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# Nicotine-mediated induction of *E-selectin* in aortic endothelial cells requires Src kinase and E2F1 transcriptional activity

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ABSTRACT

Smoking is highly correlated with enhanced likelihood of atherosclerosis by inducing endothelial dysfunction. In endothelial cells, various cell-adhesion molecules including *E-selectin*, are shown to be upregulated upon exposure to nicotine, the addictive component of tobacco smoke; however, the molecular mechanisms underlying this induction are poorly understood. Here we demonstrate that nicotine-induced *E-selectin* transcription in human aortic endothelial cells (HAECs) could be significantly blocked by α7-nAChR subunit inhibitor, α-BT, Src-kinase inhibitor, PP2, or siRNAs against Src or β-Arrestin-1 (β-Arr1). Further, chromatin immunoprecipitations show that *E-selectin* is an E2F1 responsive gene and nicotine stimulation results in increased recruitment of E2F1 on *E-selectin* promoter. Inhibiting E2F1 activity using RRD-251, a disruptor of the Rb–Raf-1 kinase interaction, could significantly inhibit the nicotine-induced recruitment of E2F1 to the *E-selectin* promoter as well as *E-selectin* expression. Interestingly, stimulation of HAECs with nicotine results in increased adhesion of U937 monocytic cells to HAECs and could be inhibited by pre-treatment with RRD-251. Similarly, depletion of E2F1 or Src using RNAi blocked the increased adhesion of monocytes to nicotine-stimulated HAECs. These results suggest that nicotine-stimulated adhesion of monocytes to endothelial cells is dependent on the activation of α7-nAChRs, β-Arr1 and cSrc regulated increase in E2F1-mediated transcription of *E-selectin* gene. Therefore, agents such as RRD-251 that can target activity of E2F1 may have potential therapeutic benefit against cigarette smoke induced atherosclerosis.



## 1. Introduction

Cardiovascular diseases are the principal causes of death in the United States, Europe, and much of Asia [1]. Extensive statistical and clinical studies have identified cigarette smoking as one of the major independent risk factor of heart diseases. According to the American Heart Association, smokers are 2–4 times more at risk of developing heart diseases than nonsmokers. Among smokers, atherosclerosis is the major form of heart disease [2,3]. Atherosclerosis is an inflammatory process that involves various cellular and molecular events and is characterized by the progressive accumulation of lipid, fibrous tissues and cell components in large and medium-sized elastic and muscular arteries [4]. This severely reduces blood flow and leads to ischemia of the heart, brain, or extremities. Among all the components of tobacco smoke, nicotine is found to have the maximum pro-atherosclerotic effects [5–9].

Exposure to risk factors, including tobacco smoke, causes endothelial dysfunction that results in the recruitment of circulating leukocytes, mainly monocytes and some T-cells to the sites of injured endothelial cells [4,10]. These monocytes adhere to the endothelial cells and migrate into the sub-endothelial space, where they differentiate into activated macrophages that are efficient scavengers of oxidized low-density lipoprotein (LDL) [10]. In the presence of increased amounts of oxidized LDL, these macrophages accumulate large amounts of cholesteryl esters in lipid droplets and become "foam cells" that form "fatty streaks"; these then have the potential to develop into complicated atherosclerotic plaques [4,10]. Studies in mouse models have shown that the atherogenesis is greatly attenuated in the absence of monocyte and macrophage recruitment to injured endothelium, even in the presence of high lipid levels [11]. This suggests that leukocyte-endothelial cell interactions are needed for the initiation and progression of atherosclerosis.

