Results and Discussion

Characterization of endothelial Nitric Oxide Synthase in Nuclear Compartments

1.0 Nitric Oxide and Breast Cancers

The exact molecular details on the genesis of breast cancer remain an enigma after several decades of intensive efforts. From a clinical perspective, there are two principal kinds of breast cancers: one type that expresses significant amounts of estrogen receptor alpha (ER-α) and remains amenable to therapy with anti-estrogens, and the second type that is unresponsive to anti-estrogen therapy, because these tumors do not contain the receptor in significant amounts. ER-α positive tumor growth is generally driven by several factors, of which the estrogen (E2) that circulates within the body plays a dominant role. Biochemical and genetic experiments in mice have implicated the loss of the genes encoding membrane micro-domain proteins, Caveolin-1 and Caveolin-2, as a risk factor in mammary tumorigenesis. Because caveolin-1 and caveolin-2 together constitute the structural proteins of the membrane micro-domain known as Caveola, which is increasingly being recognized to play roles in diverse cellular processes, including solute and lipid transport, vesicular trafficking and modulation of signal transduction, we began to focus our studies on the signal transduction aspects of an important caveola-associated protein, eNOS, in mammary epithelial cells.

A possible role of NOS proteins has been examined in the context of tumors. It has been documented that NOS activity is frequently elevated in malignant tumor tissues of breast, gastric mucosa, cervix and ovary, compared to normal or benign lesions. NOS expression and activity have been identified in human breast cancers, and eNOS expression has been found to have correlations with estrogen receptor status of the tumors, indicating that it might interface with tumor functions such as its growth, invasiveness and metastasis (217, 299, 315, 316).

Several reports have documented that caveolin proteins may undergo a substantial down regulation in many (but not all) cancers that includes breast cancers. For its natural physiological regulation, eNOS is known to critically depend upon the
presence of caveolae, suggesting that in the absence of caveolae, eNOS protein within the cells may not be efficiently targeted to the plasma membrane micro-domains, impairing its natural regulation.

Because NO can exert a range of pleiotropic effects by activating or inactivating enzymes, by affecting gene expression, or by modulating the levels of reactive oxygen species being generated within the cells, its exact role in the context of genesis, growth and metastasis of breast tumors, has not yet been precisely defined. Therefore, there existed the attractive possibility that with caveolin-1-downregulation, the eNOS was redistributed within the cells in a manner, which was less dependent on the caveolin-1 function. Secondly, since caveolin-1 is thought to inhibit unregulated NO-generation by inhibiting the eNOS enzyme activity through its scaffolding domain, its absence might deregulate the nature and the quantum of nitric oxide-generation in specific cellular compartments, affecting the mammary epithelial cells.

1.1 Atypical sub-cellular profile of eNOS expression is seen in normal and cancer cells

In order to examine if there is an atypical redistribution of eNOS in mammary epithelial cancer cells, we examined a number of cell lines derived from both normal and cancerous human breast tissues. A polyclonal antibody directed against a C-terminal epitope of human eNOS, detected the presence of this protein in human endothelial cells (HUVEC) and breast cancer cell lines MCF7, ZR-75-1 and ZR-75-30 (Figure 1.1A).
Immunofluorescence staining of cell lines with an anti-eNOS antibody (C-20)

Figure 1.1A Sub-confluent cells grown on glass coverslips were immunolabeled with a polyclonal anti-eNOS antibody, directed towards a C-terminal epitope of eNOS (C-20; 1:20), followed by a Cy3-conjugated anti-rabbit antibody (1:50; raised in donkey). Confocal micrographs were captured by a laser scanning confocal microscope, LSM 510 (Zeiss). A predominant cytoplasmic localization of eNOS is seen in all cells examined.

Although the expression level of eNOS protein in non-endothelial cells appeared to be low, it was distinctly stronger compared to results obtained with normal serum. Further, the presence of a synthetic peptide competitive to the natural protein epitope abrogated this antibody reactivity within these cells, indicating that the reactivity was specific. The protein was distinctly and predominantly cytoplasmic in its localization within the cells as seen here with endothelial (HUVEC) cells. When the same experiments were carried out using a second antibody that was directed against a different epitope of eNOS protein, this time located at its N-terminus, the cells showed a strikingly different sub-cellular distribution profile for the protein, in that, stronger signals appeared to be associated with the nuclear compartment of the cells (Figure 1.1B)
eNOS is present in the nuclei of mammalian cells

**Figure 1.1B** Confocal microscopy images of various cells of mammalian origin that were immunolabeled with a rabbit polyclonal anti-eNOS antibody (N-20; 1:20), and a Cy3-conjugated anti-rabbit antibody (1:50; raised in donkey) are illustrated. A striking nuclear localization of eNOS is seen in the cells.
Expression pattern of eNOS in MCF7 and MCF10A cells

Figure 1.1C Confocal microscopic images of MCF7, a breast cancer cell line, and MCF10A, a normal mammary epithelial cell line, stained with an anti-eNOS (N-terminal) antibody (1:20), followed by a secondary Cy3- labeled anti-rabbit antibody (1:50; raised in donkey) are shown. Both cells express comparable levels of nuclear eNOS, as seen in the quantitative analysis of the images, shown in the lower panel.

