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Resveratrol induces p53 and suppresses myocardin-mediated vascular smooth muscle cell differentiation

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Abstract

Resveratrol (RSVL), a polyphenolic antioxidant present in red wine, has been shown to provide cardiovascular protection by improving endothelial function and reducing myocardial ischemia. However, little is known about how RSVL affects vascular smooth muscle cells (VSMC) differentiation. RSVL blocks VSMC proliferation *in vitro* and neointimal formation following artery injury *in vivo*. Thus, one might expect that RSVL will promote VSMC differentiation. Unexpectedly, our results in this study show that RSVL induces VSMCs phenotypic modulation; this is characterized by suppressed transcription of SMC-specific marker genes *Tagln*, *Acta2*, *Myh11*, and *Smtn* in a dose-dependent and time-dependent manner in cultured VSMCs. Consistent with previous studies, RSVL induces the nuclear translocation of p53 and the expression of p53-responsive genes such as *Cdkn1a*, *Gadd45a*, *Gadd45*, and *Fas*. In an effort to identify the molecular mechanisms whereby RSVL represses VSMC differentiation, we found that RSVL inhibits the transcription of *Myocd* and *Srf*, the key VSMC transcriptional regulators. However, knockingdown and overexpressing p53 did not affect RSVL-induced VSMCs phenotypic modulation: this suggests that RSVL may induce VSMC dedifferentiation via p53-independent mechanisms. This study provides the first evidence showing that RSVL induces VSMC dedifferentiation by regulating *Myocd* and SRF-mediated VSMC gene transcription.

Keywords

Resveratrol; Smooth muscle cells; Myocardin; SRF; Tagln; p53

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Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

1. Introduction

Phenotypic modulation of vascular smooth muscle cells (VSMCs) plays important roles in normal blood pressure homeostasis and in the pathogenesis of vascular diseases such as atherosclerosis, hypertension, restenosis, as well as aneurysm (Iyemere et al., 2006; Orr et al., 2010). In normal adult arteries, VSMCs maintain the differentiated/contractile phenotype and express high levels of SMC marker genes, such as transgelin (*Tagln*), smooth muscle- α -actin (*Acta2*), smooth muscle myosin chain (*Myh11*) and smoothelin (*Smtn*). However, in response to pathological stimuli, contractile VSMCs undergo phenotypic modulation, as evident by the presence of a variety of distinct VSMC subtypes such as dedifferentiated, proliferative, migratory and inflammatory phenotypes in the vessel wall (Gerthoffer, 2007; Iyemere et al., 2006; Orr et al., 2010). VSMC phenotypic modulation is characterized by the downregulation of SMC-specific marker genes concomitant with the upregulation of genes regulating cell cycle, apoptosis and migration (Yoshida and Owens, 2005). It is often observed that distinct biological processes such as VSMC differentiation/anti-proliferation and proliferation/dedifferentiation are coupled during SMC phenotypic modulation.

Resveratrol (RSVL, 3,5,4'-trihydroxy-trans-stilbene) is a naturally occurring antioxidant polyphenol found in plants such as grapes, peanuts, and mulberries. It is well recognized for its presence in red wine and the association with the "French paradox" (Das and Das, 2007). Accumulating evidence shows that RSVL has direct beneficial effects on cardiovascular system, including improving endothelial function (Wallerath et al., 2002; Zhang et al., 2009), decreasing myocardial ischemic reperfusion injury (Lekli et al., 2008), inhibiting reactive oxygen species generation (Martinez and Moreno, 2000), anti-inflammation (Manna et al., 2000), inhibition of modification of low-density lipoproteins (Frankel et al., 1993), and anti-platelet aggregation (Olas et al., 2002). RSVL also has anti-cancer (Athar et al., 2009) and extending lifespan effects (Howitz et al., 2003). Thus, RSVL has a plethora of beneficial effects on diverse biological processes.

Several studies have shown that RSVL inhibits the VSMCs proliferation induced by such diverse stimuli as advanced glycation end-products (AGEs) (Mizutani et al., 2000), ox-LDL (Liu and Liu, 2004), TNF α (Lee and Moon, 2005), angiotensin II (Chao et al., 2005), and hydrogen peroxide (El-Mowafy et al., 2008). While RSVL clearly prevents VSMCs proliferation in these situations, little is known about the direct effect of RSVL on VSMCs differentiation. In this study, we used *in vitro* tissue cultures to test the hypothesis that RSVL promotes VSMCs differentiation, a process that is generally regarded as being associated with anti-proliferation. However, our results show that RSVL blocked VSMC differentiation in cultured VSMCs.

