner, in agreement with the results of Figure 5.

#### 4. Discussion

Our study points to the importance of miRNA regulation in the modulation of the levels of several tumor suppressors and TGF $\beta$  effectors by resveratrol in SW480 colon cancer cells, and provides the very first data concerning the targeting of *TGF $\beta$ 1* transcripts by *miR-663*. It provides strong support that: (a) some of the protective effects of resveratrol come from its downregulation of miRNAs over-expressed in CRCs, such as *miR-17*, *miR-21*, *miR-25*, *miR-26a*, *miR-92a-2*, *miR-103-1* and *-103-2*, or *miR-181a2*; (b) the increase of the levels of proteins encoded by putative target transcripts of these miRNAs roughly correlates with the downregulation of these miRNAs by resveratrol; (c) resveratrol upregulates *miR-663*, one of the few miRNAs targeting *TGF $\beta$ 1* transcripts; (d) resveratrol also controls TGF $\beta$ 1 levels and the rate of TGF $\beta$  signaling through SMAD2/SMAD3/SMAD4 transcription factors using *miR-663*-independent mechanisms.

MiRNA micro-arrays showed that resveratrol downregulated several miRNAs known for their strong oncogenic potentials, including *miR-21* or miRNAs from the *miR-17-92* cluster. This is an important result, for *miR-21* for example is over-expressed in several cancers, including CRCs, gliomas, as well as breast, gastric, prostate, pancreas, lung, thyroid and cervical cancers [17]. *MiR-21* has been shown to function as an onco-miR, due to its targeting of transcripts encoding key regulators of cell proliferation and apoptosis such as PTEN and PDCD4 [28-30]. Especially, PDCD4 is downregulated in a number of cancers, and its suppression in lung and colorectal cancers is associated with poor patient prognosis [31, 32]. Indeed, the levels of these two anti-tumor factors increased following resveratrol treatment of SW480 cells. Also, the downregulation of both *miR-103-1* and *miR-103-2* by resveratrol may possibly be critical to its anti-metastatic effects. Namely, these miRNAs have been recently shown to induce epithelial-to-mesenchymal transition by targeting *Dicer1* transcripts [33], in relation with the fact that a global reduction of miRNA abundance appears a general trait of human cancers, playing a causal role in the transformed phenotype [34-36]. Interestingly, the lower metastatic propensity in SW480 cells as compared with SW620 human colon cancer cells, both derived from the primary tumor and a metastasis of the same patient, respectively [37], was associated with a lower level of expression of *miR-103* [33]. Finally, we also show that the levels of several effectors of TGF $\beta$  pathway signaling, and especially TGF $\beta$ R1 and TGF $\beta$ R2, increase following resveratrol treatment in correlation with the downregulation of miRNAs whose consensus target sequences are present in their 3'-UTRs. This may be the reason why resveratrol favors the tumor suppressor, i.e., the cytostatic activity of TGF $\beta$ 1 in epitheliums [11, 13] at the early stage of cancers. Altogether, the modification of the levels of no less than 48 miRNAs following resveratrol treatment of SW480 colon cancer cells, some well known to be oncogenic but

several of them remaining essentially un-characterized at the present time, is likely to be critical for resveratrol protective effects.

Furthermore, epidemiological studies suggest that as many as 25% of all cancers may be due to chronic inflammation [38, 39]. As *miR-21* in particular is induced by inflammation [40], and resveratrol is known as an anti-inflammatory agent, the downregulation of *miR-21* by resveratrol may be an important step in controlling cell proliferation and further tumorigenesis. In this respect, it is important to note that TGF $\beta$  also functions as a purveyor of immune privilege. While beneficial in initiating and controlling immune responses and maintaining immune homeostasis, immunosuppressive pathways mediated by TGF $\beta$  may obscure immune surveillance mechanisms, resulting in failure to recognize or respond adequately to self, foreign, or tumor-associated antigens [41]. It will thus be important to investigate the possible links between the targeting of TGF $\beta$ R1 or SMAD3 and the development of local inflammation, especially considering that the maturation of *miR-21* is increased upon TGF $\beta$  signaling in a SMAD3-dependent but SMAD4-independent pathway, with SMAD3 binding to *miR-21* primary-transcript and interacting with the microprocessor complex [42].

Among miRNAs upregulated by resveratrol, we identified *miR-663*, a miRNA previously shown to be down-regulated in hormone refractory prostate cancer cells [27]. We present evidence that *miR-663* targets constructs containing *TGF $\beta$ 1-3'-UTR*, and that the levels of TGF $\beta$ 1 in SW480 cells are inversely correlated with the levels of *miR-663*. We also show that resveratrol decreases the expression of *Luc-TGF $\beta$ 1* constructs in a *miR-663*-dependent manner. However, while resveratrol expectedly sharply decreased endogenous TGF $\beta$ 1 levels in SW480 cells, this effect paradoxically remained essentially unaffected by transfecting the cells with either *pre-miR-663* or *663-I*. Of note, we previously observed the same kind of counter-intuitive result with *JunB*, whose 3'-UTR contains two putative target sites for *miR-663*. Namely, while *miR-663* overexpression reduced both the expression of *Luc-JunB* constructs and the accumulation of endogenous JunB in THP-1 monocytic cells, resveratrol showed to decrease the expression of endogenous *JunB* essentially in a *miR-663*-independent manner (Tili et al, unpublished results). Together, these results suggest that one of the effects of resveratrol may be to relocate endogenous *miR-663* along with its target transcripts in particular regions of the cell where they may become inaccessible to both ectopically expressed *miR663* and *miR-663* antisense inhibitory RNAs. In this respect, the hairpin precursor of *miR-663* has been recently shown to be specifically bound by the nuclear factor TDP-43, a Drosha-associated protein which also represents the major component of the inclusions found in the brain of patients with a variety of neurodegenerative diseases [43]. It is thus possible that TDP-43 or other similar factors may bind *miR-663* following resveratrol treatment. Further experiments should shed more light about this hypothesis. It is nevertheless also possible that resveratrol may control the expression of *TGF $\beta$ 1* and/or *JunB* through regulators possibly targeted by other miRNAs sensitive to this compound.

