Receptors & Clinical Investigation 2016; 3: e1234. doi: 10.14800/rci.1234; © 2016 by Shu-Yi Yin, *et al*.
http://www.smartscitech.com/index.php/rci

REVIEW

Molecular basis of shikonin-induced immunogenic cell death: insights for developing cancer therapeutics

Shu-Yi Yin[1,*], Ning-Sun Yang[1], Tien-Jen Lin[1, 2, 3, 4,*]

[1]*Agricultural Biotechnology Research Center, Academia Sinica, Taipei, 115-29 Taiwan, ROC*
[2]*Graduate Institute of Injury Prevention and Control, Taipei Medical University, Taipei, 110-31 Taiwan, ROC*
[3]*Department of Neurosurgery, Taipei Medical University-Wan Fang Hospital, Taipei, 116-96 Taiwan, ROC*
[4]*Graduate Institute of Sports Science, National Taiwan Sport University, Taoyuan, 333-01 Taiwan, ROC*

[*]These authors contributed equally to this work
Correspondence: Shu-Yi Yin or Tien-Jen Lin
E-mail: yinshuyi @gate.sinica.edu.tw or trlin1@hotmail.com
Received: February 25, 2016
Published online: March 28, 2016

Shikonin, a natural plant product isolated from the herb Lithospermum erythrorhizon, has been found to strongly stimulate immunogenic cell death (ICD) of tumor cells, which induced a potent immune response by dendritic cells (DCs) to suppress tumor growth and/or metastasis. Recently, specific intracellular protein targets including heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and pyruvate kinase-M2 (PKM2) have been demonstrated to act as candidate receptors for shikonin. Among them, direct binding-interference with hnRNPA1 was found to be critical for shikonin-induced immunogenicity of mammary tumor cells, which can result in strong suppression of tumor metastasis. Mechanistic studies have further revealed that specific damage-associated molecular patterns (DAMPs) associated with immunogenicity, including heat shock proteins 70 (HSP-70), calreticulin (CRT) and high mobility group box 1 (HMGB1) in tumor cell lysate (TCL), can play important and comprehensive roles in activating specific immunities of tumor cell lysate (TCL)-pulsed DCs. In this brief review article, we present these findings together and further provide a molecular mode of action as the pharmacological basis of SK-induced ICD.

Keywords: shikonin; heterogeneous nuclear ribonucleoprotein A1; immunogenic cell death

To cite this article: Shu-Yi Yin, *et al*. Molecular basis of shikonin-induced immunogenic cell death: insights for developing cancer therapeutics. Receptor Clin Invest 2016; 3: e1234. doi: 10.14800/rci.1234.

Identification of hnRNPA1 as a protein target provides pharmacological basis for pursuit of the diverse biochemical activities of SK

Shikonin (SK) and its derivatives are active components isolated from the root tissues of a traditional Chinese medicinal herb, Lithospermum erythrorhizon, and have been broadly studied as bioactive phytochemicals [1]. In our previous studies, we showed that SK can confer a broad spectrum of specific cellular and biochemical activities. These include the inhibition of promoter/transcriptional activities of the pro-inflammatory cytokines TNF-á [2] and GM-CSF [3], the blockade of splicing of TNF-a pre-mRNA [4], the induction of epithelial-to-mesenchymal transition (EMT) activity in skin wound-healing [5], and a differential effect on the genomic expression of cytokine/chemokine genes in

