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Title: MODULATION OF THE ONCOGENE-DEPENDENT TISSUE FACTOR EXPRESSION BY KINASE SUPPRESSOR OF RAS 1

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Corresponding Author: Dr Janusz Rak, MD, PhD

Corresponding Author's Institution: McGill University Health Centre Research Institute

First Author: Joanne L Yu, MD, PhD

Order of Authors: Joanne L Yu, MD, PhD; Rosie Xing, PhD; Chloe C Milsom, PhD; Janusz Rak, MD, PhD

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Materials and Methods: We assayed TF expression and tumourigenicity in mice of human cancer cell lines expressing oncogenic receptor tyrosine kinases. These cells were subjected to modulation of the kinase suppressor of ras 1 (KSR1) levels and treated with oncoprotein inhibitors in vitro and in vivo.

Results: Here we show that herceptin, AG1478 and CI-1033 inhibitors of two different members of the ErbB family of oncogenes (HER-2 and EGFR) reduce TF levels in epithelial cancer cells. In EGFR-driven A431 cells, TF upregulation is diminished upon genetic targeting of KSR1, the scaffolding protein involved in EGFR signalling. Conversely, upregulation of KSR1 in A431 cells increases their TF expression and tumourigenicity in mice. The latter property remains dependent on EGFR, as pan-Erb (EGFR) inhibitor, CI-1033 blocks TF promoter activity and tumour formation by parental and KSR1 overexpressing A431 cells.

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Original communication

MODULATION OF THE ONCOGENE-DEPENDENT TISSUE FACTOR EXPRESSION BY KINASE SUPPRESSOR OF RAS 1

Joanne L. Yu[1], Rosie Xing[2], Chloe Milsom[1], & Janusz Rak[3]

[1] University of Toronto, Toronto, ON, Canada
[2] The University of Chicago, Chicago, Illinois, USA
[3] Montreal Children's Hospital, McGill University, QC, Canada

[*]**Correspondence should be addressed to:** Janusz Rak, Montreal Children's Hospital Research Institute, 4060 Ste Catherine West, Montreal, QC, H3Z 3Z2, Canada; Tel: 514-412-4400 ext. 22342; janusz.rak@mcgill.ca

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ABSTRACT

Introduction: Tissue factor (TF) is the key trigger of the coagulation cascade and the membrane signalling receptor for coagulation protease, factor VIIa. In cancer, TF has been implicated in cancer cell survival, growth, and angiogenesis, and is upregulated as a result of oncogenic transformation.

Materials and Methods: We assayed TF expression and tumourigenicity in mice of human cancer cell lines expressing oncogenic receptor tyrosine kinases. These cells were subjected to modulation of the kinase suppressor of ras 1 (KSR1) levels and treated with oncoprotein inhibitors in vitro and in vivo.

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Key words. Tissue factor, oncogenes, ErbB1/EGFR, ErbB2/HER-2, herceptin, KSR1, cancer

Abbreviations: ErbB – avian erythroblastosis virus related oncogene; EGFR – epidermal growth factor receptor; HER-2 – human epidermal growth factor receptor 2; KSR1 – kinase suppressor of ras 1; TF – tissue factor; FACS – fluorescence activated cell sorter; AS – antisense; S – sense; DN – dominant negative;

Tissue factor (TF) emerges as an important common molecular denominator of multiple processes associated with cancer progression [1]. This 47 kDa cell-associated transmembrane protein acts as the primary high affinity receptor for the coagulation factor VIIa [2]. This interaction triggers the coagulation cascade, which involves the enzymatic activation of factor X to an active form (FXa), followed by the conversion of prothrombin (FII) to thrombin (FIIa), activation of platelets and formation of the fibrin clot [3]. In addition to these extracellular functions, the TF/VIIa complex also interacts with FXa and protease activated, G protein-coupled receptors, mainly PAR-1 and PAR-2, to elicit intracellular signals that control other aspects of hemostasis and wound healing processes [4]. Overall, these events are initiated when TF present in the extravascular compartment comes into contact with blood, as a result of vascular, or tissue injury [2]. Alternatively, TF/VIIa complexes may arise under different pathological conditions, for instance due to vascular anomalies and TF upregulation/overexpression that occurs in cancer [5].