Of note, *miR-663* has just been described as a tumor suppressor miRNA that induces mitotic catastrophe growth arrest in human gastric cancer cells [44]. While this paper do not provide the reader with a mechanistic explanation of this effect, it is tempting to speculate that the anti-tumor effects of *miR-663* may be due, at least in part, to its effects on TGF $\beta$ 1 and SMAD3 accumulation, given that TGF $\beta$ 1 has been shown to promote invasion and metastasis of gastric cancer through its activation of TGF $\beta$ R1-ALK5/SMAD3 pathway [45]. The effects of *miR-663* described here may thus be of primary importance, for TGF $\beta$ 1 has been shown to enhance angiogenesis, epithelial-to-mesenchymal transition, cell motility and metastasis at later stages of cancers [11-13]. Especially, the expression of TGF $\beta$ 1 in both tumor and plasma was found to be significantly higher in patients with metastatic colorectal



cancer, and increasing colorectal tumor stage was correlated with higher TGF $\beta$ 1 expression in tumor tissue [14]. In this respect, it should be emphasized that, in relation with the multiple levels of possible regulation of the rate of TGF $\beta$  signaling, the 307-nt long 3'-UTR of *TGF $\beta$ 1* transcripts represents a potential target for 28 miRNAs only. While two of these miRNAs have two putative target sites in *TGF $\beta$ 1* 3'UTR, this UTR contains only one target site for all the other miRNAs, but *miR-663*. Therefore, the fact that *miR-663* may potentially target 5 different sites in *TGF $\beta$ 1* 3'-UTR suggests that this miRNA could represent a critical TGF $\beta$ 1 regulator which may possibly be called upon action in emergency situations such as those when cells begin to proliferate anarchically. The multiplicity of *miR-663* targets sites in *TGF $\beta$ 1* 3'-UTR further suggests that the effects of this miRNA may be dose-dependent, which would also help to explain the intriguing fact that the expression of both *TGF $\beta$ 1* and *JunB* expression seemed to become *miR-663*-independent following resveratrol treatment of SW480 and THP-1 cells, respectively (this paper, and Tili et al, unpublished results). Of note, previous publications suggested that striking phenotypes may be driven for the most part through small changes in the cellular concentration of key factors. For instance, in the B cell compartment, *miR-150* curtails the activity of the c-Myb transcription factor in a dose-dependent fashion over a narrow range of miRNA and c-Myb concentrations [46].

Furthermore, it has been proposed that an increase in the absolute number of mRNA targets may impact the balance and/or half-life of "free" miRNA, miRNA-RISC, and miRNA-RISC-mRNA complexes [47]. Thus, given the dual role of TGF $\beta$ 1 as both a tumor-suppressor at pre-malignant stage and promoter of metastasis at malignant stage [8, 11-13], it would be interesting to determine if the impact of *miR-663* on *TGF $\beta$ 1* transcripts may vary accordingly to the stage of tumor. Finally, as SMAD2 and SMAD3 phosphorylated at both linker and C-terminal regions transmit malignant TGF $\beta$  signaling in later stages of human CRC [48], and as SMAD7 can act either as a tumor-promoter, by inhibiting the canonical TGF $\beta$  pathway, or as a tumor suppressor, especially by promoting the ubiquitination and thus the proteasomal degradation of  $\beta$ -CATENIN, a key transducer of canonical WNT signaling [7, 49], it is probable that the interactions between TGF $\beta$  signaling and miRNAs sensible to resveratrol, and especially *miR-663*, may be generally both dose- and context-dependent.

As a last remark, the fact that *miR-663*, *miR-21* and TGF $\beta$ 1 have been implicated in the regulation of cell proliferation, tumor apparition and development, metastasis formation and innate immunity, strongly suggests that the capability of resveratrol to behave at the same time as an anti-tumor, anti-metastatic, anti-proliferation and anti-inflammatory agent most probably arises from its effects on the expression of a small set of critical endogenous miRNAs having the abilities to impact the cell proteome globally.

Finally, miRNAs have the promise to become biomarkers for different stages of cancer, both for diagnosis and prognosis. Our analyses of microRNAs that change following resveratrol treatment of SW480 colon cancer cells showed that many of their putative target transcripts encode effectors of the TGF $\beta$  signaling pathway. The discovery that resveratrol may potentially allow to control the behavior of the TGF $\beta$  signaling pathway opens the possibility of using resveratrol in colon cancers where this pathway is impaired or work to favor metastasis. Furthermore, using microRNA analyses turns out to be a powerful technique not only to classify tumors but also to identify the spectrum of genes where a certain drug/chemical might act. Thus, from our experiments, it starts to become clear that the use of resveratrol would be especially beneficial in the type of cancers where the TGF $\beta$  pathway is implicated. Of note, resveratrol use would have to be carefully correlated with the stages of cancers, knowing that TGF $\beta$  can have two faces, i.e. anti- and pro-metastatic.

In conclusion, further investigation of the effects of resveratrol on miRNA populations in tumors, and especially in CRCs, may allow to improve the diagnostic and prognostic of several types of cancers, as well as to optimize the use of this molecule as an anti-tumor agent. Importantly, a critical attention will have to be given to the model organism. Namely, while resveratrol anticarcinogenic potential has been linked with data primarily from human cell culture systems, evidence that resveratrol can inhibit carcinogenesis in several organ sites emerged from results of cancer prevention and therapy studies in laboratory animal models [24]. As *miR-663* is found only in primates, our results come as a warning that studies in animal may not always allow to predict accurately the molecular effects of resveratrol in human, especially when it comes to miRNAs.