In the presence of several atherogenic stimuli, increased adhesiveness of the circulating leukocytes on the injured endothelium depends on the expression of specific cellular adhesion molecules [12,13]. These include several selectins and intercellular adhesion molecules which act as receptors for glycoconjugates and integrins

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present on monocytes and T-cells [12,14]. Knockout of *E-selectin* resulted in decreased atherosclerosis in *in vivo* models [15,16]. Moreover, increased expression of *E-selectin* is found in the endothelium of human atherosclerotic lesions [14,17–19]. Thus, reducing the expression of *E-selectin* may lead to a reduction in the leukocyte-endothelial cell interactions and may ultimately lead to a decreased initiation of atherosclerosis. Our earlier studies had shown that cytokines like TNF-α could induce apoptosis of human aortic endothelial cells [20], while inducing proliferation of aortic smooth muscle cells in an E2F1 dependent manner [21]. In the present study, we report the E2F1-mediated transcriptional upregulation of *E-selectin* expression upon nicotine exposure and the reduction of *E-selectin* expression through inhibition of E2F1-mediated transcription through a small molecule inhibitor RRD-251. This results in reduced adhesion of monocytic U937 cells to HAECs. These results show for the first time that the Rb-E2F transcriptional regulatory pathway contributes to the expression of genes involved in promoting atherosclerosis.

## 2. Materials and methods

### 2.1. Cell culture and reagents

Primary human aortic endothelial cells (Lonza) were cultured in endothelial growth medium (EGM) supplemented with endothelial growth factors and 5% FBS (Cambrex). Experiments were done on cells that were within passages 2–7. Human monocytic cell line U937 [22] was purchased from ATCC and cultured in RPMI-1640 medium supplemented with 10% FBS and used as surrogate for monocytes. Src-kinase inhibitor, PP2 and α7-nAChRs inhibitor α-bungarotoxin (α-BT), were purchased from Sigma Chemical Company. RRD-251 was synthesized as described earlier [23]. Nicotine, at a concentration (1 μM) that typically found in the blood stream of a heavy smoker, was used as stimulus [24]. Unless otherwise noted, 20 μM of RRD-251 was used in the experiments.

### 2.2. siRNA transfections

siRNA for cSrc (Src), β-Arrestin-1 (β-Arr1) and E2F1 were purchased from Santa Cruz Biotechnology Inc., 20 picomoles of siRNAs were transfected in each well of a standard 96 well plate and 100 picomoles in 60 mm dishes using Oligofectamine reagent (Invitrogen) as per manufacturer's instructions [25]. A non-targeting siRNA sequence was used as control.

### 2.3. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were conducted on $2 \times 10^7$control, nicotine or serum stimulated cells as indicated following published protocols [25–27]. Following primer sequences were used to amplify the region spanning the E2F binding sites on *E-selectin* promoter: forward 5′-TCTCCCCAGGAAAGTATTTCAAGCC-3′ and reverse 5′-GGA CAGCCCCAGACAAGCAA-3′.

### 2.4. RNA extraction and real-time reverse transcription-PCR

Total RNA was extracted and purified according to manufacturer's instructions using RNeasy Mini Kit (Qiagen). Reverse Transcription reaction was then carried out to synthesize cDNA using iScript (Bio-Rad). Real-time PCR was done with 1 μL of the reverse transcription product in a MyiQ real-time PCR detection system (Bio-Rad) by using iQ SYBR Green PCR Supermix (Bio-Rad) as described before [25,28,29]. The PCR cycling conditions used were as follows: 40 cycles of 15 s at 95 °C, 15 s at 55 °C and 20 s at 72 °C. Fold inductions were calculated using the formula $2^{-(ddCt)}$ using GAPDH as internal control genes. The gene-specific primer pairs were as follows. E-selectin-F 5′-TGAAGCTCCCACTGAGTCCA A-3′, E-selectin-R 5′-GGTGCTAATGTCAGGAGGGAGA-3′, GAPDH-F 5′-GGTGGTCTCCTCTGACTTCAACA-3′ and GAPDH-R 5′-GTTGCTGTA GCCAAATTCGTTGT-3′.

### 2.5. Monocyte adhesion assay

CytoSelect™ leukocyte-endothelium adhesion assay kit was purchased from Cell BioLabs. U937 cells were labeled with LeukoTracker™ according to manufacturer's instructions. The endothelial cells were grown to full confluency in a 96 well plate and were rendered quiescent by culturing in EGM containing 0.5% FBS for 24 h; subsequently, the cells were treated with 1 μM nicotine for 3 h. Following treatment, the endothelial layer was washed with serum-free EGM and $10^5$ labeled U937 cells were added per well. The co-culture was incubated for 60 min, unadhered cells washed off. Adhesion was visualized using an inverted florescence microscope and three different fields were counted.