This unusual distribution was not specific to mammary epithelial cells, because similar labeling patterns were obtained in A549, human lung epithelial carcinoma cells and HT-1080, fibrosarcoma cells. The nuclear eNOS could not be attributed to pathological consequences of cancer because non-transformed cells including HUVEC, MCF10A and bone marrow-derived human primary fibroblasts showed similar nuclear profiles of eNOS staining, and there was no appreciable difference in the expression levels of nuclear eNOS between the non-transformed MCF10A cells and the transformed MCF7 cells (Figure 1.1C). We could extend these results further by examining isolated purified nuclei by immunocytochemistry. As shown in Figure 1.1D, the nuclear stain for eNOS was evident again in HUVEC, HT-1080, MCF7, A549, HEK-293 (human embryonic kidney), CHO (Chinese hamster Ovary), K562 and KG1a cells. The last two cells are derived from human hematological malignancies.
eNOS is detectable in purified nuclei

Figure 1.1D Nuclei of various cell lines were isolated and were immunolabeled with a rabbit polyclonal anti-eNOS antibody (N-20; 1:20) and with a FITC-conjugated anti-rabbit antibody (1:80, raised in donkey). Images were captured on a confocal laser microscope. Nuclear localization of eNOS is seen even in the isolated nuclei.

The reactivity with the Chinese hamster protein may have been derived from the highly conserved N-terminal sequences present in eNOS of rodent origin. Nuclear eNOS was again found to be present in both estrogen receptor positive (MCF7, T47D, ZR-75-1 and ZR-75-30) and estrogen receptor negative (MDA-MB-231) breast cancer cell lines. These results together strongly suggested that the atypical distribution of eNOS observed here, was more widespread among various cell- and tissue-types, rather than being a characteristic of a restricted few cell-types. Secondly, there was no evidence to suggest that the nuclear localization of eNOS was due to a secondary consequence of cell transformation.
1.2 Nuclear eNOS is an intact protein

The discrepancy of results obtained with C-terminal and N-terminal antibodies to eNOS might derive from the expression of an aberrant form of nuclear eNOS in which the C-terminal epitope was missing, leaving the N-terminal regions intact. In this model, the immunoreactive proteins detected from the cytoplasm and the nuclei might have different molecular masses. When whole cell lysates from the tumor cells were examined in SDS-PAGE, a single immunoreactive band migrating with the expected molecular mass of ~135 kDa, was detectable in all the samples, making it unlikely that a different eNOS-related protein was present in the nuclei of the cells or an N-terminal fragment of eNOS protein, resulting from a proteolytic cleavage of eNOS, was responsible for the observed results (Figure 1.2A).

N-terminal eNOS antibody detects a single species of protein

![Figure 1.2A](image)

Figure 1.2A Immunoblot of whole cell lysates of the breast cancer cells was analysed using an antibody to eNOS that recognizes an N-terminal epitope (N-20; 1:1000). The blot was reprobed with anti-beta actin (1:5000) to confirm an equal loading of proteins. A single band of eNOS having a molecular mass of 135kDa is seen.

A formal possibility remained in that the nuclear eNOS was derived from an alternatively spliced transcript that specified a non-identical protein that fortuitously had a similar molecular mass, and shared sequences from the N-terminus of eNOS. In order to address this, we analysed the proteins isolated from purified nuclei in SDS-PAGE and probed the same western blot using four different antibodies reactive to eNOS epitopes that are located at various regions of eNOS viz N-terminus, Calmodulin-binding region, middle region (aa 597-614) and C-terminus. The data (Figure 1.2B) showed clearly that all the four antibodies reacted with the same band common to eNOS, in all three cell types examined. The reactivity with Chinese hamster protein was not surprising, because we had previously observed the nuclear staining of eNOS with the N-terminal antibody in immunofluorescence experiments, and other human specific eNOS antibodies were reactive to this rodent protein because of a high degree of evolutionary conservation at the amino acid level.
Nuclear eNOS is an intact protein

Figure 1.2B Integrity of the eNOS protein in the nuclei of mammalian cells was assessed by immunoblotting of the nuclear lysates and by probing of the same blot using antibodies recognizing various regions of eNOS: N-terminal (1:1000), C-terminal (1:1000), 597-614 aa eNOS (middle region; 1:10,000) and CaM-binding region (1:1000). A band having a 135 kDa molecular weight was detected with all four antibodies, confirming its identity as eNOS and also indicating that the protein was an intact one. Representative immunoblots are shown from experiments that were repeated independently for four times.

