

A NOVEL APPROACH OF VIROTHERAPY BASED HSF-1 shRNA IN CANCER ERADICATION

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ABSTRACT

Cancer is the second leading cause of death worldwide with continues rise mortality rate. Current cancer treatment modalities are still ineffective and associated with many side effects leading to robust research to find new specific target therapy. Heat shock factor (HSF)-1 is heat shock response mediator protein and act as transcription factor for HSP encoding gene. Many cancers have up-regulated HSP as a result of increase HSF-1 expression. Interestingly, inhibition of HSF-1 has no effect to normal cell, indicating HSF-1 as promises target therapy. RNAi is potential mechanism to block and down regulate HSF-1 which will affect many cellular processes in cancer cell. Combining RNAi base treatment with oncolytic viruses will boost the therapeutic effect of this novel treatment. Despite its potency, this modality still need further research in order to evaluate its efficacy and optimal doses to gain optimal result.

Keyword: Cancer, HSF-1, HSP, RNAi, oncolytic virus.

INTRODUCTION

Cancer is second leading cause of death worldwide accounting for 13% of all death (7.4 million deaths). Deaths from cancer are projected to continue rising, with an estimated 12 million deaths in 2030. It is estimate that cancer death reach Lung, stomach, liver, colon, and breast cancer are the most prevalent ones, giving great contribution to total cancer mortality rate. Survivability of cancer patient largely depends on cancer type in which lung cancer has the worst one. But cancer survivability also has inverse correlation with cancer stage. Nevertheless, cancer has low survivability rate especially in advanced stage.¹The high mortality rate and low rate of survivability indicates that current cancer therapy is not very effective, especially to treat advanced cancer. Current modalities include surgery, radiotherapy, and chemotherapy. But these modalities have low efficacy and associated with many side effect. For example, Radiation can cause esophagitis, induction of secondary cancer, radiation related pneumonitis, cardiotoxicity (left ventricular heart failure), extremity edema, and skin irritation.² Meanwhile chemotherapy can induce alopecia, sterility, pancytopenia, nausea and vomiting, peripheral neuropathy, and sometimes elicit anaphylaxis reaction.³ Therefore, ongoing research are still conducted in order to find specific target for cancer therapy which are non invasive and

minimal side effect. HSF-1 is a heat shock response mediator protein that acts as transcription factor. Upon activation by environmental stress, HSF-1 dissociate from its cognate binding protein heat shock protein (HSP) 90 and HSP70. Dissociated HSF-1 translocate to the nucleus where it act as transcription factor for HSP encoding gene as well as other genes that play important role to orchestrate many cellular process. Currently, researches show that many tumors, including breast, lungs, and colorectal tumor, have up regulated HSP that result from HSF-1 over expression. Interestingly, inhibition of HSF-1 has no effect on normal cell. This phenomenon makes HSF-1 as a new potential target of cancer treatment.⁴

RNAi is an efficient gene silencing system. RNAi is an evolutionary conserved, gene silencing phenomenon which small pieces of double-stranded RNA suppress expression of gene with sequence homology. This system is mediated by small interfering RNA that has capability to recognize specific site in target mRNA and degrade it. This capability arise when siRNA form a complex with other protein to form RISC in cytoplasm. RNAi system is has high silencing efficiency, about 100-1000 times more powerful than antisense oligonucleotide because siRNA is protected within RISC and therefore can act as catalyst to degrade many copies of target RNA. Currently, RNAi has been applied in cancer management. Targeting siRNA to crucial molecule like pro-survival gene *Bcl-2*, growth factor receptor, angiogenic gene, and the others would make RNAi to be powerful tools to combat cancer cell.⁵

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Since most of the proteins depend on HSP in order to achieve precise folding, silencing of HSF-1 gene will down regulate many HSP proteins including HSP90 and HSP70 which have wide range of client proteins.⁵ HSP down regulation theoretically will bring catastrophe effect on cancer cell since it alters many cellular processes and signaling. Therefore, targeting HSF-1 gene by siRNA will bring crucial implication in the development of cancer treatment. By combining siRNA with conditioned replicate adenovirus which just target cancer cell and spare normal cells will further increase the efficiency of this potential therapy as well as its safety.

Role of heat shock factor-1 and heat shock protein in cancer cells

Heat shock factor-1 is heat shock response mediator protein. There are three kinds of human HSF namely HSF-1, HSF-2, and HSF-4 but HSF-1 is the most crucial to HSR. In responses to HSR, HSF-1 activated and dissociate from HSP90 and HSP70 (**Figure 1**). Activated HSF-1 translocate to the nucleus where it binds with heat-shock element and drives the expression of target gene.³

This review highlights the molecular pathogenesis of lung cancer, the role of heat shock factor-1 in cancer cells, and general description of RNAi. As we progress, there will be description of constructing mechanism of adenovirus bearing HSF-1