### 2.6. Statistical analysis

Experiments were done in triplicate and the statistical significance evaluated using Student's *t* test.

## 3. Results

### 3.1. Nicotine induces E-selectin expression in β-Arr1-Src-regulated manner

Our earlier studies have shown that nicotine stimulation of lung cancer cells induced the expression of E2F-regulated proliferative genes in α7-nicotinic acetylcholine receptor (α7-nAChR), β-Arr1 as well as Src dependent manner [24,25]. Since nicotine has been reported to induce *E-selectin* expression, we examined whether similar molecular mechanisms are involved in its induction in HAECs. Towards this purpose, HAECs were rendered quiescent by culturing in 0.5% serum for 24 h followed by three hours of treatment with nicotine (1 μM) alone or in presence of α7-nAChR inhibitor α-BT (1 μM) or Src-kinase inhibitor PP2 (1 μM). As shown in Fig. 1A, nicotine treatment resulted in approximately 3-fold increase in the expression of *E-selectin* mRNA, as seen by RT-PCR. In agreement with previous results, we also found a significant inhibition of nicotine-induced *E-selectin* expression in α-BT treated cells [6,30] confirming its dependence on α7-nAChR mediated signaling. Interestingly, inhibition of Src by its inhibitor PP2 could completely inhibit the induction of *E-selectin* transcription by nicotine. Our earlier studies had shown a role for the scaffolding protein, β-Arr1, in the nicotine-mediated activation of Src and proliferation of lung cancer cells [24]. To ascertain whether a similar pathway was involved, siRNA to β-Arr1 or Src were transiently transfected into HAECs. Cells transfected with siRNA were rendered quiescent for 24 h followed by 3 h of treatment with nicotine (1 μM). As demonstrated in Fig. 1B, nicotine mediated induction of *E-selectin* transcription was significantly decreased in Src as well as β-Arr1 depleted cells. Depletion of Src and β-Arr1 was confirmed by RT-PCR analysis (Fig. 1C and D). This suggests that nicotine-mediated induction of *E-selectin* in HAECs involve α7-nAChR, β-Arr1 and Src dependent signaling.

### 3.2. Nicotine-induced E-selectin expression is E2F1 regulated

Our earlier studies had shown that nicotine-stimulated β-Arr1–Src activation, results in the enhanced interaction between Raf-1 kinase and retinoblastoma tumor suppressor protein, Rb. This led

