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AUF1-RGG peptides up-regulate the VEGF antagonist, soluble VEGF receptor-1 (sFlt-1)

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Abstract

The macrophage is essential to the innate immune response, but also contributes to human disease by aggravating inflammation. Under severe inflammation, macrophages and other immune cells over-produce immune mediators, including vascular endothelial growth factor (VEGF). The VEGF protein stimulates macrophage activation and induces macrophage migration. A natural inhibitor of VEGF, the soluble VEGF receptor (sFlt-1) is also produced by macrophages and sFlt-1 has been used clinically to block VEGF. In macrophages, we have shown that the mRNA regulatory protein AUF1/hnRNP D represses VEGF gene expression by inhibiting translation of AURE-regulated VEGF mRNA. Peptides (AUF1-RGG peptides) that are modeled on the arginine- glycine-glycine (RGG) motif in AUF1 also block VEGF expression. This report shows that the AUF1-RGG peptides reduce two other AURE-regulated genes, TNF and GLUT1. Three alternative splice variants of sFlt-1 contain AURE in their 3' UTR, and in an apparent paradox, AUF1-RGG peptides stimulate expression of these three sFlt-1 variants. The AUF1-RGG peptides likely act by distinct mechanisms with complimentary effects to repress VEGF gene expression and over-express the endogenous VEGF blocking agent, sFlt-1. The AUF1-RGG peptides are novel reagents that reduce VEGF and other inflammatory mediators, and may be useful tools to suppress severe inflammation.

Keywords

AUF1; hnRNP D; VEGF; soluble VEGFR-1; sFlt-1; AUF1-RGG peptide; AU-rich elements; AURE; inflammation

Introduction

Inflammation is a natural response by the immune system to insults on biological integrity by live pathogens. A central mediator of the innate immune response is the macrophage cell

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[1]. Macrophages are activated by bacterial cell wall components and by endogenous cytokines, including vascular endothelial growth factor (VEGF) [2,3] and TNF [4,5]. Both VEGF and the macrophage play a critical role in inflammatory processes including sepsis [6], synovial joint inflammation [7], atherosclerosis [8], tumorigenesis [9], corneal neovascularization [10], and nephritis [11]. A major target of VEGF is the VEGF receptor on endothelial cells. Whereas endothelial cells express both VEGF receptors, VEGFR1 and VEGFR2, macrophages only express VEGFR1 (Flt-1) [12]. The VEGFR1 receptor plays a role as a decoy receptor on endothelial cells because VEGFR1 is less active (low kinase activity, high VEGF affinity) [9]. On macrophages VEGFR1 is active, and blockade of VEGFR1, but not VEGFR2, reverses joint destruction in the K/BxN mouse model of arthritis [13]. Macrophages respond to VEGF in migration assays [12,14], and this role explains the presence of macrophages at sites of inflammation.

To suppress inflammation therapeutically, cytokines are targeted with neutralizing biologic reagents including anti-cytokine antibodies and soluble cytokine receptors. Soluble receptors retain cytokine binding affinity but lack the membrane spanning domain and intracellular signaling domain. Experimental treatments for severe inflammation, such as sepsis, include VEGF-neutralizing soluble VEGFR1 (sFlt-1) [6,15–17]. Treatment of choiroidal neovascularization [18] and murine arthritis models [15] has also been successful with sFlt-1. However, biologics, such as antibodies and soluble receptors, require injection and target only one cytokine. Treatment of monkeys with sFlt-1 expressed by adenovirus reduced experimentally induced neovascularization in the eye [19]. However, viral mediated biologicals also have risks. This project explores preliminary development of AUF1-RGG peptides that target multiple cytokines in inflammation.