The cancer related increase in TF levels is attributed to the regulatory influences of oncogenic signalling pathways [6,7], which may be coupled with the modulating role of the tumour microenvironment [8-10]. Activated K-*ras* and members of the ErbB family, such as epidermal growth factor receptor (EGFR) and EGFRvIII all upregulate TF on the surface of cancer cells [10-12] and trigger the release of TF-containing microvesicles into the circulation of tumour bearing mice [7,10]. These events contribute to tumour formation and angiogenesis by causing changes in the expression of angiogenic factors [7], or through their impact on tumour initiation [10].

Human cancers harbouring ErbB oncogenes, including ErbB2/HER-2 and EGFR, are often prone to coagulopathy and overexpress TF (e.g. as is observed in breast, lung, brain or ovarian malignancies) [13], but the extent of this connection and its molecular nature remain poorly understood. Here we show that pharmacological inhibition of two different, naturally occurring ErbB oncogenes, HER-2 and EGFR, leads to TF downregulation in the respective human cancer cell lines. The EGFR-driven upregulation of TF is abolished in cancer cells, in which the scaffolding protein known as kinase suppressor of ras 1 (KSR1) is downregulated, or inhibited. Conversely, cancer cells engineered to overexpress KSR1 exhibit increased EGFR-dependent tumourigenicity and higher levels of TF. Thus, we postulate that KSR1 is involved in the oncogenic control of both procoagulant and aggressive phenotype of cancer cells. We postulate that these parallel events may be functionally linked.

MATERIALS AND METHODS

Cell Culture and Treatments. A431 human squamous cell carcinoma line (ATCC, Manassas, VA) and SKBR3 human breast carcinoma cell line (gift from Dr. Viloria-Petit, Guelph University, ON) were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Cells were serum starved overnight prior to treatments with inhibitors. The EGFR inhibitor, AG1478 (Biomol Research Laboratories, Plymouth Meeting, PA) was used at concentrations between 1 and 10 μM, while Herceptin (Genentech, San Francisco, CA) was used at 20 μg/ml, for time intervals indicated. Both agents were prepared in phosphate buffered saline from concentrated stocks, as described earlier [7,10]. CI-1033 was made available by Pfizer Inc., Montreal courtesy of Dr. Luc Levesque. The generation and

characterization of A431 cells transfected with sense (S), antisense (AS), or dominant negative (DN) mutant of KSR1 sequences was described previously [14]. The KSR1-directed phosphorothioate-modified oligonucleotides (ODNs) were prepared in the antisense 5'-CTT TGC CTC TAG GGT CCG-3' and sense 5'-CGG ACC CTA GAG GCA AAG-3' orientation by Mobix Laboratory (McMaster University, Hamilton, ON). A range of concentrations of these ODNs was tested against A431 cells after transfection, using Oligofectamine Transfection Reagent (Invitrogen), essentially as described earlier [14]. The optimal dose (200 μM) of both ODNs was selected for further experiments on the basis of the most pronounced and specific KSR1 downregulation. TF promoter assays were performed following transient transfection of the hTF-pGL2 luciferase reporter plasmid containing the full-length (2.2kb) human TF 5' UTR, as described elsewhere [10]. Cells were treated with various inhibitors at concentrations stated in the figures.

Northern blotting. Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer. Northern blotting was performed as described previously [7,9] using a 600bp cDNA fragment for human tissue factor as a probe (a gift from Dr. Nigel Mackman, University of North Carolina). Changes in TF mRNA were quantified following normalisation to the 28 S rRNA, as indicated.

Flow cytometric detection of cell surface TF. Tumour cells were harvested using 2mM EDTA and processed as described previously [7,10]. Briefly, the single cell suspension was incubated with the sheep antibody against human TF (Affinity Biologicals Ancaster, ON, Canada) and Alexa Fluor 488 donkey anti-sheep secondary antibody (Molecular Probes, Eugene, OR), and analyzed (FACScalibur; BD Biosciences, Mississauga, ON, Canada) [10].

