Summary & Conclusion
Chapter 5: SUMMARY AND CONCLUSION

The polyamines (PAs) are low molecular weight, polycationic, ubiquitous and organic molecules. The natural PAs in mammals are putrescine (Put), spermidine (Spd) and spermine (Spm). They play multifunctional roles in cell growth, differentiation and development. PAs are reported to play important role in every strata of life ranging from bacteria to plants and mammals. Due to their polycationic nature, they interact with a diverse brigade of negatively charged macromolecules such as DNA, RNA, proteins, ATP etc. required for normal progression of cellular processes. PAs have been shown to influence cell proliferation, transcription, RNA stabilization, translational frameshifting, chromatin remodeling, histone acetylation and apoptosis. All the aforementioned processes are affected by a complex regulation of intracellular PA pool size by cumulative action of de novo synthesis, interconversion, degradation and transport of PAs. The diamine Put, an obligatory precursor for the formation of higher PAs, Spd and Spm, is synthesized from ornithine by the action of ornithine decarboxylase (ODC). These steps are catalyzed by aminopropyl transferases, namely Spd and Spm synthases (SPDSYN/SPMSYN), respectively, which add propyl amino groups to Put and Spd sequentially and such propyl amino groups are donated by the decarboxylated S-adenosylmethionine (dcSAM), which is turn synthesized from SAM by SAM decarboxylase (SAMDC).

Cancer is among the most deadly diseases in the world. A tumor is a mass of cell undergoing abnormal and uncontrolled growth. It has been established that multiple factors such as genetic, epigenetic, environmental, pathogenic provide selective growth advantage to cancer cells, thus leading to neoplastic growth. During tumorigenesis, deregulation of polyamine metabolic pathway takes place. Both ODC and SAMDC are rate-limiting enzymes known to play an equally important role in genesis and progression of cancer. Many studies have reported changes in ODC regulation at transcriptional, translational and protein degradation level during carcinogenesis. ODC has also been
documented as the mother target of c-myc, an oncogene. ODC itself can regulate p53 expression which is a tumor suppressor gene. Increased SPDSYN activity has also been documented in regenerating tissue, hormonally stimulated tissues, malignant tissues and malignant cell culture.

In order to inhibit the carcinogenesis, PA metabolic pathway has been exploited extensively by the use of chemical inhibitors, polyamine analogues, nonsteroidal anti-inflammatory drugs (NSAIDs) and antisense RNA technology. Among the chemical inhibitors, α-difluoromethylornithine (DFMO), an inhibitor of ODC, is the most exhaustively studied as a possible cancer therapy but it had limited efficacy in pre-clinical trials. But DFMO has proved to be an effective cancer chemopreventive agent in various clinical trials. Some clinical evidence for the efficacy of DFMO in curtailing certain cancer risk factors includes results of oral DFMO in reducing the occurrence of basal cell carcinoma in a recent phase III trial, and of oral doses of combination of DFMO and sunlida (NSAID) in dramatically reducing the incidence of metachronous colorectal adenomas. Since PAs play a crucial role in progression of cancer, there is a need to look for new tools.

An alternative novel strategy is the application of RNA interference (RNAi). RNAi is a natural process that results in specific gene silencing due to degradation of target mRNA with the recruitment of small interfering RNAs (siRNA) and RISC. RNAi is induced by introduction of double stranded RNA (dsRNA) produced by external or endogenous sources. As soon as dsRNA is produced or introduced in cell, it is recognized by the endonuclease Dicer and cleaved into 21-23 nucleotide long siRNA. A multienzyme complex called the RNA-induced silencing complex (RISC) recognizes the siRNA duplex and discards the sense strand to form an activated complex containing the antisense strand. The activated AGO2-RISC complex then binds to cognate target mRNA strand sharing a complementary sequence and leads to its degradation, shutting down protein synthesis. After its discovery in mammals, it was spontaneously realized that this highly specific mechanism of sequence-specific gene silencing might be harnessed to
develop a new class of drugs that interfere with disease-causing or disease-promoting genes. One of the most important advantages of using siRNA is that, compared to antisense oligonucleotides, siRNA is 10–100-fold more potent for gene silencing. A plethora of genes have been targeted utilizing RNAi that include, cell cycle genes, oncogenes and signaling pathways.

