BMT, Inc.

Technology Overview

Small interfering RNAs (siRNAs) are short, double-stranded RNAs (dsRNAs) that mediate efficient gene silencing in a sequence-specific manner. The specific cleavage of mRNA molecules targeted by siRNAs is mediated by the endogenous RNA interference (RNAi) pathway, which is present in most eukaryotic cell types. Using Drosophila melanogaster embryo lysates, Elbashir et al. identified siRNAs with 19-base-pair (bp) duplex regions and 2-nucleotide (nt) 3′ overhangs (the so-called 19+2 structure) as the most efficient triggers of sequence-specific mRNA degradation. The 19+2 siRNA structure has thus become the standard for designing gene-silencing RNA molecules for therapeutic applications.

Despite the significant promise of siRNA technology, several studies have demonstrated nonspecific effects triggered by conventional 19+2 siRNA structures. First, siRNA can silence non-target genes either by imperfect pairing between mRNA molecules and the antisense strand of siRNA or by incorporation of sense strand into RNA-induced silencing complex (RISC) that results in the cleavage of mRNAs complementary to the sense strand. Second, excess amounts of siRNAs can saturate the cellular RNAi machinery and competitively inhibit the activity of other siRNAs or microRNAs (miRNAs). Third, while siRNAs were originally designed to circumvent the dsRNA-induced innate immune response, several studies have reported that nonspecific innate immune response can be induced by siRNAs. These nonspecific effects triggered by siRNAs limit the development of siRNA as a therapeutic modality.

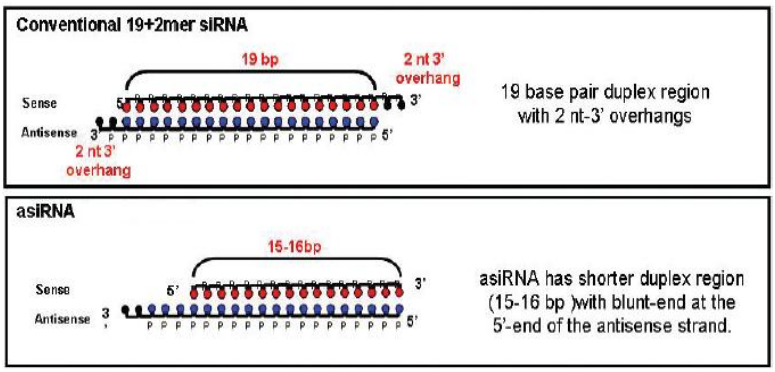

Figure 1. asiRNA structure

We have developed a novel siRNA structure termed asiRNA, which is an asymmetric shorter-duplex siRNA backbone structure with duplexes shorter than 19 bp. Importantly, this RNA duplex structure significantly reduces nonspecific effects caused by conventional 19+2 siRNA scaffold, such as sense strand–mediated off-target gene silencing and saturation of the cellular RNAi machinery.

Technology platform

- **AsiRNAs trigger efficient gene silencing comparable to conventional 19+2 siRNAs**
  - We designed and synthesized a series of siRNA structural variants and confirmed that siRNAs with duplexes shorter than 19 bp can trigger effective gene silencing in human cells (Figure 2). Gene silencing experiments using diverse target mRNAs (Figure 2 b, c, d, e) and multiple target sequence of one gene (Figure 3) revealed that asiRNA triggers efficient gene silencing comparable to conventional 19+2 siRNAs.
  - We also confirmed that asiRNAs induce gene silencing through the RNAi pathway utilized by the conventional 19+2 siRNAs through 5′-RACE analysis and other studies.

