JNBT
©2005INBDK

JNBT, Vol 2, No 1, pp. 10-16

Promising potential of allosteric ribozyme in bio-nano technology

Byung-Su Kwon and Seong-Wook Lee*

Department of Molecular Biology, Institute of Nanosensor and Biotechnology,
Dankook University, Seoul 140-714, Korea

ABSTRACT

Allosteric ribozyme exhibits a catalytic activity that is modulated by specific effector through binding to receptor sites that are distinct from the active site. The allosteric control of ribozyme activity is attractive not only from the perspective of intrinsic catalytic properties, such as structure-function relationships, but in terms of applications to bio-nano technology. Now many of allosteric ribozymes have been created by using "allosteric selection", a molecular engineering process, that combines modular rational design and *in vitro* evolution strategies. Here we describe the molecular basis, robustness and application of the allosteric ribozyme.

Key words : allosteric ribozyme, biochip, biosensor, HTS, gene regulation

Introduction

In recent years, the potential of RNA to form dynamic structures has been exploited by ribozyme engineers in the development of RNA molecular switches or 'allosteric ribozymes'. Although the study of artificial enzymes developed from studies exclusively of proteinaceous enzymes and the discovery of RNA enzymes (ribozymes) were only relatively recent(1), the rapidly developing field of RNA biotechnology seems likely to overtake that of proteins and peptides, in particular, in the field of artificial enzymes(2-5)

The basic principle of an allosteric nucleic acid sensor is shown in Figure 1. Unlike other enzyme inhibitor or enhancement mechanisms, allosteric enzyme undergoes modulation of catalytic activity in the presence of an effector molecule that binds to a receptor site that is distinct from that of the enzyme's active site(6,7). Therefore, a typical allosteric enzyme is comprised of three functional features: a catalytic center, an effector binding center, and a structurally responsive architecture that permits alterations in the function of the catalytic center upon binding of the effector. It has been known for several years that functional RNAs and DNAs can be 'evolved' in the test tube(5,8) and that *in vitro*

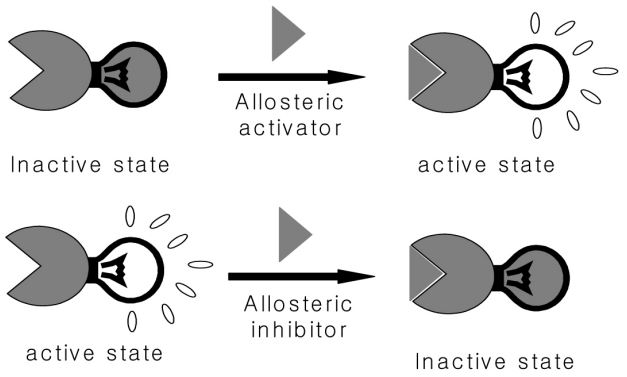

Fig 1. Schematic representation of the action of allosteric ribozymes. The activity of a ribozyme can be positively affected (allosteric activation) or negatively affected (allosteric inhibition) by conformational changes that result from ligand binding to an appended aptamer domain.

* Corresponding author :
Seong-Wook Lee
Tel : 82-2-709-2905
Fax : 82-2-798-4733
E-mail : SWL0208@dankook.ac.kr

selection can be used to isolate a diverse array of precisely folded sequences that bind specific proteins or small organic compounds(6,7). These engineered RNA biopolymers may be used as diagnostic agents or biosensors for any number of target compounds, as artificial molecular switches to control gene expression in complex systems, and as gene-inactivating agent for cancer or viral gene therapy.

Allosteric ribozyme as a biosensor component

Designing sensors has long been a focus of research as it can provide on-site, real-time detection, and quantification of target analytes of interest for civilian, clinical, and military applications(9.10).

The chemical properties of the allosteric ribozyme make it amenable for use as a switch or sensor. Allosteric ribozymes serve as self-contained biosensor elements that can be made to selectively bind an analyte and whose catalytic function can be made to respond characteristically to the analyte-binding event. The range of chemical and even physical stimuli that can be used to modulate allosteric ribozyme activity is likely to be broad, which is essential if nucleic acids and their analogs are to serve as a robust medium for biosensor development.