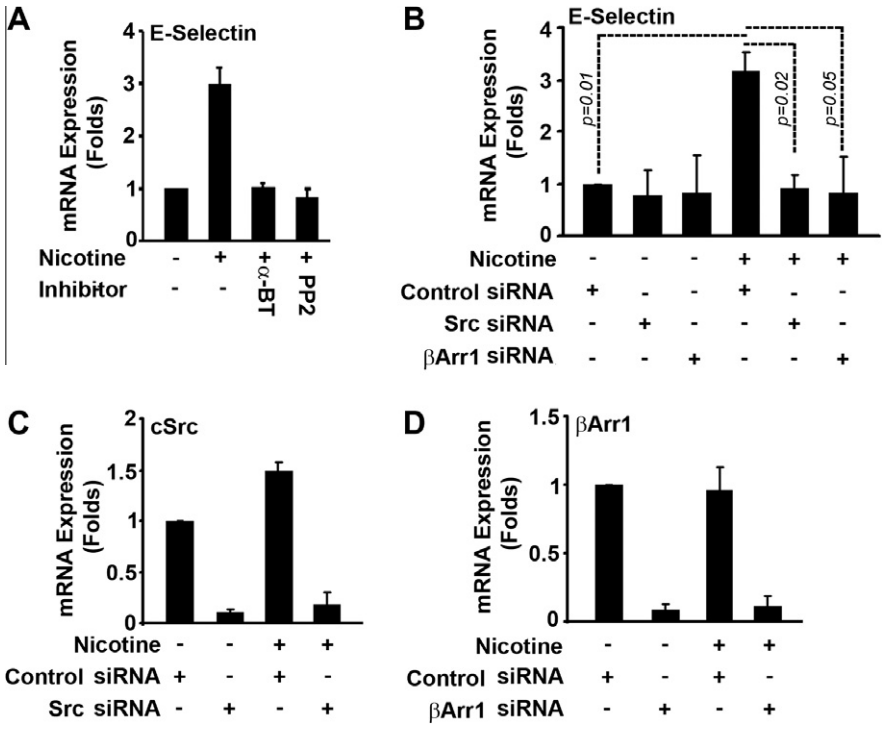


**Fig. 1.** Signal transduction dependent induction of *E-selectin* transcription in response to nicotine stimulation. (A) Nicotine-induced *E-selectin* transcription was inhibited by of α7-nAChR inhibitor α-BT or Src inhibitor PP2. Data represent the mean ± SD ($p < 0.05$). (B) Nicotine-induced *E-selectin* transcription was suppressed by β-Arr1 or Src siRNA. (C and D) Suppression of Src expression by Src siRNA (C) and suppression of β-Arr1 expression by β-Arr1 siRNA was evaluated by RT-PCR analysis.

to the phosphorylation-mediated inactivation of Rb, resulting in enhanced E2F1-mediated transcription [24,25]. To assess if similar events are involved in the nicotine-mediated induction of *E-selectin* in HAECs, we first examined the *E-selectin* promoter for E2F1-bidning sites. Analysis of the promoter regions of human *E-selectin* gene using the MatInspector program (Genomatix Software Inc.) revealed three putative-E2F-binding sites very close to the transcription start site (TSS), as shown in Fig. 2A.

To examine the possible regulation of *E-selectin* promoter by E2F1 transcription factor, HAECs were transiently transfected with E2F1-expression vector or a control vector. The expression of endogenous *E-selectin* mRNA was found to be upregulated by approximately 3-fold as compared to mock-transfected cells (Fig. 2B). This suggests that E2F1 can regulate *E-selectin* gene transcription in HAECs. Next, the binding of endogenous E2F1 on *E-selectin* promoter was determined by ChIP assays. Quiescent HAECs were serum stimulated for 3 h and ChIP assay was performed as described in materials and methods. As shown in Fig. 2C, E2F1 was recruited on *E-selectin* promoter upon serum stimulation, correlating with the transcriptional induction.

### 3.3. Inhibition of E2F1 activity by RRD-251 results in decreased E-selectin expression

The transcriptional activity of E2F1 is regulated by its physical interaction with the Rb protein [24,31,32]. Our laboratory had shown that in response the signaling kinase Raf-1 can physically interact with Rb upon nicotine stimulation, leading to its phosphorylation and inactivation in HAECs [24,25]. Furthermore, the disruption of Rb–Raf-1 interaction by a small molecule inhibitor of Rb–Raf-1 interaction, RRD-251, inhibits the expression of E2F1 target genes [23,32]. This suggests that preventing the binding of Raf-1 to Rb eventually activates Rb and results in E2F repression [31]. The possibility of using RRD-251 as an inhibitor of E2F1-mediated E-selectin expression in HAECs was explored.