## Acknowledgments

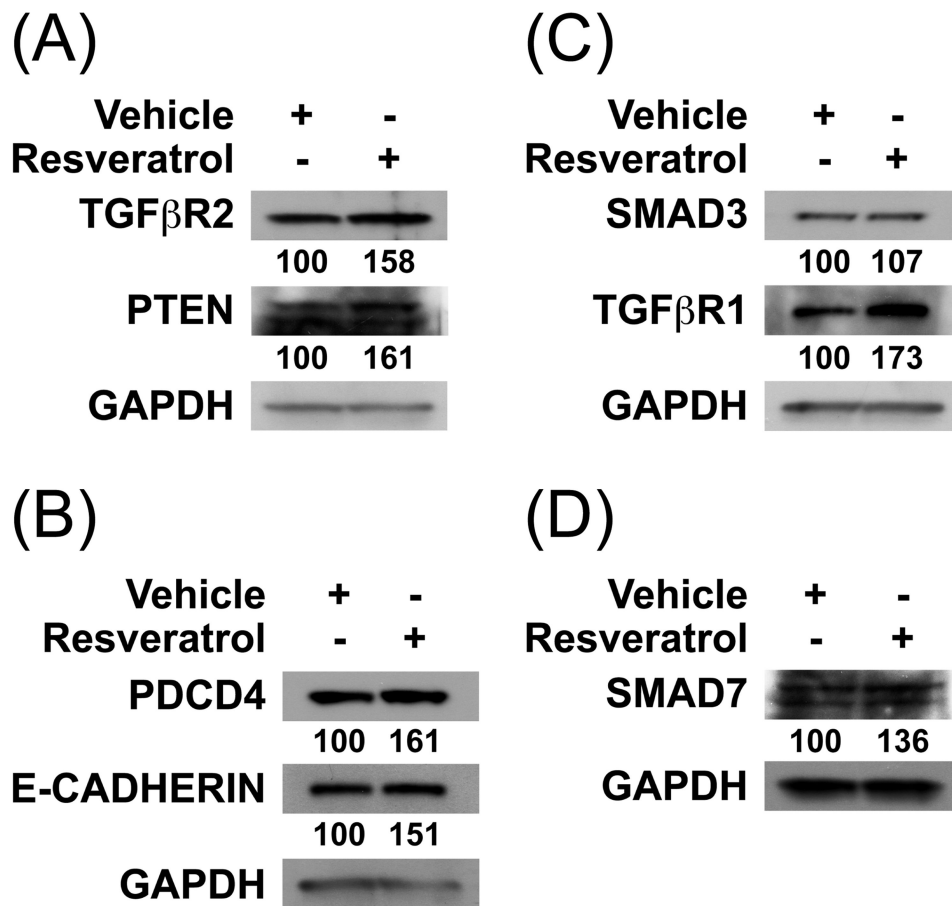
This work was funded by the Ohio State University and the French Cancer League.

## References

1. Tenesa A, Dunlop MG. New insights into the aetiology of colorectal cancer from genome-wide association studies. *Nat Rev Genet.* 2009; 10:3538.
2. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, et al. Cancer statistics, 2006. *Cancer J Clin.* 2006; 56:106–30.
3. Olsson L, Lindblom A. Family history of colorectal cancer in a Sweden county. *Fam Cancer.* 2003; 2:87–93. [PubMed: 14574157]
4. Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, et al. Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark and Finland. *N Engl J Med.* 2000; 343:78–85. [PubMed: 10891514]
5. Cheah PY. Recent advances in colorectal cancer genetics and diagnostics. *Crit Rev Oncol Hematol.* 2009; 69:45–55. [PubMed: 18774731]
6. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell.* 1996; 87:159–70. [PubMed: 8861899]
7. Giles RH, van Es JH, Clevers H. Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim Biophys Acta.* 2003; 1653:1–24. [PubMed: 12781368]
8. Massagué J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell.* 2000; 103:295–309. [PubMed: 11057902]
9. Akhurst RJ. TGF beta signaling in health and disease. *Nat Genet.* 2004; 36:790–2. [PubMed: 15284845]
10. Elliott RL, Blobe GC. Role of transforming growth factor beta in human cancer. *J Clin Oncol.* 2005; 23:2078–93. [PubMed: 15774796]
11. Massagué J. TGFbeta in cancer. *Cell.* 2008; 134:215–30. [PubMed: 18662538]
12. Padua D, Massagué J. Role of TGFbeta in metastasis. *Cell Res.* 2009; 1:89–102. [PubMed: 19050696]
13. Tian M, Schiemann WP. The TGF-beta paradox in human cancer: an update. *Future Oncol.* 2009; 5:259–71. [PubMed: 19284383]
14. Langenskiöld M, Holmdahl L, Falk P, Angenete E, Ivarsson ML. Increased TGF-beta 1 protein expression in patients with advanced colorectal cancer. *J Surg Oncol.* 2008; 97:409–15. [PubMed: 18176914]
15. Calin GA, Croce CM. MiRNA signatures in human cancers. *Nat Rev Cancer.* 2006; 6:857–66. [PubMed: 17060945]
16. Tili E, Michaille JJ, Gandhi V, Plunkett W, Sampath D, Calin GA. 2007. miRNAs and their potential for use against cancer and other diseases. *Future Oncol.* 2007; 5:521–7. [PubMed: 17927518]
17. Krichevsky AM, Gabriely G. miR-21: a small multi-faceted RNA. *J Cell Mol Med.* 2009; 13:39–53. [PubMed: 19175699]