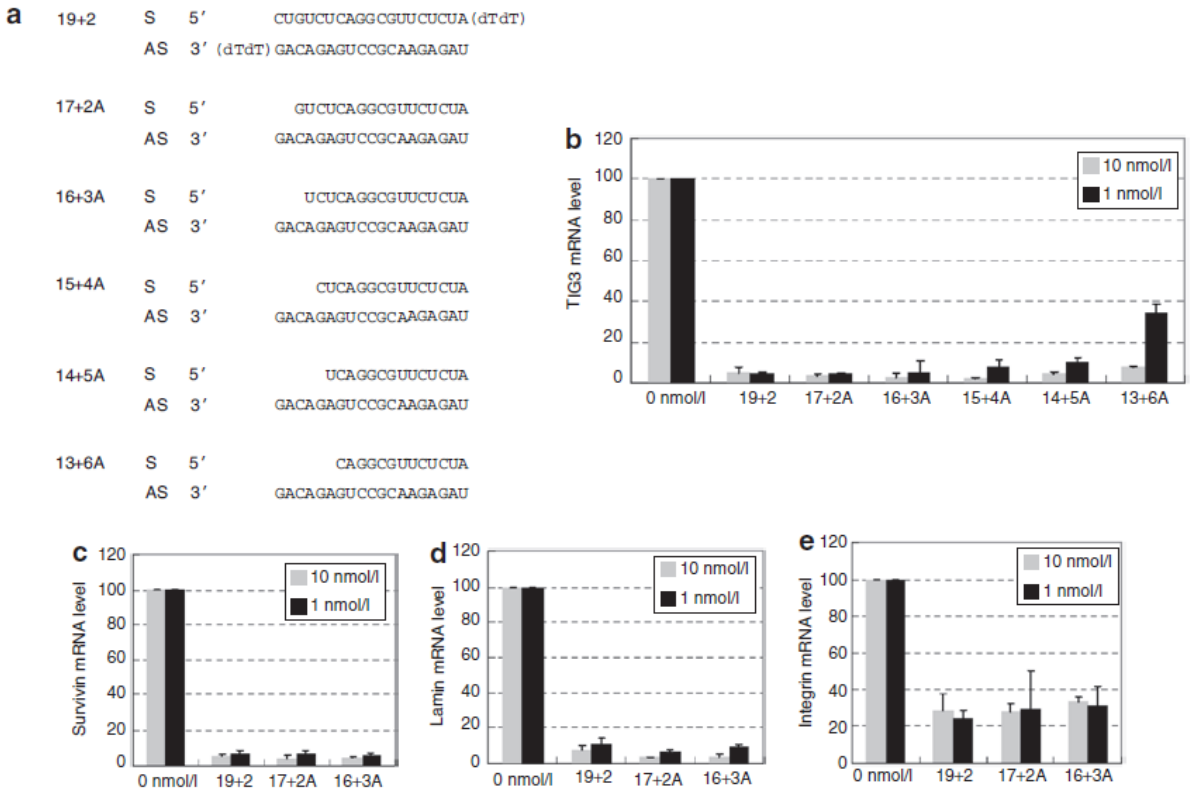

Figure 2. Gene silencing triggered by asymmetric shorter-duplex siRNAs. (a) Structures of asiRNAs that target TIG3 mRNA. (b) Activities of asiRNAs that target TIG3 mRNA in HeLa cells. (c) Activities of asiRNAs that target Survivin mRNA. (d) Activities of asiRNAs that target LaminA/C mRNA. (e) Activities of asiRNAs that target Integrin mRNA. 0 nmol/l, control with no siRNA transfected. asiRNA, asymmetric shorter-duplex siRNA; siRNA, small interfering RNA.

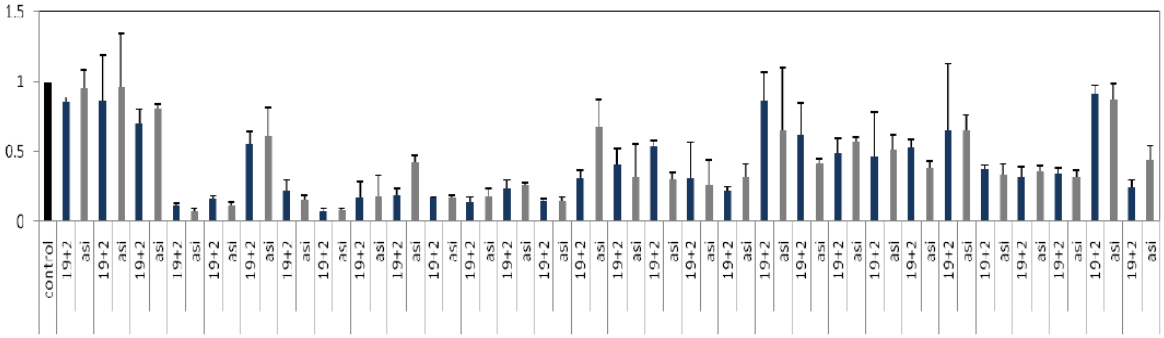

Figure 3. Comparison of gene silencing efficiency. Thirty target sequences for gene X were selected and gene silencing efficiencies of 19+2 and asiRNA were analyzed. Control, control with no siRNA transfected; asiRNA, asymmetric shorter-duplex siRNA; 19+2, conventional siRNA.

- **AsiRNAs show reduced sense-strand–mediated off-target silencing activity**

  - Off-target gene silencing is a key obstacle to the development of RNAi therapeutics and one mechanism of off-target gene silencing is the incorporation of sense strand into RISC. It is suggtested that because asiRNA structures are highly asymmetric and have sense strands whose lengths are suboptimal for effective gene silencing, these asiRNAs should yield less sense-strand–mediated off-target gene silencing than 19+2 siRNAs.
  - We confirmed that asiRNAs display less sense-strand–mediated off-target silencing than 19+2 siRNAs (Figure 4).