1.3 eNOS associated with purified nuclei generates NO in vitro

As nuclear eNOS was found to be an intact protein, it was of interest to see if it generated nitric oxide under appropriate reaction conditions. We, therefore, incubated highly purified nuclei, or protein lysates prepared from them, in assay mixtures that provided all the cofactors and the substrates required for NO generation by the enzyme, following a standard assay protocol (317). The NO generated by the enzyme was measured by the conversion of a non-fluorescent diamino-fluorescein derivative to its highly fluorescent triazole derivative by NO (308). Figure 1.3A-i shows that the protein lysates prepared from purified nuclei of MCF7 cells were capable of generating NO in a dose-dependent manner. That NO was being specifically detected, could be verified by showing that the inclusion of a NO scavenger, carboxy-PTIO, in the reaction mixture could abrogate the formation of DAF-2-dependent fluorescent product (Figure 1.3A -ii).
Spectrofluorimetric analysis using DAF-FM-chemistry shows that nuclear eNOS generates NO

Figure 1.3A (i) NOS activity in the MCF7 nuclear lysate was measured by DAF chemistry. A dose- and time-dependent increase in the DAF-fluorescence is seen. (ii) Addition of a NO-scavenger, cPTIO, reduced the fluorescence, indicating that the observed effect was nitric oxide-specific.

When the intact purified nuclei were incubated with the reaction mixture, NO-generation showed biphasic kinetics (Figure 1.3B). In the first two hours of incubation, the rate at which NO was generated was slow. The kinetics of NO-generation increased to a great extent thereafter, and continued up to four hours. The biphasic kinetics indicates that the nuclear eNOS is probably present in an enzymologically compromised form, and with incubation it gradually becomes activated and attains the competence to generate NO. A possible interpretation of this result could be that, for an efficient generation of NO, eNOS needs to be in its dimeric form, and it is possible that either the majority of nuclear eNOS is not present as dimers; or that eNOS present in the nucleus is in a complexed-form with other proteins, and its dissociation from these complexes is a slow process in vitro and after its dissociation, it becomes activated to produce NO.
Figure 1.3B eNOS-activity in the isolated nuclei of MCF7 was measured by DAF-FM chemistry: It is clearly seen that eNOS present in the intact purified nuclei has a capacity of generating NO \textit{in vitro}. The biphasic kinetics of the reaction indicates that the nuclear-eNOS is present in an enzymatically compromised form, and it slowly attains the competence to produce NO, in the presence of co-factors that are provided in the buffer.

We verified the possibility of nuclear eNOS being present in a monomeric form, by carrying out a Western blot analysis of the nuclear lysates, using a non-reducing gel. As seen in the Figure1.3C, even the non-reduced samples of nuclear lysates showed the predominant presence of the monomeric form and the stimulation of the cells with thapsigargin resulted in a slight increase in the dimeric form.

**Nuclear eNOS exists predominantly as a monomer.**

![Western Blot Analysis](image)

Figure 1.3C A western blot analysis of the nuclear lysates of MCF7: Reduced or non-reduced samples of the nuclear lysates (25 μg) were fractionated by a low temperature SDS-PAGE and were electrically transferred to a PVDF membrane. The blot was probed with an anti-eNOS antibody (N-20; 1:1000). A predominant monomeric form is seen even in the non-reduced samples along with a very small amount of dimeric form, which increased slightly with the Thapsigargin treatment.

1.4 Nuclear eNOS has an inhomogeneous distribution within the nuclei.

A feature observed in common to all the cell-types was that the staining of eNOS in the nuclear compartment was not uniform; rather, it was distributed into some major and several minor regions of punctate nature, and the nucleoli appeared to be relatively free of eNOS (Figure 1.4). An examination of the nature of eNOS-distribution within the nuclear compartment of MCF7 cells by confocal microscopy revealed that eNOS-distribution in the nucleoplasm was uniform, but appeared granular in nature, and a more intense immuno-reactivity could be seen with several nuclear spots that were apparently randomly distributed within the nucleoplasm (Figure 1.4).
The purified nuclei of MCF7 cells were immunostained for eNOS, using a polyclonal anti-eNOS antibody (N-20; 1:20) and a FITC-conjugated anti-rabbit antibody (1:50; raised in goat). Images with an optical thickness of 0.4µ were captured on the confocal laser microscope. The eNOS was seen to be distributed in an inhomogeneous fashion in the nuclei, giving a speckled appearance.