18. Aqeilan RI, Calin GA, Croce CM. miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. *Cell Death Differ.* 2010; 17:215–20. [PubMed: 19498445]
19. Rossi S, Kopetz S, Davuluri R, Hamilton SR, Calin GA. MicroRNAs, ultraconserved genes and colorectal cancers. *Int J Biochem Cell Biol.* 2009;10.1016/j.biocel.2009.05.018
20. Yang L, Belaguli N, Berger DH. MicroRNA and Colorectal cancer. *World J Surg.* 2009; 33:638–4. [PubMed: 19123024]
21. Nicoloso MS, Spizzo R, Shimizu M, Rossi S, Calin GA. MicroRNAs – the micro steering wheel of tumor metastases. *Nat Rev Cancer.* 2009; 9:293–302. [PubMed: 19262572]
22. Csiszar A, Smith K, Labinskyy N, Orosz Z, Rivera A, Ungvari Z. Resveratrol attenuates TNF $\alpha$ -induced activation of coronary arterial endothelial cells: role of NF- $\kappa$ B inhibition. *Am J Physiol Heart Circ Physiol.* 2006; 291:H1694–9. [PubMed: 16973825]
23. Shankar S, Singh G, Srivastava RK. Chemoprevention by resveratrol: molecular mechanisms and therapeutic potential. *Front Biosci.* 2007; 12:4839–54. [PubMed: 17569614]
24. Bishayee A. Cancer prevention and treatment with resveratrol: from rodent studies to clinical trials. *Cancer Prev Res.* 2009; 2:409–18.
25. Athar M, Back JH, Tang X, Kim KH, Kopelovich L, Bickers DR, et al. Resveratrol: a review of preclinical studies for human cancer prevention. *Toxicol Appl Pharmacol.* 2007; 224:274–83. [PubMed: 17306316]
26. Delmas D, Rébé C, Micheau O, Athias A, Gambert P, Grazide S, et al. Redistribution of CD95, DR4 and DR5 in rafts accounts for the synergistic toxicity of resveratrol and death receptor ligands in colon carcinoma cells. *Oncogene.* 2004; 23:8979–86. [PubMed: 15480430]
27. Lin SL, Chiang A, Chang D, Ying SY. Loss of miR-146a function in hormone-refractory prostate cancer. *RNA.* 2008; 14:417–24. [PubMed: 18174313]
28. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A.* 2006; 103:2257–61.
29. Slaby O, Svoboda M, Fabian P, Smerdova T, Knoflickova D, Bednarikova M, et al. Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. *Oncology.* 2007; 72:397–402. [PubMed: 18196926]
30. Selcuklu SD, Donoghue MT, Spillane C. miR-21 as a key regulator of oncogenic processes. *Biochem Soc Trans.* 2009; 37:918–25. [PubMed: 19614619]
31. Chen Y, Knösel T, Kristiansen G, Pietas A, Garber ME, Matsuhashi S, et al. Loss of PDCD4 expression in human lung cancer correlates with tumour progression and prognosis. *J Pathol.* 200; 200:640–6. [PubMed: 12898601]
32. Mudduluru G, Medved F, Grobholz R, Jost C, Gruber A, Leupold JH, et al. Loss of programmed cell death 4 expression marks adenoma-carcinoma transition, correlates inversely with phosphorylated protein kinase B, and is an independent prognostic factor in resected colorectal cancer. *Cancer.* 2007; 110:1697–707. [PubMed: 17849461]
33. Martello G, Rosato A, Ferrari F, Manfrin A, Cordenonsi M, Dupont S, et al. A microRNA targeting Dicer for metastasis control. *Cell.* 2010; 141:1195–207. [PubMed: 20603000]
34. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. *Nature.* 2005; 435:834–8. [PubMed: 15944708]
35. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat Genet.* 2007; 39:673–7. [PubMed: 17401365]
36. Ozen M, Creighton CJ, Ozdemir M, Ittmann M. Widespread deregulation of microRNA expression in human prostate cancer. *Oncogene.* 2008; 27:1788–9. [PubMed: 17891175]
37. Leibovitz A, Stinson JC, McCombs WB 3rd, McCoy CE, Mazur KC, Mabry ND. Classification of human colorectal adenocarcinoma cell lines. *Cancer Res.* 1976; 36:4562–69. [PubMed: 1000501]
38. Hussain SP, Harris CC. Inflammation and cancer: an ancient link with novel potentials. *Int J Cancer.* 2007; 121:2373–80. [PubMed: 17893866]
39. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature.* 2008; 454:436–44. [PubMed: 18650914]

