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Knockdown of TNFR1 by the sense strand of an ICAM-1 siRNA: dissection of an off-target effect

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ABSTRACT

Tumor necrosis factor (TNF) initiates local inflammation by triggering endothelial cells (EC) to express adhesion molecules for leukocytes such as intercellular adhesion molecule-1 (ICAM-1 or CD54). A prior study identified siRNA molecules that reduce ICAM-1 expression in cultured human umbilical vein EC (HUVEC). One of these, ISIS 121736, unexpectedly inhibits TNF-mediated up-regulation of additional molecules on EC, including E-selectin (CD62E), VCAM-1 (CD106) and HLA-A,B,C. 736 siRNA transfection was not toxic for EC nor was there any evidence of an interferon response. 736 Transfection of EC blocked multiple early TNF-related signaling events, including activation of NF-κB. IL-1 activation of these same pathways was not inhibited. A unifying explanation is that 736 siRNA specifically reduced expression of mRNA encoding tumor necrosis factor receptor 1 (TNFR1) as well as TNFR1 surface expression. A sequence with high identity to the 736 antisense strand (17 of 19 bases) is present within the 3′UTR of human TNFR1 mRNA. An EGFP construct incorporating the 3′UTR of TNFR1 was silenced by 736 siRNA and this effect was lost by mutagenesis of this complementary sequence. Chemical modification and mismatches within the sense strand of 736 also inhibited silencing activity. In summary, an siRNA molecule selected to target ICAM-1 through its antisense strand exhibited broad anti-TNF activities. We show that this off-target effect is mediated by siRNA knockdown of TNFR1 via its sense strand. This may be the first example in which the off-target effect of an siRNA is actually responsible for the anticipated effect by acting to reduce expression of a protein (TNFR1) that normally regulates expression of the intended target (ICAM-1).

INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved, regulatory pathway found in many different organisms including petunias (1), *Nuerospora* (2), *Caenorhabditis elegans* (3), *Drosophila* (4) and mammalian cells (5). Recent investigations have revealed that RNAi plays a key role in heterochromatic silencing and organization (6,7), maintenance of genetic stability (8) and protection from viral pathogens (9). Long double-stranded RNA (dsRNA), from regulatory transcription elements, transposon intermediates, or replicating viral agents, can be recognized and processed within the cell by Dicer, an endogenous RNase III-like enzyme, into short (21–23 nt) interfering dsRNA (siRNA) (10–13). These siRNAs associate with a group of cellular proteins to form the RNA-induced silencing complex (RISC) which mediates siRNA unwinding, exposure of the guide (antisense) strand, and interaction with target mRNA transcripts in a sequence-specific manner. Synthetically produced siRNA function similarly in cultured mammalian cells to silence expression of specific gene products (5). RNAi is now widely and routinely used as an experimental tool for transient gene knockdown, target discovery screens and *in vivo* therapeutic applications (14). The fundamental concern is no longer whether a gene can be silenced, but rather if the functional consequences observed are attributable to the gene being targeted. Recent reports have chronicled the phenomena of off-target effects of RNAi that result when non-specific cellular effects are generated as an unintended side effect of siRNA treatment. These off-target siRNA effects can lead to misinterpretations of the consequences of gene knockdown with the end result being the false assignment of a particular gene function to a specific target gene. The majority of off-target effects can be grouped into four categories: (i) siRNA-like, (ii) miRNA-like, (iii) immune stimulatory (interferon-like) and (iv) global (toxic) non-specific inhibition. SiRNA-like off-target effects encompass situations where partial siRNA nucleotide identity with non-targeted cellular genes (15) can lead to

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enzymatic mRNA destruction resulting in the silencing of many unintended cellular proteins. MiRNA-like effects follow from siRNA species mimicking the activity of microRNA (miRNA) which primarily block protein translation by cognate recognition of short nucleotide sequences within the 3′UTR of target genes (16,17). Translational block can lead to depressed cellular protein levels without a corresponding drop in gene transcript levels. As part of the innate immunity, mammalian cells recognize dsRNA species, such as replicative viral intermediates, and initiate an interferon stress response that includes generalized RNA degradation and protein synthesis inhibition (18). Recent findings have demonstrated that certain 21 nt siRNA are able to trigger the interferon response (19). Using a functional genomics approach, researchers found that many interferon-stimulated genes (ISG) were activated in siRNA- but not mock-transfected mammalian cells. Finally, some siRNAs appear to initiate cell injury or death, and as part of this response, cells may broadly shut down various biosynthetic functions, including transcription and translation.

ISIS 121736 (736) is a double-stranded siRNA whose antisense strand is complementary to a sequence within the coding region of intercellular adhesion molecule-1 (ICAM-1, or CD54) and 736 transfection was shown to reduce ICAM-1 expression in TNF-treated human umbilical vein endothelial cells (HUVEC) (20). We find that 736 actually exhibits broad anti-TNF activities through selective siRNA knockdown of the ICAM-1 regulator, TNFR1, via its sense strand and that the unintended effect on TNFR1 is more relevant than the direct effect on ICAM-1. While siRNA sense strand off-target silencing activity has been suggested in previous *in vitro* biochemical studies (21–23) and genome-wide screens (15), our report is the first example of an off-target effect in which the sense strand of an siRNA displays an unintended effect by silencing an 'upstream' regulator of the intended target.

MATERIALS AND METHODS

Reagents

TNF and IL-1 were obtained from R&D Systems (Minneapolis, MN, USA). Antibodies used are anti-p65 and anti-IκBα (Santa Cruz, Biotechnology, Inc., Santa Cruz, CA, USA), anti-TNFR2 (Biosource; Camarillo, CA, USA), anti-phospho SAPK/JNK, anti-phospho p38 (Cell Signaling Technologies; Beverly, MA, USA), and anti-β-actin (Sigma; St Louis, MO, USA), anti-ICAM-1-FITC, anti-E-selectin-FITC, anti-VCAM-PE, anti-HLA-ABC-FITC (Immunotech; Miami, FL, USA), anti-TNFR1-FITC, anti-AnnexinV-FITC, anti-TNFR1, anti-RIP-1, anti-TRAF2, anti-TRADD (Pharmingen, San Diego, CA, USA) and anti-ICAM-1 (R&D Systems). Hoechst dye 33258 was from Molecular Probes (Eugene, OR, USA). Unless otherwise specified, all other reagents were from Sigma Chemical Co. (St. Louis, MO, USA). Quantikine human soluble TNFR1 ELISA (R&D Systems) was used to detect sTNFR1 and was used according to the manufacturer's instructions.

Construction of mutant EGFP/TNFR1 3′UTR retroviral constructs

The EGFP coding sequence was PCR amplified from pEGFP-C1 (Clonetech, Mountain View, CA, USA) using EGFP-specific primers with a 5′-tailed BamHI site and a 3′-tailed homology domain corresponding to the most 5′ proximal sequence of the TNFR1 3′UTR. The complete TNFR1 3′UTR was amplified from HUVEC cDNA using TNFR1-specific primers. The two amplified products were annealed, PCR amplified and inserted into pCR2.1 using a TOPO TA Cloning Kit (Invitrogen). The EGFP/TNFR1 3′UTR fragment was liberated by BamHI/NotI restriction digest and sub-cloned into the multiple cloning site of pLZRS. To generate EGFP/TNFR1 736 mutants, a fragment containing the upstream EGFP/TNFR1 3′UTR sequence and a mutant 736 sequence at the 3′ end was generated by PCR. A second fragment bearing the same 736 mutant sequence linked at the 5′ end to the downstream EGFP/TNFR 3′UTR sequence was annealed to the upstream fragment, PCR amplified and sub-cloned into pLZRS. These constructs appear in Figure 6B.

Cells and cell culture

All human cell cultures were prepared under protocols approved by the Yale Human Investigations Committee. HUVEC were isolated and cultured as previously described (24) and used at passage 2 or 3 in all experiments. Cultures were uniformly positive for CD31 and contained no detectable $CD45^+$ cells. In cells treated with TNF, ICAM-1 and VCAM-1 were uniformly up-regulated and over 90% expressed E-selectin. Human dermal microvascular endothelial cells (HDMEC) supplied by the Cell Culture Core of the Yale Skin Disease Research Center were liberated from the superficial vascular plexus of the upper dermal layers of normal adult human skin (provided as discarded surgical specimens) by dermatome, by enzymatic digestion (50 U/ml Dispase; Collaborative Medical Products) for 30 min at 37°C, and then by fine mincing as described previously (25). Released cells were cultured on 10 μg/ml fibronectin-coated plastic (Falcon, Lincoln Park, NJ, USA) in EGM2-MV growth medium (Cambrex, East Rutherford, NJ, USA). Between subculture one and two, substrate-adherent cells were resuspended for isolation on a mini-MACS column by immunoselection using anti-CD-31-biotin followed by streptavidin-magnetic beads (Miltenyi Biotec, Auburn, CA, USA). In response to TNF, HDMEC uniformly up-regulate ICAM-1 and VCAM-1 expression, and over 90% express E-selectin.

To produce EGFP/TNFR1 3′UTR chimera-expressing HUVEC cell lines, retroviral supernatants were produced by transfecting Phoenix amphotropic packaging cells with EGFP/TNFR1 3′UTR retroviral constructs to generate virus supernatants which were used for HUVEC transduction as described (26). To produce over-expressing HDMEC cell lines, the cDNA for human ICAM-1 and E-selectin were ligated into the retroviral vector pLZRS

and used to prepare virus for HDMEC transduction. Cells were used between passages four and eight for the experiments described.

siRNA transfection

HUVEC or HDMEC were plated onto gelatin-coated 12-well plates at ~70% confluence. At 24 h post-plating, mixtures of OligofectAmine (Invitrogen, Carlsbad, CA, USA) to 20 µl/ml and siRNA to 50 nM were prepared in Opti-MEM I Reduced Serum Medium (Invitrogen) and these concentrated mixtures of siRNA complexes were diluted 5-fold to a 1X concentration before co-incubation with EC cultures for 6 h at 37°C. Fresh medium was added overnight and cells were re-transfected 24 h later, rested for 24 h, and then treated with 10 ng/ml human TNF for 24 h where indicated. SiRNA duplexes were obtained from Dharmacon, Inc. (Lafayette, CO, USA):

736 (AS): 5′GUGGCCUUCAGCAGGAGCUdTdT3′
736 (S): 5′AGCUCCUGCUGAAGGCCACdTdT3′
lamin (AS): 5′UGUUCUUCUGGAAGUCCAGdTdT3′
lamin (S): 5′CUGGACUUCCAGAAGAACAdTdT3′
TNFR1-1 (AS): 5′PGUACAAGUAGGUUCCUUUGUU3′
TNFR1-1 (S): 5′CAAAGGAACCUACUUGUACUU3′
TNFR1-2 (AS): 5′PUAGUAGUUCCUUCAAGCUCUU3′
TNFR1-2 (S): 5′GAGCUUGAAGGAACUACUAUU3′
LFA-3 (S): 5′CAGCCAUCGAGGACUUAUAdTdT3′
LFA-3 (AS): 5′UAUAAGUCCUCGAUGGCUGdTdT3′

The following single strand RNA oligos were purchased from Dharmacon:

