For RNAmv investigation of new drug (IND) plan, we will finish the following experiments and tests:

1. We have done cell test for senescence of human carcinoma MCF7 cells using lentiviral vector producing CDC6 shRNA. We plan to run more test to determine RNAmv made from pRNV-p16 and pRNV-CDC6 shRNA in inhibiting proliferation of MCF7 cancer cells.

2. Since 2018, we have collaboration with professor Jun Fujita of Kyoto University Medical School, Japan, to study knockdown Gankyrin to activate p53 anti-cancer activity. Gamkyin plays a role in activating proteolysis, and could stimulate degradation of anti-tumor protein p53, promoting cancer progression. In this plan, we are going to test RNAmv made with Gankyrin shRNA in protecting p53 from fast degradation, and test the stimulation effect of the Gankyrin shRNA made RNAmv on p53 anti-cancer function. Now we have made our lentiviral vector p53 that allows us to test anti-cancer function in many human cancer cell lines and in animals.

3. We will test some specific RNAmvs in human prostate cancer cell lines and in animals. These RNAmvs are made with PTEN transgene, EZH2 shRNA, and RA538 lncRNA. PTEN tumor suppressor is a specific phosphatase, and plays multiple roles in regulating cellular metabolism to control cell differentiation and inhibit cell growth. PTEN is inactivated in prostate cancer cells, while enforced expression of PTEN shows suppression of prostate cancer progression and metastasis. By using RNAmv made with PTEN teansgene, we will determine if the therapeutics can block tumor cell growth in cell lines and in animals. EZH2, enhancer of Zeste homology 2, gene product is a chromatin histone acetyl transferase. This enzyme plays a role in stimulating tumor malignant activity such invasion and destroy normal tissue, tumor cell metastasis, in many tumors, such as prostate carcinoma, lung cancer, and breast carcinoma. RNAmv made by EZH2 shRNA specific vector will have EZH2 eliminated from cancer cells and will block cancer cell malignant activities. RA538 is a cDNA isolated from retinoic acid induced differentiation of human primary esophagus carcinoma cell line, which is my PhD thesis and project 30 years ago when I was a PhD student in China. Without knowledge of non-coding RNA during that time, RA538, possesses sequences homology to human oncogene c-myc, but in anti-sense orientation, which showed inhibition of c-myc expression in cell testing, could not be recognized as a lncRNA in tumor suppression until 30 years late. Now, RA538 made RNAmv is going to be test in prostate cancer cell lines and in animals to see if the lncRNA RA538 has function in suppressing tumor malignancy.

We will complete characteristic data in terms of RNAmv size, contents, and quantitative levels before we run RNA sequencing to reveal transcriptomics of RNAmv. Large volumes of purified RNAmv will be prepared with RA538 or p53 CCD vectors by transfecting human embryonic kidney 293T cells and by

collecting conditioned medium, and by purifying the microvesicles from the conditioned medium. The purified RNAmv will be processed to have RNA concentration at 200 μg per milliliter solution. This kind of RNAmv preparation ensures the nanoparticle characterization.

The preparation will be measured on Zetasizer to determine RNA nanoparticle size, molecular weight, concentration, zeta potential, and dynamic surface structures. The application of dynamic light scattering (DLS) technology, the size of RNA microvesicles is calculated from the translational diffusion parameters. A value is obtained that refers to how a particle diffuses within the fluid as a hydrodynamic diameter. More specifically, use Di0.5 to express the size of particles. Di0.5 is based on intensities of scattered light, not on the volume of particle solution. The count rate of DLS is directly related to the concentration of RNA microvesicles (number of nanoparticles per sample). Since the count rate represents the average scattering intensity, optimal dilution of the sample would give good count rate about Kcps. A potential way to check if the size was not changing would be to confirm with the DLS and count rate results at different concentrations.

The reason that we will run RNA sequencing to detail RNAmv contents and features is that this technology provides us a far more precise measurement of levels of transcripts and the results can catalogue all transcripts, including mRNA, non-coding RNA and small RNA. The RNA sequencing technology overcomes limitations that hybridization based method has for characterizing transcritomes. These limitations are mainly high background owing to cross-hybridization, and lack of appropriate normalization methods for comparing expression levels of different samples. RNA sequencing directly determines cDNA sequences. Following sequencing, the resulting reads are aligned to a reference genome, or reference transcripts, or assembled *de novo* sequence to produce a genome-scale transcription map that consists of both the transcriptional structure and/or level of expression for each gene.

RNA sequencing enables comparisons of transcripts in RNAmv made from different cell lines, from different tumor stages, and from patient's blood samples before and after RNAmv treatment. Upon comparison, signature gene expression will be examined and identified from each RNAmv sample. Levels of signature gene transcription in different status of tumor cell progression will be analyzed to determine specific RNAmv function. For example, RNAmv made with CDC6 shRNA producing CCD vector would show CDC6 mRNA down and transcriptomic of the cell treated with RNAmv displaces changes of many RNA transcripts and changes the levels of those RNA that are affected by the knockdown CDC6 mRNA. Signature gene expression related to CDC6 function would show down or up regulation, levels of gene expression for those signature genes would indicate if tumor progression be suppressed or not.