Western blotting. Western blotting was performed as described previously [10]. Membranes were probed with the following primary antibodies: rabbit anti-human TF IgG (American Diagnostica, Greenwich, CT) and monoclonal β-actin for loading control (Sigma-Aldrich, Oakville, ON, Canada). Horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Upstate, Lake Placid, NY) were used prior to ECL detection (Amersham Biosciences, Piscataway, NJ).

Tumour analysis. Cells were injected s.c. ($2x10^6$), in 0.2 ml of PBS into immunodeficient SCID mice (Charles River, Canada). Tumour volume was calculated as described previously [7]. CI-1033 was administered daily *via* intraperitoneal (i.p.) injections at 5mg/kg, starting on day 12 post inoculation. Animal studies were conducted according to protocols approved by the institutional Animal Care Committees at McMaster and McGill Universities and in accordance with the guidelines of the Canadian Council of Animal Care (CCAC).

***Data analysis*.** All data points were generated from at least 4-5 mice per group. The results were expressed as mean ± SD. Whenever, appropriate a Student t-test was used and $p < 0.05$ was used as the threshold of statistical significance.

RESULTS

We have previously reported that TF is upregulated in cells expressing oncogenic forms of EGFR [10]. In order to extend this analysis to other oncogenic members of the ErbB family we assayed additional human cancer cell lines for TF mRNA. Thus, human cancer cells, SKBR3,

expresses endogenously high levels of the ErbB2/HER-2 oncogenic kinase, while A431 squamous carcinoma cells express high levels of EGFR, due to the *egfr* gene amplification [10]. Both cell lines produce copious amounts of TF transcript and this expression is markedly diminished upon the incubation with specific inhibitors of HER-2 (herceptin) and EGFR (AG1478), respectively (Fig. 1). This confirms and extends our observation that ErbB oncogenes drive the expression of TF in human cancer cells.

The EGFR-driven tumourigenicity of A431 cells was previously shown to depend on the status of the scaffolding protein KSR1, which contains docking sites for multiple elements of the Raf/MAPK cascade, an effector of EGFR [14]. Moreover, aggressive properties of these cells are also influenced by TF [10]. Therefore, we chose to examine whether KSR1 can influence EGFR-driven expression of TF. Indeed, in the previously characterized A431 cell lines [14], engineered to express diminished levels (A431-KSR-AS), or dominant negative mutant of KSR1 (A431-KSR-DN) the expression of TF is dramatically reduced at both, the mRNA and protein levels (Fig. 2A). Likewise, the surface expression of the TF antigen was also suppressed in these settings, as determined by flow cytometry (Fig. 2B). In addition, treatment of A431 cells with the previously validated, phosphorothioate-modified antisense oligonucleotides (ODNs) [14] lowered the levels of KSR1, as expected, and downregulated the expression of TF protein (Fig. 2C). Conversely, A431 cells transfected with the expression vector containing functional KSR1 sequence (A431-KSR-S) [14] exhibited a marked elevation in the expression of TF (Fig. 2AB). Thus, KSR1 appears to modulate the expression of TF in EGFR-driven tumour cells in a manner consistent with the previously reported impact of this protein on tumourigenicity.

In addition to its impact on TF overexpression, elevation in KSR1 activity could also translate into increased tumour formation [14]. Either of these effects could occur, via mechanisms enhancing the oncogenic effects of EGFR, or in an EGFR-independent manner, e.g. by assembly of the MAPK cascade activated by other stimuli [15]. To distinguish between these possibilities A431 and A431-KSR-S cells were tested for TF promoter activity in the presence of absence of two different EGFR inhibitors (AG1478 and CI-1033). As shown in Fig. 3 A and B, both of these agents suppressed TF gene expression in A431 cells irrespectively of their KSR1 status. This suggests that KSR1 merely amplifies the signal leading to TF upregulation downstream of EGFR, but does not generate one independently; A431-KSR-S cells were not resistant to EGFR inhibitors in this regard.