To better understand mechanisms of VEGF cytokine expression, this report explores the production of the VEGF antagonist sFlt-1. In previous work we have shown that the mRNA regulatory protein AUF1/hnRNP D suppresses VEGF gene expression [2]. In addition, we have discovered AUF1-RGG peptides that are modeled on the “RGG” regulatory domain of AUF1 [2]. The AUF1-RGG peptides reduce expression of VEGF, perhaps by activating AUF1. The AUF1 protein binds AU-rich elements (AURE) in targeted mRNA and usually decreases translation [20], but may increase translation [21] or transcription [22]. The sFlt-1 protein is encoded by multiple Variants and investigation of the mRNA of three sFlt-1 Variants revealed AURE in the 3'-untranslated regions, similar to those found in VEGF mRNA and other cytokines. To investigate whether the AUF1-RGG peptides affect AURE-dependent gene expression in genes other than VEGF, we first evaluated the effects of AUF1-RGG peptides on TNF and glucose transporter-1 AURE-regulated expression. Next, we determined that three sFlt-1 Variants with AURE in their mRNA are up-regulated when human macrophage-like U937 cells are treated with a synthesized AUF1-RGG peptide. Finally, we confirm the stimulatory effects of AUF1-RGG peptides on sFlt-1 mRNA and protein in mouse RAW-264.7 macrophage-like cells. Our results demonstrate that AUF1-RGG peptides suppress expression of cytokines, including VEGF, and increase expression of the VEGF neutralizing protein sFlt-1. The AUF1-RGG peptides likely act by different and opposing mechanisms to reduce VEGF activity. These results suggest that AUF1-RGG peptides may be valuable for designing small molecule drugs that suppress the activity of VEGF and other AURE-regulated cytokines.

Materials and Methods

Cell culture, transfection and luciferase assay

The RAW-264.7 cell line were obtained from ATCC and maintained as previously described [2]. Media was DMEM or RPMI supplemented with 10% FBS and antibiotics. Cultured cells were plated ~24 hours prior to transfection in 96-well plates with sufficient cells to

give ~40% confluence at the time of transfection. Cells were transfected with luciferase plasmid constructs at 50 ng plasmid DNA per well. Plasmid DNA was complexed with Lipofectamine-2000 in serum-free Opti-MEM media at a ratio of 3 μ L Lipofectamine-2000 per μ g DNA. After 24-hours, the luciferase activity in the lysate was determined by addition of luciferin substrate (Promega) in a Berthold luminometer. Transfections were performed in 3–6 replicate well and results reported as the mean $\pm$ standard deviation (“SD”).

Stable AUF1-RGG peptide expressing cell lines were created by transfecting RAW-264.7 cells with plasmids containing the AUF1-RGG peptide sequence and a G418 resistance gene (pcDNA-3.1 parent vector). After 24 hr, cells were brought to 500 μ g/mL G418 and incubated for 30 days. Colonies were isolated and screened by Real-Time PCR for the presence of the AUF1-RGG mRNA. Control stable cell lines (“PC-2”) were created using the pcDNA-3.1 parental plasmid. Comparable levels of mRNA in all clones were confirmed by obtaining RNA from stable cell lines and performing RT-PCR using the vector based primer pairs (“T7” sense primer and “BGH” anti-sense primer, Invitrogen). The effects of AUF1-RGG peptides on AURE-dependent luciferase production were measured by transfecting AUF1-RGG stable cell lines with luciferase reporters, described below. Reporter activity of cells transfected with AUF1-RGG peptide plasmids are expressed relative to the activity of control stable cell lines (“PC-2”). All experiments were performed two or more times.

Human monocytic U937 cells were a generous gift of Patricia Pioli or Alan Eastman and were maintained in RPMI/10% FBS plus antibiotics and split when cell density reached 1.5×10^6 per mL. To study sFlt-1 splice variants, U937 cells were treated for 24 hr with PMA (34 nM) and then treated with a synthesized AUF1-RGG peptide for an additional 72 hr. The lyophilized AUF1-3RGG peptide was suspended in PBS and added directly to U937 cells to give a final concentration of 20 μ M. The AUF1-3RGG (see Table 1) peptide contains the three RGG motifs from AUF1. The peptide was synthesized in vitro using sterile techniques, and cells were not contaminated by peptide treatment.

Plasmid construction and peptide expression

VEGF-3'UTR reporter—Construction of VEGF-3 UTR luciferase reporter was previously described [3]. Briefly, the mouse 3 UTR was PCR amplified and cloned into the 3 UTR of the luciferase gene in the pcDNA-3.1-luciferase parent vector. The VEGF construct contains bases 209-1747 of the mouse VEGF 3 UTR sequence in the GenBank reference sequence, AF317892.

TNF reporter—The human TNF reporter was created by PCR cloning into the luciferase-pcDNA-3.1 construct [described in 3]. The human TNF 3 UTR sequence corresponds to nt 1254-1529 of the NCBI Reference sequence NM_000594. The human TNF-AURE reporter contains nt 1306-1406 of the NCBI Reference sequence NM_000594. The cloned human TNF sequence contains three polymorphisms differing from the Reference sequence (1286-G T; 1355-T C; 1357-A G). The cloned human TNF AURE sequence contains two polymorphisms (1355-T C; 1381-T A).