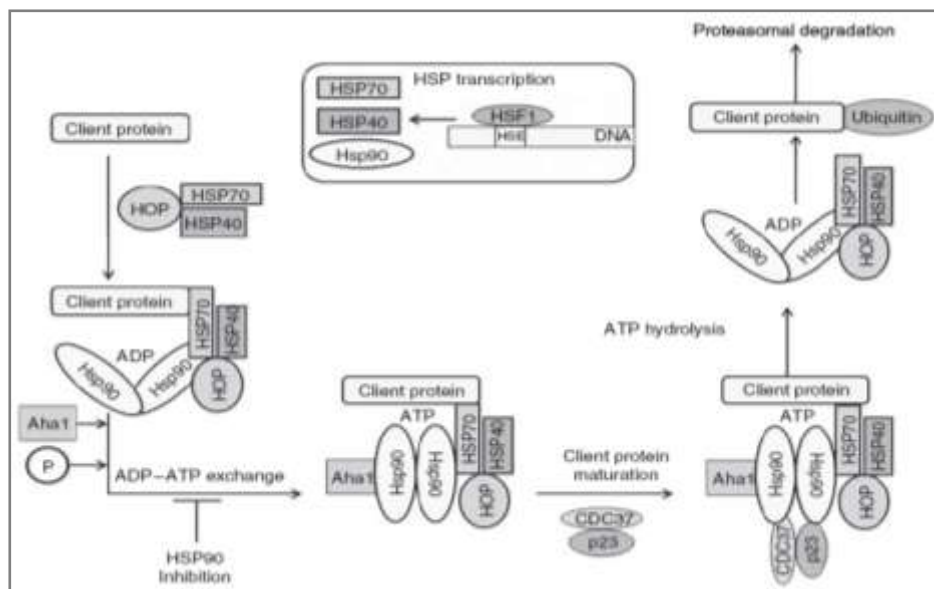


Figure 1 Heat shock response mechanism mediate by HSF-1 and heat shock proteins. HSF-1 be a transcription factor for several heat shock proteins (HSP) like HSP90, HSP70, and HSP40. These HSP's will mediate heat shock response either by stabilizing the client protein or guide it to proteosomal degradation

HSF-1 mediates the expression of many heat shock protein (HSP). Overall, most important HSP regulated by HSF-1 are HSP90, HSP70, and HSP40. Many tumors show increase level of HSP which enable them to survive in harsh environment and up regulate many crucial signaling molecule. HSP promote cell survival, growth and metastasis by allowing continued protein translation and cellular proliferation. Knocking out HSF-1 in mice showed reduced tumor development and rendered cultured cell highly refractory to transformation initiated by mutated Ras or by PDGF-B over expression.

HSF-1 depletion also decrease viability of multiple human cancer lines but has no effect on normal cells.⁶ This suggests that HSF-1 provides critical

Related to its function as anti-apoptotic protein, HSP70 have many crucial function related to cell survivability. HSP70 is known to directly bind apoptosis protease-activating factor-1 (APAF-1), prevent it to recruit caspase-9 and subsequent activation of effector caspases.⁸ It is also known to interact with procaspase-3 and 7, preventing their maturation. HSP70 inhibit cytochrome-C release from mitochondria and inhibit apoptosis inducing factor (AIF) thereby preventing induction of apoptosis pathway and nuclear fragmentation. In addition, caspase activated DNase is also known to regulate by HSP70 and HSP40.⁹

HSP70 has close relationship with pro-survival signal transducing pathway like Raf-1/ERK. Its cochaperone partner, Bag-1 is known to increase Bcl-2 anti-apoptotic protein. Overall, HSP70 increase cancer cell resistance to apoptosis.¹⁰

In addition to HSP70, HSP90 is also one of important HSP. Two type of HSP90, HSP90 α and HSP90 β , are essential for viability of eukaryotic cells. This protein is quite abundant, comprising about 1-2% of cellular protein.¹¹

HSP90 is associates with many signaling protein like steroid receptor, ligand independent transcription factor like MyoD, tyrosine kinase (v-Src), and serine/threonine kinase (Raf-1). Its main function is to ensure conformational stability of its client protein. In order to function properly, HSP90 needs to bind with ATP and undergoing conformational change to its active form.¹²

In relation to cancer cell, HSP90 has even more important role than HSP70. HSP90 has broad array of client protein that play important role in tumorigenic process like cell proliferation, growth-signal self sufficiency, limitless replication potential, angiogenesis, invasion and metastasis (**Figure 2**). Therefore many research and drugs has been developed to target this protein. But the result far from expected. Some researches reveal that this is result from up-regulation of HSP70 in response to HSP90 inhibition, rendering HSP90 inhibitors to perform their maximal effect.^{13,14}

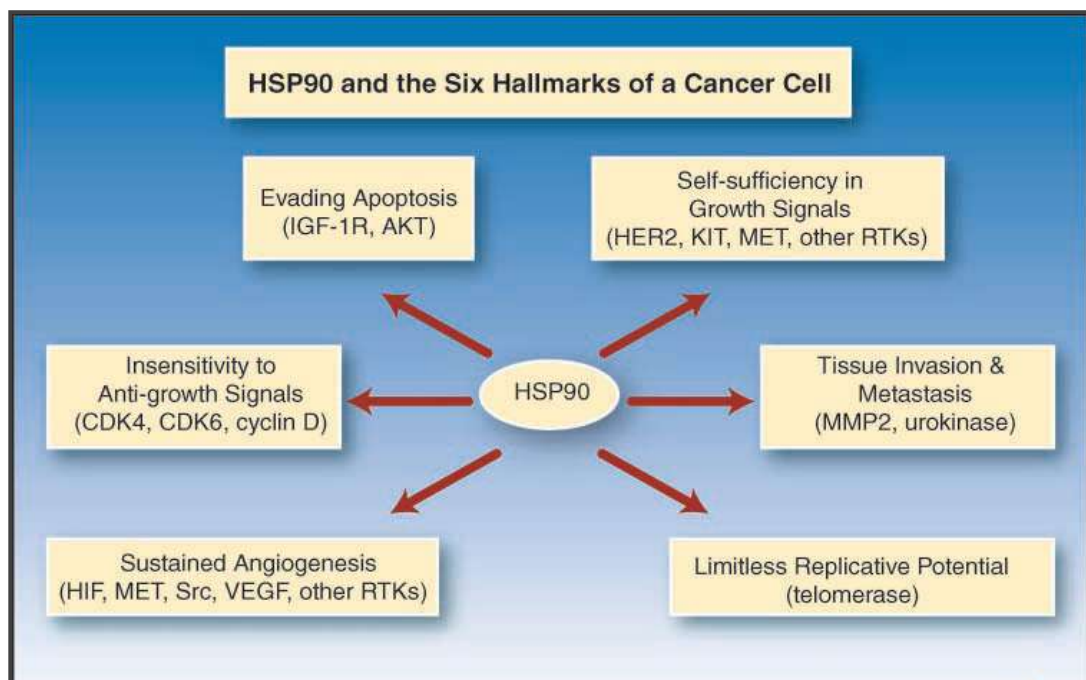


Figure 2 HSP90 and six hallmark of cancer cell. Among all HSF-1 dependent HSP, HSP90 have the most widespread effect because its broad array of client protein. It sustain almost all properties of cancer cell like antiapoptosis, self-sufficiency, limitless replication potential, angiogenesis, and tissue invasion as well as metastasis.^{13,14}