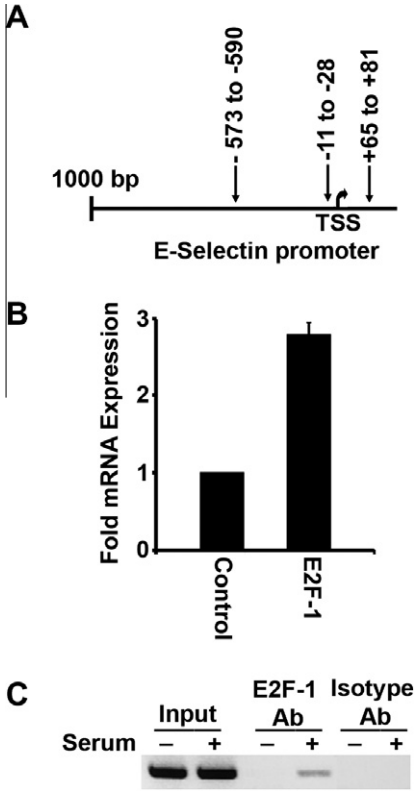


**Fig. 2.** *E-selectin* promoter is E2F1 responsive. (A) Three putative-E2F-binding sites are shown in relation to TSS. (B) *E-selectin* was significantly upregulated at transcriptional level upon transient overexpression of E2F1 in HAECs ($p < 0.05$). (C) ChIP assay showing the binding of endogenous E2F1 on *E-selectin* promoter in serum stimulated HAECs.

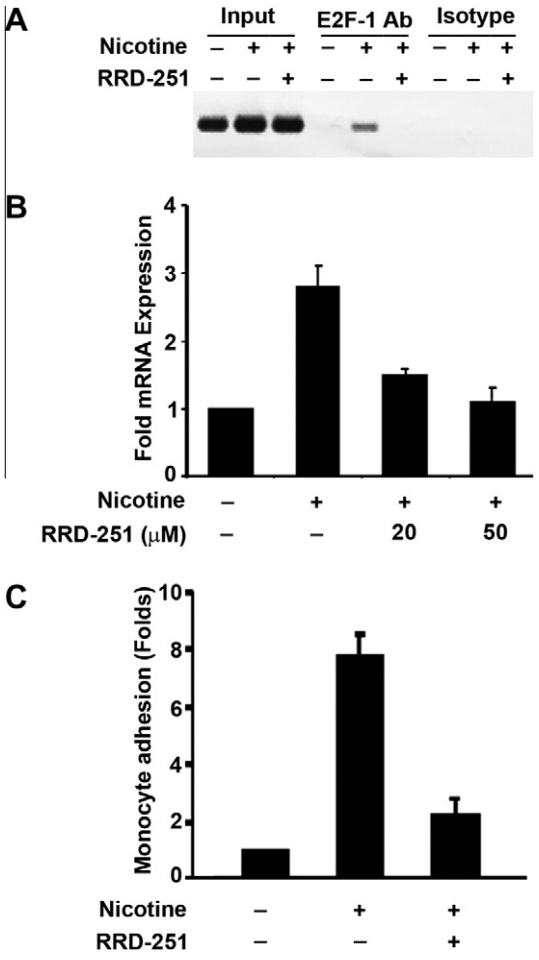


**Fig. 3.** RRD-251 inhibits nicotine mediated induction of *E-selectin* and monocyte binding. (A) Nicotine-stimulated E2F1 binding on *E-selectin* promoter was suppressed by RRD-251 as seen in a ChIP assay. (B) Nicotine-stimulated expression of *E-selectin* was significantly suppressed by RRD-251 in dose dependent manner. (C) U937 monocytic cells adhered on nicotine-stimulated HAECs, which was significantly blocked by RRD-251 ($p < 0.05$).

Quiescent HAECs were stimulated with 1 μM nicotine in the presence or absence of 20 μM RRD-251 for 3 h. Nicotine-induced recruitment of E2F1 on *E-selectin* promoter was analyzed by ChIP assay. As shown in Fig. 3A, E2F1 was recruited on *E-selectin* promoter upon nicotine stimulation. Interestingly, this recruitment was completely suppressed in RRD-251 treated cells. Real-time PCRs were conducted to examine whether the inhibition of E2F1 binding to the promoter upon RRD-251 treatment correlated with a repression of *E-selectin* expression; treatment with RRD-251 significantly suppressed the nicotine-induced expression of *E-selectin* gene (Fig. 3B), correlating with the lack of E2F1 recruitment to the promoter.