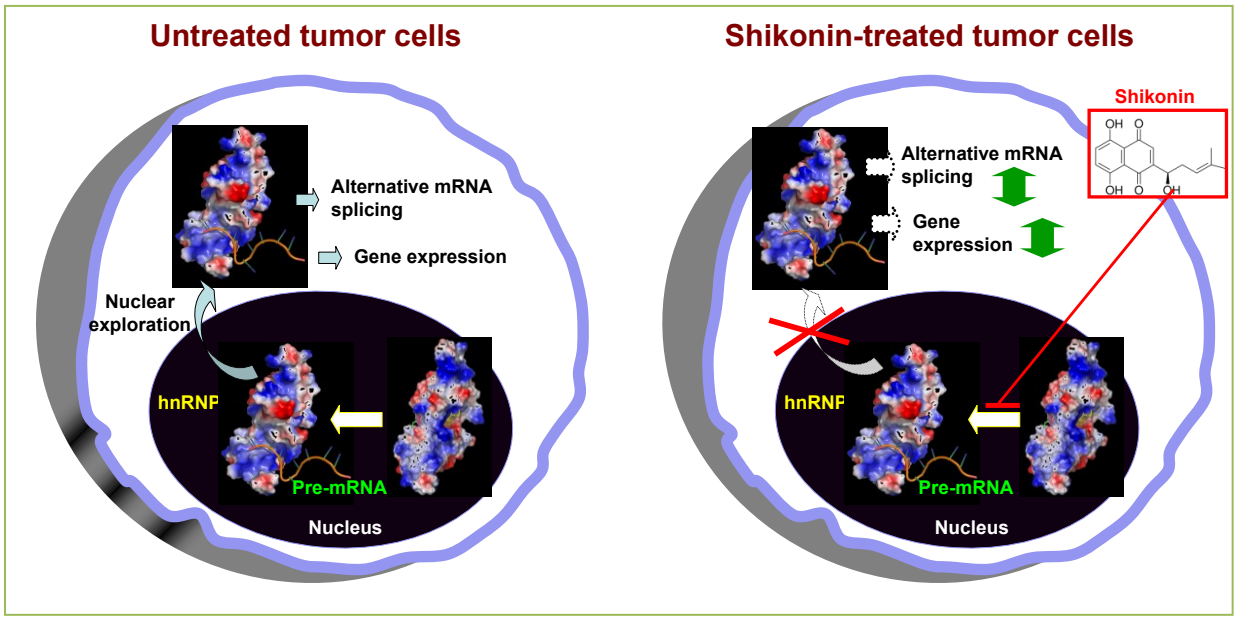

Figure 1. Mode of action for SK-mediated post-transcriptional regulation via direct binding-interference with hnRNPA1. On the left, the electrostatic surface of intracellular hnRNPA1 in complex with pre-mRNA (orange) is shown. In untreated tumor cells, hnRNPA1 mediates several post-transcriptional processes of mRNA, including nuclear exploration, alternative splicing and expression. On the right, because hnRNPA1 function was suppressed by SK, various hnRNPA1-mediated post-transcriptional processes or activities are regulated or modified in SK-treated tumor cells.

human monocytes [6]. Recently, a molecular target of SK, namely heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), was identified to play a key role in SK-induced ICD in mammary tumor cells [7]. Specifically, hnRNPA1 was shown to act as an important substrate of GzmA that can impair the nuclear export activity of newly synthesized RNA, and this resulted in a specific type of immune-mediated programmed cell death [8]. This finding along with many previous studies suggests that SK can confer a spectrum of apparently related molecular and intracellular effects on post-transcriptional processing of RNAs, including nuclear export and pre-mRNA splicing activities **(Figure 1)**. In addition, we consider that dysfunction of hnRNPA1 caused by SK can also provide a pharmacological basis for potential application of SK-induced ICD in cancer therapeutics, e.g., cancer vaccines.

Previously, in vivo and in situ treatment with SK (>50 ìg/site/mice) was found to suppress skin tissue inflammation [2] and expression of TNF-á [2, 4]. On the other hand, topical treatment with SK was found to also confer a potent stimulatory effect on EMT and many pro-inflammatory activities, such as the increase in expression of MMP2, MMP-9 and vimentin, during wound-healing of skin tissues [5]. These results indicate that SK may exhibit both pro- and anti-inflammatory activities at the tissue/organ levels. The identification of hnRNPA1 [7] may provide a plausible mechanistic explanation for this seemingly contradictory "Yin vs. Yang" regulation, and the diverse effects of SK on epithelial tissues. We suggest that by targeting hnRNPA1 activity via binding with SK the splicing and nuclear export activities of specific inflammation-related genes may be efficiently, but transiently, suppressed, and this highly temporal action may result in an interruption of the acute "cytokine storm", effecting timely control of the "resolution" of inflammation activities. On the other hand, the interaction between SK and hnRNPA1 molecules may also negatively regulate the control of specific tissue wound-healing processes, such as regulation of the expression of 200 family microRNAs [5]. These seemingly contradictory bioactivities of SK at the tissue and organ levels may hence represent an elegant control at the temporal and spatial levels of the pro- versus anti-inflammatory activities evolved in various mammalian systems, including humans.