1.5 Nuclear eNOS is phosphorylated at its Threonine 495 residue.

It is known that eNOS activity is dynamically regulated within the cells by post-translational modifications, particularly by phosphorylations of several sites that include Serine-114, Threonine-495 and Serine-1177 residues. While a phosphorylation of its Serine-1177 leads to an increased synthesis of NO, a similar phosphorylation at Threonine-495 attenuates NO synthesis, but accentuates reactive oxygen species formation (84,238,318-322).

We examined the phosphorylation status of nuclear eNOS by staining purified nuclei with the phospho-specific antibodies directed against its Serine-1177 or Threonine-495 residues. The anti-phospho-eNOS^{Ser-1177}-specific antibody did not show a significant reactivity with purified nuclei. By contrast, a phospho-eNOS^{Thr-495}-specific antibody was strongly reactive with the nuclei, indicating that, to a large extent, the nuclear eNOS was phosphorylated at its Threonine-495 residue (Figure 1.5A).

A Thr-495-phosphorylated form of eNOS is present in the nuclei

The isolated nuclei of MCF7 cells were immunostained with an anti-eNOS^{Thr-495} antibody (1:20) followed by an anti-rabbit FITC antibody (1:160; raised in donkey). It is clearly seen that the eNOS protein present in the nuclei, is phosphorylated at the Threonine-495 residue.
In order to find out if the nuclear eNOS was exclusively phosphorylated at its Thr-495 residue, we imaged the phospho- and the non-phospho forms of eNOS simultaneously, by double labeling experiments, followed by a pixel analysis of the images to examine their co-localization (Fig 1.5B & C). The data showed that nearly 75% of the eNOS was phosphorylated at the Thr-495 residue and the co-existence of both phospho-and non-phospho-forms appeared as bright yellow spots, localizable to specific structures. Non-phospho- and phospho-eNOS$^{\text{Thr}495}$ were also present in the nucleoplasm, without obvious co-localization, whereas, the nucleolar region appeared to be more selectively enriched with respect to the phosphorylated form. Thus, the nuclear eNOS comprised of an unequal mixture of both phosphorylated and non-phosphorylated forms of this enzyme; phosphorylated form appeared to be more selectively localized to nucleolar regions and both the forms were concentrated into small spots within the nucleoplasm.

**A major fraction of the nuclear eNOS is phosphorylated at its Threonine-495 residue**

![Figure 1.5B](image-url) MCF7 cells were subjected to a double immunolabeling experiment with antibodies to eNOS (1:20) and phospho eNOS$^{\text{Thr}495}$ (1:20). A co-localization of both signals is evident in the merged image.
Figure 1.5C Dot plot representation showing the extent of colocalization between the two forms of eNOS. Here the channel for FITC (native eNOS) has been represented on the X-axis, and the channel for Cy3 (eNOS T495) has been shown on the Y-axis. The colocalized pixels (seen in the third quadrant) in the nucleus (a) and in the nucleolus (b) show that most of the nuclear eNOS is phosphorylated at its Threonine-495 residue.

Because the majority of eNOS within the nucleus was phosphorylated at the Threonine-495 residue, it is suggested that Threonine-495 phosphorylation was a characteristic feature of nuclear eNOS in general, rather than being a characteristic feature of the molecules that were associated only with the nuclear speckle-like spots.

eNOS is associated with speckles in the nuclei

It is well known that the architecture of the nuclear compartment is rather complex, comprising of nucleoplasm, nucleoli, nuclear speckles and nuclear bodies: the overall biology of the nucleus is coordinated by the activities of these various nuclear sub-compartments. An important functional unit of the nucleus is the specialized structure known as “speckle”, involved in the aspects of mRNA biogenesis, including their transcription, splicing, polyadenylation, maturation and transport to the cytoplasm. These specialized bodies came to be known as speckles, because of their characteristic “speckled” staining pattern obtained, when they were probed with antibodies directed against splicing factors. Individual nuclei may have a limited
number of speckles, where the protein factors necessary for RNA splicing are concentrated. Because eNOS staining in the isolated nuclei also had a speckled appearance (Figure 1.4), we sought to examine next if eNOS was associated with the speckles that were detectable with antibodies to the splicing factors. We used a number of antibodies to molecules involved in RNA-splicing that are known to be associated with spliceosomes. Figure 1.6 A shows a typical profile of dual staining of isolated, purified nuclei from MCF7 cells with antibodies to eNOS (Green) and to spliceosome (Red). The data show that there are several features that are common to staining with both these antibodies.