The extent to which nucleic acids will serve as useful biosensor elements will depend largely on how easily RNA and DNA molecular switches can be created. There are two critical step for design of allosteric ribozyme.

The first step is to obtain analyte-specific aptamers, DNA/RNAzymes, or aptazymes. To create an allosteric ribozyme, a ribozyme is connected via a "communication module" to an aptamer that is specific for a certain ligand. The communication module couples the aptamer–ligand interaction to ribozyme activity(11,12), usually via up-regulation but sometimes via inhibition(13). The resulting RNA combines an aptamer with a ribozyme and has thus been termed an "aptazyme"(11). The activity enhancement upon ligand activation of an aptazyme is typically between 10 and 10^3; the higher end of this range is more than sufficient for practical biosensor assays. The mechanism by which ligand binding is coupled to ribozyme activation is usually not known in detail, but this is generally thought to involve structural stabilization of the catalytically active ribozyme conformation upon ligand binding(14).

The other critical step is to transfer the target-specific recognition event into a physically detectable signal. Toward this goal, conductivity(15) and radioisotope(16) or fluorescence(17,18) labeling methods have been developed. In many sensor applications, colorimetric detection can be advantageous because it can avoid difficulties associated with handling and disposing of radioisotopes, eliminate, or minimize most costs associated with instrumentation and operation in fluorescence detection, and thus make on-site, real-time detection, and quantification easier(19,20).

A number of studies have now shown that aptamers, DNA/RNAzymes, or aptazymes can often rival antibodies in the broad range of analytes they can recognize(21). At least two features make them excellent platforms for making sensors(21,22). First, the selection is *in vitro* and thus can be carried out in short time and with limited cost. Second, unlike antibodies, most aptamers, DNA/RNAzymes, or aptazymes can be denatured and renatured many times without losing the binding ability or activity and, therefore, can be processed and stored under rather harsh conditions.

Advanced biochip systems

Microarrays are a well-utilized tool in both academic and industrial research laboratories. They can be used to assess gene and protein expression (via nucleic acid or protein microarrays) to identify novel targets, and can also be used to validate the found targets at the tissue or cell scale (via tissue or cell microarrays). As it was becoming clear that catalytic nucleic acids could serve as biosensor elements, it was proposed that their combined recognition and reporting features could be harnessed to create a new biochip system based on arrays of allosteric RNA or DNA catalysts(23,24). Breaker's group has already generated a small aptazyme array that discriminated between a variety of metabolites(16). They first generated seven different aptazymes, each of which was specifically triggered by its effector, and immobilized these on a gold surface via a 5'- thiotriphosphate moiety. A 63-feature RNA array was created and used to se-

lectively and quantitatively report the presence of various analytes even when incubated in a complex chemical mixture. Further improvements in the methods to detect signal and the development of a greater number of allosteric ribozymes would permit the unification of genomics, proteomics and metabolomics assays in a single biochip format.

High-throughput drug screen system

High throughput screening (HTS) of compound libraries to identify molecules that inhibit the catalytic activity of specific kinases, helicases, proteases, and other enzymes is a major focus of pharmaceutical industry efforts. Strategies for the detection of enzyme activity in HTS formats that can readily translate to multiple targets have the potential to greatly accelerate therapeutic discovery. Because allosteric ribozyme is able to selectively recognize the common product of an enzyme reaction class and readily transduce a detectible signal, it is possible to use it to HTS. For example, protein kinases typically convert ATP into ADP upon protein phosphorylation. A highly specific ADP-sensing RNA switch or RiboReporter(25,26) has been created and used to detect and report the amount of ADP by-product. This indirectly reflects the level of protein kinase activity in a given assay. A RiboReporter that yields a fluorescent report upon activation by ADP was used successfully to identify reaction mixtures containing the protein kinase inhibitor staurosporine(25). Similarly, this allosteric ribozyme could be used in large high-throughput screens to identify new protein kinase inhibitors or to find compounds that modulate the activity of any enzyme whose activity generates or destroys ADP. Allosteric ribozymes have also been created to respond to protein targets(27) such as lysozyme and the Rev peptide from HIV(28). Furthermore, other protein-dependent allosteric ribozymes have recently been shown to be useful tools to screen for small molecules that disrupt protein–ligand interactions(29).