The effective and targeted gene delivery is the bottleneck limiting the success of gene-based drugs in clinical trials. The major limitations against the use of siRNA as a therapeutic tool are its degradation by serum nucleases, poor cellular uptake, and rapid renal clearance following systemic administration. Non-viral delivery systems as compared to their viral counterparts are considered more favorable in therapy because of their acceptable safety profiles and the relatively more convenient preparation technique. Polymeric carriers in particular are attractive since they are amenable for optimal design via chemical modification techniques. Poly (lactide-co-glycolide) (PLGA) is an FDA approved polymer used effectively in a number of therapeutic devices owing to its biocompatibility and biodegradability. PLGA nanoparticles have been reported to deliver many cancer drugs like doxorubicin, cisplatin successfully. It was also used to target STAT3 specific siRNA to dendritic cells. The ideal delivery system should be able to deliver siRNA specifically into tumor cells. There are some proteins which as such or their mutated forms are expressed specifically on tumor cells. Targeted drug delivery systems harness these receptors. Various targeting molecules like monoclonal antibodies or aptamers (APTs) specific to these tumor-specific proteins have been utilized. Mucin is such a glycoprotein whose aberrant glycoforms are expressed specifically on various types of cancer. DNA APTs have been designed and characterized for specifically binding to MCF7 carcinoma cells.

In the present study, RNAi technique has been employed to silence genes of PA biosynthetic pathway to ameliorate the growth of oral (KB) and breast (MCF7 and MDA MB 231) carcinoma cells through targeted delivery of siRNAs by APT conjugated siRNA PEG-PLGA nanoparticles (ASPN). To optimize the small interfering nucleic acid
(siNA)-based drug for therapeutic purpose, three different variants of ODC- specific siNA i.e. siRNA, LNA modified siRNA (LNA), siHybrid [asRNA-ssDNA (siHasRNA) and asDNA-ssRNA (siHasDNA)] were transfected to KB and MCF7 carcinoma cell lines in a dose- and time-dependent manner. Three different concentrations (12.5, 25 and 50 nM) were used for transfection and the cells were analyzed at 24, 48, 72, 96, 120, 144 and 166 h post-transfection. It was observed that ODC-siRNA exhibited better silencing efficacy as compared to ODC-siLNA, ODC-siHasRNA and ODC-siHasDNA as analyzed at ODC transcript and PA level. The ODC mRNA was decreased in all the siNA-treated cells with increasing concentrations of siNA. The maximal silencing was observed at 50 nM concentration. At PA level, Put was decreased up to non-detectable level at 50 nM concentration. A considerable decrease in Spd and Spm level was also observed. With the advancement of time the suppression of ODC gene expression is increased up to 48 h and then the ODC mRNA titer starts increasing 4th day onwards while the suppression in polyamine level was observed up to 72 h. Gene silencing brought about by application of siRNA is transient in nature. As soon as the effector molecule is utilized, the gene expression and product formation revert back to normalcy. In our case also, after 48 h in case of mRNA and 72 h in case of PA level, the transcript and PA level started reverting back to normal. Although, the mRNA level decrease in both the cell lines was comparable, there was a considerable difference observed in PA level and cell growth inhibition. At 50 nM concentration and 48 h of incubation, there was about 60% reduction in cell viability in case of KB cells as compared to 66% in MCF7 cells. This difference in cell growth inhibition could be attributed to different regulatory pathways of PA in different cell lines. However, there was no difference observed at transcript, PA level or cell viability in unrelated siRNA-treated cells as compared to untreated cells.

It is known that PA levels are elevated in almost all forms of cancer but the gene responsible for the over-expression might vary from one cell line to another. Therefore, SAMDC and SPDSYN genes were silenced using siRNA specific to them and the effect on cell viability was monitored. The mRNA titers of SAMDC and SPDSYN genes were
lowered with increasing siRNA dose in siSAMDC- and siSPDSYN-treated cells respectively. At the product level, there was a significant decrease in total PAs as compared to the control. In MCF 7 and KB cells, siSAMDC and siSPDSYN treatment resulted in higher total PA decline than siODC treatment while in MDA MB 231 cells there was no statistically significant difference observed among the three targeted genes. When cell viability was checked in siRNA-treated cells, there was a significant decrease observed as compared to the control. Similar to PA level, siSAMDC- and siSPDSYN-treated cells exhibited more cell growth inhibition than siODC-treated cells in case of MCF7 and KB cells. In MDA MB 231, the cell growth inhibition was comparable in all the siRNA-treated cells. Further, the PA biosynthetic genes were targeted in combination with each other. In case of MCF 7 cells, maximum cell growth inhibition was observed in cells treated with siSAMDC in combination with siODC and siSPDSYN when targeted simultaneously. In MDA MB 231 cells, highest cell growth inhibition was observed in cells treatment with the combination of siSPDSYN with siODC and siSAMDC resulted in while in KB cells the siSAMDC and siSPDSYN combination proved to be the best. However, when all the three genes were targeted simultaneously the growth inhibition was low when compared to single and double gene targeting.