First, spliceosome antibodies revealed the distribution of their antigens to distinct speckles in the nucleoplasm, with a very little staining seen in the nucleolar regions. Second, the eNOS antibody staining was also localized to nucleoplasm, with a very little stain being present on the nucleoli. The staining pattern for eNOS within the nucleoplasm was of a granular appearance, comprising of very fine as well as large granules of heterogeneous size, which were similar to speckles. Since all the granular spots seen with anti-spliceosome antibody were relatively larger in size, but fewer in number, it gave indications that eNOS was associated with several more “mini-speckles” that were apparently free of (antigenic) spliceosomal proteins. This becomes clear from the merge of the two images, where it is seen that the spots containing eNOS alone were comprised of small and large granular spots, whereas the spliceosomal proteins associated with eNOS were confined to relatively large-sized granular spots.

**eNOS containing speckled regions were identified as spliceosomes**

![Image](image_url)

**Figure 1.6 A** A double immunolabeling of the isolated nuclei of MCF7 cells was carried out using the antibodies against spliceosome (red) and eNOS (green). Optical sections of 0.4µ thickness were imaged using a Confocal microscope. A clear colocalization of these proteins is evident as yellow coloured spots in the merged image.
A dot plot has been depicted here to show the extent of colocalization between the two proteins in a selected nucleus (marked with a white circle). The FITC-channel (eNOS) is represented on the X-axis and the Cy3-channel (spliceosomes) is shown on the Y-axis. The colocalized pixels are seen in the third quadrant, as blue dots.

Similar results were obtained when the whole MCF7 cells were probed with the same antibodies, indicating that the observations made with purified nuclei did not arise due to any non-specific reasons associated with nuclear preparations (Figure 1.6 b).

Colocalization of eNOS to spliceosomes was again evident when whole MCF7 cells were imaged with an antibody directed against a specific spliceosome-associated protein SC-35 (Figure 1.6C). Like in the case of whole spliceosome-related antibody, it was again seen that the eNOS protein was apparently distributed in the nuclear compartment, in two pools. A pool having considerable amounts of eNOS present in the nucleoplasm, which was apparently free of SC-35, and the second one, where eNOS appeared to be a component of speckled bodies, as revealed by its colocalization with SC-35. There were relatively few speckles that contained eNOS, but had no SC-35. The results can be explained in several ways. These data are consistent with models: i) the speckles having no reactivity with SC-35 may contain...
other splicing factors undetectable by SC-35 antibody; ii) during the assembly of speckles, eNOS is recruited prior to the recruitment of SC-35 or other spliceosome-related proteins, and the speckles containing only eNOS here represent these early complexes.

**Colocalization of eNOS with splicing factor**

![Colocalization of eNOS with splicing factor](image)

**Figure1.6C** Subconfluent MCF7 cells, grown on coverslips, were subjected to double immunolabeling using an anti-eNOS antibody (N-20; 1:20) and an anti-SC-35 antibody (1:1000). The secondary antibody used for eNOS was an anti-rabbit FITC-labelled antibody (1:100; raised in goat) and that for SC-35 was an anti-mouse Cy3-labelled antibody (1:40; raised in donkey). A strong colocalization of these two molecules is seen in the nucleus, as yellow coloured speckles in the merged image. The dot plot in the middle panel represents the colocalized pixels in its third quadrant. The lower panel shows an image from an identical experiment, where DAPI was used to demarcate the nuclear region.

1.7 eNOS associated with SC-35 is phosphorylated at Threonine 495.

We examined the phosphorylation status of eNOS with respect to the Threonine-495, to determine if the distribution of the phosphorylated form was in any way different from that of the total eNOS. The staining profiles obtained in these experiments were
virtually similar to what was seen earlier using the anti-eNOS antibody. Quantitation of the co-localization by pixel analysis showed that the association of SC-35 with Phospho-eNOS^{Thr495} was >85% whereas, the association of SC-35 with total eNOS was only to the extent of ~70%. These results underscore that phosphorylation at Threonine-495 probably leads to a better colocalization with SC-35.

**Association of phospho-eNOS^{Thr495} and SC-35 in the nuclear speckles**

![Image of co-localization](image)

**Figure 1.7A**

a) MCF7 cells were processed for double immunolabelling with a monoclonal anti-SC-35 antibody (red) and an anti-phospho-eNOS^{Thr495} antibody (green). A strong colocalization of the molecules is seen as yellow-coloured spots in the merged image. DAPI was used to demarcate the nuclear region.

b) The comparative dot plots of the colocalized pixels of eNOS and SC-35 (left panel) and p-eNOS^{Thr495} and SC-35 (right panel) are illustrated. It is seen that SC-35 pre-dominantly colocalizes with the T495-phosphorylated form of eNOS.