Allosteric ribozyme as a gene knock-down tool

Scientists have been working on strategies to selectively turn off specific genes in diseased tissues for the past thirty years. Also the demand for better tools to assign gene function has been made imperative by the advent of genomics, a field that within a few years has produced numerous monumental advances from the complete sequence of *S. cerevisiae* (30) to a draft sequence of the human genome. (31,32).

The overwhelming advances of the last few years in the field of nucleic acid-based technologies laid the basis for the development of gene knock-down. Currently, the best characterized of this group of RNA-mediated gene regulation pathways is the post-transcriptional gene silencing mechanism known as RNA interference. RNAi is a process by which double-stranded, 21-nucleotide, siRNAs post-transcriptionally inhibit gene expression by targeting homologous mRNA for degradation. RNAi is speculated to be an evolutionarily conserved mechanism for host plant and animal cells to defend against foreign or invading nucleic acids, such as transposons, viruses or aberrant mRNA, thereby functioning as a genomic immune system(33). The biochemical pathways of RNAi are still being investigated(33,34), but fundamental steps include endogenous double-stranded RNA (dsRNA) incorporation into an RNA-protein complex that includes a helicase, a kinase and a type III ribonuclease, DICER, which processes long dsRNAs into 21 to 25-nucleotide, double-stranded siRNA with a two to three nucleotide 3′-overhang. Although DICER appears to be important for RNAi in lower organisms, it has not been detected in mammalian systems, which explains the non-specific interferon induction and cell death in mammalian cells treated with siRNA constructs longer than 30 base pairs. Helicase-unwound single-stranded RNA becomes part of the larger RNA-induced silencing complex and guides the complex to target mRNA. The bound siRNA/mRNA duplex is finally cut by an RNA-induced silencing complex endoribonuclease, thereby inactivating translation. Because it offers efficient gene knock-down, relatively low cost, and easy to make, the use of siRNA is the most popular and will continue to expand. If so, dose RNAi supplant the use of antisense, ribozymes, DNAzymes, and related approaches for many applications? RNAi is very efficient and useful, but each system have the various unique

potential use. Especially ribozyme has a several advantage superior to siRNA.

The first, short inhibitory RNAs have not been effective against intron target sites and may not be effective against RNAs that are exclusively nuclear, such as spliceosomal RNAs. Ribozymes, on the other hand, can be designed to target introns and nuclear-localized RNAs. These agents may be more useful when it is necessary to selectively down regulate a sequence derived from a gene family of highly homologous sequences in which only the introns have grossly different sequences.

The second, in choosing a method for targeted knock-down of gene expression, an important consideration has to be the potential for off-target, non-sequence specific effects. So far it is unclear whether siRNA elicit the off-target effect or not, but there is a report in the literature of a multitude of off-target effects by synthetic siRNAs(35). In contrast, ribozymes are much more sensitive to polymorphisms at the cleavage site (though relatively less so in the hybridizing arms, depending upon position) and have therefore been used for discriminating between single nucleotide polymorphisms(36).

To the end, the most important feature of ribozyme is that it is possible to regulate its activity. The hammerhead ribozyme is a metalloenzyme and that the RNA component, in particular the region known as the catalytic core, is merely used as a scaffold for capture of metal ions at a site adjacent to the scissile bond(37,38). A slight change in the conformation of the catalytic core of a hammerhead ribozyme can easily prevent bound metal ions from participating in the chemical cleavage reaction or can prevent the metal ions from binding to the appropriate site, with a resultant dramatic loss of catalytic activity. By using of this character of hammerhead ribozyme, Taira and co-worker generated a novel allosterically controllable ribozyme, the maxizyme, which has a dimeric structure(39,40) and specially cleaves BCR-ABL mRNA inducing apoptosis in cultured CML cells. They have succeeded in allosterically controlling the activity of the heterodimer by using one of the two substrate-binding regions as sensor arms. Only in the presence of the correct target sequence does the heterodimer form the cavity that can catalytically capture indispensable Mg2+ ions. The maxizyme remained in an inactive conformation in the presence of normal ABL mRNA or in the absence of the junction sequence.