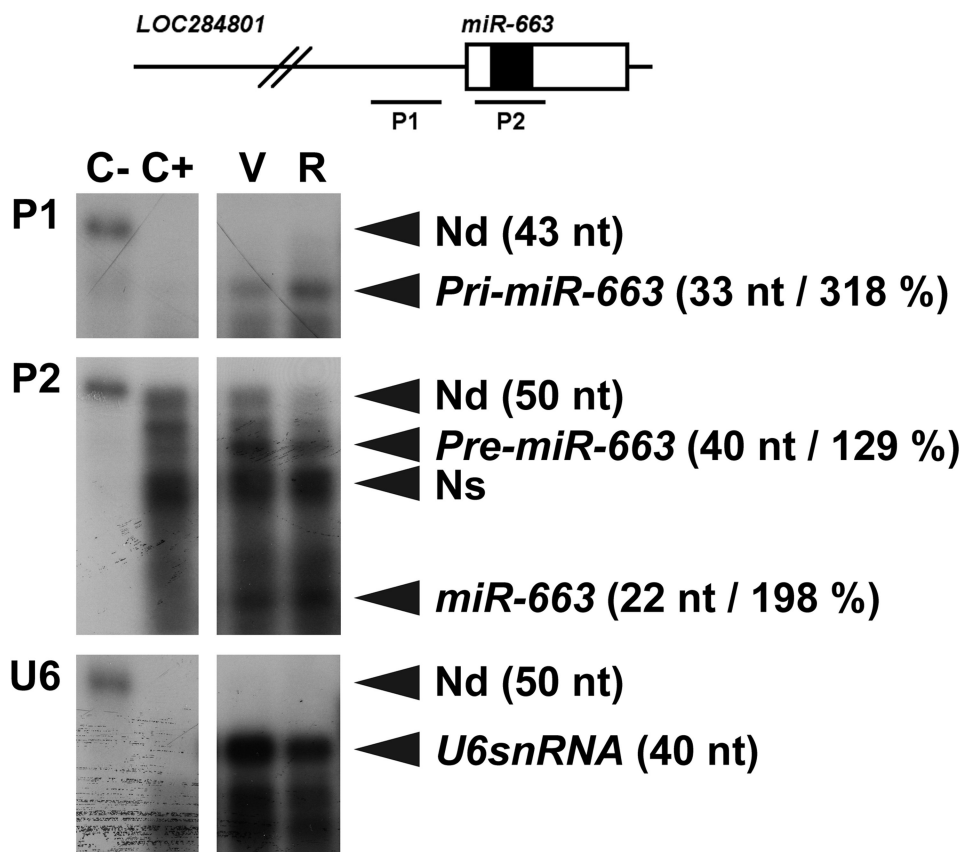
40. Schetter AJ, Heegaard NH, Harris CC. Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis*. 2010; 31:37–49. [PubMed: 19955394]
41. Wahl SM, Wen J, Moutsopoulos N. TGF-beta: a mobile purveyor of immune privilege. *Immunol Rev*. 2006 Oct.;213, 213–27. [PubMed: 16972906]
42. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature*. 2008; 454:56–61. [PubMed: 18548003]
43. Buratti E, De Conti L, Stuani C, Romano M, Baralle M, Baralle F. Nuclear factor TDP-43 can affect selected microRNA levels. *FEBS J*. 2010; 277:2268–81. [PubMed: 20423455]
44. Pan J, Hu H, Zhou Z, Sun L, Peng L, Yu L, et al. Tumor-suppressive miR-663 gene induces mitotic catastrophe growth arrest in human gastric cancer cells. *Oncol Rep*. 2010; 24:105–12. [PubMed: 20514450]
45. Yoo YA, Kang MH, Kim JS, Oh SC. Sonic hedgehog signaling promotes motility and invasiveness of gastric cancer cells through TGF-beta-mediated activation of the ALK5-Smad3 pathway. *Carcinogenesis*. 2008; 29:480–90. [PubMed: 18174246]
46. Xiao C, Colado DP, Galler G, Thai TH, Patterson HC, Wang J, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell*. 2007; 131:146–59. [PubMed: 17923094]
47. Kuchen S, Resch W, Yamane A, Kuo N, Li Z, Chakraborty T, et al. Regulation of microRNA expression and abundance during lymphopoiesis. *Immunity*. 2010; 32:828–39. [PubMed: 20605486]
48. Matsuzaki K, Kitano C, Murata M, Sekimoto G, Yoshida K, Uemura Y, et al. Smad2 and Smad3 phosphorylated at both linker and COOH-terminal regions transmit malignant TGF-beta signal in later stages of human colorectal cancer. *Cancer Res*. 2009; 69:5321–30. [PubMed: 19531654]
49. Yan X, Liu Z, Chen Y. Regulation of TGF- $\beta$  by Smad7. *Acta Biochim Biophys Sin*. 2009; 41:263–72. [PubMed: 19352540]



**Figure 1. The levels of factors encoded by putative target transcripts of miRNAs downregulated by resveratrol increase correspondingly**

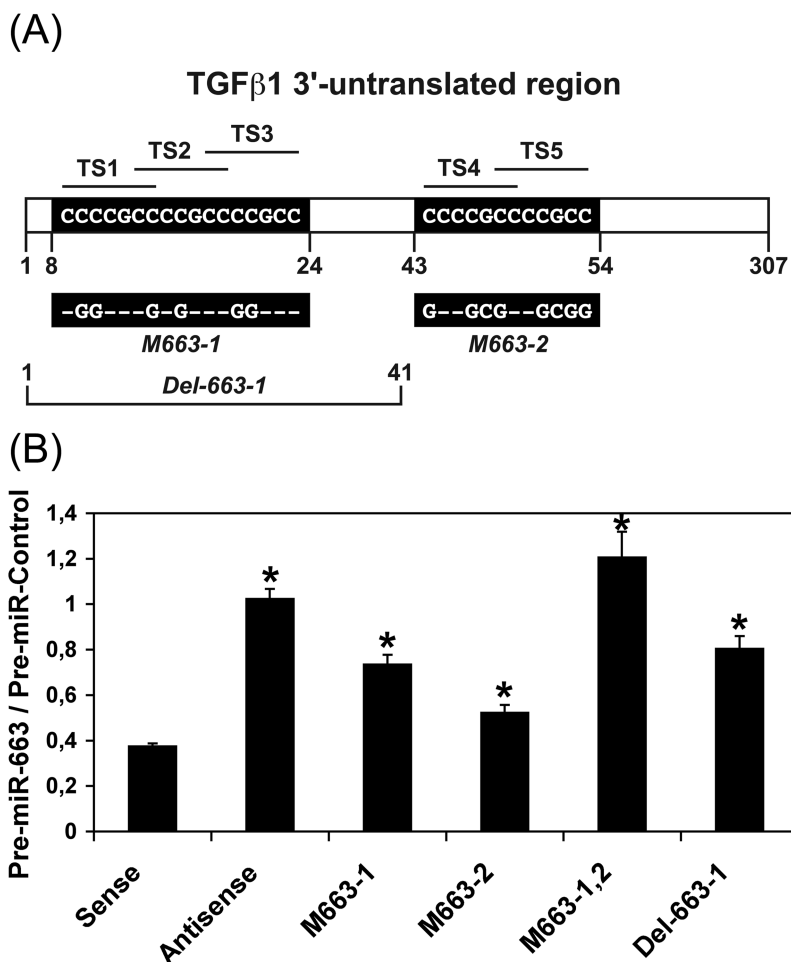
The levels in SW480 cells of tumor suppressors as well as key effectors of TGF $\beta$  signaling pathway were assessed by Western blotting using the indicated antibodies. The relative levels following resveratrol treatment are given in percent of the corresponding control vehicle sample. Panels A to D were from different gels.