736 (AS): 5′GUGGCCUUCAGCAGGAGCUdTdT3′
736 (S): 5′AGCUCCUGCUGAAGGCCACdTdT3′

[also synthesized as 2′-*O*-methyl modified versions of 736 (AS) and 736 (S)]
736 5′deoxy (S): 5′deoxy-AGCUCCUGCUGAAGGCCACdTdT3′
736/M4 (S): 5′AGCGCCUGCUGAAGGCCACdTdT3′
736/M6 (S): 5′AGCUCAUGCUGAAGGCCACdTdT3′
736/M16 (S): 5′AGCUCCUGCUGAAGGACACdTdT3′
736/M15/16 (S): 5′AGCUCCUGCUGAAGUACACdTdT3′
736/Bulge (S): 5′AGCUCCUGACUAAGGCCACdTdT3′

Quantitative RT-PCR

Knockdown of siRNA-transfected, TNF-treated EC was assessed by qRT-PCR 24 h after the last transfection. Total RNA was isolated using the RNeasy column system (Qiagen, Valencia, CA, USA) and reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). For real-time qPCR, cDNA, $MgCl_2$ and primers were added to Platinum SYBR Green qPCR SuperMix (Invitrogen). Samples were amplified for 35 cycles on a iCycler thermocycler (Bio-Rad): 50°C for 2 min, 95°C for 2 min, 95°C for 15 s, 62°C for 15 s, 72°C for 15 s and 72°C for 5 min. cDNA input levels for each sample were normalized to levels of β-actin amplified with actin-specific primers. Threshold cycles for each sample were compared to control (mock-transfected) samples after β-actin normalization. Primers used in qRT-PCR reactions were:

β-actin Forward: 5′ATGGGTCAGAAGGATTCCTAAGTG3′
β-actin Reverse: 5′CTTCATGAGGTAGTCAGTCAGGTC3′
OAS1 Forward: 5′TCAGAAGAGAAGCCAACGTGA3′
OAS1 Reverse: 5′CGGAGACAGCGAGGGTAAAT3′
PKR Forward: 5′GCCTTTTCATCCAAATGGAATTC3′
PKR Reverse: 5′GAAATCTGTTCTGGGCTCATG3′
TNFR1 Forward: 5′GCCAGGAGAAACAGAACACC3′
TNFR1 Reverse: 5′CTCAATCTGGGGTAGGCACA3′
TNFR2 Forward: 5′GCCAACATGCAAAAGTCTTCTGTA3′
TNFR2 Reverse: 5′CAGGTGCAGATGCGGTTCTGTT3′
TRADD Forward: 5′CACTCGGTGCCGCTGCAACT3′
TRADD Reverse: 5′TCAGATTTCGCAGCGCATCCT3′
TRAF2 Forward: 5′ACTGCTCCTTCTGCCTGGCCAGCAT3′
TRAF2 Reverse: 5′CAGGTGCATCCATCACTGGGACAGA3′
RIP-1 Forward: 5′GGAGATTGGTGGGACGAGTT3′
RIP-1 Reverse: 5′TCCCAGATTTTCCCTGATTG3′
EGFP Forward: 5′CCTACGGCGTGCAGTGCTTCAGC3′
EGFP Reverse: 5′CGGCGAGCTGCACGCTGCGTCCTC3′

FACS analysis

siRNA-transfected HUVEC expression of ICAM-1, E-selectin, VCAM-1 and MHC class I were determined by FACS analysis (FACSort, Becton Dickinson) after immunostaining with saturating concentrations of directly labeled antigen-specific or isotype-matched monoclonal antibodies. To measure apoptosis, siRNA-transfected HUVEC were collected and stained with FITC-labeled anti-AnnexinV or isotype-matched antibodies for 30 min on ice. The same cells were stained with 5 µg/ml propidium iodide for 10 min and analyzed for fluorescence using the BD FACSort.

Immunocytochemistry

To assess p65 translocation, siRNA-transfected HUVEC monolayers were washed twice with PBS (1× PBS containing Ca^{++} and Mg^{++}) and fixed for 30 min. at RT with 4% paraformaldehyde/PBS. Monolayers were washed again with PBS and treated for 30 min. with staining buffer (0.5% BSA, 2 mM EDTA in PBS) containing 0.1% Triton X-100 and 0.5% donkey serum. Rabbit anti-human-p65 antibody was added to HUVEC

monolayers, and incubated at 4°C overnight. Donkey anti-rabbit IgG Alexa Fluor 488 (Molecular Probes) was added and again incubated overnight.

Cellular growth curve

siRNA-transfected or mock-transfected EC were collected and replated at 1000 cells/well in multiple gelatin coated 96-well plates. At the indicated times, cell monolayers were washed with PBS, fixed in ethanol and treated with a fluorescent nucleic acid stain, Hoechst 33258 at 100 μg/ml for 30 min. Nucleic acid fluorescence (excitation = 360 nm and emission = 460 nm) was monitored using a Cytofluor I automated fluorescence plate reader at gain = 80 and reported as relative fluorescence units (RFU).

Western blot analysis

HUVEC were transfected with siRNA in gelatin-coated 12-well plates. Cells were treated with or without TNF or IL-1 for various times and whole cell lysates were prepared. Protein concentrations were determined using BCA Protein Assay (Pierce, Rockford, IL, USA). Ten (10) μg of each lysate was resolved using SDS-PAGE, proteins were transferred to Immobilon-P PVDF (Millipore, Bedford, MA, USA) and the membranes were blocked in 5% non-fat milk (Bio-Rad, Hercules, CA, USA). Antigen-specific antibodies and HRP-conjugated secondary antibody (Pierce) were used in conjunction with a Femto SuperSignal chemiluminescent substrate (Pierce) to detect target proteins.

RESULTS

736 Inhibits TNF-α-induced effects on human endothelial cells

In vitro screening of a panel of anti-ICAM siRNA molecules performed by Vickers *et al.* (20) revealed an active siRNA compound, ISIS 121736 (hereafter designated 736), that was effective at inhibiting TNF-mediated induction of ICAM-1 on cultured HUVEC. We conducted a BLAST search designed to identify non-targeted transcripts with identity to 736 and found no perfect matches to 736 in the RefSeq database. Human zinc finger protein 3 (Accession #BC011887) displayed 17 contiguous homologous base pairs with 736, but was unaffected by 736 transfection (data not shown). In the course of our independent study analyzing functional effects of ICAM-1 expression on human endothelial cells (EC), we confirmed the activity of 736 identified in Vickers *et al.* (Figure 1). Specifically, we found that TNF-induced ICAM-1 cell surface expression was inhibited by 77% when compared to lamin siRNA-transfected HUVEC using FACS analysis. However, tests for siRNA specificity revealed that 736 transfection unexpectedly inhibited a number of other TNF-inducible cell surface proteins. E-selectin was inhibited by 71%, VCAM-1 by 81% and MHC class I by 40% compared to lamin siRNA-transfected HUVEC (Figure 1). Similar results were observed for transfected HDMEC suggesting that both EC types are similarly affected by 736 activity (data not shown). These results were surprising since none of these TNF-responsive genes have significant homology with 736 and so we chose to investigate further the mechanism of action of 736.

First, to determine whether the 736 off-target activity was due to a general cytotoxic effect we assessed the activity of this siRNA on cell growth by plating 736 and lamin siRNA-transfected HDMEC into 96-well plates and monitoring their growth rates using a Hoechst fluorescent dye assay. The growth rates of transfected cells were compared to the growth of mock-transfected (OligofectAmine alone) or lamin siRNA-transfected HDMEC. All cells grew progressively and no difference in growth rates among the three treated groups was detected over a 7-day period (Figure 2A). To determine whether 736 induced cell death, the levels of apoptosis or necrosis in siRNA-transfected and control HUVEC cultures was measured. Mock-transfected and 736 or lamin siRNA-transfected HUVEC were harvested 24 h post-transfection and tested for AnnexinV staining or propidium iodide (PI) exclusion by FACS analysis (Figure 2B). AnnexinV staining was low in all three groups and was not elevated in 736-transfected cells compared to lamin siRNA-transfected cells. Although some PI uptake was detected, there was no increase in PI incorporation in 736 versus lamin siRNA-transfected cells.

An earlier sign of cell injury is activation of TNFα converting enzyme (TACE) and subsequent shedding of surface TNFR1, which could cause TNF unresponsiveness (27). To ascertain whether TNFR1 was present in the media of transfected cells, an ELISA was used to detect soluble or shed TNFR1 (sTNFR1) in mock- or siRNA-transfected HUVEC conditioned media. Untreated HUVEC cultures spontaneously shed 25–30 pg/ml of sTNFR1 over a 24-h period while cells treated for 15 min with histamine, a known TACE activator (28), increased the production level to 65 pg/ml sTNFR1 over the same time period (data not shown). HUVEC transfections were performed for periods of 1, 4 and 48 h and culture media was collected after 24 additional hours of undisturbed growth. Conditioned media from mock- or lamin siRNA-transfected cells maintained similar levels of sTNFR1 as the untreated HUVEC control cells (25–30 pg/ml) whether they were transfected for 1, 4 or 48 h (Figure 2C). Cells transfected with 736 siRNA also released similar levels of sTNFR1 (~25 pg/ml) when transfected for 1 or 4 h, and showed diminished levels of sTNFR1 (5 pg/ml) in the culture media after 48 h of transfection, ruling out an effect due to receptor shedding.

Many double-stranded siRNA are detected by intracellular proteins and can produce an interferon stress response through the activation of cellular stress response genes such as 2′-5′-oligoadenylate synthetase 1 (OAS1) and protein kinase R (PKR). These genes can trigger a wide array of cellular responses that could account for the unique phenotype of 736 siRNA-transfected cells. To rule out this possibility, gene-specific oligonucleotide primers were designed to monitor the induction of OAS1 and PKR mRNA in siRNA-transfected cells using real-time qPCR. PolyI:C, a potent stimulator of the interferon