Like a previously stated, several groups recently succeeded in creating allosterically controllable ribozymes whose activities can be regulated in a useful way by allosteric effector molecules. In some cases, the allosteric effector molecule is a nucleic acid (DNA or RNA) and in other cases it is a small chemical compound. If *trans*-cleavage concept of maxizyme is conjugated with the allosteric ribozyme regulated by a small chemical compound or a nucleic acid, novel class of gene knock-down allosteric ribozyme would be developed.

Allosteric ribozyme as a gene expression regulator

Investigations of the mechanisms involved in appropriate, developmentally regulated tissue-specific gene transcription have laid the foundations for transgenic and gene-therapy technologies directing specific induction or ablation of genes of interest in a tissue-restricted manner. This technology has further evolved to allow for temporal control of gene expression and ablation. Useful gene regulation system such as tissue-specific promoter, tetracycline regulatory(41) and Cre-loxP system(42) have been developed and used at various fields. However, these system is insufficient to meet all of requirements. So novel and powerful system for temporal and spatial gene regulation is required.

Recently, a novel mode of RNA-mediated genetic regulation has been discovered that requires no auxiliary protein factor. RNA serves as the sole molecular switch that modulates transcription, translation or RNA processing through conformational changes prompted by direct interaction with a specific cellular metabolite(43,44). These natural RNA switches show a wide range of target specificities and affinities. For examples, coenzyme B12(45), flavin mononucleotide (FMN)(46), thiamine pyrophosphate (TPP)(47), S-adenosylmethionine (SAM)(48), lysine(49), guanine(50), and adenine(51) have been characterized. The discovery of natural riboswitches stimulated the study of natural allos-

teric ribozyme-switches. In 2004, the first of natural metabolite-sensing allosteric ribozyme switches reported(52). Breaker and coworker have identified a new class of ribozymes that cleaves the messenger RNA of the glmS gene in Gram-positive bacteria. The ribozyme is activated by glucosamine-6-phosphate (GlcN6P), which is the metabolic product of the GlmS enzyme. Additional data indicate that the ribozyme serves as a metabolite responsive genetic switch that represses the glmS gene in response to rising GlcN6P concentrations.

The examples of natural riboswitches and engineered allosteric ribozymes provide evidence that RNA has sufficient functional potential to maintain a complex metabolic state without the need for proteins. Moreover, riboswitches open fire on new possibility of gene regulation. Actually Mulligan and coworker showed that RNA-based gene regulation system can effectively work in mammalian cells via the incorporation of sequences encoding self-cleaving RNA motifs into the transcriptional unit of a gene or vector(53). When correctly positioned, the sequences lead to potent inhibition of gene or vector expression, owing to the spontaneous cleavage of the RNA transcript. Administration of either oligonucleotides complementary to regions of the self-cleaving motif or a specific small molecule results in the efficient induction of gene expression, owing to inhibition of self-cleavage of the messenger RNA. Efficient regulation of transgene expression is shown in a variety of mammalian cell lines and live animals. This study provide an important 'proof of-principle' for gene regulation strategies based on the modulation of RNA processing.

Conclusion

RNA and DNA can be created to function as an allosteric enzyme, wherein distinct effector-binding and catalytic domains interact dynamically to control catalysis. These features of the allosteric ribozyme provide new opportunities as novel genetic-control elements, biosensor components, or precision switches for use in nano-biotechnology via targeted molecular sensing. So creation of allosterically controllable ribozymes is of great current interest and will be actively developed.