We examined next, if the phosphorylation of Threonine-495 residue was related to its interaction with SC-35. As shown in Figure 1.7, the eNOS associated with SC-35 was both phosphorylated and non-phosphorylated at its Threonine-495 residue. However, a quantitation of this association by pixel analysis of the data revealed that there were several molecules of eNOS, both phosphorylated and non-phosphorylated ones that were not associated with SC-35. However, a relatively more number of SC-
35 molecules were associated with the phosphorylated form of eNOS, than with the non-phosphorylated one.

In order to examine if the association of eNOS with splicing protein was specific, we also examined if it was associated with other nuclear and nucleolar proteins, such as C23-Nucleolin and B23-Nucleophosmin. Fluorescence imaging of C23-Nucleolin showed it to be nuclear, and to a much greater extent being nucleolar, as reported by several workers (323) But when it was imaged together with eNOS in the same cells, it was not found to be colocalized with eNOS (Figure 1.7B). Only in occasional fields, a colocalization of these two molecules was seen in the nucleolus.

**eNOS and Nucleolin do not colocalize within the nuclear compartment.**

![Image](image1.png)

**Figure 1.7 B a)** Double immunofluorescence staining of isolated nuclei of MCF7 cells with an anti-nucleolin antibody (1:20) and an anti-eNOS (N-20; 1:20) antibody. The secondary antibodies conjugated to Cy3 and FITC, respectively, were used. No appreciable colocalization is seen in the nuclear compartment; **b)** occasionally, a nucleolus having a colocalization of these two molecules was observed.

B23/ Nucleophosmin protein in MCF7 cells showed a characteristic staining pattern, where the protein was mostly associated with the nucleolar compartments (Figure
However, on several occasions the B23 distribution pattern was found to be punctate and the protein was distributed over cytoplasmic, nuclear and nucleolar compartments (Figure 1.7C- lower panel). Interestingly, in the latter, eNOS was found to be clearly colocalized with B23/ Nucleophosmin in all the sub-compartments. The exact identity of these fine granular sub-compartments distributed over cytoplasm, nuclei and nucleoli has not been known yet, but an attractive possibility is that they may represent the nucleolus-derived foci (NDF) that have been identified in the mitotic phase of cells, which contain the B23/ Nucleophosmin protein and 45S precursors of ribosomal RNA and processing proteins (324).

**eNOS and B23 colocalize in the nucleus and the nucleolus.**

a) A nucleolar localization of B23 is seen in the nuclei of MCF7 that were immunostained with an anti-B23 antibody (1:20), followed by an anti-goat FITC-labelled secondary antibody (1:100; raised in donkey).

b) Isolated nuclei were immunostained with antibodies against eNOS (green) and B23 (red), and subsequently with appropriate fluorescently labeled secondary antibodies. The third square in this panel shows an overlay of the images obtained with the indicated antibodies (yellow staining indicates colocalization).

c) The dot plot analysis shows a complete colocalization of the two molecules in the nucleolar region (the third quadrant - blue dots)

*Figure 1.7C a) A nucleolar localization of B23 is seen in the nuclei of MCF7 that were immunostained with an anti-B23 antibody (1:20), followed by an anti-goat FITC-labelled secondary antibody (1:100; raised in donkey). b) Isolated nuclei were immunostained with antibodies against eNOS (green) and B23 (red), and subsequently with appropriate fluorescently labeled secondary antibodies. The third square in this panel shows an overlay of the images obtained with the indicated antibodies (yellow staining indicates colocalization). c) The dot plot analysis shows a complete colocalization of the two molecules in the nucleolar region (the third quadrant - blue dots)*
Colocalization of eNOS and B23 in the nucleus

**Figure 1.7D** Isolated nuclei of MCF7 were processed for double immunolabeling with antibodies to B23 and eNOS. A strong colocalization of the proteins is seen. A dot plot analysis depicting the colocalized pixels is shown in the lower panel.

Figure 1.7D shows the quantitative aspects of colocalization between eNOS and B23/Nucleophosmin, assessed from double labeling experiments. The marked cell in the figure as an example shows that virtually >98% eNOS-containing pixels were colocalizable with B23/Nucleophosmin, whereas only two thirds of all B23/Nucleophosmin pixels were associated with eNOS. This result implies that eNOS function within the nucleus may be related, at least partly to the B23/Nucleophosmin function.

**1.8 eNOS is physically associated with SC-35 but not with B23/Nucleophosmin.**

Since eNOS was found to be colocalized with SC-35 as well as with B23 in the nuclear compartments, it raised questions if these proteins physically interacted with each other. In order to resolve this, we analyzed the immunoprecipitates of nuclear lysates done using eNOS antibody with SC-35 and B23/Nucleophosmin antibodies in western blots. The results showed that SC-35 protein was pulled down by eNOS antibody (Figure 1.8), but it failed to pull down B23/Nucleophosmin. These results
show clearly that eNOS does not interact directly, or indirectly through protein intermediates, to form a complex with B23/ Nucleophosmin, despite its apparent co-localization in the nuclear sub-compartments. In order to verify the eNOS–SC-35 interaction, we also conducted the reciprocal experiment, where the eNOS protein was probed after an immunoprecipitation of nuclear lysates using SC-35 antibody. The results clearly showed the presence of eNOS-specific protein bands.