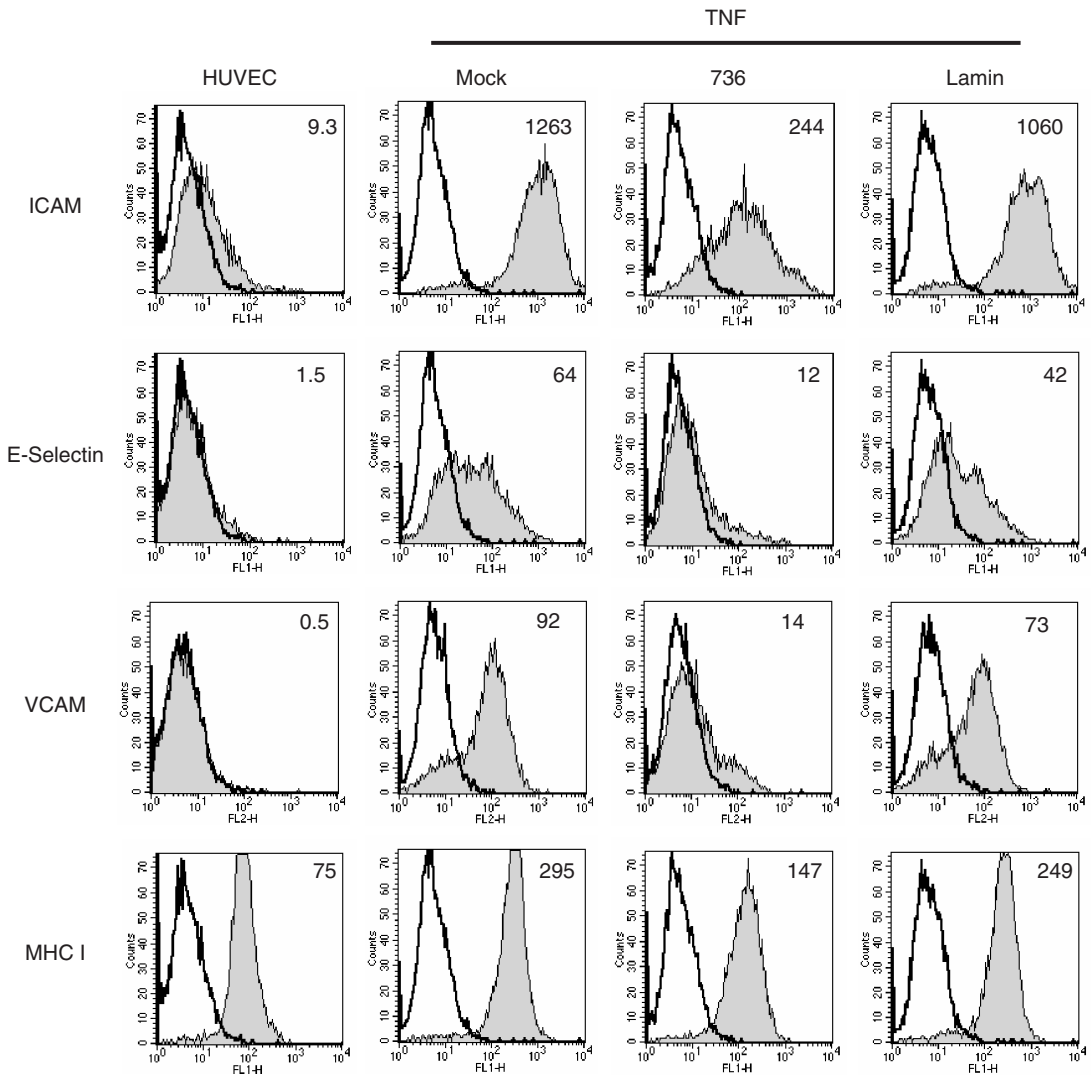

Figure 1. 736 Inhibits TNF-mediated HUVEC antigen expression. HUVEC were mock-transfected or transfected with 10 nM 736 or lamin siRNA on consecutive days and treated with 10 ng/ml TNF for 24 h. HUVEC were immuno-stained using fluorescent antigen-specific antibodies for E-selectin, ICAM-1, VCAM-1 and MHC I and analyzed by flow cytometry. Filled histograms represent antigen-specific staining and empty histograms represent isotype control staining. Corrected MFI in the upper right hand corner = antigen-specific MFI minus isotype MFI. Representative of one of three experiments with similar results.

response, elicited a 2299-fold increase in OAS1 message, but there was no induction of OAS1 observed in 736 or lamin siRNA-transfected HUVEC compared to mock-transfected controls (Figure 2D). PKR transcripts in Poly I:C treated HUVEC increased by 12.4-fold while HUVEC transfected with 736 or lamin siRNA showed no PKR induction when compared to mock-transfected cells. These analyses show that interferon response genes are not induced by 736 and are unlikely to be responsible for the observed 736 siRNA phenotype.

736 Inhibits TNF, but not IL-1 signaling

To further analyze the effect of 736 on TNF-induced proteins, we examined specific signal transduction pathways. NF-κB is a critical messenger for transducing inflammatory signals in EC. The three surface antigens we found to be negatively regulated by 736 transfection are all up-regulated through NF-κB signaling. Therefore, we examined if 736 transfection interfered with NF-κB activation. We initially used p65 (RelA) nuclear translocation as a measure of NF-κB activation. HUVEC were siRNA-transfected and rested for 24 h. TNF was added to the HUVEC cultures at varying concentrations and incubated for 15 min after which the cells were fixed and prepared for p65 (RelA) immunocytostaining. p65 was diffusely localized within the cytoplasm of HUVEC cells in cultures where TNF had been withheld, but had markedly translocated and accumulated in the nucleus of TNF-treated cells (Figure 3A). Lamin siRNA-transfected cultures treated with TNF displayed a similar pattern, with nearly all the p65 localized to the nucleus, suggesting normal NF-κB activation. Some translocation in 736- treated cells was detectable, but the number of cells exhibiting nuclear staining and overall signal

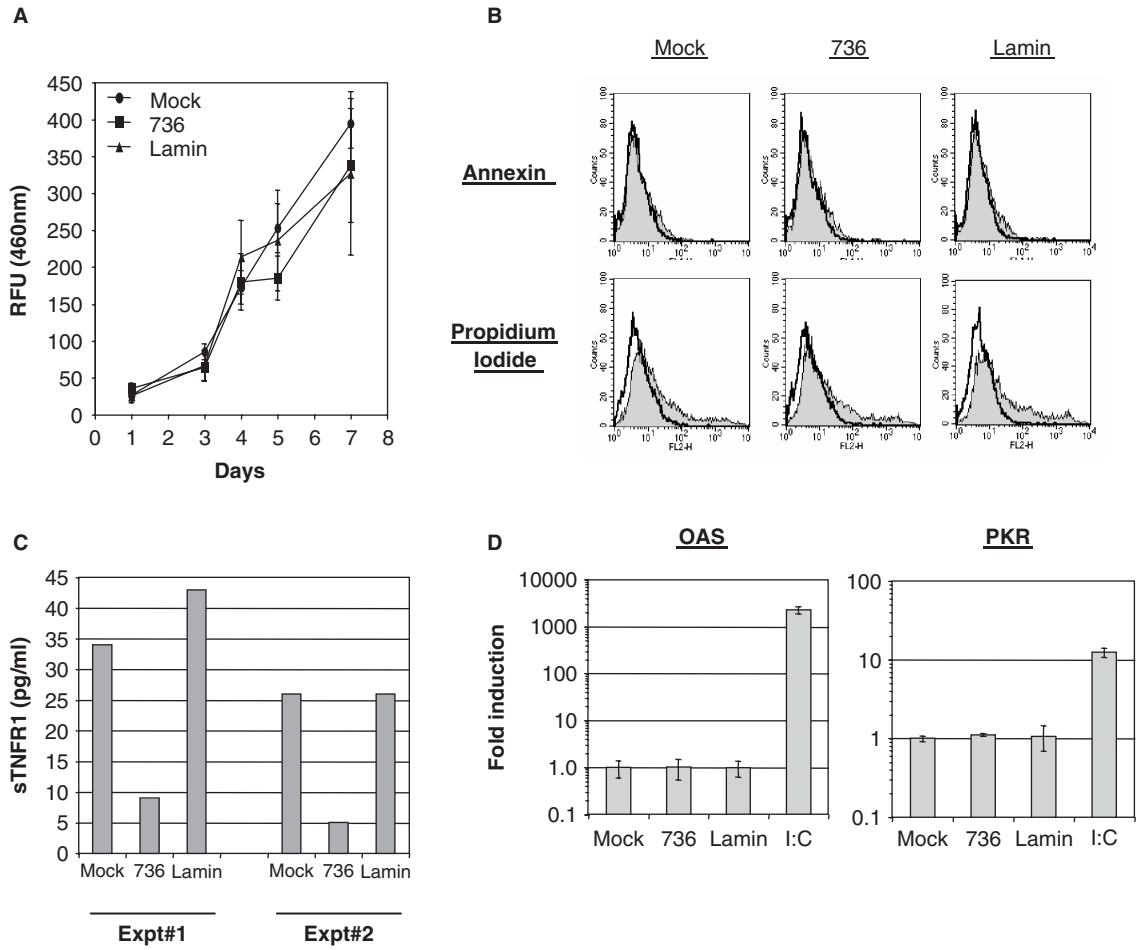

Figure 2. 736 siRNA is not cytostatic or cytotoxic, and does not induce an interferon response. (**A**) siRNA-transfected or mock-transfected HDMEC were collected and replated at 1000 cells/well in multiple 96-well plates. At the indicated times, cell monolayers were washed, fixed in ethanol and treated with the fluorescent nucleic acid stain, Hoechst 33258. Fluorescence (emission = 460 nm) was monitored using a Cytofluor I automated fluorescence plate reader and reported as relative fluorescence units (RFU). (**B**) siRNA-transfected HUVEC were collected and stained with AnnexinV-specific Ab or propidium iodide (PI). Fluorescence, indicating apoptosis or necrosis, was measured by flow cytometry. (**C**) HUVEC were siRNA transfected for 1, 4 or 48 h. Culture supernatants were collected 24 h after transfection and tested for soluble TNFR1 (sTNFR1), a measure of early toxicity, by ELISA. (**D**) HUVEC were transfected twice on consecutive days with siRNA or treated with 100 ug/ml PolyI:C for 24 h. qRT-PCR was performed on cDNA from each culture using OAS- and PKR-specific primers. Values were normalized using actin controls. Representative of one of two or more experiments with similar results.

intensity was generally diminished. Although indicative of NF-κB inhibition, this assay is not easily quantifiable. Therefore, we also measured degradation of IκBα by immunoblotting. IκBα associates with NF-κB heterodimers within the cytoplasm where it masks nuclear localization signals (NLS). Proteolytic degradation of IκBα following pro-inflammatory cytokine treatment (e.g. TNF and IL-1) leads to unmasking of NLS, translocation and transactivation of NF-κB responsive genes. To determine whether 736 inhibition of p65 translocation functioned at the level of IκBα degradation, HUVEC cultures were siRNA transfected, treated with TNF, and subjected to western blot analyses to gauge the level of IκBα degradation. At the level of 0.1 ng/ml TNF, IκBα degradation was essentially complete in mock- or lamin siRNA-transfected cultures, but 736 transfection prevented complete degradation of IκBα (Figure 3B). These results indicate that TNF signaling is intact in mock and lamin, but not in 736 siRNA-transfected cells.