**Figure 2. Resveratrol increases the levels of *miR-663* as well as those of *pri-miR-663* and *pre-miR-663* differentially**

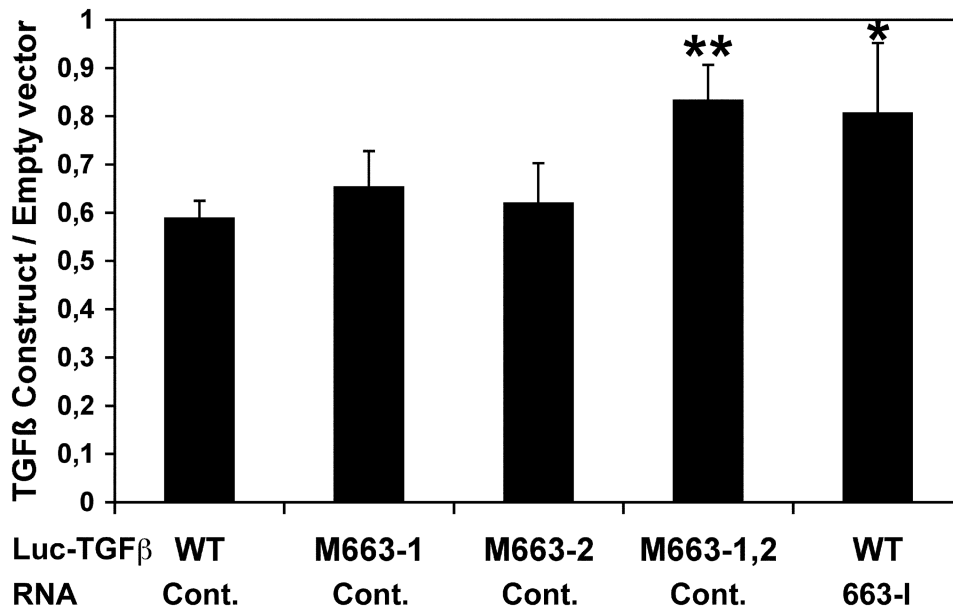
Total RNAs (0.6  $\mu$ g) extracted from SW480 cells treated for 14 hours with either the vehicle (V) or resveratrol (R) were hybridized with a radiolabeled RNA antisense probe (P1, P2 or U6) in the presence of 4.4  $\mu$ g of yeast tRNA. The schematic structure of the *LOC284801* locus, whose transcripts represent *miR-663* primary transcripts, is presented on the unscaled top drawing. Open and filled boxes represent *miR-663* precursor (*pre-miR-663*) and mature *miR-663*, respectively. P1 protects a 33 nt-long fragment starting 184 nt upstream of *pre-miR-663*. *Pre-miR-663* and *miR-663* respectively generate 40 and 22 nt-long protected fragments from P2, which corresponds to one of the miRNA micro-array probes. The relative intensity of the signal following resveratrol treatment is given between parentheses in percent of the vehicle sample, calculated using *U6snRNA* as an internal control. The efficiency of RNase digestion was assessed in parallel (two left lanes). Samples and controls were from the same gels. C-, no RNase (15 fold dilution); C+, RNase; Nd, non digested; Ns, non specific.



**Figure 3. *MiR-663* targets *TGF $\beta$ 1* transcripts**

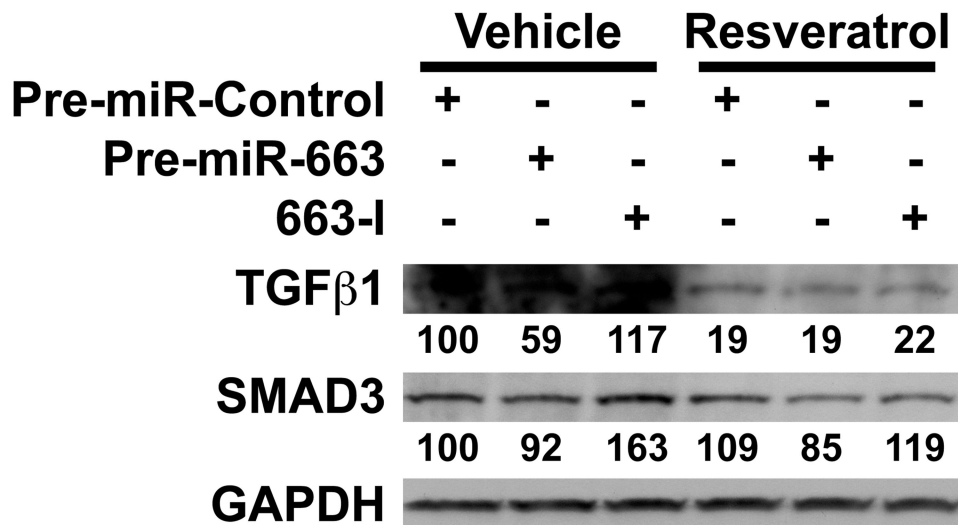
(A) Schematic representation (not to scale) of the 307 nt-long 3'-untranslated region of *TGF $\beta$ 1* transcripts. The positions of the two sets of overlapping *miR-663* target sites (TS1 to TS5) are given in nucleotides. Their sequences (CCCCGCC) are given in black boxes. Nucleotides modified in the two single mutant constructs (*Luc-TGF $\beta$ M663-1* and *Luc-TGF $\beta$ M663-2*) are given below the figure (dashes correspond to conserved nucleotides). *Luc-TGF $\beta$ M663-1,2* contains both sets of mutated nucleotides. The first 41 nucleotides of *TGF $\beta$ 1* 3'-untranslated region is lacking in *Luc-TGF $\beta$ Del-663-1*.

(B) Effects of *pre-miR-663* on *Luc-TGF $\beta$*  constructs. SW480 cells were transfected with wild type *Luc-TGF $\beta$*  (WT) constructs both in sense and antisense orientation, with mutated *Luc-TGF $\beta$*  constructs, (M663-1, M663-2, and M663-1,2), or with a *Luc-TGF $\beta$*  construct bearing a deletion of the first 41 nt of *TGF $\beta$ 1* 3'-UTR (Del-663-1), along with either a Control RNA (Pre-miR-Control) or *pre-miR-663*. The sequences of the two mutated series of overlapping *miR-663* target sites are given in panel A. Values represent the mean  $\pm$  standard deviation ( $n = 4$ ). \*, Significantly different from Pre-miR-Control,  $P < 0.001$  ( $df = 6$ ).