References

(1) Gesteland RF, Cech TR, Atkins JF (1999) The RNA World. Cold Spring Harbor Laboratory Press.
(2) Famulok M (1999) Oligonucleotide aptamers that recognize small molecules. Curr. Opin. Struc. Biol. 9: 324–329.
(3) Roberts RW, Ja WW (1999) In vitro selection of nucleic acids and proteins: What are we learning? Curr. Opin. Struc. Biol. 9: 521–529.
(4) Li Y, Breaker RB (1999) Deoxyribozymes: new players in the ancient game of biocatalysis. Curr. Opin. Struct. Biol. 9: 315–323.
(5) Wilson DS, Szostak JW (1999) In vitro selection of functional nucleic acids. Annu. Rev. Biochem. 68: 611–647
(6) Gold L, Polisky B, Uhlenbeck O, Yarus M (1995) Diversity of oligonucleotide functions. Annu. Rev. Biochem. 64: 763-797.
(7) Osborne SE, Ellington AD (1997) Nucleic acid Selection and the challenge of combinatorial chemistry. Chem. Rev. 97: 349-370.
(8) Breaker RR (1997) In vitro selection of catalytic polynucleotides. Chem. Rev. 97: 371-390.
(9) Gizeli E, Lowe CR (1996) Immunosensors. Curr. Opin. Biotechnol. 7: 66-71.
(10) Hesselberth J, Robertson MP, Jhaveri S, Ellington AD (2000) In vitro selection of nucleic acids for diagnostic applications. Rev. Mol. Biotechnol. 74: 15-25.
(11) Robertson MP, Ellington AD (2000) Design and optimization of effector-activated ribozyme ligases. Nucleic Acids Res. 28: 1751-1759.
(12) Kertsburg A, Soukup GA. (2002) A versatile communication module for controlling RNA folding and catalysis. Nucleic Acids Res. 30: 4599-4606
(13) Tang J, Breaker RR (1997) Rational design of allosteric ribozymes. Chem. Biol. 4: 453-459.
(14) Soukup GA, Breaker RR (1999) Nucleic acid molecular switches. Trends Biotechnol. 17: 469–476

(15) Fahlman RP, Sen DJ (2002) DNA conformational switches as sensitive electronic sensors of analytes. J. Am. Chem. Soc. 124: 4610-4616.
(16) Seetharaman S, Zivarts M, Sudarsan N, Breaker RR (2001) Immobilized RNA switches for the analysis of complex chemical and biological mixtures. Nat. Biotechnol. 19: 336-341.
(17) Nutiu R. Li YJ (2003) Structure-switching signaling aptamers. J. Am. Chem. Soc. 125: 4771-4778
(18) Li JJ, Fang X, Tann W (2002) Molecular aptamer beacons for real-time protein recognition. Biochem. Biophys. Res. Commun. 292: 31-40.
(19) Stojanovic MN. Landry DW (2002) Aptamer-based colorimetric probe for cocaine. J. Am. Chem. Soc. 124: 9678-9679.
(20) Elghanian R, Storhoff JJ, Mucic RC, Letsinger RL, Mirkin CA (1997) Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles. Science 277: 1078-1080.
(21) Jayasena SD (1999) Aptamers: An emerging class of molecules that rival antibodies in diagnostics. Clin. Chem. 45: 1628-1650.
(22) Mei SHJ, Liu Z, Brennan JD, Li YJ (2003) An efficient RNA-cleaving DNA enzyme that synchronizes catalysis with fluorescence signaling. J. Am. Chem. Soc. 125: 412-420.
(23) Marshall KA, Ellington AD (1999) Training ribozymes to switch. Nat. Struct. Biol. 6: 992–994.
(24) Hesselberth J, Robertson MP, Jhaveir S, Ellington AD (2000) In vitro selection of nucleic acids for diagnostic applications. J. Biotechnol. 74: 15–25.
(25) Srinivasan J, Cload ST, Hamaguchi N, Kurz J, Keene S, Kurz M, Boomer RM, Blanchard J, Epstein D, Wilson C, Diener JL (2004) ADP-specific sensors enable universal assay of protein kinase activity. Chem. Biol. 11: 499-508.
(26) Ferguson A, Boomer RM, Kurz M, Keene SC, Diener JL, Keefe AD, Wilson C, Cload ST (2004) A novel strategy for selection of allosteric ribozymes yields RiboReporterTM sensors for caffeine and aspartame. Nucleic Acids Res. 32: 1756-1766.
(27) Najafi-Shoushtari SH, Mayer G, Famulok M (2004) Sensing complex regulatory networks by conformationally controlled hairpin ribozymes. Nucleic Acids Res. 32: 3212-3219.
(28) Hesselberth JR, Robertson MP, Knudsen SM, Ellington AD (2003) Simultaneous detection of diverse analytes with an aptazyme ligase array. Anal. Biochem. 312: 106-112.
(29) Hartig JS, Najafi-Shoushtari SH, Grune I, Yan A, Ellington AD, Famulok M (2002) Protein-dependent ribozymes report molecular interactions in real time. Nature Biotechnol. 20: 717-722.
(30) Dujon B *et al.* (1997) The nucleotide sequence of Saccharomyces cerevisiae chromosome XV. Nature 387: 98-102.
(31) Venter J (2001) The sequence of the human genome. Science 291: 1304-1351.
(32) International Human Genome Sequencing Consortium (2001) Initial sequencing and analysis of the human genome. Nature 2001 409: 860-921.
(33) Plasterk RH (2002) RNA Silencing: The Genome's Immune System. Science 296: 1263-1265.
(34) Hannon GJ (2002) RNA interference. Nature 418: 244-251.
(35) Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS (2003) Expression profiling reveals off-target gene regulation by RNAi. Nat. Biotechnol. 21: 635–637.
(36) Lewin AS, Drenser KA, Hauswirth WW, Nishikawa S, Yasumura D, Flannery JG, LaVail MM (1998) Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa. Nat. Med. 4: 967–971.
(37) Walter NG, Burke JM (1998) The hairpin ribozyme: structure, assembly and catalysis. Curr. Opin. Chem. Biol. 2: 24-30.
(38) Carola C, Eckstein F (1999) Nucleic acid enzymes. Curr. Opin. Chem. Biol. 3: 274-283.
(39) Kuwabara T, Warashina M, Tanabe T, Tani K, Asano S, Taira K. (1998) A novel allosterically trans-activated ribozyme, the maxizyme, with exceptional specificity in vitro and in vivo. Mol. Cell. 2: 617-627.