2. Materials and Methods

2.1. Reagents

Resveratrol (RSVL, # R5010) and actinomycin D (ActD, #A1410) were purchased from Sigma. The antibodies against p53 (#sc-6243, Santa Cruz), β -actin (#4967, Cell Signaling Technology) and GAPDH (#sc-47724, Santa Cruz) were used in this study.

2.2. Cell culture

PAC1 cells (a pulmonary artery-derived SMC line) (Rothman et al., 1992) were maintained in DMEM (Invitrogen) with 10 % FBS at 37 °C with 5 % CO₂. PAC1 cells at 80% confluence were treated with vehicle (DMSO) or RSVL (freshly made) at different dosage for different time. 10T1/2 cell line is an embryonic mesenchymal progenitor cell line (#CCL-226, ATCC).

2.3. Realtime PCR

Total RNA from PAC1 cells was extracted and purified using RNeasy Kit (Qiagen). cDNAs were synthesized using the Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed using the StepOnePlus system (Applied Biosystems) in the presence of SYBR Green. snRNA U6 was used as the internal control, and all PCR primers were designed to cover at least 2 exons (Table 1).

2.4. mRNA stability analysis

To study the role of mRNA stability in mRNA expression regulation by RSVL, PAC1 cells were treated with either DMSO or RSVL (50 μ M) in the presence of the general transcription inhibitor ActD for 1, 3, 6, 12, and 24 hours, respectively. Realtime PCR was used to determine the level of mRNA expression.

2.5. Luciferase assay

The SMC promoter-linked luciferase reporters (50 ng) including Tagln, Acta2, Myh11, 4xTagln CArG and MyoE8 (Myocd enhancer driven) (Creemers et al., 2006; Li et al., 1997; Li et al., 1996; Wang et al., 2001; Yoshida et al., 2003) were transiently transfected with 1 ng CMV-Renilla Luciferase Reporter (Promega) into 10T1/2 or PAC1 cells using the Lipofectamine and Plus transfection reagents (Invitrogen) for 24 hours. The promoter activities were determined by the firefly luciferase activity relative to the internal control renilla luciferase activity using the dual luciferase assay system described by the manufacturer (Promega, Madison, WI).

2.6. Preparation of whole cell lysate and nuclear extracts

Whole cell lysate of PAC1 cells was prepared by using M-PER Mammalian Protein Extraction Reagent (Pierce) with Halt Protease Inhibitor Cocktail (Pierce). NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) plus Halt Protease Inhibitor Cocktail (Pierce) were used to prepare the nuclear extracts. Protein was measured by Qubit fluorometer (Invitrogen).

2.7. Western blot

Equal amounts of proteins were subjected to electrophoresis on 4–12% Bis-Tris NuPAGE Mini-gel (Invitrogen), followed by transfer to PVDF membrane (Millipore). The membrane was blocked with 5% non-fat milk for 1 hour and incubated with primary antibody overnight at 4 °C. After incubation with secondary conjugated antibody for 1 hour, the membrane was subjected to enhanced chemiluminescence detection using SuperSignal West Pico Chemiluminescent Substrate (Pierce). The protein expression levels were quantified by the Adobe Photoshop software.

2.8. p53 silencing by small interfering RNA

p53 silencing was achieved by using Dicer-Substrate siRNA duplexes (IDT). PAC1 cells were transfected with p53 siRNA duplex or scrambled siRNA duplex (src, the universal negative control) at 100 nM by using DharmaFECT3 (Dharmacon). After 48 hours, RSVL and vehicle were added for another 24 hours followed by RNA isolation and realtime PCR assay.

2.9. Statistical analysis

At least three independent experiments were performed for all assays. The values presented are means $\pm$ standard errors of the means (SEMs). Statistical analysis was performed using

one-way ANOVA with post test Dunnett. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. RSVL induces VSMC dedifferentiation

To determine the effects of RSVL on VSMC differentiation, we examined the effect on VSMC morphology and the expression of VSMC markers in VSMCs (PAC1 cells, a rat pulmonary arterial derived VSMC line) treated with RSVL at 40 μ M and 80 μ M for 24 hours (Fig. 1). RSVL did not significantly affect the morphology of cultured VSMCs (Fig. 1A). Consistent with the antiproliferatory effect of RSVL on VSMCs (Poussier et al., 2005), the density of the VSMCs appeared to be suppressed with increased concentration of RSVL (Fig. 1A). Unexpectedly, RSVL decreased mRNA levels of VSMC marker genes such as *Tagln*, *Acta2*, *Myh11*, and *Smtn*, in a time- and dose-dependent manner (Fig. 1B and C). Because RSVL at the concentration of 50 μ M for 24 hours consistently inhibited VSMC marker gene expression, the experiments described below were performed under this condition.