We then tested the specificity of the effect on TNF responses in EC. IL-1 shares many effects on EC with TNF, including ICAM-1 induction, but utilizes a distinct receptor and assembles a different early signaling complex. While 736 was able to retard IκBα degradation in TNF treated cells, we did not observe the same inhibitory effect in IL-1-treated cells (Figure 3B). At IL-1 doses that resulted in complete IκBα degradation in mock- and lamin siRNA-transfected cultures (0.1ng/ml), IκBα was also completely degraded in 736 siRNA-transfected cells. These data indicate that TNF receptor signaling and IκBα degradation is impaired in 736 siRNA-transfected HUVEC, but signaling through IL-1 receptor remains intact and is not affected by 736 treatment. We also examined IκBα proteolytic degradation in human dermal fibroblasts and, as in HUVEC, IκBα was completely

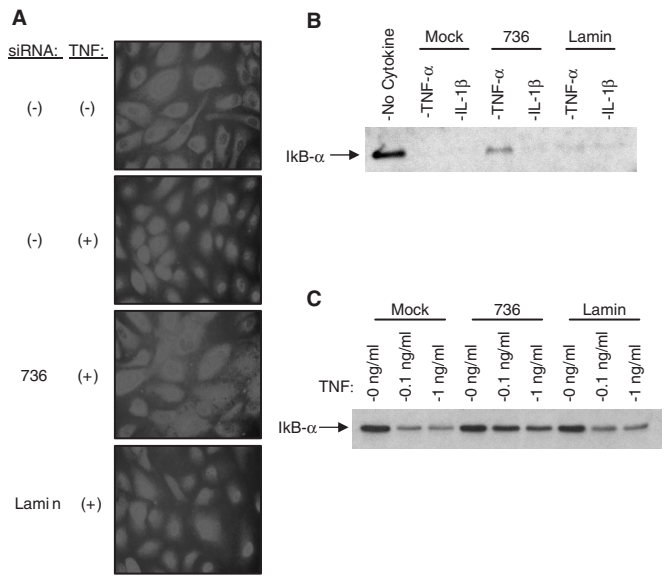

Figure 3. 736 Inhibits NFκB translocation and IκB-α degradation of TNF-treated HUVEC. (**A**) Cells were transfected twice on consecutive days with 736 or anti-lamin siRNA and treated with 10 ng/ml TNF for 15 min. Cultures were washed, fixed *in situ* with paraformaldehyde, and immunostained with a NF-κB p65-specific antibody. (**B**) HUVEC were transfected twice on consecutive days with 736 or anti-lamin siRNA. Cells were treated for 30 min. with 0.01 ng/ml TNF or IL-1. Whole cell lysates were prepared, resolved on SDS-PAGE, and immunoblotted with an IκB-α-specific antibody. (**C**) Human fibroblasts were siRNA transfected and subsequently treated with TNF [0, 0.1, and 1.0 ng/ml] for 30 min. Cell lysates were immuno-blotted with an IκB-α–specific antibody. Representative of one of two experiments with similar results.

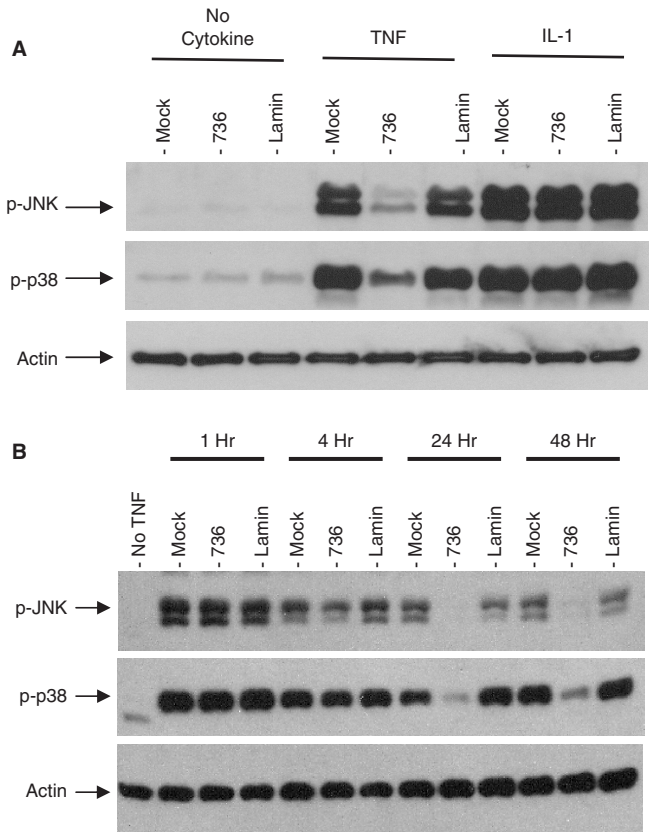

Figure 4. JNK and p38 MAPK activity is suppressed in 736-treated HUVEC. (**A**) HUVEC were transfected twice on consecutive days with 736 or lamin siRNA. Cells were treated for 30 min. with TNF or IL-1. whole cell lysates were prepared, resolved on SDS-PAGE and immunoblotted with phospho-JNK and phospho-p38-specific antibodies. (**B**) Transfected HUVEC were TNF-treated (30 min) at various times after initial exposure to siRNA when lysates were collected for SDS-PAGE and immunoblotting with phospho-JNK and phospho-p38-specific antibodies. Representative of one of two experiments with similar results.

degraded in TNF-treated cells transfected with lamin siRNA, but was significantly protected in 736 siRNA-transfected cells (Figure 3C).

In addition to signaling through NF-κB, TNF and IL-1 also activate JNK and p38 MAPK. We examined the ability of 736 to interfere with these pathways in a TNF-specific manner. For this purpose, HUVEC were mock- or siRNA-transfected then rested for 24 h and treated with either TNF or IL-1 for 20 min. Cell lysates were resolved by SDS-PAGE and immunoblotted using phospho-JNK or phospho-p38 MAPK antibodies to detect activated JNK or p38 MAPK, respectively. Little or no activated JNK was detected in the absence of cytokine addition (Figure 4A). Addition of IL-1 to mock-transfected or siRNA-transfected cultures led to a significant accumulation of phospho-JNK. However, 736 transfection inhibited the response to TNF so that cells treated with TNF displayed significant levels of phospho-JNK only in mock- or lamin siRNA-transfected cells. In contrast to IL-1 treated cells, after simulation with TNF there was a marked decrease in the level of activated SAPK/JNK in 736 siRNA-transfected cultures. In addition, 736 siRNA-transfected cells were limited in their ability to phosphorylate p38 MAPK following TNF treatment, but not after IL-1 stimulation. Collectively, these data suggest that 736 transfection reduces TNF signaling in human EC and dermal fibroblasts but does not impair IL-1 signaling.

TNFR1 message and protein levels are reduced in 736-transfected cells, but not other TNF signalosome components

To ascertain how long after siRNA exposure TNF signaling defects were detectable, cells from siRNA-transfected HUVEC were collected at 1, 4, 24 and 48 h after transfection. Lysates were immunoblotted for phospho-JNK and phospho-p38 MAPK. SAPK/JNK phosphorylation was only modestly reduced after 4 h of 736 siRNA-transfection, but was almost entirely absent at 24 h after exposure to 736 and remained undetectable up to 48 h after transfection (Figure 4B). p38 MAPK phosphorylation defects caused by 736 were slightly slower to develop and less persistent than SAPK/JNK defects. p38 MAPK phosphorylation was unaffected at 4 h and was almost completely absent at 24 h, but became detectable again at 48 h post-transfection. These data suggest that 736 may affect the expression of one or more components of the TNF signaling apparatus. We then measured the mRNA and protein levels of TNFR1 and other TNF signalosome components. First, whole cell

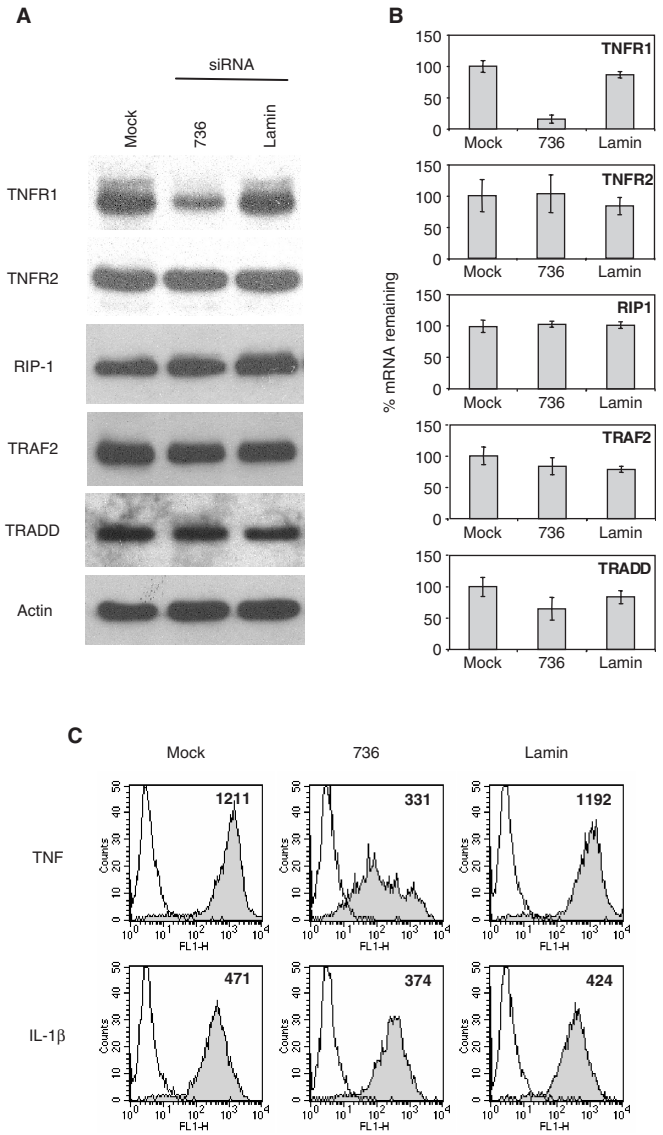

Figure 5. 736 siRNA selectively targets TNFR1 mRNA and protein and selectively inhibits TNF responses. (**A**) HUVEC were mock-transfected or transfected with 736 or lamin siRNA. Cell lysates were prepared 24 h after the last transfection, resolved by SDS-PAGE, and immunoblotted with antibodies specific for TNF receptor signaling proteins. (**B**) HUVEC were transfected with 736 or lamin siRNA. cDNA from each culture was analyzed by qRT-PCR using TNF signalosome-specific primers. Values were normalized using actin controls and converted to '% mRNA Remaining' relative to mock expression levels. Representative of one of two experiments with similar results. (**C**) HUVEC were transfected with 736 or lamin siRNA. Cells were then stimulated for 24 h with either TNF (10 ng/ml) or IL-1β (50 ng/ml), immuno-stained using fluorescent antigen-specific antibody for ICAM-1 and analyzed by flow cytometry.

lysates from siRNA-transfected HUVEC were resolved by SDS-PAGE and immunoblotted with antibodies specific to five important signalosome members: TNFR1, TNFR2, RIP-1, TRAF-2 and TRADD. TNFR1 protein levels were substantially reduced when compared to mock- or lamin siRNA-transfected HUVEC (Figure 5A). Other signalosome components, including RIP-1, TRAF2 or TRADD, were not affected at the protein level by 736 siRNA-transfection. There was also no effect on TNFR2 protein levels.

