Summary and Conclusions

Targeting of eNOS into various sub-cellular locations within the cell plays an important role in human physiology. We have addressed the following questions in this study: a) whether eNOS localization into the nuclei of human cells was a major mode of its regulation or was it only a minor and a specialized mode of regulation that takes place in exceptional cases; b) what were the possible molecular attributes of the eNOS protein that effect its distribution between the nuclear and cytoplasmic compartments of the cell; and c) what physiological consequences might ensue with an disturbed nuclear distribution of eNOS within the cellular environment. The experiments carried out and the results discussed in this thesis have been largely successful in providing explanations to all the three questions.

First, we made an important and a novel observation that eNOS was present in the nuclei of several types of human cells, both normal and cancerous, under the conditions of a steady state growth in cell culture. Although human umbilical vein endothelial cells (HUVEC) have been subjected to numerous studies earlier, and the eNOS localization in these cells has been concluded to be cytoplasmic in nature, the data presented and discussed in this thesis provided convincing evidence for the first time that physiologically relevant amounts of eNOS can be found in the nuclear compartments of HUVEC as well, testifying that the nuclear localization of eNOS is a more general phenomenon, rather than being an exceptional or a sporadic one, and its function in this compartment must be taken into consideration while interpreting the role of eNOS in the physiology of vascular, cardiac, renal and other systems.

The characterization of eNOS protein from the purified nuclei revealed that the nuclear eNOS was largely phosphorylated at the Threonine-495 residue and existed predominantly as a monomer. A major fraction of nuclear eNOS was associated with nuclear speckles, thought to contain spliceosomes and to be involved in the RNA-metabolism. Association of eNOS with spliceosomes/speckled bodies was specific, because it was found to colocalize with spliceosomal proteins as determined by confocal laser scanning microscopy and this observation was supported adequately by biochemical experiments that clearly demonstrated its physical interaction with
specific spliceosomal constituents such as SC-35. We demonstrated that the nuclear eNOS retained its ability to generate NO \textit{in vitro} when supplemented with the necessary cofactors and substrates. The kinetics of NO generation from the extracts of purified nuclei showed distinct signatures of enzyme-activation \textit{in vitro} in a temporal sequence and indicated that the eNOS present in the nuclei may not have been primed to generate optimal amounts of NO. This is in agreement with our independent finding that the protein exists largely as monomers. We showed further that an appreciable amount of eNOS was present in the nucleolar compartment of MCF7 cells by direct biochemical experiments.

In this work, we have identified that eNOS is targeted to the nuclei and the nucleoli of cells by a complex mechanism, wherein at least five structural motifs present within the protein cooperated. Of these five, three motifs were directly involved in the process by functioning as nuclear and nucleolar localization signals (NLS), and the other two motifs acted as target sequences of phosphorylation by protein kinases to regulate the function of these three nuclear and nucleolar localization signals. The kinase-regulated sequences centered around the Threonine-495 and Serine-114 residues. More interestingly, three of the four conserved serine residues flanking the sequence WRRKRKE motif in NLS1 appeared to be the targets of post-translational modifications, because their single or multiple substitutions by Alanine or Asparagine resulted in sub-cellular localizations that deviated from that of the wild type. We have provided a working model for explaining the role of post-translational modifications of these serine residues in regulating nuclear and nucleolar targeting. Although this model was useful in explaining a majority of the observations, it was insufficient to explain some of them. This indicates that the actual process is probably more complex than that visualized in the model. In keeping with this view, we found that the EGFP-T495-NLS cargo protein contained potential sites for O-GlcNAc modification. Therefore, the switch regulating nuclear and nucleolar traffic may not be bilateral (e.g. phosphorylation vs non-phosphorylation), but may involve three states, \{(Unmodified) – (Phosphorylated) – (O-GlcNac modified)\}, making the regulation more sophisticated than envisaged here. Further work will be required to resolve this issue in more detail.

Another interesting feature of regulation noted in our work was that the nuclear localization of the EGFP-NLS cargo protein was strongly dependent upon the
phosphorylation status of the Threonine-495 residue. In the absence of a Threonine-495 motif, NLS3 could target the cargo into nuclei, but its distribution between nucleolus and nucleus was distinct. Moreover, a significant fraction of cells also showed cytoplasmic localization of the EGFP-NLS3 cargo. Similarly NLS1 alone, or in combination with NLS2, was capable of improving the nuclear partition of the cargo. Because the presence of Threonine-495 motif in the absence of NLS sequences is not known to promote entry of cargo to nuclei, these results provide strong evidence that NLS1, NLS2 and NLS3 acted primarily as signals for nuclear entry whereas, Threonine-495 motif, most likely, was responsible for the nuclear retention of the cargo. Finally, by using a heterokaryon assay, we demonstrated that eNOS is equipped with signals that promote its shuttling between nucleus and cytoplasm; in other words its nuclear localization is dynamically regulated.