**Nuclear eNOS physically interacts with SC-35**

![Figure 1.8](image.png)

**Figure 1.8** The nuclear lysate of MCF7 was prepared and an immunoprecipitation was carried out independently with anti-SC-35 and anti-eNOS antibodies. The immunoprecipitates were subjected to a western blot analysis by probing the blots with anti-eNOS antibody for the SC-35-IP (left panel), and with an anti-SC-35 antibody for the eNOS-IP (right panel). It is clearly seen that these two molecules physically interact *in vivo*.

2. Identification and Characterization of Nuclear Localization Signals (NLS) in eNOS protein

A large number of proteins enter into and exit the nucleus as a part of their physiological function. It has been recognized now that a short sequence motif of amino acids in a cargo protein, usually comprising of a cluster of basic amino acids such as arginine and lysine, may form a recognition and binding motif for the receptors of nuclear import machinery, nuclear importins alpha and beta, to guide the cargo through the nuclear pores to the cellular nucleus. Such short peptide motifs have come to be known as nuclear localization sequences (NLS). One of the early NLS to have been recognized was associated with the large T antigen of SV40 virus comprising of the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val (PKKKRKV) (325). That the NLS motifs can work autonomously was recognized from experiments, where cytoplasmic proteins such as pyruvate kinase or *E.coli*-derived beta galactosidase showed a nuclear localization in cells, after the short NLS motif was artificially
tagged to their carboxy termini. Chelsky et al. arrived at four amino acid consensus sequence of Lys-Arg/Lys-X-Arg/Lys for the NLS after examining a number of monopartite NLS (326). Subsequently it was recognized that NLS can be more complex in nature, when it was identified in Xenopus nucleoplasmin that its functional NLS was bipartite in nature. In this sequence, mutational analysis showed that the necessary basic amino acids, **KR** and **KKK** of the sequence AVKRPAATKKAGQAKKKKLD were separated by ten other amino acids, each of which could tolerate substitution without compromising the nuclear localization of this protein. (327). Presently, examples of several other such bipartite motifs are known, and in many cases of viral proteins, multiple NLS motifs may cooperate for the nuclear entry of the protein cargo. More complex architecture of NLS is possible in some cases, as seen with the Adeno virus IVa2 protein. Its nuclear localization sequence was mapped to the amino acids 432-449, comprising the sequence **LNDRDRWSRAYRARKTPK**, where the string of basic amino acids (green) were punctuated by the presence of acidic (orange), hydrophilic or bulky amino acids (blue) (328) Taken together, NLS function may be associated with classical amino acids containing basic amino acids.

Nuclear localization of a protein could be constitutive, as seen with the basic proteins such as histones, or the migration of a cytoplasmic protein to the nucleus can be induced by hormones, by small signaling molecules or by signals that orchestrate modification of the phosphorylation status of specific sequence motifs. Indeed, a conditional or a regulated entry into the nucleus can have a profound role in cellular modulation of proliferation, metabolism and signal transduction. For example, in the absence of the hormone, the Glucocorticoid receptors remain in cytoplasm forming a complex with HSP90, but the steroid hormone-binding releases the receptor from HSP90 and helps in its translocation to the nucleus to activate specific gene-expression, by binding to specific target DNA sequences in the nucleus. Elevated cyclic nucleotide, cAMP, can bind to the regulatory subunit and release a catalytically active cAMP-dependent protein kinase, which can migrate to the nucleus. There it may activate cAMP-response element binding-protein by phosphorylation, thereby inducing specific gene expression. These are examples, where the affinity of the cargo for the cytoplasmic anchoring protein is lowered as a result of phosphorylation. Many
transcription factors, signal-transducing molecules such as ERK1, ERK2 are dependent upon phosphorylation for their nuclear entry. In some of these cases, the affinity of the cargo is increased to a nuclear anchor or a retention protein. There are cases where nuclear entry of molecules is inhibited by phosphorylation of residues that are present in the proximity of the NLS, because it may increase the affinity of the cargo to a cytoplasmic anchor. Thus, nuclear localization can be regulated by multiple pathways, where a suitable post-translational modification may alter the affinity of the protein either to a cytoplasmic or to a nuclear anchor, affecting its equilibrium and redistribution between the two compartments. As an illustration, when casein kinase II phosphorylates Serine\textsuperscript{111} and Serine\textsuperscript{112} residues of SV-40 T-antigen in a signal transduction-dependent manner (Fig 2.1), it has a positive effect on the nuclear localization and retention, because the phosphorylation accelerates nuclear import and helps the retention of the antigen by increasing its affinity to the nuclear envelope protein NLS-BP. By contrast, when CDK/p34\textsuperscript{cdk2} phosphorylates Threonine-124 in a cell-cycle-dependent manner, the phosphorylation affects nuclear trafficking in a negative manner, because this phosphorylation decreases the maximal accumulation of the antigen in the nucleus and increases the affinity of a cytoplasmic anchor for this protein (329).