General description of RNA interferences

RNA interference (RNAi) is an accurate and potent gene silencing methods which first documented in nematode *Caenorhabditis elegans*. Effector molecules in RNAi are called silencing triggers, a nucleic acids that initiate the assembly of protein-

RNA complexes that repress expression of specific target genes by reducing their rates of transcription, the stability of their mRNA, or the translation of their mRNA into protein. The best studied RNAi molecule is double stranded RNA which can be produced by bidirectional transcription, transcription of inverted repeat or "hairpin" sequence, or physically introduced

into the cell by injecting, soaking, or even feeding dsRNA expressing bacteria.¹⁵

siRNA, the effector molecule of RNAi is 21-25 bp long RNA. The silencing capability of RNAi is initiated by introducing of dsRNA, synthetic siRNA or shRNA into cellular target. This small RNA guide silencing effector complexes like RNA-induced silencing complexes (RISC) or RNA-induced initiation of transcriptional gene silencing (RITS) complex. The main player in this complex is argonaute family protein that act as siRNA docking and also degrade target RNA.¹⁶

Mechanism of construction and delivery of HSF1 shRNA

shRNA is constructed by connecting sense and antisense strands of siRNA duplex by loop sequence allowing it to fold back when being transcribed. RNA polymerase III promoter is often used to drive shRNA transcription. Consecutive thymidines are added at 3'end which will recognize by RNA polymerase III as terminator sequence. RNA polymerase promoter U6 small nuclear RNA and ribonuclease P H1 subunit gene are the most frequently used promoters in many research to drive shRNA transcription.¹⁷ They are located at 5'end without cis acting regulatory element and initiate transcription at single position.¹⁸ After assembling the sequence, shRNA is converted into duplex DNA which will insert into viral genome. Highly effective shRNA has length ranging from 19-29 nt. But shRNA above this range has more effective silencing effect probably because increased probability to be recognized by dicer or allowing generation of several overlapping siRNA.¹⁹

The biggest challenge in RNAi therapy is about how to effectively deliver siRNA encoding gene into cancer cell. Several methods have been developed and can be grouped into viral and non-viral-based delivery. Non-viral-based delivery consists of direct intravenous delivery, lipid-based complex delivery, dynamic polyconjugates delivery and protein-conjugation-based delivery.²⁰ But this method is not effective because its low bioavailability, require large amount of siRNA, and also tend to sequester in non-target organ like liver and spleen. In contrast, viral-based delivery offer some advantage like penetrating ability, target cell/organ specificity, and because it deliver plasmid containing gene, offer long term effect. Viral-based delivery also can be sequestering in non-target organ, but recent development of less antigenic viral carrier has been solving this problem. In addition, this methods is the most suitable to deliver shRNA.²¹

There some viruses that has been evaluated as shRNA delivery vector. They include adenovirus, adeno-associated virus, retrovirus, lentivirus, and baculovirus.²¹ Among them, adenovirus is the most promising one because it is easy to modify its

genomes and tropism.²² Recent development of conditioned replicate adenovirus that restrict adenovirus replication just in cancer cell further boost its position not just as viral vector but also as therapeutic agent.²³

Currently, there are 53 serotype of adenovirus in human. Among them, adenovirus serotype 5 (Ad.5) of species C is the most well characterize. This virus has a number of features that make them attractive as a platform of oncolytic virus construction. It is not associated with any serious disease and its genome is well characterized, allowing for relatively easy genetic modification. It is also grown in high titer in culture. Most importantly, antibody that usually develop toward non-replicating adenovirus does not attack replicate one, especially Ad.5, which make it relatively stable in bloodstream and allowing systemic administration to target metastatic tumor.²⁴

Constructions of adenoviral expressing HSF1 shRNA involve deletion of E3 region and replacing it with HSF1 shRNA gene (**Figure 3**). Adenovirus has E1A gene that control viral replication.²⁵ The gene is preserve but its promoter is replace by PEG-3 promoter. This promoter is isolated from progressive tumor of E11-NMT mice.²⁶ E11 is embryonic tumor that occurs after mutant serotype 5 adenovirus infections. Meanwhile E11-NMT is a product from E11 clone and selected based on aggressiveness and higher proliferation capability. Hybrid subtraction technique shows that DNA is C-terminal mutant of GADD-34. PEG-3 promoter from minimum region (-118 until +194) is placed upstream from adenoviral E1A gene. PEG-prom is used because of its tumor selectivity. This promoter requires transcription factors PEA-3 (-104) and AP-1 (+8) which are over express only in cancer cell.

Ad-PEG-E1A-HSF1shRNA is constructed by using 2 combinations of shuttle vector, pE1.2 and pE1.3.²⁷ Transgenic band of pE1.2 contains CMV promoter and *HSF1shRNA* meanwhile pE1.3 contain PEG-promoter and *E1A* gene. Both shuttle vectors will be ligated by various restriction endonuclease enzymes (*AlwNI*, *BstAPI*, *DraIII*, *PfIMI*) at multiple cloning site (MCS) which result in cleavage of CMV- *HSF1shRNA* strand and PEG-prom-E1A strand. The product of this process is single strand containing PEG-prom-E1A and *HSF1shRNA*. Both of these gene are translocated into adenoviral plasmid, yielding Ad.PEG-E1A-*HSF1shRNA* which ready to be processed and infected into target cell.