(40) Amontov S, Nishikawa S, Taira K (1966) Dependence on Mg2+ ions of the activities of dimeric hammerhead minizymes. FEBS Lett. 386: 99-102

(41) Hillen W, Berens C (1994) Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annu. Rev. Microbiol. 48: 345-369.

(42) Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science 265: 103-106.

(43) Nudler E, Mironov AS (2004) The riboswitch control of bacterial metabolism. Trends Biochem. Sci. 29: 11-17.

(44) Winkler WC, Breaker RR (2003) Genetic control by metabolite-binding riboswitches. Chem. BioChem. 4: 1024-1032.

(45) Nou X, Kadner RJ (2000) Adenosylcobalamin inhibits ribosome binding to btuB RNA Proc. Natl. Acad. Sci. USA 97: 7190-7195.

(46) Winkler WC, Cohen-Chalamish S, Breaker RR (2002) An mRNA structure that controls gene expression by binding FMN. Proc. Natl. Acad. Sci. USA 99: 15908-15913.

(47) Mironov AS, Gusarov I, Rafikov R, Lopez LE, Shatalin K, Kreneva RA, Perumov DA, Nudler E (2002) Sensing small molecules by nascent RNA: a mechanism to control transcription in bacteria. Cell 111: 747-756.

(48) McDaniel BAM, Grundy FJ, Artsimovitch I, Henkin TM (2003) Transcription termination control of the S box system: Direct measurement of S-adenosylmethionine by the leader RNA. Proc. Natl. Acad. Sci. USA 100: 3083-3088.

(49) Kochhar S, Paulus H (1996) Lysine-induced premature transcription termination in the lysC operon of Bacillus subtilis. Microbiology 142: 1635-1639.

(50) Mandal M, Boese B, Barrick JE Winkler WC, Breaker RR (2003) Riboswitches control fundamental biochemical pathways in Bacillus subtilis and other bacteria. Cell 113: 577-586.

(51) Mandal M, Breaker RR (2004) Adenine riboswitches and gene activation by disruption of a transcription terminator. Nat. Struct. Mol. Biol. 11: 29-35.

(52) Winkler WC, Nahvi A, Roth A, Collins JA, Breaker RR (2004) Control of gene expression by a natural metabolite-responsive ribozyme. Nature 428: 281-286.

(53) Yen L, Svendsen J, Lee JS, Gray JT, Magnier M, Baba T, D'Amato RJ, Mulligan RC (2004) Exogenous control of mammalian gene expression through modulation of RNA self-cleavage. Nature 431: 471-476.

(Received Jan 15, 2005; Accepted Feb 25)