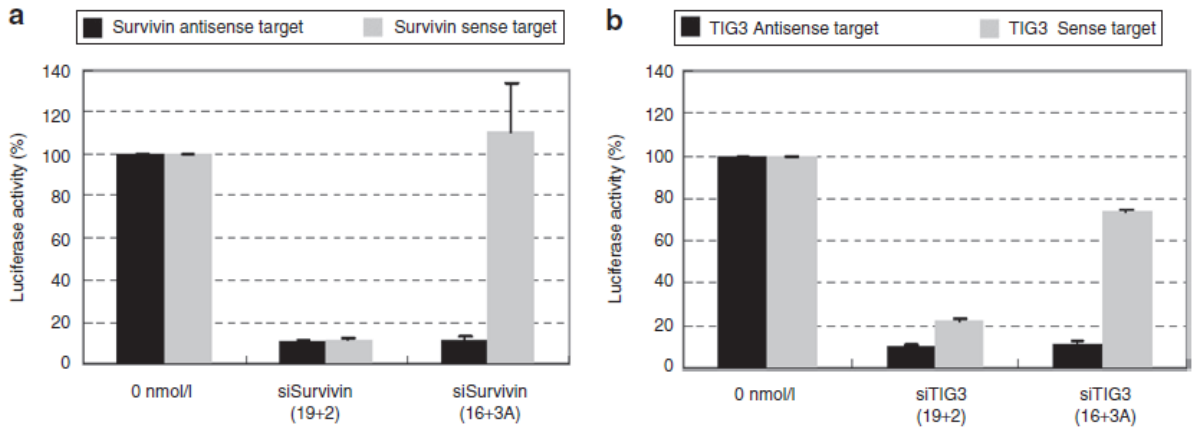

Figure 4. AsiRNAs show reduced sense-strand–mediated off-target gene silencing. HeLa cells were transfected with a luciferase reporter plasmid that carried either a Survivin AS, Survivin sense, TIG3 AS, or TIG3

sense-target sequence, without (0 nmol/l) or with 10 nmol/l of Survivin or TIG3 19+2 or 16+3A siRNAs. Luciferase activity was measured 48 hours after transfection. (a) Gene-silencing activities of sense and AS strands of siSurvivin variants. (b) Gene-silencing activities of sense and AS strands of siTIG3 variants. asiRNA, asymmetric shorter-duplex siRNA.

- Genome-wide study using DNA microarray also confirmed that asiRNA showed lower off-target effect (Figure 5).

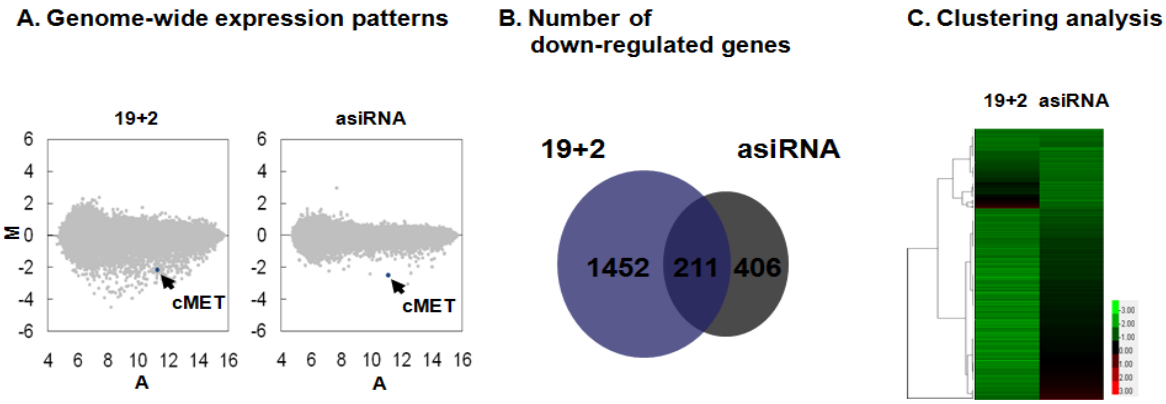

Figure 5. Genome-wide study using DNA microarray.

- **AsiRNAs have less or no inhibition of exogenous siRNA and endogenous miRNA activity**

  - Recent reports have documented another unexpected complication of RNAi-mediated gene silencing: saturation of the RNAi machinery (e.g., Ago2) by exogenously introduced siRNAs, which can result in competition between two siRNAs.
  - We assessed the ability of the asiRNA to saturate the RNAi machinery by measuring the competition potency of these siRNAs when co-transfected into cells along with another siRNA. We confirmed that asiRNA have less competition potency than conventional siRNA (Figure 6 a, b).
  - It has been also shown that siRNAs compete with endogenous miRNAs to inhibit miRNA function. Above mentioned observation that asiRNAs have little or reduced competition with 19+2 structures (Figure 6 a,b) suggests that cellular RNAi machinery saturation may be reduced or prevented with our asiRNA structures.