Because of its small size and immunological properties, Ad.PEG-E1A-*HSF1shRNA* can be administered by intratumoral injection, intravenous, or inhalation.²⁸ Intratumoral injection of adenoviral vector has been applied in some research. Sarkar et. al. injects 1×10^8 colony forming unit (cfu) into breast cancer xenograft (T47D) and prostate cancer (DU-

145).^{29,30} On the other hand, Luo et. al., administered 2×10^8 cfu into BEL7404 tumor cells. The viral dose is comparable to tumor size but the principle is to infect

100 pfu to every tumor cell because in this concentration 90% of the tumor cell is thought to be infected.³¹

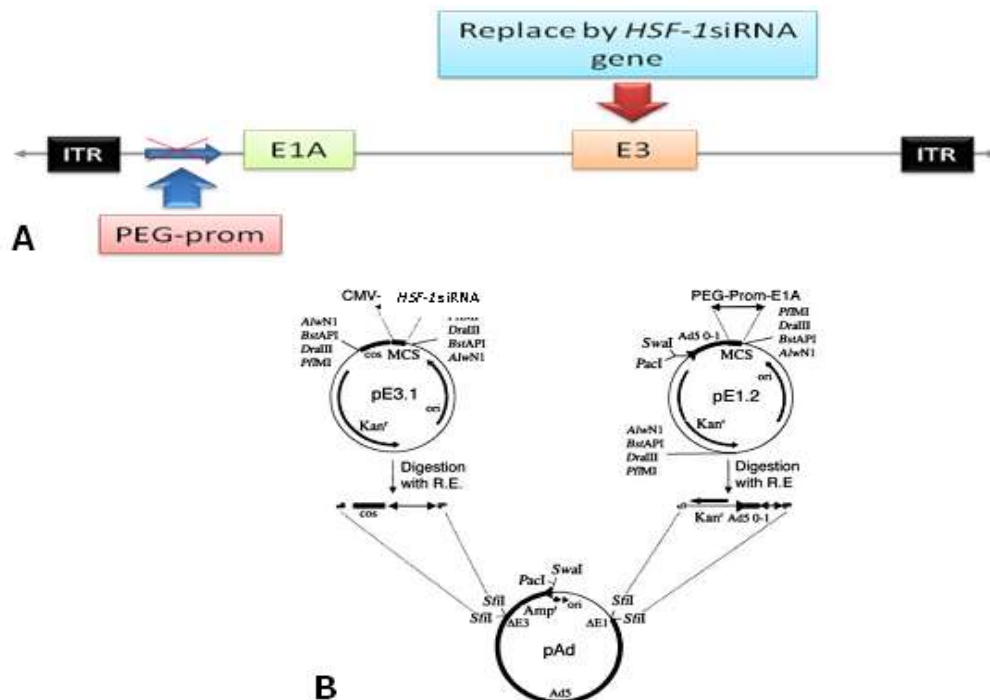


Figure 3 A. Ad.PEG-E1A-*HSF1shRNA* gene sequences. E3 gene encode capsid protein is replaced by HSF-1shRNA encoding gene. The E1A gene that control viral replication is preserve but its promoter is substituted by PEG-promoter which enable the virus to replicate specifically in cancer cells.²⁶ **B** Ad.PEG-E1A-*HSF1shRNA* gene is constructed by using shuttle vector pE1.2 carrying PEG-Prom-E1A and pE3.1 carrying HSF-1siRNA which will integrate into pAd.²⁷

Mechanism of adenovirus homing and its internalization to cancer cell

Adenovirus has icosahedral capsid consist of three main proteins (hexon [II], penton base [III], and knob fiber [III]). Beside these proteins, there are also minor proteins like protein VI, VIII, IX, IIIa, and VIa. Adenoviral infection can be divided into 2 phases, initial phases where adenovirus enter the cell and translocate its genome to the nucleus. Second phase, which called end phases consist of viral genome expression (PEG-E1A-HSF1 siRNA).³²

Internalization of serotype-5 adenovirus is mediated by *coxsackie* and *adenovirus receptor* (CAR), integrin, and *heparan sulfate glycosaminoglycan* (HS-GAGs) (**Figure 4**). Internalization process is initiated by formation of RGD peptide bond between penton base with integrin from $\alpha\beta3$ and $\alpha\beta5$ vitronectin receptor family. This complex activates phosphoinositide (PI) 3-kinase, $p130^{CAS}$, Rho GTPase, and *Raf/mitogen activated protein kinase* (MAPK).³³ These molecules induce actin rearrangement in the intracellular surface of plasma membrane which will mediate adenoviral internalization. Peptide complex RGD and integrin facilitate the formation of high affinity binding between fiber knob and CAR. CAR is 46 kDa trans

membrane protein and belongs to immunoglobulin family. This interaction releases virus into cytoplasm. Then protease will degrade protein VI, which connect capsid with core protein, and freeing viral core component. Viral genome will set its way to the nucleus. This process is mediated either by viral core protein (TP protein, protein V, VII, and μ -peptide) and cellular protein p32. p32 is thought to mediate cellular transportation between nucleus and mitochondria but can also mediate viral genome translocation.³⁴

In the nucleus, viral genome is directed to nuclear matrix where TP protein forms a complex with CAD pyrimidine. Then, the lamin B protein complexes with p32 which dissociates viral DNA from p32. The viral genome integrates with host genome and ready to be express by transcription and translation.^{35,36}

Mechanism of HSF-1 gene silencing by shRNA

As tumor cell express the viral protein, it also express siRNA gene in viral genome. shRNA gene is expression is same like common transcriptional model but it will fold as a result from folding strand within it. shRNA then being transported from nucleus to the cytoplasm by exportin-5. In cytoplasm, shRNA will cleave by dicer, a type III ribonuclease. This yield a small 21 nucleotide double stranded RNA composed

of two stranded, guide stranded and passenger stranded. Strand selection is dictated by

thermodynamic stability in which guide strand is located at less stably base pair in 5' end.³⁷