Next, qRT-PCR analysis of transfected HUVEC was performed to determine whether TNFR1 and other signalosome component mRNA levels were affected by 736 siRNA-transfection. RNA from transfected cells was reverse transcribed and used as a template for a series of qRT-PCR reactions with TNFR1, TNFR2, RIP-1, TRAF-2 and TRADD gene-specific primers. The results were normalized for cDNA input using β-actin specific primers. 736 siRNA-transfected cells showed a substantial reduction in TNFR1 mRNA compared to mock- or lamin siRNA-transfected cells (Figure 5B). TNFR2, RIP1 and TRAF2 mRNA levels were essentially unchanged across all three transfected groups, while TRADD transcript levels were modestly depressed in one of two experiments. To test whether 736-mediated reduction of TNFR1 levels, and not a direct effect on ICAM-1, was responsible for depressed ICAM-1 induction, we tested whether 736 would inhibit ICAM-1 levels if stimulated by receptors other than TNFR1. We measured the levels of ICAM-1 induction following TNF or IL-1 treatment of 736-transfected HUVEC. As seen before, TNF-induced ICAM-1 cell surface expression was reduced in 736-tranfected cells, but, in contrast, ICAM-1 levels were only slightly affected in IL-1 stimulated cells (Figure 5C), indicating that 736 did not directly reduce ICAM-1 levels. These data suggest that TNFR1, rather than ICAM-1, is the principle target of 736 siRNA, and explains its general inhibitory effect on the TNF, but not on the IL-1-induced inflammatory response.

Alignment of siRNA 736 with human TNFR1

Following our determination that 736 transfection-reduced TNFRI protein and transcript levels, we performed an alignment analysis (LALIGN: www.ch.embnet.org) on both strands of 736 siRNA and human TNFR1 mRNA (Accession number BC010140). We found 17 of 19 nt homology between the sense strand of 736 (736/sense) and TNFR1 mRNA at a position within the 3′UTR of TNFR1 that is 6 nt downstream of the polyA site (nt 2083–2101; Figure 6A). Mismatches at position 7 and position 18 of the sense strand, neither of which were G:U wobble mismatches, left two homologous strings of 6 and 10 bases. No other homologies of >10 nt were found for 736 and TNFR1 mRNA. Based on this observation, we hypothesized that 736/sense might target the homologous sequence within the 3′UTR of TNFR1 and cause TNFR1 knockdown via an siRNA mechanism. To test this idea, we constructed three separate EGFP-based chimeric transgenes (Figure 6B). In each case, the coding sequence for EGFP was linked to the 3′ UTR of TNFR1 using recombinant PCR techniques. Each of the three chimeric transcripts had a unique 19-base pair sequence at the putative 736 target site. EGFP/TNFR1-3′UTR bears the wild-type 736 target site derived from the 3′UTR of TNFR1 (17 of 19 nt homology), EGFP/Scramble contains a scrambled version of the 736 target site (2 of 19 nt homology) and EGFP/736AS has a perfect homology (19 of 19 nt) with the sense strand

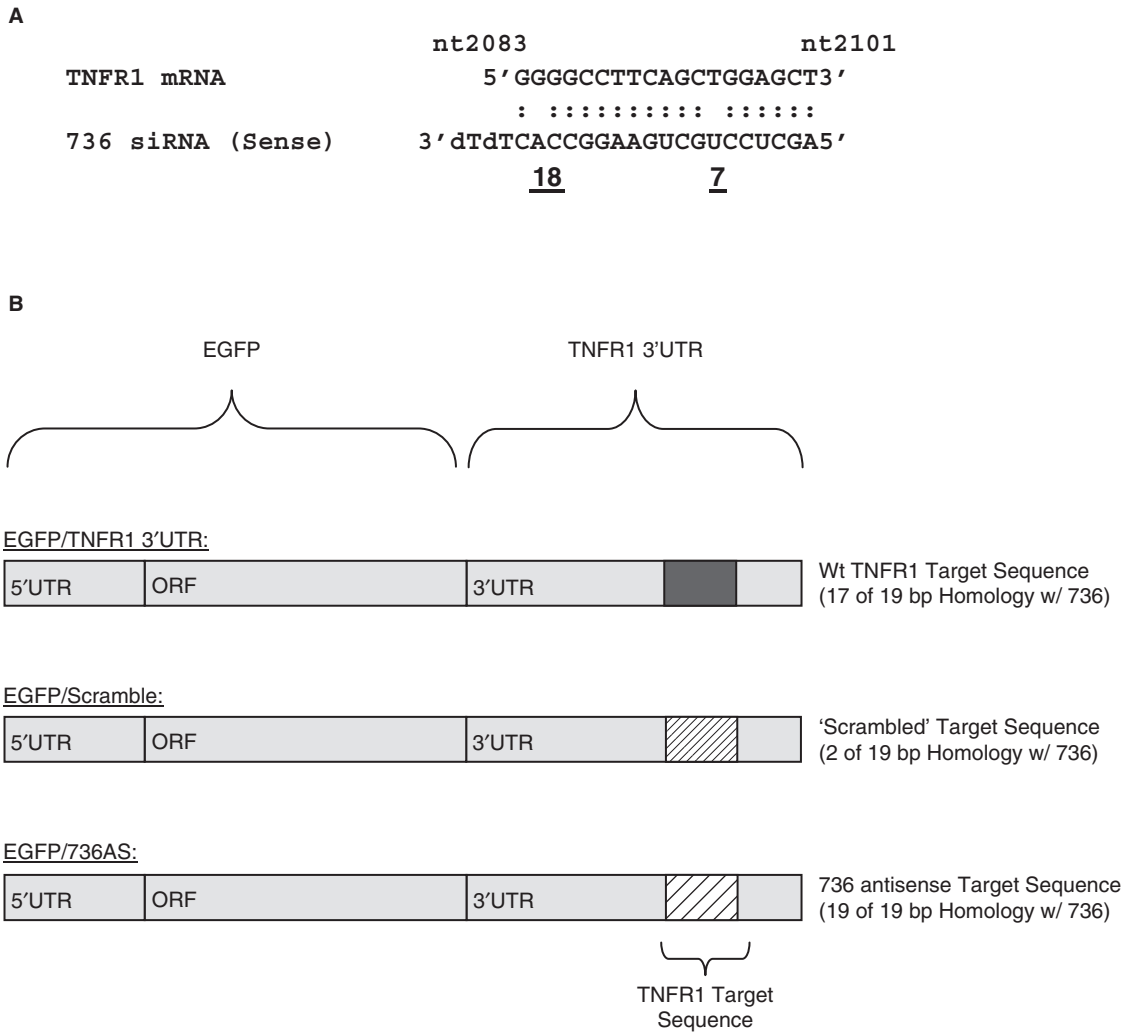

Figure 6. 736 Sequence homology and location with TNFR1. (**A**) Base pair homology comparison of 736 siRNA sense strand with TNFR1 mRNA. (**B**) Chimeric EGFP constructs indicating the location of the 736 target sequences and extent of sequence homology with 736.

of 736. Each of these three chimeric transcripts were inserted into the retrovirus, pLZRS, and used to produce retroviral vectors capable of expressing the chimeric transcripts in human EC. After being transduced with each of these viral vectors, HUVEC that uniformly expressed each chimeric transcript were prepared by sterile FACS sorting based on their EGFP expression. The three chimeric EGFP cell lines were siRNA-transfected using our standard protocol, and EGFP transcript expression was analyzed by qRT-PCR using EGFP gene-specific primers. In addition, we analyzed EGFP reporter protein expression by FACS. In cells expressing EGFP/TNFR1-3′UTR, mock- and lamin siRNA-transfected cells showed no EGFP knockdown, while 736 siRNA-transfected cells showed a >50% knockdown compared to mock-transfected cells (Figure 7A). EGFP/736AS HUVEC cells were unaffected by mock or lamin siRNA transfection, but were sensitive to 736 activity when compared to mock-transfected cells. In contrast, 736 was not able to knockdown EGFP/Scramble transcript levels. EGFP reporter protein levels mirrored EGFP transcript levels in siRNA-transfected cells. EGFP/TNFR1-3′UTR and EGFP/736AS cells expressed lower levels of EGFP reporter protein when transfected with 736 siRNA as compared to mock- and lamin siRNA-transfected cells and EGFP expression in EGFP/Scramble cells was unaffected by 736 siRNA (Figure 7B). Lamin siRNA knockdown of lamin transcripts was confirmed in all experiments (data not shown). These results support the conclusion that an intact 19 nt TNFR1 3′UTR target sequence is necessary and sufficient for the observed 736 effects on TNFR1 expression that are most consistent with an siRNA mechanism.

736 Sense strand activity is inhibited by chemical and sequence modification

In our chimeric EGFP transcript studies, we were able to establish the critical importance of the TNFR1 3′UTR target sequence for 736-mediated TNFR1 silencing. To determine whether other anti-TNFR1 siRNA, targeting alternative transcript regions, produce a similar

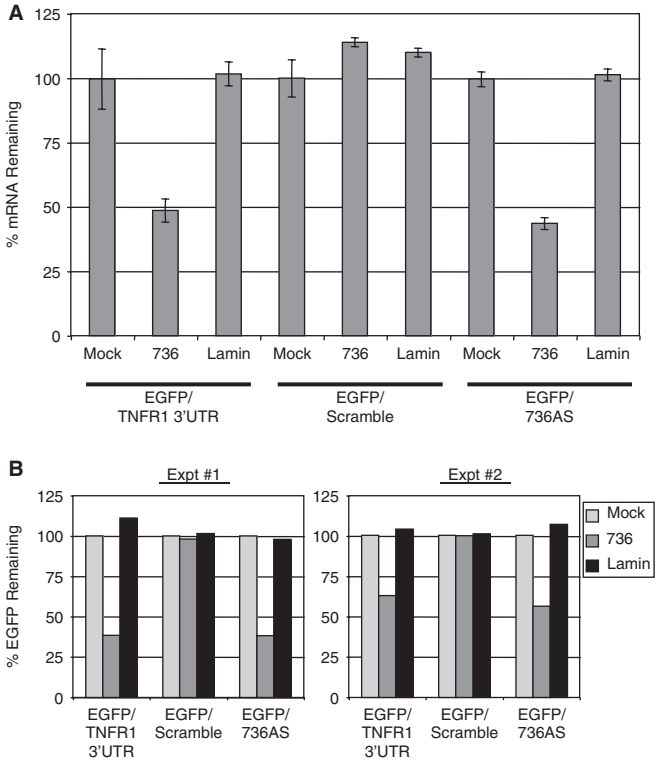

Figure 7. TNFR1 3′UTR Target sequence determines off-target activity of the sense strand of 736. EGFP/TNFR1 3′UTR chimeric constructs were designed to contain wild-type TNFR1, or scrambled or 'complete' homology to the sense strand of 736. These constructs were inserted into retroviral vectors and used to produce HUVEC cell lines which express the target sequences. Transduced cell lines were transfected on back to back days with 736 or lamin siRNA or with Oligofectamine alone (mock). (**A**) qRT-PCR was performed on cDNA from each culture using EGFP-specific primers. Values were normalized using actin controls and converted to '% mRNA Remaining' relative to mock treatment. Representative of one of two independent experiments with similar results. (**B**) Cells were collected 24 h after the last transfection and analyzed for EGFP reporter expression by flow cytometry. Corrected MFIs were converted to '% GFP Remaining' relative to mock treatment. Two independent experiments are represented.

functional phenotype as 736 we purchased two commercially available siRNA reagents, TNFR1-1 and TNFR1-2. We tested 736, TNFR1-1 and TNFR1-2 for functional activity by their ability to inhibit TNF-mediated induction of EC cell surface antigens. TNF-mediated expression of E-selectin, ICAM-1 and MHCI were decreased 29, 51 and 30%, respectively, by 736 siRNA transfection compared to lamin controls (Table 1). TNFR1-1 and TNFR1-2 siRNA transfection produced a similar, but more pronounced decrease in antigen induction. TNFR1-1 inhibited E-selectin, ICAM-1 and MHC I by 70, 75 and 43%, respectively, and TNFR1-2 by 70, 78 and 58%, respectively. This demonstrates that 736 displays a similar functional effect as other TNFR1 silencing siRNA.