- We tested whether asiRNA structures have less inhibition of endogenous miRNA activity, and found that the corresponding asiRNA structure showed less inhibition of miRNA activity (Figure 6 c),

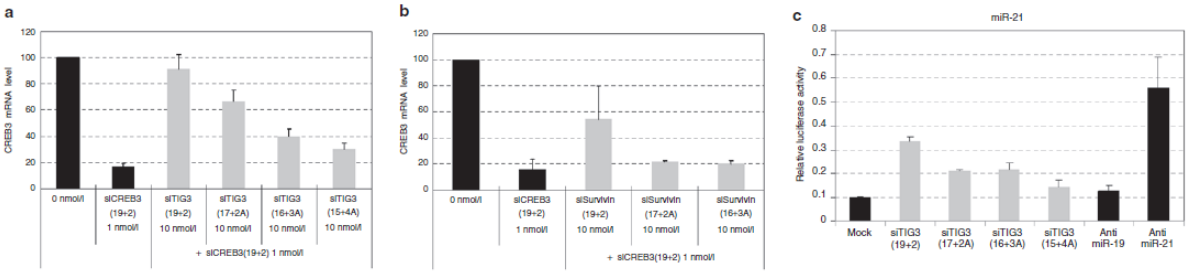

Figure 6. AsiRNAs have less or no inhibition of the activities of other siRNAs or miRNAs. (a) siRNAs that target CREB3 mRNA and competitor siRNA variants were co-transfected and CREB3 mRNA levels were analyzed. (b) HeLa cells were transfected with siCREB3(19+2) with or without Survivin siRNAs (Survivin 19+2, Survivin 17+2A, or Survivin 16+3A). (c) Changes in the gene-silencing activity of miR-21 by transfection of exogenous siRNAs into HeLa cells. HeLa cells were transfected with a luciferase reporter plasmid that contained a miR-21 binding site and one of several siTIG3 variants. Luciferase activity of the control luciferase reporter was set as one. anti-miR-19 and anti-miR-21, HeLa cells treated with an antagomir against miR-19 or miR-21, respectively.

- **Alleviated innate immune response by asiRNA administration into model cell lines.**

  - One of major issues in RNAi therapy is innate immune response triggered by siRNA treatment.
  - In 2008, Ambati group from University of Kentucky discovered that intraocularly injected naked siRNAs could block angiogenesis in mouse models of age-related macular degeneration regardless of its sequence. Further studies revealed that this effect was caused by non-specific innate immune signaling activation (TRL3 activation).
  - Upon the release of this report, the Phase 3 human clinical trial of an siRNA drug, Opko Health's bevasiranib, has been terminated.
  - We paid attention to the Ambati group's observation that the non-specific TLR3 activation by siRNAs is dependent on the RNA duplex length, and hypothesized that asiRNA with shorter-duplex structure might alleviate TLR3 activation. Indeed, we confirmed that the level of activated TLR3 is reduced in asiRNA treated model cell lines (bEND3 and TLR3 over-expressed HEK293 cell line)

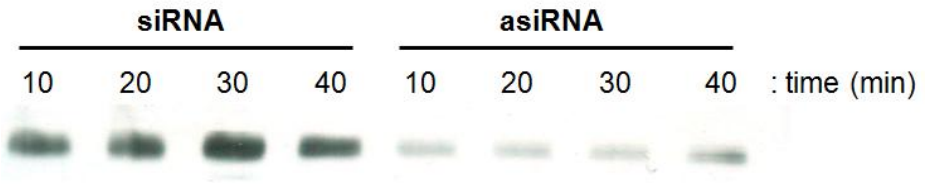

Figure 7. TLR3 activation upon either siRNA or asiRNA treatment. siRNA or asiRNA was treated to TLR3 over-expressing HEK293 cells and TLR3 activation was analyzed by phospho-TLR3 specific antibody.

Stage of development

- **ASI001: anticancer asiRNA therapeutics**

  - lead asiRNA developed for anticancer target X

  - Xenograft model study in progress

- **ASI002: anti-AMD asiRNA therapeutics**

  - lead asiRNA developed for anti-AMD target X

  - animal model study will be conducted in J. Ambati's group at U of Kentucky

Specific Patent Information

U.S.A Application & Resistration	
PCT Application	**Novel siRNA structure for minimizing off-target effects and relaxing saturation of RNAi machinery and the use thereof. (PCT/KR2008/007530**)