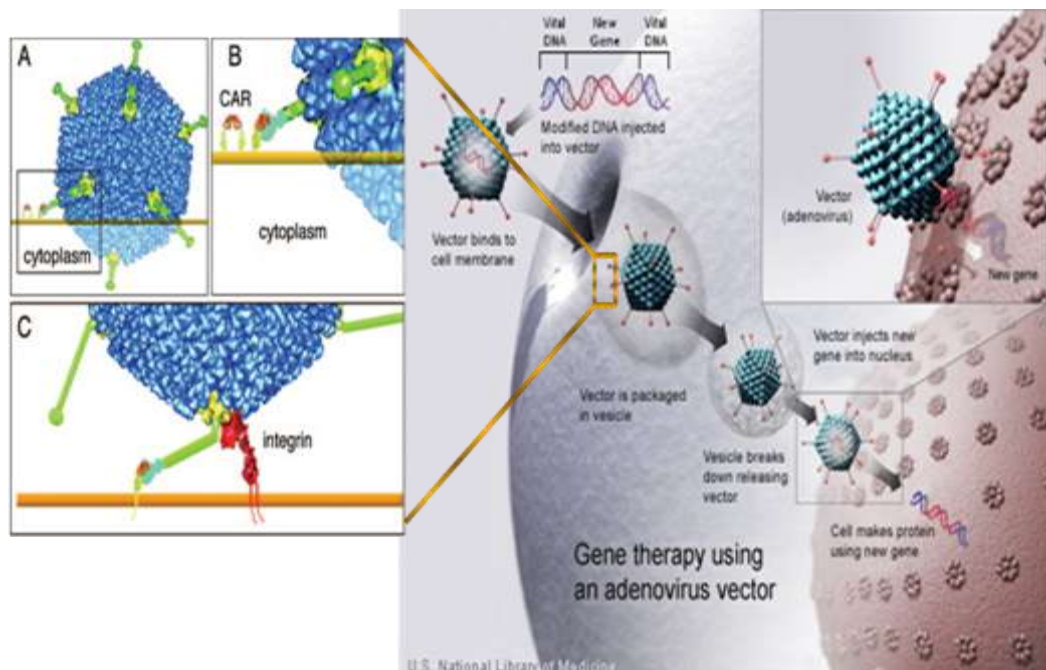


Figure 4. Adenovirus internalization process. Internalization process begin with binding of viral fiber knob with CAR and integrin. After internalization, viral release and capsid degradation ensue until viral genome set it way into the nucleus.³³

In cytoplasm, double stranded siRNA forms a complex with Dcr-2 and R2D2. This complex is called RNA-induced silencing complex (RISC) loading complex (RLC) (**Figure 5**). RLC function to unwind dssiRNA, releasing guide strand from passenger strand which will be degraded. Then, siRNA will be recruited into

holo-RISC a large protein complex containing TSN, Dcr-1, VIG, dFMR1, and Ago2. This recruitment is mediated by Dcr-2 and R2D2 interaction. The PAZ domain of Ago2 recognizes 3' end of siRNA and begin to unwind dssiRNA, cleave the passenger strand and eject it from complex. The final product is functional 80S RISC that has capability to cleave target RNA.³⁸

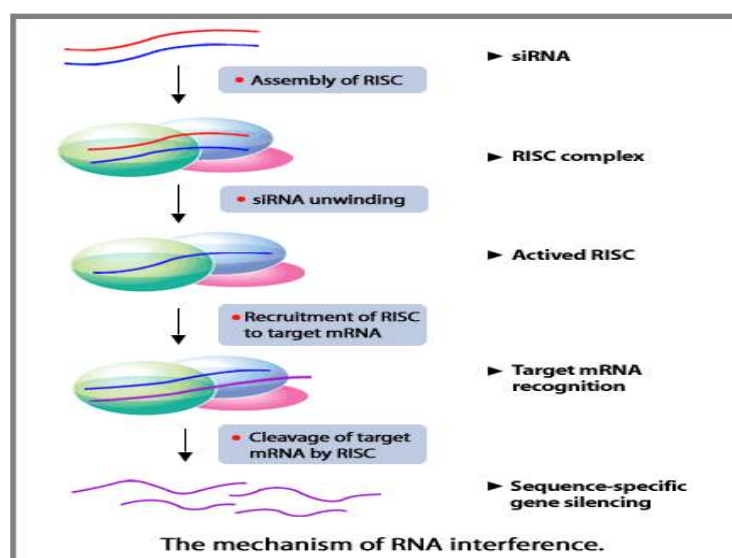


Figure 5. Mechanism of integration of siRNA into RISC complex. After cleavage, double-stranded siRNA congregate with proteins like Dcr-2 and R2D2, forming RISC complex. With subsequent dissociation of guide strand, RISC is activated ready to degrade target mRNA.³⁸

Once loaded into RISC, small RNA guides at least three distinct mode of silencing (**Figure 6**).^{39,40} Most often, small RNA will direct RISC to target mRNA and its 5' half will form base pairing complementary the target mRNA. Next, piwi domain of Ago2 protein will catalyze cleavage process. This domain has structural homolog with RNase H and requires Mg^{2+} as cofactor. The cleavage process is very precise: phosphodiesterase activity is mediated at siRNA residues 10 and 11 (from

5'end) producing 5'monophosphate and 3'hydroxyl terminus. These fragments are subject of oligouridylation process and can mediate exonucleolytic targeting which will complete the degradation process. After cleavage of target mRNA is complete, siRNA depart intact with RISC and guide it to the next target mRNA. This shows that RISC is a multiple turnover enzyme.