T495-NLS and Syngene-NLS proteins provided valuable clues to understand possible functions of nuclear eNOS. Using cells expressing these two proteins, we could clearly establish that the nuclear eNOS is a source of reactive oxygen species (ROS) under specific conditions, and is capable of generating high levels of oxidative stress in nuclear and nucleolar compartments. To the best of our knowledge, this is the first clear demonstration that significant amounts of ROS can arise in the nuclear and the nucleolar compartments by the action of eNOS. Generation of ROS within the confines of nuclear compartment has wide ranging ramifications. An increased oxidative stress generated within the proximity of genetic material was expected to lead to increased oxidative damage to DNA that can affect both deoxy ribose sugar and bases. In support of this, we have provided convincing evidence that there were increased 8-Oxo 2’-deoxy Guanine (8-OxoG) contents in the nucleic acids of the cells that retained T495-NLS and Syngene-NLS cargo protein in their nuclei and nucleoli, as compared to the host cells. Because an increased 8-Oxo 2’-deoxy Guanosine can pair with both cytosine and adenine, it is thought to induce increased transversions of G:C to T:A and foster a mutagenic environment during DNA-replication. Increased ROS in cells have been implicated in a wide variety of pathologies that includes cancer, ageing, diabetes and kidney diseases. Our results therefore provide a novel and specific experimental basis to understand, to measure and to monitor the role of nuclear eNOS in the generation of oxidative stress and the various pathologies that it may induce.
Role of oxidative stress in tumor biology remains controversial. We examined some of the tumor-specific characters of MCF7/T495-NLS and MCF7/Syngene-NLS cells, since these cells exhibited higher ROS levels as compared to the parental cell line. Interestingly, we observed that though these cells had a compromised growth in soft agar and an attenuated invasion in matrigel, their abilities to form tumor in vivo were vastly different. When introduced into an immuno-compromised mouse host, the MCF7/T495-NLS cells formed tiny or no tumors, while the same numbers of MCF7/Syngene-NLS cells formed large aggressive tumors, which were distinct from the parental cells. The cells grown in vitro or those retrieved from the mouse tumors had acquired distinct characteristics of “mammary stem-cells”. Their conflicting behavior in vivo indicates that the MCF7/T495-NLS cells were prone to a quiescent stem cell fate, whereas the MCF7/Syngene-NLS cells were highly proliferative. Because these characteristics are not detected in vitro, it is possible that the distinctive in vivo characteristics are modulated by the micro-environmental factors. It will be interesting to determine the exact reason for this difference. Our data strongly supports the notion that the nuclear eNOS through ROS generation and spliceosome association is capable of modulating the expression of specific genetic programs that may contribute to cell differentiation and stem cell functions.

In conclusion, our study reveals that:

1. Significant amounts of eNOS are present in the nuclei of mammalian cells, a part of which may exist in a physical association with the splicing factors such as SC-35. A small amount of eNOS is also present in the nucleoli.

2. eNOS is a nucleo-cytoplasmic shuttling protein, and this trafficking is regulated by at least three nuclear localization signals, with phosphorylation of Thr-495 residue serving as a critical determinant for its nuclear retention.

3. The nucleolar localization of eNOS is regulated by the post-translational modifications in the serine residues flanking the NLS-1. NLS-2 motif may co-operate with NLS-1 in this process.

4. Nuclear eNOS exists predominantly as a monomer, and its activity seems to be kept under control by its interactions with other molecules. However, if a dimer is formed, especially under deregulated conditions of CaM homeostasis, it may favour superoxide-formation over the nitric oxide-production.

5. The role of nuclear eNOS in modulating the overall redox state of cells in both nuclear and cytoplasmic compartments is indicated by our demonstration that
the ROS production from eNOS is coupled to the ROS produced by xanthine oxidase in nuclei, the elements of mitochondrial respiratory chain in the cytoplasm and NADPH-oxidase in the membrane and cytoplasm.

6. Elevated oxidative stress arising from the uncoupled nuclear eNOS may lead to severe consequences like aggressive tumor formations as a result of a) possible mutagenic changes induced in the nucleic acids or b) by its promotion of stem cell characteristics in tumor cells.