### 3.4. Suppressed adhesion of monocytes on endothelial cells by E2F1 and Src inhibition

Attempts were made to assess whether induction of *E-selectin* by nicotine had an effect on the adhesion of monocytes on HAECs. Specifically, monocyte adhesion assay was performed on nicotine-stimulated HAECs in presence or absence of RRD-251 at indicated doses. There was a significant increase in the number of monocytes that bound to the nicotine-stimulated endothelial cells (Fig. 3C). Interestingly, the adhered monocytes were significantly reduced in presence of RRD-251 (Fig. 3C). These results suggest that repressing the transcriptional activity of E2F1 by RRD-251 leads to a decrease in the expression of *E-selectin* which suppressed monocyte binding on nicotine-stimulated HAECs.

The direct role of E2F1 and Src in monocyte–endothelial cell interaction was confirmed by targeting the endogenous expression of these proteins using siRNA approach. Monocyte adhesion assays were performed on nicotine-stimulated HAECs, after transient transfection of a non-targeting control siRNA, E2F1 siRNA or Src siRNA. Upon Nicotine stimulation, HAECs, transfected with Src1 siRNA or E2F1 siRNA showed a significant decrease in the number of adhered monocytes as compared to control siRNA transfected cells. The representative images from two different experiments are shown in Fig. 4A. The average number of adhered monocyes from three different fields of two different experiments were counted and represented as histogram in Fig. 4B. These results revealed a pivotal role Src-kinase and E2F1 functions in the monocyte adherence on HAECs.

## 4. Discussion

Pathophysiological effects of cigarette smoke components are associated with cardiovascular diseases like atherosclerosis. While recent studies have demonstrated that the expression of cell-adhesion molecules is regulated by cigarette smoke condensate as well

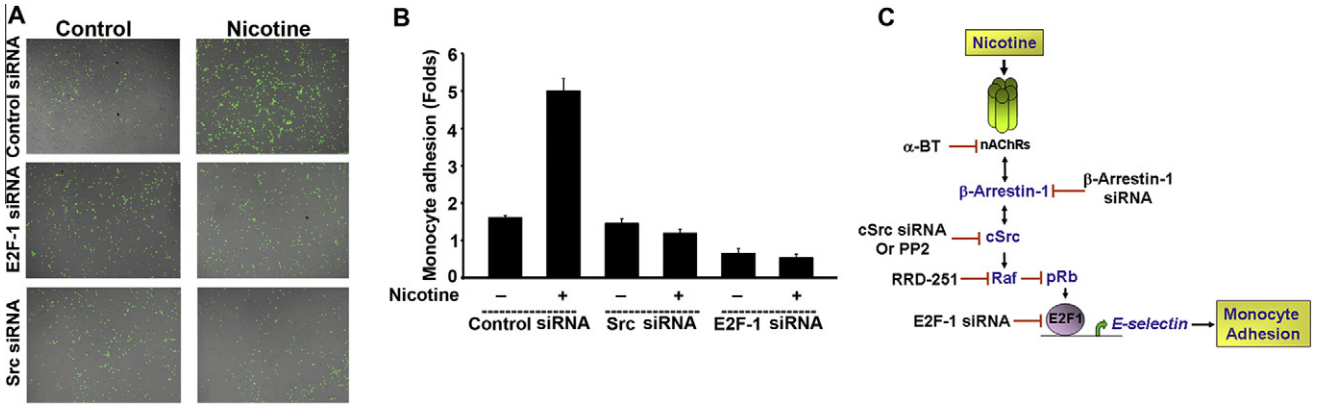


**Fig. 4.** Adhesion of monocytic cells on HAECs is dependent on Src and E2F1 activity. (A and B) siRNA mediated depletion of Src or E2F1 results in suppressed adhesion of U937 cells on nicotine-stimulated HAECs. The image is a composite of fluorescent U937 cells adhered on a confluent monolayer of endothelial cells. (B) Bar graph representing the average number of U937 adhered on the HAECs. (C) Schematic of nicotine-induced monocyte adhesion on endothelial cells. Nicotine stimulation results in α7-nAChR/β-Arr-1/Src/Raf-1–Rb/E2F1-mediated transcriptional upregulation of *E-selectin* in HAECs. Targeting β-Arr1, Src or E2F1 suppressed the expression of *E-selectin* which results in decreased monocyte adhesion.

