The Production of AJS2011: a novel gene transfer vehicle carrying therapeutic RNA

RNTein Biotech Lab., Inc.
Dedicated to human cancer gene therapy

Recent research results show that despite the high integration load, tumorigenesis is not affected with insertions of human immunodeficiency virus type 1 (HIV-1) derived lentiviral vector (Montini et al., 2006). So far, there is no adverse event reported regarding lentiviral vector induced tumorigenesis in animal models. Moreover, the integration site selection or preference by lentiviral vectors shows no significant overrepresentation in the genomic regions and in Gene Ontology (GO) database, which is different from oncogenic retroviral vectors of which the integration sites show some trends to transcription units of genome, and the activation of those genes related to cell cycle progression. The recombinant lentiviral vectors, therefore, has low genotoxic potential, providing a major scientific rationale for advancing these vectors to clinical trials.

As a pioneer work, the RNTein Biotech Lab., Inc., based in Los Angeles, California, USA, and the Green Life Star of Tokyo, Japan, have launched an unofficial clinical trial of *CDC6* silencing on treating advanced cancer patients since 2006, and some promising results were reported on UICC 2008 World Cancer Congress in Geneva, Switzerland. This is a first clinical trial of using recombinant lentivirus vector in the world. A short hairpin double-stranded RNA (shRNA) specific against *CDC6* mRNA, which is encoding an essential human replicating protein Cdc6, is produced from a lentiviral vector, AJS2001, which infects most human cells including cancer cells. The *CDC6* shRNA recognizes *CDC6* mRNA and provokes the target mRNA degradation in a way of RNA interference (RNAi), which causes cancer cells but not normal somatic cells replicative senescence, and confers the cancer suppression.

It is intrigue that the effect of AJS2001 is far beyond “one-on-one” combat with cancer cells, which has been quite obvious in Tokyo clinical trial. The fact that limited amounts of lentiviral particles in AJS2001 fighting overnumbered cancer cells has prompted us to identify a new type of bionanoparticles. To advance the ongoing clinical trial, the RNTein Biotech Lab., Inc. has initiated a new project, that is the treatment of advanced cancer patients using AJS2011, a new type of bionanoparticles carrying therapeutic RNA. These multiple membrane vesicle units carrying therapeutic RNA enter the target cells and deliver the payloads, spreading therapeutic RNA-mediated cancer suppression effect in overnumbered cancer cells.

AJS2011, the latest biotechnology development, shows two features:

First, HIV-1 accessory protein Vpu is expressed and incorporated in this new type of bionanoparticles

Human embryonic kidney 293T cells or genetic engineered human cervical carcinoma HeLa/Tat cells are transfected with lentiviral vector plasmid DNA (Figure I) together with envelope protein expressing vector. Inside cells, virions are assembled with synthesized lentiviral RNA and viral proteins, and the virions with the compacted viral RNA and proteins are then enveloped and fuse with cellular membrane, and budding out of the cells becoming infectious viral particles. Lentiviral RNA and viral proteins, and some cellular RNA and proteins can also be incoporated into nanoscale endocytic membrane vesicles, or exosomes with the aid of viral protein Vpu and some cellular protein transportation system called endosomal sorting complexes required for transport (ESCRT), and envelope protein sorting factor tetherin, or CD317 (Sauter et al., 2010).

Viral protein Vpu plays an essential role in lentiviral RNA and viral protein-carrying virion or exosome formation and releasing. Vpu enhances HIV-1 release by suppressing tetherin- or CD317-mediated tethering the virions to cell membranes (Sauter et al., 2010).

Vpu also functions to modulate the compatibility of pseudotyped virions, preventing other viral envelop proteins from occupying HIV-1 budding sites. Vpu regulates accumulation of some envelop proteins within ESCRTs. These structures have several features consistent with an endosomal viral or virus-like particle (VLP) assembly domain: they contain protein complexes including proteolytically processed viral matrix protein; the tetraspanins CD63; and AIP 1/ALIX, a cellular cofactor for viral budding and for exosome releasing.

Vpu is related to so-called “channel-pore” dualism that the sequence of Vpu from HIV-1 can be aligned with host ion channels and a toxin. It has been found that the efficiency of Vpu-mediated virus budding or exosome releasing is inversely correlated with membrane potential polarization because Vpu interacts destructively with host background K (+) channels, destabilizing the electric field across a budding membrane.

Second, some native human chromatin regulatory sequences are integrated in vectors that are used for generating bionanoparticles.

The expression of human telomerase reverse transcriptase (hTERT) gene, encoding the catalytic subunit of telomerase, is a feature of most cancer cells. The activation of the hTERT promoter requires native chromatin context and/or distal regulatory elements. Research results show that there are two distinct E-box elements, one proximal, the other distal in the hTERT 5’ upstream sequences. The proximal E-box downstream to transcription initiation site functions as a transcriptional repressor when associated with specific transcriptional or regulatory factor(s) in most somatic cells. However, this transcriptional repression activity is lost in some human cancers. The hTERT

downstream E-box and the regulatory factors bound on it would function as a specific switch to control transgene expression in cancer cells, but not in normal somatic cells.