SK-induced ICD of tumor cells can efficiently activate various immunities of DC-based vaccine and suppress metastatic activities of tumor cells

Immunogenic cell death (ICD) is a cell death modality that can effectively stimulate an immune response against dying or dead cell antigens, in particular when they are derived from cancerous cells [9, 10]. The molecular phenotype of ICD has been characterized by changes in damage-associated molecular patterns (DAMPs) associated with immunogenicity, including heat shock proteins (HSP), calreticulin (CRT), high mobility group box 1 (HMGB1), glucose-related protein (GRP) and others [11-14]. Among

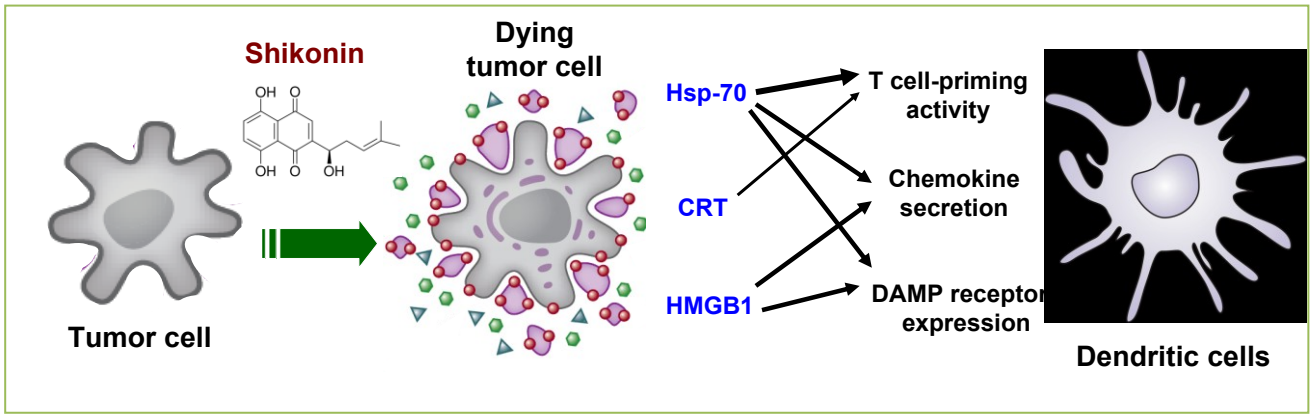

Figure 2. Diagrammatic presentation of various stimulatory activities of specific DAMP components in SK-treated TCL, resulting in activation of a spectrum of DC immunities. Three specific DAMP constitutes in SK-treated TCL (HSP70, CRT and HMGB1) have been demonstrated to play different and comprehensive roles in stimulating important DC immunities, including T cell-priming, chemokine secretion and DAMP receptor expression activities. Among them, HSP70 is shown as most critical for the anti-metastatic activity of DCs, which can be activated by SK-treated TCL [16].