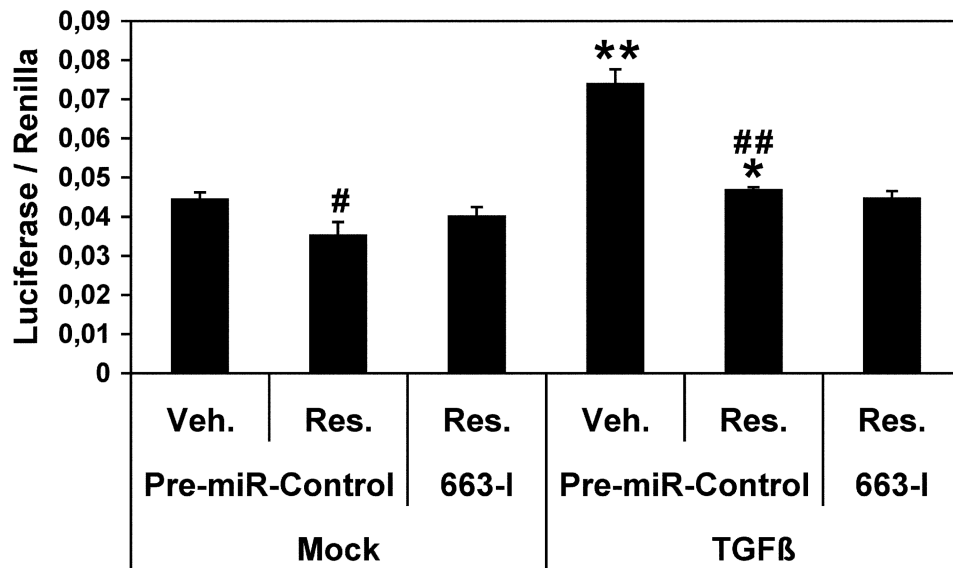


**Figure 4. Resveratrol downregulates *TGFβ1* transcripts through *miR-663***

SW480 cells were transfected with wild type (WT) or mutated (M663-1, M663-2, and M663-1,2) *Luc-TGFβ* constructs, along with either a Control RNA (Cont.) or a *miR-663* antisense inhibitory RNA (663-I) before resveratrol treatment. Bars show the ratios of the relative levels of expression of the different constructs to those of the empty *pGL3-Control* vector. Values represent the mean ± standard deviation (n = 4). \* and \*\*, Significantly different from the corresponding WT / Control, \*,  $P = 0.05$ , \*\*,  $P = 0.017$  (df = 6).



**Figure 5. Resveratrol decreases the levels of TGF $\beta$ 1 mainly in a *miR-663*-independent manner** SW480 cells were transfected with either a control RNA (pre-miR-Control), *pre-miR-663* or an antisense *miR-663* inhibitory RNA (*663-I*) before resveratrol treatment. The levels of TGF $\beta$ 1 and SMAD3 were then assessed on Western blots. The relative levels of TGF $\beta$ 1 and SMAD3 are given in percent of the corresponding Pre-miR-Control Vehicle-treated samples.



**Figure 6. Resveratrol decreases the expression of a *SMAD2/SMAD3/SMAD4* luciferase reporter construct**

After cotransfection with the *SMAD2/SMAD3/SMAD4* luciferase reporter plasmid along with either a Control RNA (pre-miR-Control) or a *miR-663* antisense inhibitory RNA (*663-I*), SW480 cells were mock-treated or treated with TGF $\beta$  for 14 hours, and subsequently treated with the vehicle (Veh.) or resveratrol (Res.) for another 14 hours. The Firefly luciferase activity was measured 48 hours after transfection and then normalized to the *Renilla* luciferase activity. Values represent the mean  $\pm$  standard deviation ( $n = 6$ ). \* and \*\*, TGF $\beta$ 1-treated significantly different from the corresponding Mock-treated, \*,  $P < 0.03$ , \*\*,  $P < 0.002$ ; # and ##, Resveratrol-treated significantly different from the corresponding Vehicle-treated, #,  $P < 0.03$ , ##,  $P < 0.005$  ( $df = 10$ ).



Table 1

Human microRNAs whose levels changed significantly ( $P < 0.05$ ) following resveratrol treatment of SW480 cells, as determined on miRNA microarrays.

* <i>hsa-miR</i>	Resveratrol / Control	Parametric P values	* <i>hsa-miR</i>	Resveratrol / Control	Parametric P values
206	2.748	0.0004106	26a	0.391	0.0153595
560	2.924	0.0005005	594	0.622	0.01711
194-2	2.435	0.0005049	30a-3p	0.374	0.0175082
181a2	0.32	0.0012911	615	3.113	0.0175787
639	2.467	0.0016422	663	1.934	0.0183065
1	11.106	0.0024567	17	0.432	0.0208079
801	3.117	0.0027415	565	0.505	0.0234496
21	0.437	0.0040256	103-1	0.481	0.0263589
424	0.492	0.004825	565	1.762	0.0269349
196a1	0.237	0.0063464	103-2	0.608	0.028218
659	5.35	0.0063984	340	2.975	0.0282464
323	1.926	0.0066204	363*-5p	2.568	0.028538
572	1.972	0.0072194	631	0.47	0.0293983
560	2.084	0.0082416	638	1.712	0.0320884
657	0.473	0.0096236	494	2.038	0.032807
92a-2	0.504	0.0101895	30d	0.587	0.0339924
23a	0.236	0.0108057	622	2.724	0.0363927
16-1	0.588	0.0110056	23b	0.484	0.0366713
25	0.586	0.0117457	102	0.547	0.0414935
146a	0.591	0.0121974	574	1.501	0.0441683
497	1.691	0.0126286	205	0.595	0.0472807
29c	0.58	0.0127887	629	0.53	0.0485583
30c-1	2.204	0.0138886	146b-5p	17.667	0.0490768
100-1/2	0.423	0.0147436	30e-5p	0.496	0.0494118

\* Human microRNAs were arranged based upon increasing P values.