Chemical modification of RNA oligos can have significant consequences for siRNA activity and can be used to identify strand utilization of an siRNA duplex. We have used two chemical modification strategies to determine the role that the sense strand of 736 plays

Table 1. Comparison of 736 with other TNFR1 siRNA species

siRNA	E-selectin	ICAM-1 (MFI)	MHC I
Mock	72	892	242
Lamin	70	1058	224
736	50	522	155
TNFR1-1	21	267	127
TNFR1-2	21	232	94

HUVEC were transfected with indicated siRNA. Cells were treated with TNF for 24 h immunostained with E-selectin, ICAM-1 and MHCI, and analyzed by flow cytometry.

in TNFR1 knockdown. First, we exploited the fact that 5′ phosphorylation of the guide, or targeting, strand is required for efficient siRNA activity. Therefore, capping the sense strand of 736 with a terminal 5′ modification that inhibits phosphorylation should result in the inactivation of 736 siRNA. We have prepared a hybrid 736 siRNA with an unmodified antisense strand and a 5′deoxy cap on the sense strand [736(5′Cap)]. We compared the activity of this duplex with wild-type 736 by transfecting HUVEC and measuring E-selectin and ICAM-1 induction following TNF treatment (Figure 8). 736 inhibited E-selectin by 62% and ICAM-1 expression by 55% compared to lamin controls, while 736(5′Cap) actually showed increased E-selectin expression while only inhibiting ICAM-1 by 15%. Next we used limited 2′-*O*-methyl modification of RNA, which is known to inhibit siRNA strand activity (29), to determine the strand utilization for 736. Specifically, we introduced 2′-*O*-methyl modifications at the 5′ end (positions 1 and 2) of the sense or antisense strands of 736 and prepared chemically modified, hybrid siRNA duplexes (Figure 9A). HUVEC were transfected with wild-type or modified siRNAs and tested for TNFR1 expression by immunoblotting (Figure 9B) and for TNF-inducible protein expression by FACS (Figure 9C). A control siRNA, LFA-3, had no effect on TNFR1 protein expression. Transfection with unmodified, wild-type 736 siRNA (736/WT) resulted in a pronounced decrease in TNFR1. 2′-*O*-methylation of the antisense strand when paired with unmodified sense strand (736/AS) largely preserved this siRNA effect whereas modification of the sense strand, alone (736/S) or in combination with modification of the anti-sense strand (736/S + AS), inhibited protein knockdown. Similarly 2′-*O*-methylation of the antisense strand (736/AS) alone produced a marked reduction of ICAM and E-selectin, comparable to the effect of transfection with 736/WT. This result confirms that the unmodified sense strand is sufficient to produce the anti-TNFR1 effects of 736. When the sense strand of 736 was modified (736/S) we did observe a modest reduction in ICAM protein expression, but there was no effect on E-selectin. A direct but modest anti-ICAM activity of the unmodified 736 antisense strand is likely to be responsible for this result. These data support the assertion that an unmodified, phosphorylated 736 sense strand can be utilized to promote RNAi activity and is both necessary and sufficient for effective TNFR1 knockdown.

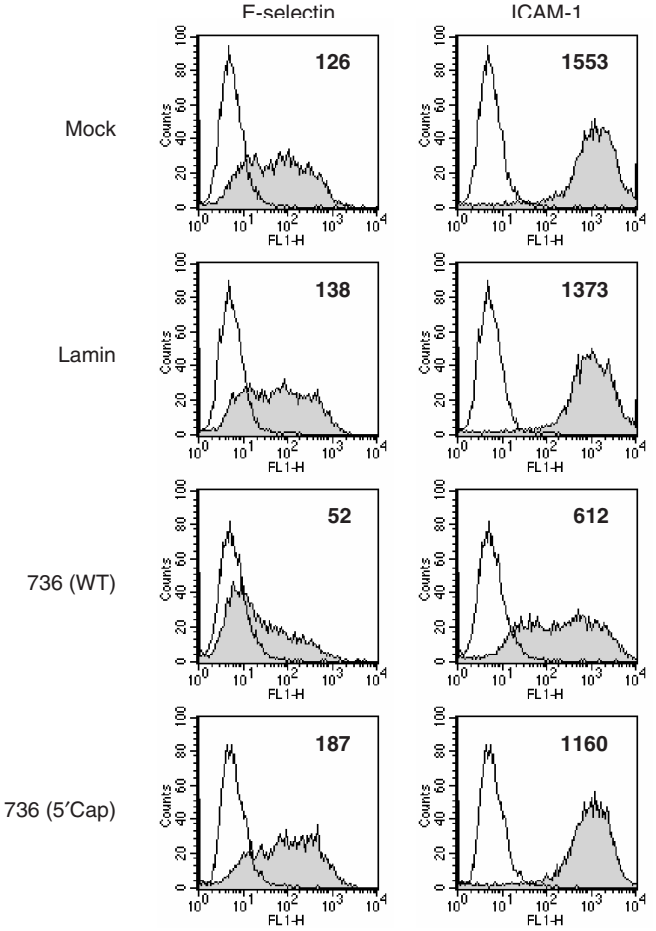

Figure 8. 5′ phosphorylation of the 736 sense strand is required for effective TNFR1 knockdown. Hybrid 736 siRNA duplexes were prepared by annealing wild-type 736 antisense RNA oligos with either wild-type 736 sense strand oligos [736 (WT)] or 5′deoxy capped 736 sense strand oligos [736 (5′Cap)]. HUVEC were mock-transfected or transfected with 10 nM lamin, 736 (WT), or 736 (5′Cap) siRNA on consecutive days and treated with 10 ng/ml TNF for 24 h. HUVEC were immuno-stained using fluorescent antigen-specific antibodies for E-selectin and ICAM-1 and analyzed by flow cytometry. Filled histograms represent antigen-specific staining and empty histograms represent isotype control staining. Corrected MFI in the upper right hand corner = antigen-specific MFI minus isotype MFI. Representative of one of two experiments with similar results.

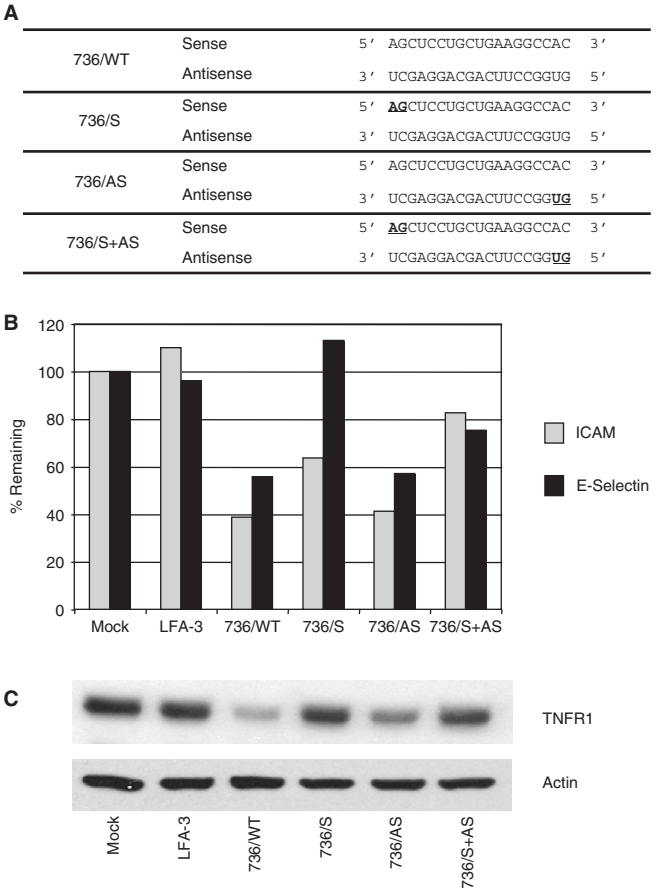

Figure 9. 2′-*O*-methyl modification of the 736 sense strand eliminates TNFR1 knockdown. (**A**) Hybrid 736 siRNA duplexes were prepared by annealing unmodified 736 RNA oligos (sense or antisense) with complementary RNA oligos modified by 2′-*O*-methylation, (such hybrid duplexes are indicated as 736/S or 736/AS). Dual modified duplexes (736/S + AS) and wild-type 736 (736/WT) were also prepared. 2′-*O*-Me modified bases are in bold and underlined. (**B**) HUVEC were mock-transfected or transfected with 10 nM LFA-3, an irrelevant siRNA, 736/WT, 736/S, 736/AS or 736/S + AS siRNA on consecutive days and treated with 10 ng/ml TNF for 24 h. HUVEC were immuno-stained using fluorescent antigen-specific antibodies for ICAM-1 and E-selectin and analyzed by flow cytometry. Corrected MFIs were converted to '% Remaining' relative to mock expression. (**C**) Hybrid siRNA-transfected HUVEC lysates were analyzed by western blot for the expression of TNFR1 and β-actin. Representative of one of two experiments with similar results.

These experiments cumulatively show that the sense strand of 736 is capable of mediating TNFR1 knockdown without full complementarity with its target sequence. A question arises whether 736 is functioning as an attenuated (mismatched) siRNA or as miRNA. SiRNA activity is usually dependent on thermodynamic stability throughout the target sequence, while miRNA activity is critically dependent on nucleotides 2–8 at the 5′ end of the strand. This so-called 'seed' region is extremely sensitive to mismatches while the remaining portion of the sequence may, or may not, participate in a compensatory role to stabilize the miRNA-target duplex (30).

To clarify the sequence-dependent activity of the 736 sense strand, we prepared a series of siRNA duplexes with an unaltered antisense strand paired to variously mutated 736 sense strands (see Table 2 for sequences). Using these hybrid siRNA, we were able to compare 736 sequence-dependent activity for sense strands bearing 0, 2, 3, 4 and 5 nt mismatches with the TNFR1 target sequence. In Table 2, we show that the introduction of mismatches that destabilize duplex formation and increase free energy of hybridization with the TNFR1 target sequence is inversely related to a reduction in TNFR1 cell surface expression and TNF-mediated induction of ICAM. Notably, single mutations in the 736 sense strand were inhibitory whether they were introduced within the potential miRNA 'seed' region (see 736/M4 and 736/M6) or whether they were more proximal to the 3′ region. In addition, we engineered a 736 sense strand with an intact potential 8 nt 5′ 'seed' region and a 3-nt canonical bulge at positions 9–11 (Table 3). When duplexed with

Table 2. 736 Sense strand knockdown activity is directly related to thermodynamic stability with TNFR1 target sequence

TNFR1 mRNA:	3′ TCGAGGTCGACTTCCGGGGdTdT 5′	ICAM (MFI)	TNFR1 (MFI)	ΔG (kcal/mol)	Number of mismatches
TNFR1	5′ AGCUCCAGCUGAAGGCCCCdTdT 3′	187	5.2	−26.8	0
736	5′ AGCUCCUGCUGAAGGCCACdTdT 3′	276	6.8	−22.0	2
736/M4	5′ AGCGCCUGCUGAAGGCCACdTdT 3′	708	13.7	−19.6	3
736/M6	5′ AGCUCAUGCUGAAGGCCACdTdT 3′	633	10.9	−18.6	3
736/M16	5′ AGCUCCUGCUGAAGGACACdTdT 3′	817	11.6	−18.2	3
736/M15/16	5′ AGCUCCUGCUGAAGUACACdTdT 3′	963	17.7	−15.6	4
Lamin		986	21		

Hybrid 736 siRNA duplexes were prepared by annealing wild-type 736 antisense RNA oligo with a TNFR1 mRNA complementary oligo (TNFR1), wild-type 736 sense RNA oligo (736) and four other mismatched 736 sense strand oligos. Mismatches of the sense strand with the TNFR1 target sequence are underlined and noted in the table. Free energy (ΔG) calculations were made with the help of the DINAMelt Web application (www.bioinfo.rpi.edu).
HUVEC were transfected with hybrid siRNA containing wild-type 736 antisense oligo and mismatched 736 sense oligo or with lamin siRNA. After transfection, cells were stimulated for 24 h with TNF (10 ng/ml) and immuno-stained for ICAM-1 and cells were immuno-stained with TNFR1-specific antibody without TNF treatment.