Given the linkage between both TF and KSR1 and EGFR-driven tumorigenicity we have also inoculated both A431 and A431-KSR-S cells subcutaneously into immunodeficient (SCID) mice and the animals received daily treatment with the pan-Erb inhibitor, CI-1033. In our hands this agent effectively and irreversibly blocks EGFR in vitro and in vivo [16]. As shown in Fig. 3, A431-KSR-S cells, as expected, exhibited a greater aggressiveness and faster tumour growth relative to their parental A431 counterparts. However, both types of tumours eventually emerged and were efficiently suppressed by the CI-1033 treatment. This suggests that KSR1 exerts its effect on A431 cells in a manner dependent on the kinase activity of the EGFR oncogene and not independently. Thus, the nature of the impact of KSR1 on tumor formation parallels that on TF expression.

DISCUSSION

Our study contains several novel observations. We provide the first piece of direct evidence that in addition to EGFR [10], the HER-2 oncogene can also impacts the expression of TF in human cancer cells. This can be reversed by exposure to clinically relevant blocking agents, notably Herceptin, a drug widely used in cancer treatment [17]. In contrast to some other anticancer therapies, such as chemotherapy and certain antiangiogenic agents (e.g., bevacizumab, thalidomide, SU5416) [18,19], Herceptin/trastuzumab rarely provokes hemostatic perturbations [20]. Our observations presented here could suggest that the lower risk of coagulopathy associated with Herceptin treatment may be due (at least in part) to downregulation of TF in cancer cells. It should be kept in mind, however, that TF is regulated not only by oncogenes but also by a multiplicity of other factors, including differentiation pathways [10], cell-cell inderactions [9] and tumour microenvironment [8]. Therefore, the impact of herceptin on the procoagulant events in vivo requires further evaluation.

Our experiments also shed new light on the signalling pathway that mediates the upregulation of TF downstream of ErbB oncogenes. Indeed, this report is the first to suggest that the scaffolding protein KSR1 may be involved in regulating TF levels by oncogenic EGFR. This is consistent with studies pointing to the involvement of the MEK/Erk1/2 cascade in control of TF expression [21]. However, it should also be borne in mind that the kinase [14,22,23] scaffolding functions [15,24,25] and other functions of KSR1 are still poorly understood and therefore the exact nature of the linkage between this protein and TF remains to be established. Indeed, even though one of the main functions of KSR1 is thought to be the in the physical assembly of the elements of the raf/MEK/MAPK cascade downstream of ras and EGFR, it is possible that additional proteins

interacting with KSR1 (e.g. C-TAK, 14-3-3, PP2A, NM23) [15] may also play a role in TF regulation, since inhibition of the enzymatic function of MEK was not always shown to lower TF expression in various cancer cell lines [10].

TF regulation is complex [26] and often dependent on the signalling circuitry unique to a particular cell type [5]. For example, in normal endothelium, TF can be triggered by pathways involving protein kinase C (PKC), MAPK and several transcription factors (EGR-1, NFκB, AP1 and NFAT) [27-30]. In cancer cells, the effects of oncogenic EGFR on TF are mediated through an NFκB dependent mechanism [10], as well as by processes involving AP-1 and c-Jun N-terminal kinase (JNK) activation [12]. Since, the TF promoter also contains functional sites for other transcription factors, including SP1 and Egr-1, other pathways may also be involved downstream of EGFR [26]. In this regard, the available experimental evidence suggests that TF can be regulated by the PI3K/Akt pathway following the loss of the tumour suppressor gene *PTEN*, especially in glioma [8]. It is unclear whether KSR1 contributes to TF regulation by these MAPK-unrelated modules.

Although the predominant transforming defects in A431 cells are the result of the amplified EGFR, other types of cancer cells may control TF expression *via* more complex oncogenic mechanisms, including mutations of K-*ras* and losses of tumour suppressor genes (e.g. p53) [7]. It is noteworthy that while *KSR1-/-* mice (unlike *TF-/-* mice [31]) are viable [24,32,33], they are also resistant to H-*ras*-driven epithelial tumourigenesis and exhibit cutaneous anomalies reminiscent of EGFR deficiency [32]. It would be of interest to explore the TF status in this context.