The transient luciferase assays in PAC1 cells showed that RSVL significantly inhibited promoter activities of SMC marker genes (Fig. 1D). Studies of the transcriptional control of SMC-specific gene expression have highlighted the importance of the CArG box in SMC gene regulation (Miano et al., 2007): each SMC-specific marker gene promoter used in the luciferase assays contains the key CArG box regulatory element. Transient transfection assays also showed that RSVL markedly suppressed the luciferase activities driven by the 4 copies of CArGnear boxes in the *Tagln* promoter (Fig. 1D), suggesting that RSVL inhibits CArG-mediated VSMC gene transcription.

3.2. RSVL activated p53 signaling in VSMCs

It is well established that tumor suppressor p53 is involved in RSVL-induced apoptosis on cancer cells (Athar et al., 2009) and anti-proliferation on VSMCs (Mnjoyan and Fujise, 2003; Wang et al., 2006). A recent study demonstrated that p53 down-regulates the expression of myocardin, the master differentiation-inducing transcription factor, and thus inhibits SMC differentiation (Molchadsky et al., 2008). Therefore, it is conceivable that p53 may mediate RSVL-induced VSMCs phenotypic modulation.

As the first step to investigate the mechanism underlying RSVL-induced VSMCs phenotypic modulation, we examined p53-mediated signaling in response to RSVL in PAC1 cells. Consistent with the anti-proliferative effects of RSVL, we found by Western blot assays that RSVL induces p53 expression in both nuclear and whole cell extracts (Fig. 2A). The transcription of several p53-responsive genes such as *Cdkn1a*, *Gadd45a*, *Gadd45b*, and *Fas* was also upregulated 3–15 folds, although the expression of *Tp53* mRNA was not affected (Fig. 2B). This result confirms that p53 signaling is activated in RSVL-treated PAC1 cells.

3.3. RSVL downregulated the transcription of VSMC master regulators myocardin and SRF

Extensive studies have been carried out to characterize the molecular mechanisms that regulate SMC differentiation. The serum response factor (SRF)-mediated transcriptional regulatory network plays a central role in regulating SMC gene expression (Miano et al., 2007). SRF recruits myocardin, a potent SMC transcriptional co-activator, and binds to the CArG box to activate the transcription of an array of SMC contractile genes (Wang et al., 2001). Since RSVL downregulated the CArG box-mediated transcription of SMC differentiation marker genes, we next investigated the effects of RSVL on the expression of

Myocd and *Srf*, two key regulators for SMC differentiation: we found that RSVL markedly downregulated the mRNA levels of *Myocd* and *Srf* (Fig. 3A, 3B). The expression of *Myocd* is reduced about 90% 24 hours after RSVL treatment.

The regulation of gene expression involves transcriptional and posttranscriptional mechanisms. It was reported that RSVL decreases *Cyp11a1* mRNA stability and hence inhibits *Cyp11a1* expression (Lee and Safe, 2001). However, there were studies showing that mRNA stability was not involved in RSVL-induced angiotensin II type 1 receptor (*Agtr1a*) downregulation in VSMCs (Miyazaki et al., 2008). We examined the effect of RSVL on the mRNA stabilities of *Myocd* and *Srf*. ActD is known to inhibit *de novo* mRNA synthesis. The mRNA degradation rate of *Myocd*, *Srf*, and *U6* (the internal control) were similar in the presence and absence of RSVL treatment (Fig. 3C), suggesting that RSVL does not affect the stability of *Myocd* and *Srf* mRNAs. Therefore, RSVL represses the transcription of *Myocd* and *Srf*. Consistent with this result, RSVL significantly decreased *Myocd* enhancer driven promoter activities (MyoE8-luc) in a dose-dependent manner by luciferase assay in PAC1 cells and 10T1/2 cells (Fig. 3D). This result suggests that RSVL inhibited *Myocd* and *Srf* expression at the transcriptional level.