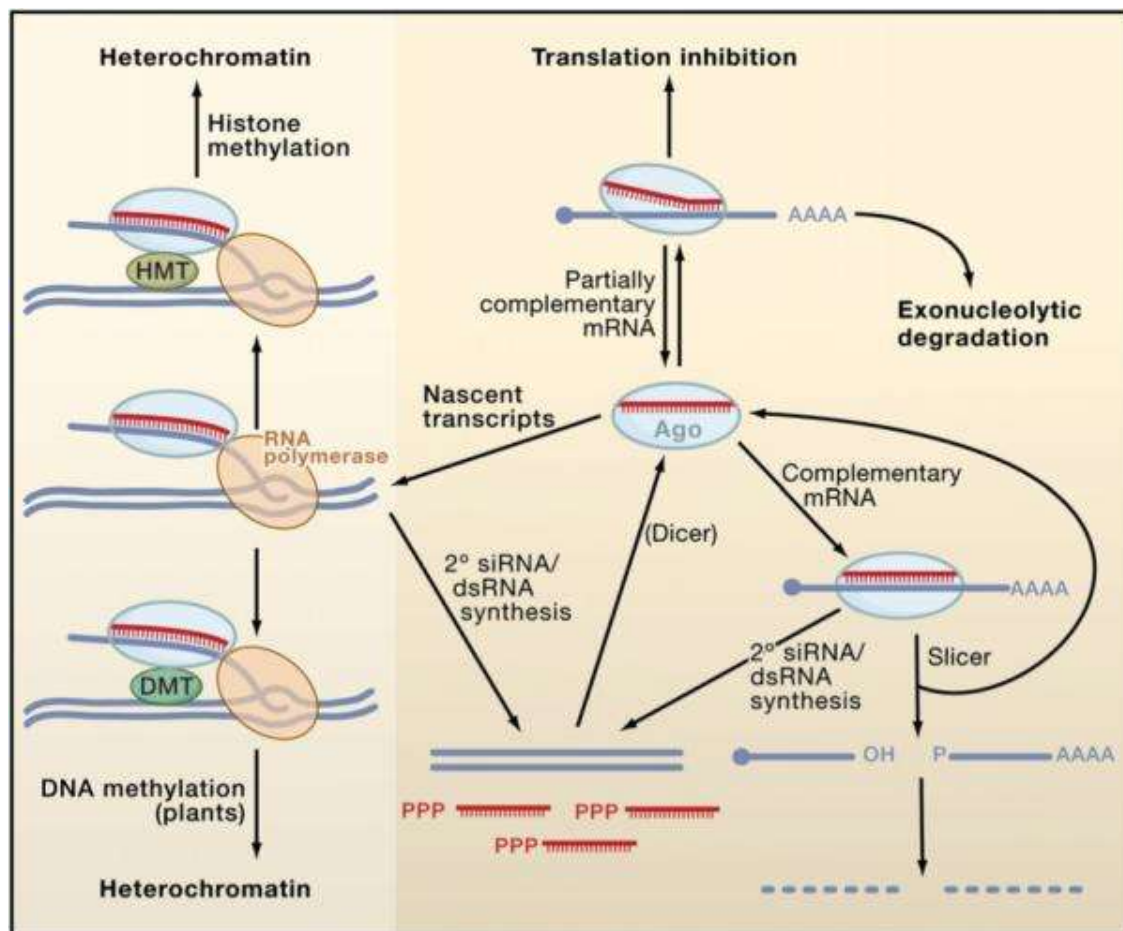


Figure 6. Mechanism of gene silencing by siRNA. siRNA shut down gene expression by several mechanism. Most notably is degradation of target mRNA which have fully complemter sequence to it. It also able to alter gene transcription by induce heterochromatin formation or disrupt translational process if there are some mismatch between siRNA and target mRNA¹⁵

siRNA is not always complement the target mRNA completely. Sometimes, it partially mismatches. Partial mismatch between siRNA and mRNA suppress the endonucleolytic activity of PIWI domain of Ago2. siRNA still capable to suppress gene expression but by using different mechanism. The post transcriptional silencing mechanism mediated by RISC is the same between siRNA and miRNA. The precise mechanism is still on debate but there are three proposed mechanisms. First, RISC probably competes with eIF4E for binding in mRNA 5'cap. Without eIF4E, initiation of transcription will not occur. Second, RISC stimulate

dead-enylation of mRNA tail. In this case, translation is suppress because the cap and PABP-1 free tail of deadenylate mRNA is unable to circularize. The last proposed mechanism stated that RISC block association of 60 S ribosomal subunit with 40 S preinitiation complex. This result from physical interaction between human Ago2 with eIF6 (which function in 60 S ribosomal subunit biogenesis and maturation and prevent premature association between 60 S and 40 S) and 40S ribosomal subunit.⁴¹

Finally, siRNA also capable to suppresses gene expression in transcriptional level by forming

repressive heterochromatin.⁴² In this case, siRNA form another complex called RNA-induced initiation of transcriptional gene silencing (RITS) consist of Ago1 and chromodomain protein Chp1 and Tas3. siRNA guide RITS complex to specific chromosomal loci such as centromere repeats. Then, nascent transcript will be recognizing by siRNA, facilitated by direct interaction between RITS and RNA polymerase II. This association promotes histone H3 methylation on lysine 9 (H3K9) by histone methyl transferase (HMT) leading to recruitment of chromodomain containing swi6 and chromatin compaction. Engagement of nascent transcript by siRNA also activates RNA-dependent RNA polymerase complex (RORC). RORC has RdRP subunit which will produce secondary siRNA and reinforce gene silencing

siRNA is very effective in knocking down the target gene and even in small amount can completely shut down the expression of target gene. This will result in complete down regulation of HSF-1. Since HSF-1 act as primary transcription factor for HSP90, HSP70, and HSP40, its absence will result in complete silencing of those gene. This means cancer cells will not be able to mount heat shock response, lost many functional proteins, and succumb from accumulation of miss folded protein which finally drives the cell to apoptosis.