In the test, hTERT gene 5’ upstream regulatory sequence is inserted in the vectors used for generating AJS2011 (Figure I). The hTERT 5’ upstream sequences play a role in controlling lentiviral RNA transcription and processing. RNA transcription peoducts from the hTERT 5’ upstream region may stimulate and activate RNA splicing and shuffling. This reveals high levels of viral antigen protein p24 in AJS2011 producing HeLa/Tat cells (Figure II).

In addition, AJS2011 bionanoparticles are able to carry such hTERT 5’ upstream sequence-dependent RNA processing in their “infected” cells. HeLa cells are incubated with RNA-containing bionanoparticles at 37°C and 5% CO2 for 16 hours, and total cellular RNA samples are prepared. The RNA samples are subjected to reverse transcription (RT) and then, the RT products are examined in limited PCR amplification with p32 radioactivity incorporated. The PCR products with radioactivity signals are detected on agarose gel (Figure III). It is evident that RNA processing in HeLa cells incubated with bionanoparticles made from cells with pTHTN transfection is over 100-time higher than that with bionanoparticles made from cells transfected with pTHTH, which has a large piece of fragment deleted from hTERT 5’ upstream regulatory sequences, and with pTCTH, in which a regulatory sequence from cytomegalovirus (CMV) enhancer and promoter has been added in the lentiviral vector (Figure I and III).

The biological function of long non-coding RNA, or large intervening non-coding RNA (lincRNAs) could be the molecular mechanism of this super active RNA processing. LincRNAs may function as host response to virus infection, some has unique expression signatures associated with virus infection. Regarding to cancer cell proliferation, the specific lincRNA, MALAT1 (for metastasis associated lung adenocarcinoma transcript 1), is found to regulate pre-mRNA, that is RNA splicing and shuffling (Tripathi et al., 2010). MALAT1 plays a role in recruiting important proteins, including pre-mRNA splicing factors, to the site of gene transcription products. MALAT1 RNA sequences contain many motifs for associating with SR splicing proteins. It is interesting that depleting cells of MALAT1 or over-expressing the splicing factors to which it can bind led to the same alteration in the splicing of a large number of pre-mRNAs in the cells. In contrast to MALAT1, RNA from the hTERT 5’ upstream sequences may stimulate splicing proteins interact with freshly transcribed products, resulting in high levels of processed mRNAs.

AJS2011, the new type of bionanoparticles meet many gene delivery vehicle requirements that are critical in clinical applications. The surfaces of outside membranes of purified AJS2011 are grafted with hydrophilic poly (ethylene glycol) (PEG), the process called PEGylation. AJS2011 can be present in bloodstream from 1-2 hours without grafiting to as long as 1-2 weeks with PEGylation. The PEGylated bionanoparticles are resistant to different kinds of protease degradation, ensuring the delivery of therapeutic RNA to the target cells. Masked on the surfaces, the novel bionanoparticles can be administrated into body multiple times while minimizing the

chance of immune reactions. Therefore, PEGylated AJS2011 becomes more stable carrying and delivering therapeutic RNA without being disassembled or destroyed by proteases or other factors inside cells or in bloodstream from different tissues.

The AJS2011-mediated gene transduction has been examined in tissue cultures (Figure IV). Human endonuclease FEN-1 has been found in the bionanoparticles. Studies show that FEN-1 functions as a cellular help factor important for HIV-1 cDNA maturation and chromosome integration (unpublished data). The c-myc tagged FEN-1 are expressed in 293T cells, and the AJS2011 bionanoparticles are then prepared and introduced into HeLa cells. Then, the cells are subjected to indirect immunofluorescent straining using antibody against c-myc. Cells with AJS2011 bionanoparticles in and have c-myc-tagged FEN-1 delivered are showing positive c-myc-tag staining. Because the PEGylated bionaoparticles are so stable and active that they would carry out “gene transduction” by entering into more cells in a way of sperading or transpassing. Processed by color evaluation, “gene transduction” efficiency via AJS2011-mediated RNA delivery can be evaluated according to the variations of color absobence over background value (Figure IV), which could be transformed into high-throughput analysis.

As we have tested, AJS2011 bionanoparticles show high efficiency in gene delivery and gene transduction while the novel type of bionanoparticles are safe with low genotoxcity and low immune reactions. We predict that AJS2011 bionanoparticles are reliable in clinical applications.

Refernces:

Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration
Eugenio Montini, Daniela Cesana, Manfred Schmidt, Francesca Sanvito, Maurilio Ponzoni, Cynthia Bartholomae, Lucia Sergi Sergi, Fabrizio Benedicenti, Alessandro Ambrosi, Clelia Di Serio, Claudio Doglioni, Christof von Kalle & Luigi Naldini Nature Biotech. 24 (6), 687-696 (June 2006)

Tetherin: holding on and letting go.
Daniel Sauter, Anke Specht, & Frank Kirchhoff Cell 141 (3), 392-398 (30 April 2010)

The Nuclear-Retained Noncoding RNA MALAT1 Regulates Alternative Splicing by Modulating SR Splicing Factor Phosphorylation
Vidisha Tripathi Jonathan D. Ellis, Zhen Shen, David Y. Song, Qun Pan, Andrew T. Watt, Susan M. Freier, C. Frank Bennett, Alok Sharma, Paula A. Bubulya, Benjamin J. Blencowe, Supriya G. Prasanth, & Kannanganattu V. Prasanth Mol. Cell.39(6):925-38 (24 September 2010)