various ICD inducers, SK can effectively induce ICD activity of tumor cells and increase the expression of DAMPs in the resultant tumor cell lysate (TCL), which could then be used to stimulate strong anti-tumor activity in a DC-based cancer vaccine approach [15-17]. Recently, the targeting of hnRNPA1 by SK has been proven to play a key role in SK-induced ICD in mammary tumor cells and the derived anti-metastatic activities of vaccinated DCs [7]. Moreover, three specific ICD proteins were shown to exhibit distinguishable or comprehensive activities in activating DCs **(Figure 2)**. Specifically, HSP70 and CRT can mediate a key role in SK-TCL-induced DC immunity resulting in both $CD4^+$ and $CD8^+$ T cell proliferation in vitro. HSP70 is the most important component, followed by CRT, in facilitating DC-induced immunity that suppresses metastasis of mouse 4 T1 mammary tumors. Only HSP70, but not CRT or HMGB1, is effective in suppressing both granulocytic and monocytic MDSC populations in vivo. Both HSP70 and HMGB1, but not CRT, are essential for activating the expression of three key DAMP-associated receptors, CD91, TLR2 and TLR4, on test DCs. Finally, each of the three ICD components can exhibit distinguishable stimulatory activities in stimulating the secretion of chemokines in test DCs [16]. Based on these recent findings, we believe that research is urgently needed to explore and employ phytochemicals for ex-vivo induction, engineering or/and reconstitution of custom-made or re-constituted DAMPs. For cancer vaccines or other clinical cancer applications, these ex-vivo assembled tumor-derived DAMPs and specific immune cell (e.g., DC) preparations could be applied as tailor-made combination therapeutics for specific individual patients as personalized medicines, aiming at optimizing the DC functions for priming of T cells against specific metastatic tumor cells.

In related studies, SK and its analogs have been shown to act as potent inhibitors of a tumor-specific pyruvate kinase-M2 (PKM2) [18], a suggested molecular target for disrupting glucose metabolism in cancer cells [19, 20], or proteasome subunits in cancer cell [21]. However, the possible role of these candidate targets in activating ICD of tumor cell has not been reported. We believe it is important for future studies to comparatively investigate the possible coordinated roles of hnRNPA1, PKM2 and proteasome activities in expression and regulation of various ICD components or features in SK-treated tumor cells. We consider this information will provide additional networking and mechanistic modes of action for SK in activating tumor cell ICD. Since shikonin can confer an act without a genetic modification, it is drastically different from most other ICD inducers, including doxorubicin, mitoxantrone, oxaliplatin, UVC irradiation and anthracyclines [13, 22-27].

A selected therapeutic approach may be needed for application of SK in the development of cancer therapy

In addition to the strong stimulatory effect of shikonin on ICD of tumor cells [13, 15, 18, 22], many previous studies have also indicated that in vivo treatment of SK could also be considered as an approach for suppressing tumor growth or metastasis [1, 21, 28, 29]. However, up to now the candidate cellular targets identified for SK are not specific for cancer cells. In addition, accumulating evidence also shows that SK does not exhibit differential cytotoxicity to malignant cells, as compared to non-malignant or normal cells. Our in vivo studies have shown that SK treatment (via both intravenous and intraperitoneal injections) could significantly suppress tumor growth (B16 or 4T1 xenografts) only at a relatively high dosage (5 mg/kg/day), as compared with other clinically used anticancer drugs (e.g., Docetaxel, Doxorubicin and MG-132). In addition, a decrease in mouse body weight was

also detected (20-30%) for SK-treated mice. These results thus suggest that the strategy of using the "cytotoxicity of SK on tumor cells" will most likely not be competitive as a chemotherapy drug. In contrast, SK has been considered as a highly efficient inducer for activating ICD of various treated carcinoma cells [13, 15, 22]. For clinical and biotechnology application, the combination of SK-treated tumor cell lysate (SK-TCL) and dendritic cell (DC)-based vaccines have been shown to induce strong in vivo anti-tumor activity including anti-metastatic activities [15, 22]. We therefore suggest that the SK-induced immunogenicity in tumor cells and the resultant activation of the immune system in the host patient may be a compelling approach for cancer immunotherapy.

Recently, we also observed that topical treatment of mouse skin with SK conferred a potent stimulatory effect on EMT activity and effectively suppressed the expression of associated microRNAs (200 family microRNAs) during the subsequent skin wound-healing activity [5]. In normal somatic tissues, epithelial-to-mesenchymal transition (EMT) activity is not only instrumental in facilitating wound-healing activity but also in promoting tissue fibrosis [30-32]. In addition, hnRNPA1 has been shown to play an important role in control of specific splicing activity of tyrosine kinase receptor (Ron), and can thus further promote the MET activity [33]. Taking these findings together, we contemplate that SK-induced EMT and the related changes in microRNA expression in vivo may also reflect the dysfunction of hnRNPA1 activity [7]. Therefore, the application of SK in promoting tissue wound-healing activity after clinical resection of tumors in cancer patients may constitute another avenue of research to aid the recovery of cancer patients.