Effect of HSF-1 gene silencing to cancer cell

The direct effect of HSF-1 gene silencing is complete down regulation of its target gene like HSP90, HSP70, and HSP40.⁶ Since heat shock proteins are important to guide protein folding, their absence will result in miss folding of their client protein. Because HSP90 and HSP70 are the two major heat shock protein, their absence will shut down heat shock response completely.

Of all HSP, HSP90 is the most important in many solid tumor as well as hematological malignancy.¹⁴ HSP90 has wide variety of client protein that have important role in cell survival, proliferation, expression of growth factor receptor, invasion, and metastasis. Because of that, most of the effect of HSF-1 down regulation is likely caused by HSP90 down regulation.

Alteration of tumor survivability

Cancer cells live in harsh hypoxic and nutrient deprived environment where normal cell will not survive.³ This is because cancer cells over express some of the protein that play important role in cell survivability. AKT, Raf, and epidermal growth factor receptor (EGFR) are protein that mediate cancer survivability.⁴³ AKT and Raf act as relay protein downstream from tyrosine kinase receptor and G-protein coupled receptor. Because most growth factors receptor is tyrosine kinase receptor, it is likely

that these protein mediate many prosurvival signal elicited by growth factors receptor.

EGFR is one of growth factor receptor. EGFR is up regulated in 10% to 40% of lung adenocarcinoma.⁴⁴ This receptor is also upregulated in small cell lung carcinoma whether in lower frequency. Most of the mutation occurs in kinase properties of the receptor so it can activate itself even in the absence of signal. This receptor mediated prosurvival signaling by PI3K-AKT pathway.

AKT, EGFR, and Raf are client protein of HSP90. Down regulation of HSP90 results in missfolding of these protein which make them non-functional. PI3K-AKT pathway is known play important role in regulation of Bcl-2 and Bcl-xL prosurvival protein so down regulation of this signaling pathway will make cancer cell more vulnerable to apoptosis induction.⁴⁴ Down regulation of EGFR also contribute in apoptosis sensitization in cancer cell because it will decrease stimulation to PI3K-AKT and Ras-Raf signaling pathway.

HSP-70 down regulation also has very significant effect on apoptosis induction because HSP70 is known as powerful anti-apoptotic agent.⁸ HSP70 inhibit many important proteins in apoptosis pathway include caspase-3,-7, and -9, APAF-1, apoptosis inducing factor (AIF), caspase-induced DNase (CAD), as well as c-Jun N-terminal kinase (JNK) mediated pathway. In addition, HSP70 cochaperone Bag-1 increase activity of Bcl-2 and Raf-1/ERK signaling pathway that prefer cell survivability. Therefore, down regulation of HSP70 will ablate the inhibition toward apoptosis pathways as well as decrease prosurvival molecule such as Bcl-2 which make cancer cell very vulnerable to apoptosis.

Induction of unfolded protein responses and apoptosis
HSF-1 gene silencing will result in almost complete halt in its expression. The absence of HSF-1 impairs the cancer cell to mount HSR as a result from decrease expression of HSP90, HSP70, and HSP40. These HSP are the major chaperones that act to rescue the folding process of many proteins. Thus its absence will result in accumulation of missfolded protein either in cytoplasm and endoplasmic reticulum.

Accumulation of miss folded proteins trigger the activation of a process known as unfolded protein response (UPR).⁴⁵ Unfolded proteins will bind Bip which formerly associated with three inactive UPR sensors activating transcription factor-6 (ATF6), inositol-requiring enzyme -1 (IRE1), and PKR-like ER protein kinase (PERK).⁴⁶ When Bip dissociate from them, it activate these protein, elicit UPR signaling pathway. Activated PERK dimerises and autophosphorylate and activate eukaryotic translation initiation factor 2 α subunit (eIF2 α). This protein will halt general translational process and also phosphorylate ATF4.⁴⁷ On the other hand, IRE1 posses RNase activity and cleave 26 bp intron from X-box-

binding protein 1 (XBP1) mRNA enabling it to be translated into functional protein. Meanwhile, ATF6 transits to the cis-golgi compartment where it cleaves by site-1 protease (S1P) and site-2 protease (S2P). The cleaved N-terminal translocate to the nucleus where, together with ATF4 and XBP1, it activate transcription of UPR response gene.

In addition to UPR, missfolded proteins also trigger apoptosis mediated by UPR sensors described above.⁴⁷ IRE1 will recruit c-Jun-N-terminal inhibitory kinase (JIK) and TRAF2. These complex activated ASK1/JNK signaling pathway that drive the apoptosis process. Recruitment of TRAF2 also releases procaspase-12 from ER. When convert into its active form, caspase-12 will cleaved procaspase-9 into caspase-9. Next, caspase-9 will mediated cleavage of effectors caspase like caspase-3, caspase-7, and caspase-10.

On the other hand, ER stress, resulting from excessive accumulation of missfolded proteins, induce conformational alteration of Bax and Bak which allows Ca^{2+} ion to diffuse out from ER.⁴⁶ Ca^{2+} bind to associated protein m-calpain which will cleave procaspase-12 into caspase-12. Ca^{2+} also induces mitochondria-dependent apoptosis by releasing cytochrome-c to cytoplasm and allowing it to form APAF-1 with caspase-9 and activate effectors caspases. Meanwhile, activated PERK and ATF6 phosphorylate CEBP homologous protein (CHOP) which will down regulate Bcl-2, thus increase the vulnerability of cancer cell to apoptosis.

Inhibition of angiogenesis

Angiogenesis is important event that ensure tumor survivability once it reach certain size. Hypoxia-inducible factor-1 (HIF-1) is the chief regulator of many angiogenic genes.⁴⁸ This protein consists of 2 subunits. Alpha subunit is the oxygen sensing meanwhile β -subunit is constitutively expressed. Alpha subunit will dimerises with β -subunit before it translocates into nucleus and induce transcriptional process of many angiogenic genes.