Next, the cell morphology of PA depleted cells was studied. The PA depleted cells demonstrated characteristic features of apoptosis with aberrant morphology, blebbing of plasma membrane, occurrence of apoptotic bodies and reduced size. To confirm that the mechanism of cell death was apoptosis, FACS was employed. Approximately 9 to 12 folds increase in apoptosis was observed in case PA depleted MCF 7 cells and 5 to 10 folds in case of MDA MB 231. Further, the cell migration activity of carcinoma cells was studied after treatment with 12.5 nM concentration of siSAMDC. It was observed that the sub-lethal concentration of siSAMDC was able to inhibit the migratory property of cancer cells.
Then, the expression profile of different gene involved in cell cycle and apoptosis in PA depleted cells was studied. The results exhibited that the PA depletion lead to deregulation of cell cycle- and apoptosis related genes. Of all the genes studied, 9 were found to be down-regulated while 10 were up-regulated in treated cells as compared to the control. E2F4, p53, p21 and p27 are cell cycle antagonist genes and were found up-regulated thus inhibited normal progression of cell cycle in PA depleted cells. Apart from these, BAX, FAS, BAK, ATM and ATR which are apoptosis inducing genes were also up-regulated. In contrast to this, genes required for cell cycle progression like cyclin E, cyclin T1, cyclin T2, cyclin F and cyclin D were down-regulated. Apart from these, PCNA which is an important cofactor of DNA polymerase was also down-regulated. Thus, confirming the role of PA in DNA synthesis.

To extend the application of siRNA-drug therapy to in vivo application, the present study also developed a siRNA loading PEG-PLGA nanoparticle (NP) conjugated with MUC1 DNA APT as the targeting ligand for anti-cancer drug delivery. The siRNA loaded PEG-PLGA nanoparticles were prepared by Double emulsion solvent evaporation technique. The APT was conjugated to the surface of PEG–PLGA nanoparticles using peptide bond formation by EDC/NHS technique. The conjugation was confirmed by FTIR and zeta potential. The resulted ASPN was observed to be uniformly spherical in shape as confirmed by TEM with a particle size of 231nm and zeta potential of - 23 mV. The encapsulation efficiency of ASPN was observed to be 66%. The ASPN were found to be effective in protecting siRNA from nuclease degradation. The MUC1 APT-mucin mediated recognition and internalization significantly promoted the cellular association of ASPN in MCF7 cells as demonstrated by specific GFP expression inhibition by siGFP. The cytotoxicity of siRNA delivered by ASPN was comparable to the ones delivered by commercial transfection reagent, lipofectamine 2000. The long-circulating property imparted by PEG and MUC 1 APT would probably result in rapid, long-term, and accurate in vivo tumor targeting. However, the same needs to be validated experimentally.
In conclusion, the siRNAs designed to target the PA biosynthetic genes, *ODC*, *SAMDC* and *SPDSYN* resulted in cell growth inhibition in oral (KB) and breast (MCF7 and MDA MB 231) cancer cell lines. Our results suggested that more than one gene can silenced be simultaneously by siRNAs along with reduction in siRNA concentration. The mechanism of cell death was apoptosis. At molecular level, PA depletion leads to increased expression of pro-apoptotic and cell cycle inducing genes while the anti-apoptotic and cell cycle inhibitors were down-regulated. Our results also demonstrated that ASPN could encapsulate, protect and specifically deliver siRNA to MCF 7 cancer cells. The observations made in this study prove that PA biosynthesis pathway plays an important role in the progression of oral and breast cancer, and silencing *ODC*, *SAMDC* and *SPDSYN* genes using RNAi technique may be a novel therapeutic option for abrogating cancer growth.