GLUT1 reporters—The human GLUT1 reporter was created in the pGL3 luciferase reporter (Promega) as previously described [23]. The human 3 UTR sequence corresponds to nt 2005-2998 of the NCBI Reference sequence NM_006516. The human GLUT1-AURE reporter contains nt 2231–2265 of the NCBI Reference sequence NM_006516.

AUF1-RGG peptide expression constructs—The construction of the human AUF1-RGG expression constructs has been described [2]. Plasmids expressing peptides modeled

on the RGG domain of AUF1 (AUF1-RGG peptides) were created by PCR amplification and TA-cloning into pcDNA-3.1 (Invitrogen). Amino acid sequences are shown in Figure 1. Methionine start codons were introduced in AUF1-RGG constructs, and all clones were DNA sequenced to confirm the integrity of the construct.

Localization of AURE in sFlt-1 3'UTR sequences

The number of AURE in human sFlt-1 Variants were identified. Three classes of AURE have been described [26]. *Class I.* The Class-I AURE's contain multiple non-contiguous AUUUA pentamers that may or may not be found in groups. *Class II.* The Class-II AURE's contain multiple closely spaced AUUUA pentamers or U/A-U/A-AUUUA-U/A-U/A nonamers ("U/A" may be either U or A). *Class III.* The Class-III AURE's are adenosine and uridine rich elements but have few or no AUUUA pentamers or nonomers. The NCBI "Reference" files containing sequences for Variants 1–3 are NM_002019, NM_001159920, NM_001160030, respectively. The number of Class-I pentamers and Class-II nonamers were located and counted for the three sFlt-1 Variants expressed in U937 cells. AURE sequences longer than 15 nt and more than 80% A+U are listed as "Class-III."

Protein measurement by ELISA

Levels of mouse sFlt-1 protein in RAW-264.7 stable cell lines were measured by ELISA. Cells were rinsed, lysed in lysis buffer and sFlt-1 levels determined as described by the manufacturer (R&D Systems).

RNA preparation and Real-Time PCR

Human sFlt-1—The levels of human sFlt-1 mRNA were determined using Real-Time PCR. Total RNA was isolated from one million PMA-induced U937 cells that were not treated or treated once with the synthesized AUF1-3RGG peptide (20 µM) (sequence shown in Figure 1) for three days. Total RNA was obtained (RNeasy, Qiagen) and quantitated. One µg of RNA was DNase treated and converted to cDNA using reverse transcriptase (Fermentas). Fifty ng equivalents were PCR amplified using standard conditions (BioRad CFX-96, SYBR detection). Threshold levels (Ct) of PCR products were normalized to human β -actin mRNA measured in the same sample. The size of PCR products was determined on DNA agarose gels and PCR product integrity was confirmed by melting curve analysis. The three sFlt-1 isoform Variants contains unique 3' UTR sequences. The chromosomal DNA corresponding to each Variant 3' UTR sequences are non-identical and are separated by at least 30,000 nt on chromosomal DNA (NCBI Reference sequence NG_012003). Primers were designed to cross the splice junction that includes the last coding exon and the 3' UTR. Primer pairs were designed using "Primer3" (<http://frodo.wi.mit.edu/primer3/>), and were unique to each sFlt-1 Variant. Levels of human sFlt-1 were normalized to levels of human actin mRNA measured in the same sample.

Mouse sFlt-1—A primer pair was designed for the only known mouse sFlt-1 mRNA sequence reported (NCBI Reference sequence D88690) such that one primer includes the 3' UTR sequence. Mouse sFlt-1 was normalized to levels of mouse actin mRNA measured in the same sample.

Primer sequences are as follows:

human sFlt-1-Variant-1-SE: 5'-gaaagagtcacagaagaggatgaag-3' ;

human sFlt-1-Variant-1-AS: 5'-catttttcggataaagagggttaat-3' ;

human sFlt-1-Variant-2-SE: 5'-atagctccaataaagttgggactg-3' ;

human sFlt-1-Variant-2-AS: 5'-gagagaaaacagcctttttgttg-3' ;

human sFlt-1-Variant-3-SE: 5 -atgtatacacaggggaagaaatcct-3 ;
human sFlt-1-Variant-3-AS: 5 -atgacgatggtgacgttgatgat-3 ;
human sFlt-1-Variant-4-SE: 5 -gtatccctcaacctacaatcaagt-3 ;
human sFlt-1-Variant-4-AS: 5 -aaggaaatggaagtagttggaagag-3 ;
human -actin-SE: 5 -attaaggagaagctgtgctacgtc-3 ;
human -actin-AS: 5 -atgatggagttgaaggtagtttcg-3 ;
mouse sFlt-1-SE: 5 -actcaagattactccatcactctgaac-3 ;
mouse sFlt-1-AS: 5 -tacttttcaaatgagtcctttaatgttt-3 ;
mouse actin-SE: 5 -accaacagcagacttcaggat-3 ;
mouse actin-AS: 5 -agactggcaagaaggagtggtaa-3 .