Overall, our study adds KSR1 to the emerging list of mediators involved in the oncogene-dependent (de)regulation of TF that could impact angiogenesis, coagulopathy and tumour progression. A more practical dimension of these studies is related to the possibility of monitoring tumour progression and therapeutic responses through levels of TF. For instance, in pancreatic cancer, coagulopathy is relatively common, TF expression is upregulated [34] and the use of KSR1 targeting agents is being explored as a therapeutic strategy [14,35]. Our study suggests that it might be possible to predict the effects of these agents by assessing the levels of TF-positivity in tumour tissue, and that circulating in the blood of cancer patients as TF-containing microvesicles. These implications are currently under study.

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CONFLICT OF INTEREST

Authors declare no conflict of interest

FIGURE LEGENDS

Figure 1. Inhibitors of ErbB oncoproteins downregulate tissue factor (TF) expression by cancer cells. **A.** The impact of the specific HER-2 inhibitor (herceptin) on TF mRNA levels expressed by HER-2-driven breast cancer cells (SKBR3). The drug was incubated with cultured cells at the concerntrations indicated for 24 and 48 hours causing a time dependent TF downregulation without signs of toxicity; **B.** Expression of TF mRNA in A431 cells driven by the amplification of the oncogenic EGFR is suppressed in the presence of small molecule inhibitor of the A431 kinase (AG1478). Numbers indicate the change in signal intensity expressed as fold reduction compared to untreated controls (1.00) and normalized to the 28S rRNA loading control.

Figure 2. Impact of KSR1 status on EGFR-driven tissue factor expression in cancer cells. **A.** Suppression of TF protein (upper panel) and mRNA production in EGFR-driven A431 cancer cells stably expressing KSR1 antisense (AS) or dominant negative (DN) sequences. In contrast, upregulation of KSR1 leads to a marked increase in TF production; **B.** Changes in surface expression of the TF antigen in A431 cells engineered to alter their KSR1 status (FACS analysis, designations as in panel A); **C.** Impact of anti-KSR1 oligonucleotides on the expression of TF by A431 cells. Transient downregulation of KSR1 is paralleled by reduced expression of TF protein (see text)

Figure 3. EGFR dependent effects of KSR1 in TF expression and tumourigenicity. **A-B.** TF promoter activity in A431 cells and their KSR1 overexpressing counterparts (A431-KSR-S). In

both cell lines blockade of EGFR activity with two different agents (AG1478 and CI-1033) suppresses TF promoter activity. **C.** A431 cells overexpressing KSR1 (A431-KSR-S) form subcutaneous tumours more rapidly then their parental counterparts. In spite of this increase in aggressiveness both A431 and A431-KSR-S tumours are highly sensitive to the EGFR (pan-Erb) inhibitor - CI-1033. Tumours were initiated by injection of 2×10^6 on day 0 and daily i.p. injections of the drug (5 mg/kg) commenced when the tumours reached palpable sizes (200-300 mm^3), i.e. on day 12 for A431-KSR-S cells and on day 15 for A431 cells (* - significant at $p < 0.05$; see text for details).

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Original communication

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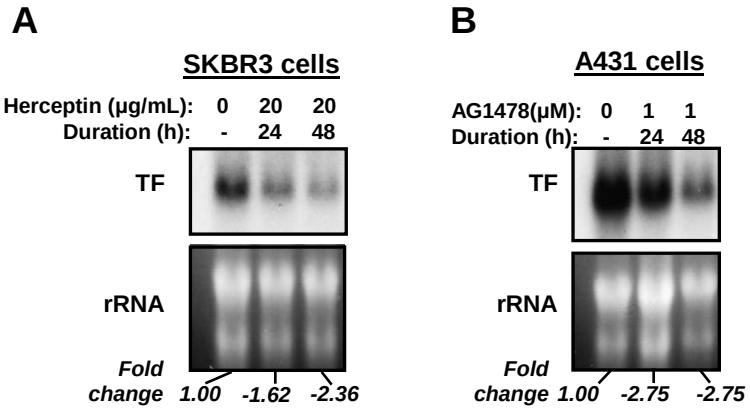
Joanne L. Yu[1], Rosie Xing[2], Chloe Milsom[1], & Janusz Rak[3]