HSF-1 is HSP90 client protein and it down regulation prevents HIF-1 folding into functional protein and directing it to proteasomal degradation. Decrease number of HIF-1 result in decrease expression of important angiogenic mediator like nitric oxide synthase (NOS) and vascular endothelial growth factor (VEGF), thus prevent increase in vascular permeability and endothelial cells migration. There are also down regulation in matrix metalloproteinase-2 (MMP-2) and urokinase-type plasminogen activator receptor that function in cellular matrix metabolism. Two glycolysis enzymes, phosphoglycerate kinase-1 and lactate dehydrogenase are also down regulate since their genes are regulate by HSF-1, thus decrease the ability of cancer cells to survive in hypoxic condition.⁴⁹ Overall, down regulation of HSP90 result in nonfunctional HSF-1 which result in angiogenesis inhibition from many cellular aspects.

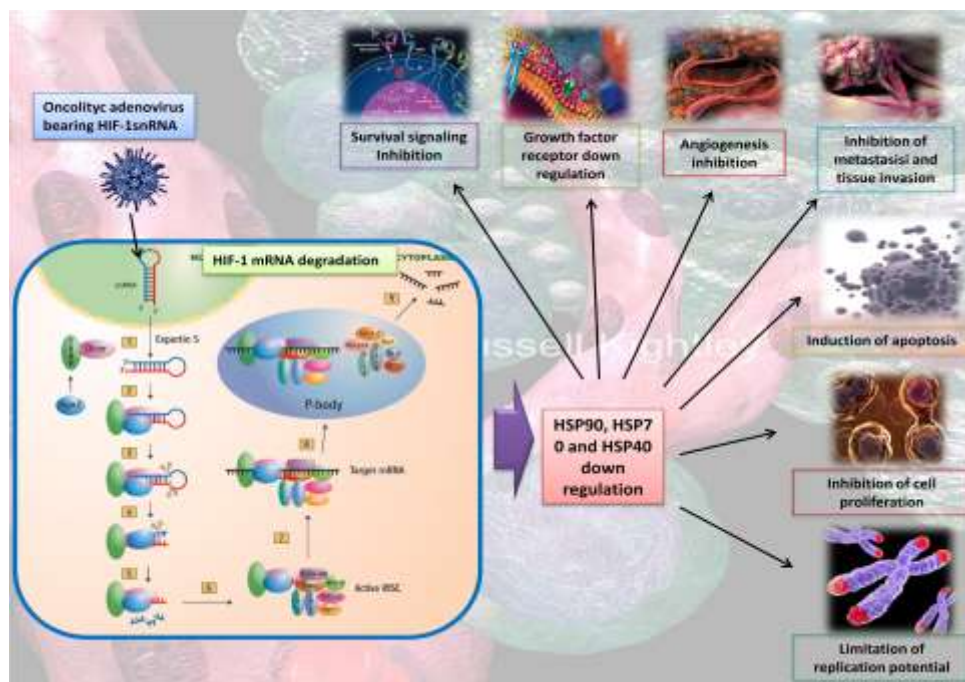


Figure 7 Summary of the effect of HSF-1 gene silencing by shRNA to cancer cell. Because HSF-1 is the main transcription factor for major HSP's, it have broad array of effects that disrupt almost all properties of cancer cell.^{6,8,14}

Inhibition of Cell Proliferation

Cancer cell retain capability to proliferate excessively as result from up regulation of cell cycle regulated protein cyclin-dependent kinase (CDK) or signaling molecule that influence it. In addition other cell cycle regulator like retinoblastoma and E2F are also takes a part. Retinoblastoma is regulated by CDK4 and when phosphorylate, it release E2F which will activate cell cycle progression from G1 to S phase.

In addition, cancer cell usually over express human telomerase reverse transcriptase (hTERT) that will maintain the length of the telomere and enable cancer cell to proliferate excessively without reduce it chromatin length.⁵⁰

Down regulation of HSP90 as a result of decrease expression of HSF-1 targets CDK4 to proteasomal degradation.⁵¹ Non functional CDK4 loses its ability to phosphorylate Rb, preventing its dissociation from E2F. As result, cancer cell will remain in G1 phase and unable to progress into S phase, prevent it to proliferate. In addition, non functional hTERT result in progressive shortening of the telomere and limit the cancer cell proliferation potential.⁵² Thus, HSF-1 gene silencing is able to inhibit cell division by targetting CDK4 and hTERT to proteasomal degradation.

Inhibition of cancer cell invasion and metastasis

HSP90 client protein, proto-oncogenes Met and MMP-2 are required for metastatic process. Gene product of Met, p190^{Met} is a hepatocyte growth factor/scatter factor (HGF/SF) receptor. Activation of this receptor increase cellular proliferation, migration, and invasion of cancer cells. Metastasis also requires cell adhesion to extracellular matrix, digestion of the matrix, and migration of the cancer cells. These processes are mediated by MMP-2. Down regulation of HSP90 decrease the number of active p190^{Met} and impair MMP-2 maturation which will inhibit cancer cell invasion and metastasis.⁵³

CONCLUSION

HSF-1 plays a crucial role in lung cancer cell to maintain many cellular processes and signaling. HSF-1 mediates its effect by acting as transcription factor that regulate HSP encoding gene as well as molecules that orchestrating many cellular process. HSF-1 siRNA is integrated into adenovirus genome, which will act as carrier and cancer terminator virus. siRNA silence HSF-1 gene by forming RISC complex which will recognize and degrade HSF-1 mRNA. siRNA also capable to halt transcription process of HSF-1 mRNA and direct silencing HSF-1 by induce the the formation of heterochromatin. Silencing of HSF-1 by siRNA will bring catastrophic effect to cancer cell since it halts prosurvival signaling, induce apoptosis, inhibit cell proliferation, and inhibit angiogenesis as well as invasion and metastasis as a result from HSP down

regulation. Despite its broad array of promising effect, application of HSF-1 siRNA in cancer treatment still need further research in order to confirm its efficacy and establish the optimal dose required to elicit its effect.

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