3.4. RSVL-induced VSMCs phenotypic modulation is mediated through p53-independent mechanisms

To explore the role of p53 in RSVL-induced VSMCs phenotypic modulation, we knocked down p53 using p53 siRNAs. Comparing with the scramble siRNA (Scr), p53 siRNA efficiently knocked down p53 protein (>70%, Fig. 4A) and mRNA (> more than 80%, Fig. 4B). The nuclear p53 level is significantly lower after p53 silencing in RSVL cells than in the control cells (scr treated in the presence of RSVL) (Fig 4A). p53 siRNA also efficiently knocked down p53 mRNA in the presence of RSVL to the same extend as in the absence of RSVL (Fig.4B). Under this condition, p53 knockdown decreased the mRNA level of its responsive gene *Cdkn1a*. RSVL-induced upregulation of *Cdkn1a* was also repressed by p53 knockdown (Fig. 4B), suggesting that RSVL induces the expression of *Cdkn1a* via p53-mediated signal pathway.

On the contrary, silencing p53 did not prevent RSVL-induced mRNA downregulation of SMC master regulators *Myocd* and *Srf* nor SMC-specific marker genes such as *Tagln*, *Acta2*, *Myh11*, and *Smtn* (Fig. 4B).

Although RSVL did not affect p53 mRNA silencing and protein downregulation induced by p53 siRNA, the p53 protein level after p53 siRNA treatment in the presence of RSVL was higher than that after Scr siRNA treatment in the absence of RSVL (Fig. 4A). Therefore, we cannot exclude the possibility that RSVL-induced expression of p53 might mediate the inhibitory effect of RSVL on the VSMC marker gene expression. To exclude this possibility, we overexpressed p53 in PAC1 cells by transfection. The Western blot assay showed that the nuclear p53 protein level increases more than 4-fold (Fig. 4C); this could mimic the increased p53 translocation induced by RSVL (Fig. 2A). Interestingly, overexpression of p53 did not significantly change the basal *Myocd*-enhancer driving promoter activity nor RSVL-induced downregulation of *this* promoter activity (Fig. 4D). Taken together, these results indicate that RSVL induces p53 activation in VSMCs and promotes VSMCs phenotypic modulation through p53-independent mechanisms.

4. Discussion

This study demonstrated that RSVL downregulates the transcription of SMC key regulators *Myocd* and *Srf*, and hence decreases SMC maker gene expression. VSMCs phenotypic

switch from contractile to synthetic phenotype play important roles in the pathogenesis of vascular diseases such as atherosclerosis and aneurysm.

RSVL is known for its beneficial cardiovascular and cancer preventive effects. However, RSVL may also have adverse pathological effect such as renal toxicity in rat (Crowell et al., 2004). The present study shows that RSVL induces VSMC dedifferentiation *in vitro*. Since SMC dedifferentiation is usually regarded as a pathological process participating in the pathogenesis of cardiovascular diseases, such as hypertension, atherosclerosis, and aneurysm, it is possible that RSVL may have the potential of inducing VSMCs phenotypic modulation *in vivo*, thus aggravating the pathogenesis of cardiovascular diseases.

In this study the cell models used to carry out the VSMC phenotypic modulation induced by RSVL were cultured PAC1 cells (a rat pulmonary artery-derived SMC line), and 10T1/2 cells (a mouse embryonic mesenchymal progenitor cell line). The PAC1 cells has been shown to retain the VSMC phenotype even after extensive passages as evident by the spindle shaped morphology, the expression of functional surface receptors for angiotensin II, norepinephrine, and alpha-thrombin as well as the expression of smooth muscle markers such as isoforms of alpha-actin, myosin heavy chain, myosin regulatory light chain, and alpha-tropomyosin (Rothman et al., 1992). Nevertheless, it should be noted that cultured VSMC lines, VSMC progenitor cell line, as well as primary VSMCs could behave differently from VSMC and their progenitor cells *in vivo*. In view of this, in future studies the potential adverse effect of RSVL on the vascular system should be examined using animal model of vascular diseases.

VSMC dedifferentiation is characterized by the down regulation of VSMC contractile gene expression, which has been associated with the pathogenesis of vascular diseases such as atherosclerosis and aneurysms (Ailawadi et al., 2009; Lenk et al., 2007; Regan et al., 2000; Shanahan et al., 1994). However, the functional roles of VSMC dedifferentiation in VSMC phenotypic modulation remain unknown. Studies of SM22 knockout mice showed that SM22 deficiency promotes VSMC inflammation in response to high fat diet and carotid artery injury *in vivo* (Feil et al., 2004; Shen et al., 2010). Therefore, downregulation of cytoskeleton proteins may play an active role in promoting VSMC phenotypic modulation in response to injury. Consistent with this notion, the down regulation of smooth muscle cell marker genes occurs early before aneurysm formation (Ailawadi et al., 2009). More evidence suggests that VSMC dedifferentiation may be a phenotype precondition for VSMC remodeling into other subtypes in response to environmental stimuli.