Results

Effects of AUF1-RGG peptides on VEGF gene expression in stable cell lines

In previous work we showed that transient transfection of plasmids expressing AUF1-RGG peptides represses endogenous VEGF protein and VEGF-3 UTR-dependent reporter expression [2]. The amino acid sequences of AUF1-RGG peptides are shown in Table 1. Sequences N- and C-terminal to the three RGG motifs were included because the minimal peptide sequence has not yet been determined. The “AUF1-Ex6+8” and “AUF1-QRGG” plasmids were used to create G418-resistant RAW-264.7 stable cell lines. Control G418-resistant cell lines (“PC-2”) were made using the “empty” pcDNA-3.1 plasmid. We next examined if AUF1-RGG stable cell lines affect 3 UTR-dependent expression, as found with transient transfection [2]. Cells were transfected with the VEGF-3 UTR reporter [2]. The AUF1-Ex6+8 and AUF1-QRGG stable cell lines reduced expression of the VEGF-3 UTR reporter by 65% and 80%, respectively (PC-2 control – 1.00 ± 0.05 ; AUF1-Ex6+8 – 0.35 ± 0.03 -- $p < 0.0001$; AUF1-QRGG – 0.19 ± 0.04 -- $p < 0.0001$; representative of at least two experiments).

Effects of AUF1-RGG stable cell lines on TNF-3'UTR and GLUT1-3'UTR reporters

Like VEGF, the tumor necrosis factor and glucose transporter-1 genes are regulated by a 3 UTR-dependent mechanism controlled by AURE [5,23,24]. To determine if AUF1-RGG peptides affect other AURE-regulated genes, AUF1-RGG stable cells were transfected with TNF full-length 3 UTR (TNF-FL) or GLUT1 full-length 3 UTR (GLUT1-FL) luciferase reporter plasmids. As shown in Figures 1-A and 1-C, the activity of full length TNF and GLUT1-3 UTR reporters was decreased in AUF1-RGG stable cell lines by greater than 70%, as compared to the PC-2 control cell line. To determine if AUF1-RGG peptides act on AURE sequences, reporters bearing the AURE of TNF or GLUT1 AURE were transfected into stable cell lines. Reporter activity (TNF-AURE and GLUT1-AURE) was reduced by 65–70% (Figures 1-B and 1-D).

AUF1-RGG peptides regulate sFlt-1

The sFlt-1 protein inactivates circulating VEGF protein, and studies on gene expression of VEGF in inflammatory diseases also consider gene expression of sFlt-1 [25]. Multiple variants of human sFlt-1 have been identified [16]. Primer pairs for PCR were designed that are unique to the mRNA of the four known human sFlt-1 Variants. Real-time PCR was used to measure Variant mRNA in human macrophage-like U937 cells. Without PMA induction, U937 cells did not produce detectable levels of any sFlt-1 Variant (data not shown). With

PMA-induction, three Variants are expressed in human U937 cells. When PMA-induced U937 cells were not treated or treated with in vitro synthesized AUF1-3RGG peptide, sFlt-1 mRNA increased 5–12-fold for all three sFlt-1 Variants (Figure 2). To determine if the 3' UTR of the three variants contain AURE, we analyzed the NCBI Reference files for the three human sFlt-1 3' UTR sequences. The AURE elements in mRNA may be grouped into three classes [26], and many AURE from all three classes are found in the 3' UTR of sFlt-1 Variants 1, 2 and 3 (Table 2). The location of the three classes of AURE are shown in Figure 3. Both scattered and clustered patterns are evident in the 3' UTR regions. Finally, we determined if the AUF1-3RGG peptides expressed in mouse macrophage-like RAW-264.7 stable cell lines affect mouse sFlt-1 protein and mRNA levels. As shown in Table 3, sFlt-1 protein levels in AUF1-Ex6+8 cells were ~150-fold higher than in the PC-2 control cell line. Levels of sFlt-1 in the AUF1-QRGG cell line were ~35-fold greater than in the PC-2 cell line. Levels of sFlt-1 mRNA were increased (~12-fold) in the AUF1-Ex6+8 cell line, as compared to the PC-2 control cells (Table 3).