**Table 2**  
**Putative mRNA targets of human microRNAs whose levels were affected by resveratrol treatment of SW480 cells**

Putative target mRNA	Main functions of the encoded factors	Role in oncogenesis	<i>hsa-miR</i> - downregulated by resveratrol	<i>hsa-miR</i> - upregulated by resveratrol
TGFβ1	Binds TGFβR1, TGFβR2 and TGFβR3 / Cytostatic / Induces EMT / Anti-inflammatory / Activation of <i>hsa-miR-21</i> maturation / Reduces the immune response	Tumor-suppressor at pre-malignant stage / Pro-metastasis at malignant stage		663
TGFβ3	Binds TGFβR1, TGFβR2 and TGFβR3	Not clear / Possibly tumor suppressor in skin / Inhibits colon metastasis in TGF m 1 <sup>-/-</sup> Rag2 <sup>-/-</sup> mice	29c / 631	
TGFβR1 / ALK5	SMAD2,3 phosphorylation / TGFβ1-3 signal transduction	Mutated in CRCs	21 / 181a2	1 / 206
TGFβR2	TGFβR1 phosphorylation and activation / TGFβ1-3 signal transduction	Mutated in CRCs	17 / 23a / 23b / 103-1 / 103-2 / 181a2	
TGFβR3 / BETAGLY CAN	Sequestration of TGFβ1-3 (secreted form) / Presentation of TGFβ2 to TGFβ receptors (trans-membrane form) / Loss facilitates EMT in neoplastic cells / TGFβ-independent effects?	Downregulation correlated with tumor progression or metastasis	16-1 / 23a / 23b / 103-1 / 103-2 / 424	1 / 206 / 497
BAMBI	TGFβ decoy receptor impairing the formation of TGFβR1-TGFβR2 complexes / Related to TGFβR1, but lacks the intracellular kinase domain / Overexpressed in metastasis	Increases cellular growth / Enhances WNT signaling and inhibit of TGFβ-SMAD signaling	17 / 146a / 205	146b-5p
SMAD2	Regulatory SMAD phosphorylated and relocated in the nucleus in association with SMAD4 under TGFβ signaling / Transcriptional regulation	Mutated in CRCs	16-1 / 25 / 30d / 30e-5p / 92a-2 / 146a / 205 / 181a2 / 424	30c-1 / 146b-5p / 206 / 340
SMAD3	same as SMAD2	Mutated in CRCs / Overexpressed in late stage sporadic CRCs / pSMAD3L mediates Mesenchymal cell invasion	16-1 / 23a / 23b / 103-1 / 103-2 / 146a / 424	1 / 206 / 146b-5p / 497 / 663
SMAD4	Interacts with phosphorylated regulatory SMADs and relocates them the nucleus / Mediates TGFβ canonical signaling / Transcriptional regulation / Lost or decreased in colon cancer	Tumor suppressor	16-1 / 23a / 23b / 25 / 26a / 92a-2 / 146a / 205 / 424	1 / 206 / 146b-5p / 206 / 340 / 497
SMAD7	Inhibitory SMAD / Interference with regulatory SMADs activation / Negative regulator of BMP and TGFβ signaling pathways / Decreases WNT signaling by recruiting SMURF2 to βCATENIN / Inhibition of NF-κB signaling	Oncogenic by inhibition of TGFβ-mediated growth inhibition / Tumor-suppressor by inhibition of TGFβ-induced metastasis and WNT signaling	16-1 / 17 / 21 / 25 / 92a-2 / 181a2 / 424	340 / 497
SMURF1	E3 ubiquitin ligase / Ubiquitinates SMAD1 and SMAD5 / Degradation of type I	Not clear	16-1 / 17 / 25 / 29c / 92a-2 / 103-2 / 340 / 424 / 659	1 / 206 / 194-2 / 103-1 / 497

Putative target mRNA	Main functions of the encoded factors	Role in oncogenesis	<i>hsa-miR</i> - downregulated by resveratrol	<i>hsa-miR</i> - upregulated by resveratrol
	receptors, regulatory SMADS, SMAD4 and SMAD7			
SMURF2	E3 ubiquitin ligase / Ubiquitinates SMAD1 and SMAD2 / Degradation of type I receptors, regulatory SMADS, SMAD4 and SMAD7 / SMAD2-SMURF2 complexes mediate the degradation of the transcriptional corepressor SnoN	Highly expressed in lymph node metastases / Decreases WNT signaling by ubiquitinating $\beta$ CATENIN	16-1 / 21 / 424	
DICER1	RNase III / Pre-miRNA processing in cytoplasm / <i>miR-103</i> validated target	Inhibition drifts epithelial cancer toward mesenchymal fate	16-1 / 17 / 21 / 23a / 23b / 25 / 29c / 92a-2 / 103-1 / 103-2 / 181a2	1 / 206
PTEN	PI3K phosphatase / Inhibition of cell proliferation	Tumor suppressor	17 / 23a / 23b / 25 / 26a / 29c / 92a-2 / 103-1 / 103-2 / 181a2 / 205	494
PDCD4	Upregulated during apoptosis / Inhibits cell proliferation and tumor angiogenesis pathways / <i>miR-21</i> validated target / Downregulated in CRCs	Tumor suppressor	16-1 / 17 / 21 / 23a / 23b / 160 / 181a2 / 424	340 / 497
MLH3	Mut-L homolog 3 / DNA Mismatch repair	Tumor-suppressor	16-1 / 23a / 23b / 29c / 30d / 30e- / 5p / 103-1 / 103-2 / 424	1 / 206 / 30c-1 / 497
MSH2	Mut-S homolog 2 / DNA Mismatch repair	Tumor-suppressor	21	
MSH3	Mut-S homolog 3 / DNA Mismatch repair	Tumor-suppressor	17	
E-CADHERIN / CDH1	Adherens Junctions / Maintenance of epithelial phenotype / Lost in EMT and metastasis	Inhibitor of invasion	17 / 23a / 23b / 25 / 92a-2 / 103-1 / 103-2	