The phenotypic modulation of different VSMC subtypes is controlled by complex regulatory mechanisms involving the regulatory circuits of transcription factors and the crosstalks of intracellular pathways (Yoshida and Owens, 2005). Myocd, and SRF are the key regulators in coordinating the transcriptional regulation of VSMC marker genes. The expression of *Myocd*, and *Srf* has been associated with VSMC phenotypic modulation in response to a variety of intracellular signal pathways and extracellular stimuli such as treatment of PDGF-BB, cyclosporine and loss of tensile stress (Deaton et al., 2009; Garvey et al., 2010; Liu et al., 2005; Wang and Olson, 2004; Yoshida and Owens, 2005; Zheng et al., 2010). To explore the molecular mechanisms mediating RSVL-induced VSMC phenotypic modulation, we examined the expression of *Myocd* and *Srf* in response to RSVL. We found that RSVL suppresses the transcription of *Myocd*, and *Srf* without affecting their mRNA stabilities (Fig. 3).

Although the direct molecular target of RSVL has not yet been identified, RSVL affects the activation of a variety of intracellular signal pathways. The present data showed that RSVL induces p53 activation in VSMCs. Unlike the recent study showing that p53 decreased

myocardin expression and hence inhibited differentiation from mesenchymal cells to SMC (Molchadsky et al., 2008), p53 activation does not appear to participate directly in the RSVL-induced VSMC phenotypic modulation (Fig. 4). The effect of p53 on Myocd-mediated SMC differentiation may depend on the cell models.

To determine the molecular mechanisms underlying RSVL-induced VSMCs phenotypic modulation, we also explored the potential participation of several RSVL regulated targets such as *Sirt1* and β -catenin in SMC phenotypic modulation (Gracia-Sancho et al., 2010) (Zhou et al., 2009). We found that knockingdown *Sirt1* or β -catenin is not sufficient to block RSVL-induced VSMC dedifferentiation by the siRNA knockdown approach (data not shown). Further studies are needed to understand the molecular mechanisms underlying RSVL-induced VSMCs phenotypic modulation.

It is worth noting that microRNAs (miRNAs) have emerged as central players in governing the transcriptional regulatory programs essential for cardiovascular development and diseases (Liu and Olson, 2010). It is possible that RSVL may regulate the expression of miRNAs to inhibit VSMC marker genes expression. It is likely that genome-wide gene profile analyses using microarray and microRNA arrays may reveal the molecular mechanisms mediating RSVL-induced VSMC phenotypic modulation.

5. Conclusions

In summary, this study provides the first evidence demonstrating that RSVL induces VSMCs dedifferentiation via p53-independent mechanisms using both gain-of-function and loss-of-function approaches *in vitro*. Since VSMC phenotypic modulation is prevalent in the pathogenesis of vascular wall remodeling in cardiovascular diseases, these results point to the possibility of RSVL having potential adverse effects on the vascular system under certain conditions in spite of its cardiovascular protective activities.

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Fig. 1.

RSVL suppresses the transcription and promoter activities of VSMC marker genes in cultured VSMCs (PAC1). (A) Effects of RSVL on VSMCs morphology. (B and C) RSVL significantly downregulated mRNA levels of SMC marker genes in dose- and time-dependent manners by q-RT-PCR assay. (D) RSVL (at 50 μ M for 24 hrs) markedly inhibited promoter activities of CARG box-containing promoter activities. * $p < 0.05$ and ** $p < 0.01$ vs vehicle; ## $p < 0.01$ vs Control.

Fig. 2.

RSVL activates p53 signaling in VSMCs. (A) Western blot showed the increased p53 protein expression and nucleus translocation induced by RSVL at 50 μ M (left panel). NE and WC indicate nuclear extract and whole cell lysate, respectively. Quantification of p53 protein levels in WC is shown in the right panel. ** $p < 0.01$ vs Vehicle. (B) PAC1 cells were treated with 50 μ M RSVL for 24 hours. RSVL did not affect *Tp53* mRNA expression but markedly enhanced mRNA expression of p53-responsive genes including *Cdkn1a*, *Fas*, *Gadd45a* and *Gadd45b* by qRT-PCR. * $p < 0.05$ and ** $p < 0.01$ vs U6.