Table 3. 736 Sense strand knockdown activity is not improved by incorporation of a bulge

TNFR1 mRNA:	3′ TCGAGGTCGACTTCCGGGGdTdT 5′	ICAM (MFI)	ΔG (kcal/mol)	Number of mismatches
736	5′ AGCUCCUGCUGAAGGCCACdTdT 3′	360	−22.0	2
736/M15/16	5′ AGCUCCUGCUGAAGUACACdTdT 3′	906	−15.6	4
736/Bulge	5′ AGCUCCUGACUAAGGCCACdTdT 3′	879	−12.0	5
Lamin		1129		

Hybrid 736 siRNA duplexes were prepared by annealing wild-type 736 antisense RNA oligo with wild-type 736 sense RNA oligo (736) and two mismatched 736 sense strand oligos. HUVEC were transfected with indicated siRNA. Cells were treated with TNF for 24 h immunostained with ICAM-1-specific antibody, and analyzed by flow cytometry. Mismatches of the sense strand with the TNFR1 target sequence are underlined and noted in the table. Free energy (DG) calculations were made with the help of the DINAMelt Web application (www.bioinfo.rpi.edu).

the wt antisense 736 strand, this siRNA, having five mismatches and a greatly lowered thermodynamic stability with the TNFR1 target, exhibited a loss of activity similar to other mutated strands, including a 4-nt mismatched strand, 736/M15/16 (Table 3). Taken together, these observations support a sequence-dependent requirement for 736 activity by an siRNA mechanism. An miRNA mechanism for TNFR1 silencing in this system seems unlikely based upon our mutagenesis studies and the effect on TNFR1 mRNA and protein levels.

TNFR1 complementarity with ICAM-1 does not regulate ICAM-1 or TNFR1 expression

Natural antisense transcripts (NAT), like miRNA and siRNA, are naturally occurring RNA species known to negatively regulate gene function by inhibition of transcription, RNA splicing and protein translation. In this report, we have identified and characterized an siRNA target sequence in the 3′UTR of TNFR1 which has 18 of 20 nt complementarity to a site within the coding sequence of human ICAM-1 (an additional homologous base pair between ICAM-1 and TNFR1 mRNA). Because of this complementary sequence relationship, we investigated whether TNFR1 transcript sequences might regulate ICAM-1 mRNA levels by an NAT mechanism, or vice versa. Because TNFR1 overexpression is toxic for EC we used our EGFP chimera transduced cell lines, which express steady state levels of EGFP/TNFR1 chimeric mRNA. These cells were treated with graduated amounts of TNF to induce ICAM-1 expression and cell surface ICAM-1 levels were measured by FACS to determine whether ICAM-1 levels were affected by TNFR1 sequences. All three cell lines responded to TNF treatment by up-regulating cell surface ICAM-1 levels. EGFP/TNFR1-3′UTR expressed slightly higher ICAM-1 levels than the other two lines (Figure 10A), and EFGP/736AS cells had nearly identical ICAM-1 protein levels as EGFP/Scramble. We conclude from this, and a similarly conducted experiment, that ICAM-1 protein expression is not negatively affected by the presence of complementary EGFP/TNFR1 chimeric mRNA sequences.

To further investigate the possibility of a natural antisense regulatory relationship between ICAM-1 and TNFR1, we tested whether HDMEC that stably express high levels of ICAM-1 have lower levels of surface TNFR1 expression. HDMEC from the same parental line were transduced with an empty retroviral vector (mock) or virus coding for ICAM-1 or E-selectin. By FACS immunostaining, mock and E-selectin cell lines expressed low basal ICAM-1 levels (MFI = 21 and 18, respectively) while the ICAM-1 cell line expressed high levels on their cell surface (MFI = 5884; Figure 10B). TNFR1 levels for mock and E-selectin HDMEC were MFI 15.6 and 11.6, respectively, while ICAM-1 HDMEC had a more profoundly reduced TNFR1 MFI of 7.7. As a control, we also evaluated TNFR2 levels in these cell lines and found the same expression pattern as TNFR1. Mock and E-selectin lines had TNFR2 MFI of 26.4 and 25.2, respectively, and ICAM-1 HDMEC had a reduced

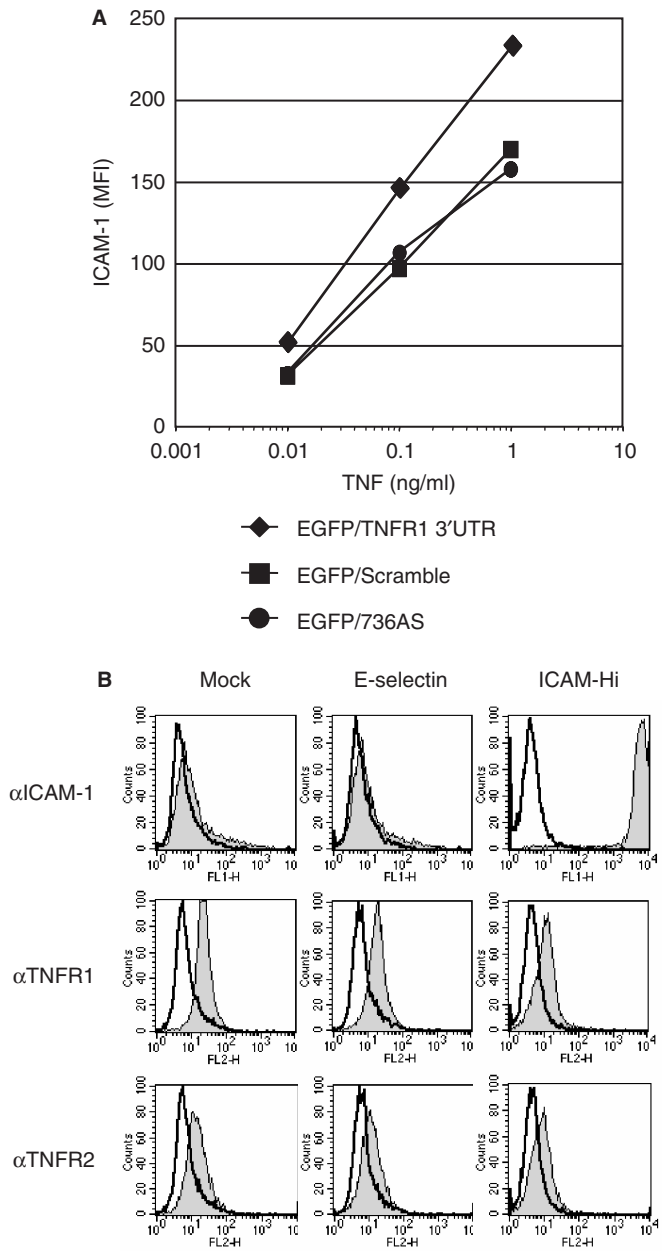

Figure 10. Complementarity between TNFR1 and ICAM-1 does not activate a natural antisense transcript (NAT) activity. (**A**) HUVEC cell lines expressing EGFP/TNFR1 3′UTR chimeric constructs were treated for 24 h with increasing concentrations of TNF to induce ICAM-1 expression. ICAM-1 expression levels were determined by FACS using an ICAM-1-specific antibody. (**B**) HDMEC cell lines overexpressing E-selectin and ICAM-1 were tested to determine basal TNFR1 cell surface levels. Cells were immunostained with fluorescently labeled antibody specific for ICAM-1, TNFR1 and TNFR2 then analyzed by FACS. Representative of one of two independent experiments with similar results.

MFI of 15.3. A similar reduction of receptor expression was observed in two additional independent sets of transductants. We conclude that ICAM-1 mRNA or protein does appear to reduce TNFR1, but the effect is not specific and we cannot conclude that the complementary sequence in TNFR1 or ICAM-1 act as endogenous natural antisense agents.

DISCUSSION

The present study examines an unexpected off-target effect of an siRNA molecule, 736, previously identified in a screen targeting ICAM-1(20). This study credited 736 for silencing TNF-stimulated ICAM-1 via an siRNA mechanism. We independently confirmed that 736 siRNA effectively silenced ICAM-1 expression in TNF-treated EC, but found, unexpectedly, that ICAM-1 expression induced by IL-1 was silenced much less effectively (Figure 5C). We interpret this to suggest that direct targeting of ICAM-1 mRNA is not the primary cause of 736 inhibitory activity. Instead, the majority of ICAM-1 knockdown as well as the global off-target effects of 736 on TNF-induced protein expression (E-selectin, VCAM-1 and MHC I) can be attributed to direct off-target silencing of TNFR1 by the 736 siRNA sense strand. We demonstrate this fact with three separate lines of investigation. First, we show that with EGFP/TNFR1 chimeric transcripts, bearing a wild-type 736 target site or a scrambled 736 target site, only transcripts with an intact target sequence are sensitive to 736-mediated silencing (Figure 7A and B). Next, we show that inhibition of 736 sense strand phosphorylation, which is known to affect RISC utilization, significantly impedes the off-target silencing of TNFR1 (Figure 8). In addition, we demonstrate by 2′-*O*-methyl chemical modification of individual siRNA strands that the 736 sense strand, and not the antisense strand, is utilized to silence TNFR1 protein expression and inhibit global TNF-mediated adhesion molecule induction (Figure 9B and C). Both observations strongly suggest that RISC strand utilization of the sense strand is the primary mechanism of the observed off-target effect. And lastly, we prepared hybrid 736 siRNA duplexes with the 736 antisense strand and sense strands containing mismatches to the TNFR1 target (Tables 2 and 3). Introducing one or more mismatches between the sense strand and the target significantly decreased silencing activity in a duplex-stability and free energy dependent manner. In addition, by correcting the two intrinsic mismatches between 736 sense strand and TNFR1 we improved duplex stability and siRNA silencing (Table 2).