Discussion

Severe inflammation in complex diseases, such as sepsis, results from the attack by macrophages and other immune cells on invading bacteria or other pathogens [6]. The immune system mounts a powerful defense, producing of strong mediators of cell activation, including tumor necrosis factor (TNF) [4] and VEGF [6]. Vascular permeability increases with VEGF production, leading to septic shock [6]. The immune response that produces biological activators, like TNF and VEGF, is tightly controlled at multiple levels of gene expression. A family of mRNA regulatory proteins control cytokine gene expression by acting on existing mRNA molecules to rapidly alter translation of mRNA into protein [27]. The AUF1 protein recognizes sequence elements in the 3' UTR of mRNA and increases or decreases translation [20]. In order to develop novel therapies that reduce severe inflammation, this report investigates the role of AUF1-RGG peptides in regulation of cytokine gene expression in macrophages.

A common feature of cytokine and growth factor mRNA's is the presence of AURE in the 3' UTR region [26,28,29]. Genomic analysis shows that AURE are found in 5–8% of human genes [29], and AUF1 controls expression of AURE-regulated genes [20]. In this report we investigate whether AUF1-RGG peptides also act on multiple targets in macrophages. Using AUF1-RGG stable cell lines, we show that VEGF, TNF and GLUT1 3' UTR reporters are all suppressed by AUF1-RGG peptides. Reagents that have complementary effects on cytokines such as VEGF and TNF may be useful in complex diseases. Of course, as the range of affected genes increases, there are chances of deleterious side effects. Trials in disease models are needed to determine if the AUF1-RGG peptides show promise for treating complex pathologies where multiple cytokines are targeted.

A curious discovery about mRNA regulatory proteins is that one protein may have opposing effects on translation in different systems. Studies of AUF1 show that in most cases AUF1 destabilizes mRNA and decreases gene expression [2,20]. However, AUF1 may also stimulate translation of some targets, such as IL-10 [21]. The present study shows that AUF1-RGG peptides both decrease (VEGF, TNF, GLUT1) and increase (sFlt-1) gene targets that have AURE enriched mRNA. Although the mechanism whereby AUF1-RGG peptides act remains to be determined, studies have established that AUF1 forms dimers, and these dimers initiate formation of a mRNA degradation complex [20]. We suspect that AUF1-RGG peptides decrease VEGF mRNA levels by promoting AUF1 dimer formation. This process explains why VEGF mRNA is decreased by AUF1 and AUF1-RGG peptides. It is possible that a different subset of degradation complexes exist that control sFlt-1 mRNA. Activation of AUF1 would cause disassembly of these complexes and release sFlt-1

mRNA from the degradative pathway. Alternatively, AUF1-RGG peptides may increase translation of sFlt-1 mRNA by another mechanism. The AUF1-RGG peptides may promote access of sFlt-1 mRNA to polyribosomes or alter AUF1 mediated mRNA shuttling into the nucleus [30]. A recent report shows that AUF1 also acts in the nucleus where it binds and activates the promoter of the Tert telomerase [22]. The AUF1-RGG peptides may affect transcription of sFlt-1, although preliminary images of FITC-labeled AUF1-RGG peptides show only cytoplasmic localization (data not shown). In summary, the results reported here demonstrate that AUF1-RGG peptides both decrease VEGF expression and increase expression of the VEGF blocking protein, sFlt-1, in the same cells. These opposing actions have a complementary effect to decrease VEGF activity in macrophages.

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Abbreviations

AURE	AU-rich element
AUF1	the AUF1/hnRNP-D mRNA binding protein
AUF1-RGG	arginine-glycine-glycine region of AUF1
GLUT1	glucose transporter-1
RGG	arginine-glycine-glycine
sFlt-1	soluble VEGF receptor-1 (sVEGFR1)
TNF	tumor necrosis factor- VEGF, vascular endothelial growth factor
UTR	untranslated region of mRNA

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Highlights

- The AUF1-RGG peptides are novel reagents that are modeled on the RGG regulatory region of AUF1
- AUF1-RGG peptides decrease expression of VEGF and stimulate expression of soluble VEGFR-1
- AUF1-RGG peptides act on AU-rich elements in mRNA

Figure 1. Effects of AUF1-RGG peptides on TNF and GLUT1 3 UTR-regulated gene expression Luciferase reporter plasmids expressing the full-length TNF (TNF-FL) (A), the AURE of TNF (TNF-AURE) (B), the full-length GLUT1 (GLUT1-FL) (C), or the AURE of GLUT1 (GLUT1-AURE) (D) were transfected into AUF1-RGG stable RAW-264.7 cells (“Ex6+8” or “QRGG”). Results are normalized to luciferase readings from “PC-2” control stable cells transfected with the same reporter. All results were $p < 0.003$, compared to the PC-2 control. Results are the mean $\pm$ SD for six wells and are representative of two or three experiments.

