The Application of "Bi-functional" Short Hairpin RNA in

A Furin-shRNA and GM-CSF (FANG) Autologous Vaccine for Cancer Therapy

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Background: A proprietary autologous tumor cell cancer vaccine using **F**urin shRNA **an**d **G**M-CSF (**FANG**) has been developed at Gradalis. IND approval (BB-IND 14205, 12-02-2009) has been obtained and a phase I clinical trial opened. FANG expression vector consists of two functional units - GM-CSF cDNA and furin bi-functional shRNA - in order to augment tumor antigen expression with GM-CSF transgene and attenuate immunosuppressive activities of transforming growth factors (TGF) β1 and β2 via furin bifunctional shRNA. Furin belongs to a family of proprotein convertases and is able to activate all TGFβ isoforms by proteolytic cleavage. High levels of furin expression have been demonstrated in virtually all cancer cell lines. The presence of furin in tumor cells contributes significantly to the maintenance of tumor-directed TGF-β1 peripheral immune tolerance. Hence, silencing of furin expression represents a novel and attractive approach for optimizing immunosensitization. A proprietary tandem "bifunctional" small hairpin RNA (shRNA) was employed to silence gene expression of furin and subsequent protein expression of secretory TGF-β1 and TGF-β2. The "bi-functional" shRNA cassette is highly effective and durable in contrast with its separate individual components and more potent than siRNA duplex with same target sequence.

Methods: A miR30-based bi-functional shRNA cassette against human furin along with cDNA of human GM-CSF was cloned into the pUMVC3 vector. FANG knockdown of furin expression and the resulting inhibition of production of mature "active" TGF-β1 and -β2 proteins was investigated using the human non-small cell lung cancer cell line (H460) and the human colon cell line (HCT-116). Processed mature shRNA and cleavage product of furin mRNA were examined by stem-loop RT-PCR and RACE-PCR, respectively. GM-CSF, TGF-β1 and TGF-β2 protein expression were measured by ELISA. Furin, GM-CSF, TGF-β1 and TGF-β2 mRNA expression were measured by quantitative RT-PCR.

Results: Mature shRNA of bi-shRNA targeting furin was detected 24 hours after transfection and its sequence was confirmed. RACE-PCR analysis indicated that furin mRNA was cleaved right in the center of target region as expected. Moreover, TGF-β1 expression was inhibited by 94%, while TGF-β2 expression was reduced by 81% 4 days after transfection. GM-CSF expression was raised more than ~20,000 fold in cells transfected with FANG plasmid. Furthermore, a 40% reduction of furin expression was found 3 days after transfection.

Conclusions: These results demonstrate the inhibitory effects of FANG on furin and TGF-β isoforms, which lays the foundation for future clinical studies of FANG vaccine in cancer patients.