Fig. 3.

RSVL suppresses the transcription of SMC master regulators myocardin/SRF. (A and B) mRNA levels of *Myocd* and *Srf* in response to RSVL in dose- and time-dependent manners were measured by the qRT-PCR assay. ** $p < 0.01$ vs Control; ## $p < 0.01$ vs Vehicle. (C) Effect of actinomycin D (ActD) on RSVL-induced *Myocd* and *Srf* mRNA downregulation was determined by the qRT-PCR assay. The mRNA expression levels in VSMCs before adding ActD were set as 100 %. n.s.: not significant. (D) The effects of RSVL dosages on *Myocd* enhancer (MyoE8) driven promoter activities were measured by the luciferase assays at 24 hours in both 10T1/2 cells and PAC1 cells. * $p < 0.05$ and ** $p < 0.01$ vs Vehicle.

Fig. 4. RSVL induces VSMCs phenotypic modulation through p53-independent mechanisms in PAC1 cells. (A) the effect of p53 silencing on nuclear p53 protein level in the presence or absence of RSVL (50 μ M for 24 hours) by Western blot (left panel) with the quantification of nuclear p53 protein level change after being normalized by the internal control β -actin (right panel). (B) The effect of knockingdown p53 by p53 siRNA on RSVL-induced VSMC dedifferentiation. (C) The expression level of the nuclear p53 protein level after pCEP4-p53 transfection was measured by Western blot (left panel) with the quantification of the nuclear p53 protein level (right panel). (D) The effect of pCEP4-p53 transfection on *Myocd* enhancer driven promoter activities in the presence and absence of RSVL. V and R indicate

the vehicle and resveratrol respectively. Mock and p53 refer to pCEP4 and pCEP4-p53 plasmids respectively.

Tab.1

Sequences of primers used for real-time PCR

Gene	Sequence	Amplicon size (bp)	GenBank accession #
U6	Forward 5'-CTCGCTTCGGCAGCACATATACTA-3'	92	NR_002752
	Reverse 5'-CGCTTCACGAATTTGCGTGTATC-3'		
Acta2	Forward 5'-ATCTTTTCCATGTCGTCCAGTTG-3'	310	NM_031004
	Reverse 5'-GAGAAGCCCAGCCAGTCG-3'		
Tagln	Forward 5'-TCCTTCCAGCCACAAACGACCAA-3'	92	NM_031549
	Reverse 5'-CTTGGACTGCACTTCACGGCTCAT-3'		
Myh11	Forward 5'-AATGCCCTCAAGAGCAAACCTCAG-3'	122	NM_001170600
	Reverse 5'-TCCCGAGCATCCATTTCTTCTTCA-3'		
Smtn	Forward 5'-TACATCCAGGAGTTCTACCGCTGTCT-3'	180	NM_001013049
	Reverse 5'-CGATTGCACATAGGTGAAGACGCA-3'		
Srf	Forward 5'-AAAGAAACCGTGAAGGCAACA-3'	224	NM_001109302
	Reverse 5'-ACAGCACCGTCTGGGACCGT-3'		
Myocd	Forward 5'-CAGTGAAGCAGCAAATGACTCGG-3'	230	NM_182667
	Reverse 5'-GTCGTTGGCGTAGTGATCGAAGG-3'		
Tp53	Forward 5'-GCACGTACTCAATTTCCCTCAAT-3'	64	NM_030989
	Reverse 5'-AACTGCACAGGGCATGTCTTC-3'		
Cdkn1a	Forward 5'-GGACATCTCAGGGCCGAAA-3'	64	NM_080782
	Reverse 5'-CGGCGCTTGAGTGATAGA-3'		
Gadd45a	Forward 5'-ACACTGTGTGCTGGTGACGAA-3'	80	NM_024127
	Reverse 5'-CCCGGCAAAAACAAATAAGTTG-3'		
Gadd45b	Forward 5'-CCTGGTCACGAAGTGCATACAG-3'	67	NM_001008321
	Reverse 5'-TAACTGGCCACCTCCACCAA-3'		
Fas	Forward 5'-ATGCTTCTCTGTGACCACTGTTA-3'	67	NM_139194
	Reverse 5'-CAAGGCTCAAGGATGTCTTCAA-3'		