Most RNAi silencing of off-target genes can be loosely grouped into four general categories: immunostimulatory or interferon-like, miRNA-like, siRNA-like or toxic. We first looked at the interferon response to determine whether 736 was triggering a cellular stress response that led to generalized RNA degradation and protein synthesis inhibition. We do not believe that the off-target effect of 736 falls into this category because the levels of OAS1 and PKR mRNA were not increased in 736 siRNA-transfected cells (Figure 2D). miRNA-like off-target effects, the reduction of protein levels in the absence of mRNA knockdown, cannot account for the off-target activity of 736 because TNFR1 mRNA is significantly reduced in 736-treated cells (Figure 5), suggesting an siRNA-like degradation of TNFR1 transcript and subsequent reduction of translational activity. We did not conduct functional genomic expression profiling to directly address the additional possibility that 736 siRNA causes a transcriptome-wide effect of gene knockdown

as would be seen if the effects of 736 were mediated through cell injury, but we did show that EC transfected with 736 siRNA had similar growth rates, levels of apoptosis, and levels of necrosis as control- or mock-transfected cells (Figure 2). In addition, we can reasonably attribute TNF signaling defects in 736 siRNA-transfected EC specifically to the silencing of TNFR1 message since protein and mRNA expression of other members of the TNF signalosome (RIP-1, TRAF-2 and TRADD) remain unchanged (Figure 5) and since IL-1 signaling is unaffected in 736 siRNA-transfected cells (Figures 3B and 4A). Based on our findings with chemical modifications and sequence alterations as well as the observation that mRNA reduction is sufficient to account for the effects on TNFR1 protein, we believe that an siRNA-like effect rather than an miRNA-like effect can best account for these data. Taken together, these observations support the conclusion that the 736 off-target effect is due to TNFR1 mRNA knockdown by the sense strand via an siRNA-like mechanism.

To our knowledge, the present study is the first detailed functional and mechanistic characterization of an RNAi off-target effect being mediated by an siRNA sense strand that leads to the silencing of a protein (TNFR1) that normally regulates expression of the intended target (ICAM-1). Previous studies have suggested that sense strands of siRNA are capable of entering the RISC complex and participating in RNAi silencing (21,22). In addition, Jackson *et al*. published a report showing that off-target effects can be mediated by a sense strand RNAi mechanism (15). In a comprehensive functional genomic expression screen, these authors used sequence comparison of genes to identify siRNA off-target silencing consistent with the sense strand of an siRNA, but these important observations were not directly confirmed by mutagenesis studies. An important aspect of this work is the unintended knockdown of an 'upstream' effector of the intended target. Other studies have reported unintended knockdown of 'downstream' proteins in the course of screening for 'upstream' effectors. For example, Lin *et al*. (23) used an siRNA library against protein kinases in an effort to identify 'upstream' effectors of Hif-1α protein stabilization and found, by directly measuring Hif-1α levels, that several siRNAs in their library directly targeted Hif-1α mRNA. However, there is no evidence that the intended kinase targets are actually upstream regulators of HIF-1α. In contrast, our study was designed to target a 'downstream' protein, ICAM-1, and lead to an unexpected silencing of an established 'upstream' regulator of the ICAM-1 pathway, TNFR1.

In this report, we have investigated the mechanism by which 736 contributes to specific silencing of TNFR1 in some detail. Because 736 has incomplete homology with TNFR1 and is targeting the 3′UTR, both being characteristic of miRNAs (31), it was necessary to ask whether 736 uses a more siRNA-like or a more miRNA-like mechanism to silence TNFR1. Recent reports have indicated that siRNA species can silence mRNA expression in a similar fashion as miRNA (16) and that miRNA can catalyze the enzymatic degradation of mRNA (32). Promiscuous silencing mechanisms make it difficult to assign an siRNA or miRNA mechanism to 736 with absolute certainty, but we favor a classical siRNA mechanism based on the criteria of (i) efficient mRNA degradation, (ii) absence of a functional canonical miRNA 'seed' region by mutational analysis, (iii) intact cleavage site and (iv) thermodynamic duplex stability. First, our data clearly show that TNFR1 mRNA is reduced by the sense strand of 736, a prerequisite for siRNA-like activity (Figure 5B). Degradation of mRNA leads to protein loss (Figure 5A) and diminished functional response to TNF (Figure 5C). miRNA silencing is dependent on target base recognition by a canonical 5′ seed region within the miRNA strand (33) and nt 2–7 are most critical. But, the sense strand of 736 only has a continuous string of 5 bp identity at its 5′ end with TNFR1 in this region (Figure 6) making it a less than optimum miRNA seed region candidate. The Lin *et al*. (23) 7 bp miRNA and Let-7 miRNA (34) both use a 7-nt seed region for silencing, but we are unaware of smaller sequence motifs that can mediate efficient miRNA target silencing. Furthermore, mutagenesis studies of functional seed regions have shown that miRNA activity is profoundly dependent on seed region duplex stability (30). We tested whether the 5′ region of the 736 sense strand was sufficient to independently silence TNFR1 by introducing mismatches in the 3′ region of the 736 sense strand (Table 2). The intact 5′ region of 736 is significantly less active when a single mismatch is introduced at position 16 from the 5′ end (736/M16), and entirely inactive when two mismatches are introduced outside the seed region (736/M15/16). These data suggest that the 7 nt 5′ region of 736 sense strand is not sufficient to mediate silencing. We also introduced mismatches into the 736 sense strand that would produce a siRNA:target bulge at positions 9–11 (Table 3). Bulges spanning the 'slicer' endonuclease cleavage site are often present in miRNA (31) and may serve to recruit additional components of the miRNA machinery (35). With intact 5′ and 3′ 736 sense strand regions and a central canonical bulge (736/Bulge), the modified 736 RNA produced diminished, not enhanced, RNAi activity (Table 3). We reason from this experiment that a bulge structure in 736 does not positively enhance recruitment or activation of miRNA components. We may also interpret these results as a loss of siRNA function, demonstrating a strict requirement for an intact, duplexed cleavage site for optimal 736 silencing activity. Finally, we observe that any introduction of siRNA:target mismatches inevitably destabilized the duplex and lowered the free energy of hybridization (Tables 2 and 3). It is widely acknowledged that thermodynamic duplex stability greatly influences RNAi activity, whether via an siRNA-like or miRNA-like mechanism (30,36). Indeed, we observed that 736 knockdown of TNFR1 is increased when the two intrinsic mismatches between 736 sense and TNFR1 mRNA are corrected (Table 2), supporting the notion that the sense strand of 736 functions as an attenuated 'classic' siRNA. Thus, 736 sense strand activity does not depend on classical miRNA-like criteria, but the dominant factor influencing 736 silencing activity appears to be the free

energy and thermodynamic stability of the siRNA:target duplex.

The complementarity of the transcripts encoding ICAM-1 and TNFR1 raised the possibility that these mRNA species could act as natural antisense transcripts (NAT). NAT are recognized as an important mechanism for transcriptional regulation in mammals (37) with recent reports predicting the existence of NAT for 20% of human mRNAs (38) and 72% of mouse mRNAs (39). NAT are known to act at various stages including inhibition of transcription, RNA splicing and protein translation. NAT and cognate transcript duplexes can serve as a substrate for Dicer leading to the destruction of target transcript and generation of siRNA that would further suppress gene expression (40). To detect a potential antisense relationship between ICAM-1 and TNFR1, we first compared the levels of ICAM-1 expression in response to stimulation with known amounts of TNF in our EGFP/TNFR1 transgene expressing HUVEC. We found no differences in ICAM-1 induction whether the TNFR1 complementarity site was present or absent. To test the converse relationship, we determined whether elevated expression of ICAM-1 in HDMEC cell lines could lower TNFR1 cell surface levels by triggering TNFR1 mRNA degradation in an antisense fashion. Here we did observe a reduction of TNFR1 mRNA in ICAM-1 transduced lines, but since TNFR2 was also depressed in these cells (Figure 8) we were unable to conclude that TNFR1 transcript can negatively regulate ICAM-1, or vice versa. Further study into the possible natural antisense relationship between TNFR1 and ICAM-1 is warranted, but more sensitive methodologies, including qRT-PCR and *in vitro* RISC cleavage assays will likely be more instructive.

Much recent effort has been devoted to identifying siRNA cross-reactivity at the design stage. Computational database homology searches *in silico* can be used to identify potential off-target effects of prospective siRNAs based on homology with non-targeted genes (41,42). BLAST searches of candidate siRNAs will invariably identify partial homologies with known mRNA sequences (43). The question of whether to 'disqualify' prospective siRNAs based on sequence homology remains controversial because no standard criteria exists today to confidently eliminate prospective siRNAs based on partial homology with non-targeted genes. Early studies indicated that single base pair mismatches between siRNA and cognate target transcripts completely eliminated RNAi activity (36). The current Editorial Policies of the Journal of Biological Chemistry, based on the 2003 recommendations published in Nature Cell Biology (44), state that a 1 or 2 base pair mismatch may be introduced as a control to demonstrate siRNA specificity. More recent investigations have revealed that RNAi activity is more promiscuous than previously thought (45). Du *et al.* systematically mutagenized a previously characterized siRNA and found that siRNA complementarity requirements can be highly degenerative (46), and efficient silencing occurs even with 3 base pair mismatched siRNAs (47). Moreover, even homology stretches as short as 7 nt have now been shown to mediate off-target silencing (23). Indeed, we report here that the sense strand of 736, having a 2-base pair mismatch, is capable of significant off-target knockdown of a TNFR1. Our study provides experimental and functional evidence that siRNAs with partial homology to non-targeted transcripts can produce unexpected and misleading off-target effects. Developing highly reliable computational predictors for off-target effects will have significant value in the RNAi field.

In the absence of clear computational standards, off-target effects caused by sequence similarities can be managed by adhering to strict experimental target validation protocols. Many excellent commentaries have been published that outline guidelines for experimentally validating siRNA specificity (48–50). Guidelines for insuring siRNA specificity usually include the following tenants; (i) monitor both mRNA and protein levels of target genes to insure that protein levels agree with observed loss-of-function phenotypes, (ii) rule out interferon responses, (iii) identify the most effective siRNAs and titrate them to lowest effective concentrations to limit siRNA off-target and interferon responses, (iv) confirm loss-of-function phenotype with multiple siRNA's to the same target and (v) perform 'rescue' experiments with siRNA-resistant target genes that fail to produce the loss-of-function phenotype (44). An additional control drawn from these data would be to monitor siRNA effects on non-target genes that are closely related to, or activated similarly as the targeted transcript. For example, the off-target effects of 736 became apparent because we measured E-selectin and VCAM-1 levels in our initial siRNA screens, both of which are activated by TNF in a similar manner as ICAM-1.

In summary, we have characterized an siRNA compound composed of an antisense strand intended to silence human ICAM-1 whose sense strand exhibited an off-target effect against TNFR1. We establish that 736-mediated knockdown of TNFR1 is not due to an miRNA-like off-target mechanism or to an immunostimulatory/interferon stress response, and we provide experimental confirmation that the sense strand of 736 functions as an siRNA leading to degradation of TNFR1 mRNA. This may be the first example in which the off target effect of an siRNA is actually responsible for the anticipated effect by acting to reduce expression of a protein (TNFR1) that normally regulates expression of the intended target (ICAM-1).

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