Conflicting interests

The authors have declared that no conflict of interests exist.

Acknowledgements

This work was supported by funding from the National Science Council (NSC 104-2320-B-001-004) and an institutional research grant from Academia Sinica, Taiwan (99-Academia Sinica Investigator Award-12). We also thank Ms. Miranda Loney of Academia Sinica for professional manuscript editing.

Abbreviations

CRT: calreticulin; DAMPs: damage-associated molecular patterns; EMT: epithelial-to-mesenchymal transition; GM-CSF: granulocyte-macrophage colony-stimulating factor; HMGB1: high-mobility group box 1 protein; hnRNPA1: heterogeneous nuclear ribonucleoprotein A1; HSP-70, heat shock proteins 70; ICD: immunogenic cell death; MMP: Matrix metalloproteinases; PKM2: pyruvate kinase-M2; SK: shikonin; TCL: tumor cell lysate; TNF-α: tumor necrosis factor-α; TLR: Toll-like receptor.

References

1. Chen X, Yang L, Oppenheim JJ, Howard MZ. Cellular pharmacology studies of shikonin derivatives. Phytotherapy research: PTR 2002; 16:199-209.
2. Staniforth V, Wang SY, Shyur LF, Yang NS. Shikonins, phytocompounds from Lithospermum erythrorhizon, inhibit the transcriptional activation of human tumor necrosis factor alpha promoter in vivo. The Journal of biological chemistry 2004; 279:5877-5885.
3. Su PF, Staniforth V, Li CJ, Wang CY, Chiao MT, Wang SY, *et al*. Immunomodulatory effects of phytocompounds characterized by in vivo transgenic human GM-CSF promoter activity in skin tissues. Journal of biomedical science 2008; 15:813-822.
4. Chiu SC, Yang NS. Inhibition of tumor necrosis factor-alpha through selective blockade of Pre-mRNA splicing by shikonin. Molecular pharmacology 2007; 71:1640-1645.
5. Yin SY, Peng AP, Huang LT, Wang YT, Lan CW, Yang NS. The Phytochemical Shikonin Stimulates Epithelial-Mesenchymal Transition (EMT) in Skin Wound Healing. Evidence-based complementary and alternative medicine: eCAM 2013; 2013:262796.
6. Chiu SC, Tsao SW, Hwang PI, Vanisree S, Chen YA, Yang NS. Differential functional genomic effects of anti-inflammatory phytocompounds on immune signaling. BMC genomics 2010; 11:513.
7. Yin SY, Jian FY, Chen YH, Liu CI, Wang AH, Chen YR, *et al*. Immunogenicity of mammary tumor cells can be induced by shikonin via direct binding-interference with hnRNPA1. Oncotarget 2016 (Under revision).
8. Rajani DK, Walch M, Martinvalet D, Thomas MP, Lieberman J. Alterations in RNA processing during immune-mediated programmed cell death. Proceedings of the National Academy of Sciences of the United States of America 2012; 109:8688-8693.
9. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. Nature reviews Immunology 2009; 9:353-363.
10. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. Annual review of immunology 2013; 31:51-72.
11. Garg AD, Krysko DV, Vandenabeele P, Agostinis P. DAMPs and PDT-mediated photo-oxidative stress: exploring the unknown. Photochemical & photobiological sciences: Official journal of the European Photochemistry Association and the European Society for Photobiology 2011; 10:670-680.
12. Garg AD, Nowis D, Golab J, Vandenabeele P, Krysko DV, Agostinis P. Immunogenic cell death, DAMPs and anticancer therapeutics: an emerging amalgamation. Biochimica et biophysica acta 2010; 1805:53-71.
13. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer

therapy. Nature reviews Cancer 2012; 12:860-875.

14. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, *et al*. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nature medicine 2007; 13:54-61.
15. Chen HM, Wang PH, Chen SS, Wen CC, Chen YH, Yang WC, *et al*. Shikonin induces immunogenic cell death in tumor cells and enhances dendritic cell-based cancer vaccine. Cancer immunology, immunotherapy: CII 2012; 61:1989-2002.
16. Lin TJ, Lin HT, Chang WT, Mitapalli SP, Hsiao PW, Yin SY, *et al*. Shikonin-enhanced cell immunogenicity of tumor vaccine is mediated by the differential effects of DAMP components. Mol Cancer 2015; 14:174.
17. Lin TJ, Liang WM, Hsiao PW, M SP, Wei WC, Lin HT, *et al*. Rapamycin Promotes Mouse 4T1 Tumor Metastasis that Can Be Reversed by a Dendritic Cell-Based Vaccine. PloS one 2015; 10:e0138335.
18. Chen J, Xie J, Jiang Z, Wang B, Wang Y, Hu X. Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. Oncogene 2011; 30:4297-4306.
19. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, Fleming MD, *et al*. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 2008; 452:230-233.
20. Spoden GA, Mazurek S, Morandell D, Bacher N, Ausserlechner MJ, Jansen-Durr P, *et al*. Isotype-specific inhibitors of the glycolytic key regulator pyruvate kinase subtype M2 moderately decelerate tumor cell proliferation. International journal of cancer Journal international du cancer 2008; 123:312-321.
21. Yang H, Zhou P, Huang H, Chen D, Ma N, Cui QC, *et al*. Shikonin exerts antitumor activity via proteasome inhibition and cell death induction in vitro and in vivo. International journal of cancer Journal international du cancer 2009; 124:2450-2459.
22. Lin TJ, Lin HT, Chang WT, Mitapalli SP, Hsiao PW, Yin SY, *et al*. Shikonin-enhanced cell immunogenicity of tumor vaccine is mediated by the differential effects of DAMP components. Mol Cancer 2015; 14:174.
23. Menger L, Vacchelli E, Adjemian S, Martins I, Ma Y, Shen S, *et al*. Cardiac glycosides exert anticancer effects by inducing immunogenic cell death. Science translational medicine 2012; 4:143ra199.
24. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, *et al*. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. Nature medicine 2009; 15:1170-1178.
25. Fucikova J, Kralikova P, Fialova A, Brtnicky T, Rob L, Bartunkova J, *et al*. Human tumor cells killed by anthracyclines induce a tumor-specific immune response. Cancer research 2011; 71:4821-4833.
26. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, *et al*. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. Science 2011; 334:1573-1577.
27. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, *et al*. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nature medicine 2007; 13:1050-1059.
28. Hisa T, Kimura Y, Takada K, Suzuki F, Takigawa M. Shikonin, an ingredient of Lithospermum erythrorhizon, inhibits angiogenesis in vivo and in vitro. Anticancer research 1998; 18:783-790.
29. Long S, GuangZhi Y, BaoJie G, Wei X, YanYong H, YingLi W, *et al*. Shikonin derivatives protect immune organs from damage and promote immune responses in vivo in tumour-bearing mice. Phytotherapy research: PTR 2012; 26:26-33.
30. Hay ED. An overview of epithelio-mesenchymal transformation. Acta anatomica 1995; 154:8-20.
31. Levayer R, Lecuit T. Breaking down EMT. Nature cell biology. 2008; 10:757-759.
32. Zeisberg M, Kalluri R. Fibroblasts emerge via epithelial-mesenchymal transition in chronic kidney fibrosis. Frontiers in bioscience: a journal and virtual library 2008; 13:6991-6998.
33. Bonomi S, di Matteo A, Buratti E, Cabianca DS, Baralle FE, Ghigna C, *et al*. HnRNP A1 controls a splicing regulatory circuit promoting mesenchymal-to-epithelial transition. Nucleic acids research 2013; 41:8665-8679.