Figure 2. Effects of the AUF1-3RGG peptide on sFlt-1 Variant mRNA

The effects of treatment with a synthesized AUF1-3RGG peptide on expression of sFlt-1 mRNA levels was measured by treating U937 with AUF1-3RGG peptide (20 μ M) for three days. The mRNA levels (Ct) were measured by real-time PCR and expressed as fold change relative to cells not treated with peptide. Results are normalized to levels of the control mRNA, β -actin. Shown are the mean and range of duplicate samples.

Figure 3. Localization of AURE in the 3 UTR of sFlt-1 Variant mRNA

Three classes of AURE are shown in the 3 UTR of sFlt-1 Variants 1–3 (see Table 2). Diagrams shown relative lengths of the three 3 UTR Class I AUUUA pentamers are shown by gray caretts (“v”). Class II nonomers are shown by gray arrows (“ ”). Black bars designate Class III AURE. Longer AURE stretches are shown with wider bars.

Sequence of the RGG domain in AUF1

Table 1

Shown is the sequence of AUF1 peptides that include the RGG domain. The RGG domain includes the three RGG repeats found in the C-terminus of AUF1. Arginines in the RGG domain are enzymatically dimethylated (data not shown). Two peptides (“AUF1-Ex6+8” and “AUF1-QRGG”) are expressed in mammalian expression plasmids. Each peptide contains an initiating methionine residue (not shown). One peptide (“AUF1-3RGG”) was synthesized under sterile conditions. Amino acid numbering of AUF1 (p37 isoform) is shown on the left and right of peptide sequences.

RGG domain		
AUF1-Ex6+8	234-	EIKVAMSKEQYQQQQQWGS RG GFAGRARG RG GDQQSGYGKVS RRGG HQNSYKPY -287
AUF1-QRGG	245-	QQQQQWGS RG GFAGRARG RG GDQQSGYGKVS RRGG HQNSYKPY -287
AUF1-3RGG	252-	SRGG FAGRARG RG GDQQSGYGKVS RRGG H -280

AURE in Soluble Flt-1 mRNA Variants

Table 2

Three classes of AURE have been reported [26], and are described in Materials and Methods. Shown here are the nucleotide length of the 3' UTR region for each sFlt-1 Variant. The number of AURE in each class are shown for sFlt-1 Variants 1, 2, and 3. The sFlt-1 Variant 4 (not shown) was not expressed in U937 cells.

sFlt-1 Variant	3' UTR length (nt)	Class-I AURE pentamers	Class-II AURE nonamers	Class-III AURE A+U-rich
Variant 1	2824	9	0	10
Variant 2	4153	13	6	18
Variant 3	484	1	0	4

Table 3
Effects of AUF1-RGG peptides on expression of soluble Flt-1

Levels of sFlt-1 were measured in stable cell lines expressing no AUF1-RGG peptide (PC-2) or AUF1-RGG peptides (sequences shown in Table 1). **Protein.** Protein levels (pg/mL) of sFlt-1 were measured by ELISA in cell lysates from control or AUF1-RGG stable cell lines (AUF1-Ex6+8 or AUF1-QRGG). Results are representative of two experiments, each performed in triplicate ($p < 0.01$ as compared to PC-2 control for both AUF1-Ex6+8 and AUF1-QRGG). **mRNA.** Levels of mouse sFlt-1 mRNA were measured in cDNA prepared with RNA from PC-2 or AUF1-Ex6+8 stable cells. Results are representative of two experiments. Levels of sFlt-1 mRNA were normalized to actin mRNA ($p < 0.005$ as compared to PC-2 control). (nd not determined)

Cell line	Protein (pg/mL $\pm$ SD)	mRNA (fold change $\pm$ SD)
PC-2	254 $\pm$ 43	1.00 $\pm$ 0.77
AUF1-Ex6+8	38430 $\pm$ 2840	11.85 $\pm$ 3.16
AUF1-QRGG	8489 $\pm$ 1735	(nd)