as its addictive component, nicotine [23,30,31,33–40], the underlying mechanisms are not completely understood. The elevated expression of these adhesion molecules at the site of inflammation is mainly due to the transcriptional induction of their respective genes. Mainly, transcription factor NF-κB has been reported as a dominant regulator of transcription for adhesion molecules in response to various cytokines [41,42]. NF-κB is found to upregulate the adhesion molecules ICAM-1 and VCAM-1 expression in nicotine-stimulated human umbilical vein endothelial cells (HUVECs). Nicotine is also shown to transcriptionally induce the expression of *E-selectin* in HUVECs [43]; however, the transcription factors involved in *E-selectin* gene regulation has not been studied earlier.

Our laboratory had previously reported the physiological effect of nicotine stimulation on human microvascular endothelial cells from lung (HMECLs) as well as HAECs [24]. Upon treatment with 1 μM nicotine we have shown a robust increase in Src phosphorylation, Rb–Raf-1 interaction and E2F1 activation which in-turn results in transcriptional upregulation of E2F1 target genes involved in cell proliferation as well as angiogenic tubule formation [24]. Since, previous studies form our laboratory have elaborated the nAChRs-β-Arr1-Src dependent activation of E2F1 in nicotine-stimulated cells, we hypothesized that *E-selectin* upregulation may also be E2F1 mediated in HAECs. Therefore, we explored the role of nicotine-stimulated E2F1 transcriptional activity in the regulation of *E-selectin* expression and whether or not it can be targeted to inhibit the endothelial cells-monocytes interaction.

The transcriptional activity of E2Fs is regulated by its physical interaction with the Rb family members [23,24,30]. In quiescent endothelial cells, E2Fs 1, 2 and 3 are bound to Rb and their transcriptional activity is suppressed. However, in response to growth factors or nicotine stimulation, Raf-1 interacts with Rb within 2 h that initiates a priming phosphorylation of Rb in HAECs [23,24]. This priming phosphorylation facilitates subsequent hyper-phosphorylation and complete inactivation of Rb and disassociation from E2F1 [23,24], enabling free E2F1 to transcribe its target genes. Disruption of Rb–Raf-1 interaction by RRD-251 is associated with downregulation of trascriptional targets of E2F1 involved in cell proliferation and angiogenesis [24,32,44,45]. While we found hat RRD-251 prevents the dissociation of Rb from E2F1 on proliferative promoters, we find that RRD-251 could block the recruitment of E2F1 on *E-selectin* promoter.

Increase in expression of various cell-adhesion molecules such as *E-selectin* leads to increased binding of monocytes to endothelial cells, which is an important event in the initial stages of atherosclerosis [4,10]. Here we show that repressing the level of E2F1 through an agent such as RRD-251 was able to decrease the number of bound monocytes to the endothelial layer (Fig. 3C). Our recent studies also showed that RRD-251 could affect TNF-α mediated induction of aortic smooth muscle proliferation, which contributes to the formation of atherosclerotic plaques [21]. Further, E2F1 was also found to be necessary for TNF-α to induce apoptosis in aortic endothelial cells, through the induction of p73 gene [20]. Therefore, it thus appears that the Rb-E2F pathway can also contribute to multiple facets of atherosclerotic process, by affecting TNF-α mediated apoptosis of HAECs and proliferation of AOSMCs, as well as by affecting nicotine mediated induction of *E-selectin* gene and monocyte-endothelial cell interaction.

In conclusion, we show that E2F1 contributes to nicotine-induced expression of *E-selectin* in HAECs in a α7-nAChR/β-Arr1/Src/Raf-1–Rb/E2F1 dependent manner, as shown in Fig. 4C. Results further suggest that repressing the activity of E2F1 or Src might be a novel therapeutic approach for cigarette smoke induced atherogenesis. Therefore, novel agents like RRD-251, which can target the transcriptional activity of the E2F1 may have potential benefits as therapeutic agents against atherosclerosis as well, in addition to cancer.

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