[1] University of Toronto, Toronto, ON, Canada
[2] The University of Chicago, Chicago, Illinois, USA
[3] Montreal Children's Hospital, McGill University, QC, Canada

CONFLICT OF INTEREST

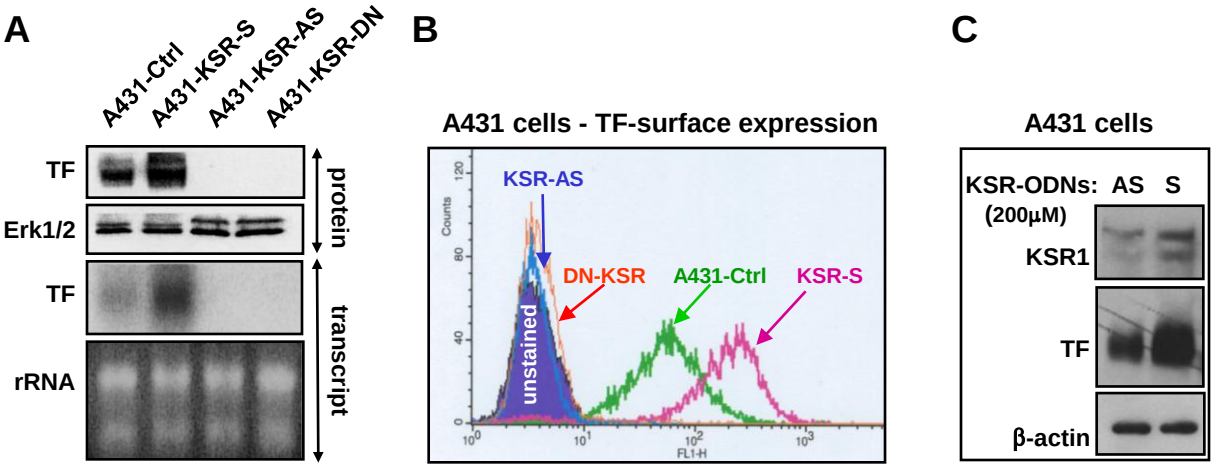
Authors declare no conflict of interest

Figure 1



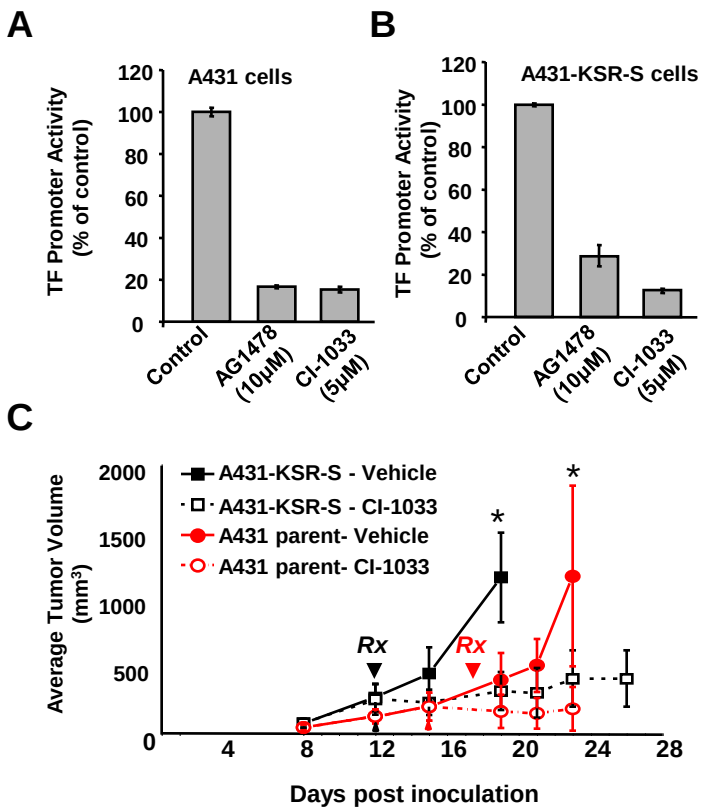
Yu (Rak) et al Figure 1

Figure 2



Yu (Rak) et al Figure 2

Figure 3



Yu (Rak) et al Figure 3