![Figure 2.1](image)

Figure 2.1 Amino acid sequence of a region of SV40 T-antigen that can positively and negatively influence nuclear migration and retention by a phosphorylation-dependent manner is depicted. The target residues are identified with a dot and the essential NLS sequence has been marked in green.

Despite favourable factors for nuclear import and retention, some proteins may exit nuclei immediately after their import, because they contain nuclear export sequences. Therefore, it is clear that the trafficking and the distribution of a protein to the nuclear compartment depends upon several factors: a) that allow binding of the cargo to nuclear importins (alpha or beta) with high affinity; b) that decrease affinity of the cargo to its cytoplasmic anchor protein; c) that increase the affinity of cargo to its nuclear anchoring protein and finally d) the presence and the nature of nuclear export sequences that may be present.

A common strategy to delineate the presence of NLS in a nuclear protein is to examine regions containing short clusters of basic amino acids to see if they can
successfully effect nuclear import, when tagged to cytoplasmic proteins. Since NLS may not be obvious from inspection; it is advantageous to generate constructs by recombinant DNA procedures and to express collinear fusions of selected regions of a target protein sequence in the cells to examine if it will direct the nuclear import of a suitable cytoplasmic reporter such as Green Fluorescence protein.

We, therefore, decided to generate chimeric protein-expressions of part eNOS amino acid sequences in cells, by constructing in-frame fusion constructs with an enhanced Green fluorescence protein (EGFP). Our strategy for generating these fusions was based on the availability of restriction sites of three enzymes, BamHI, Bgl II and KpnI on c-DNA of eNOS sequence. The distribution of these restriction sites and their relative position with respect to the known domain organization of eNOS is shown in Figure 2.2.

**Anatomy of Endothelial nitric oxide synthase**

The c-DNA of eNOS (obtained from IMAGE consortium, USA) had its initiation codon at nucleotide 294 and the termination codon at nucleotide 3905. A full-length eNOS sequence cloned in pCI-Neo flag plasmid was found to distribute the protein between cytoplasm and nucleus (Figure 2.2 B), supporting the earlier data of its detection in the nuclear compartment.
Figure 2.2 (B) A full length eNOS (aa 1 to 1204) was cloned in a plasmid vector pCI-Neo-flag that generated a fusion protein eNOS-FLAG. MCF7 cells were transiently transfected with this plasmid and the localization of eNOS was detected by an anti-FLAG antibody. It is seen that eNOS-FLAG fusion protein distributes in both cytoplasm and nucleus.

Figure 2.3 Relative position of restriction sites in c-DNA of eNOS used for constructing chimeric expression constructs with EGFP

In order to map the putative nuclear localization sequence (NLS) in the eNOS c-DNA sequence, we analysed two internal BamHI fragments (from 853-2024 and 2024 to 2978; Figure 2.3), generating in frame fusion proteins with EGFPC. While the EGFPC-(Bam853-2024) did not show a clear localization of the fusion protein in the nucleus, the EGFPC-(Bam 2024-2978) showed a 2-3 fold higher number of transfected cells to harbor the EGFP protein in their nuclei. These results alluded to the possibility that if eNOS has a NLS sequence, it may be present either partly or fully within this segment. We also examined the EGFP fusions of a large C-terminal KpnI-KpnI segment (2498-4144) that contained the 468 C-terminal amino acids, a Bgl II to KpnI (1802-2498) fragment that contained 505-735 amino acids from the central region. In both cases, a nuclear localization of the chimeric protein was observed, but the Bgl II-Kpn I (2498) sequence showed more number of cells with nuclear fluorescence and many of them had GFP-fluorescence in their nucleoli. In contrast, the sequence containing C-terminal (KpnI-KpnI; 2498-4144) chimera showed only a nuclear fluorescence. These results indicated that eNOS may have NLS sequences distributed in two non overlapping regions of its sequence and even alluded to the possibility that it may, in addition, contain a motif that is capable of targeting eNOS protein to nucleolar compartment. Therefore, a strong possibility arose that the putative NLS of eNOS may be resident within the BamHI-KpnI region (2024-2498) and perhaps a second one within the C-terminal 468 amino acids. These results are summarized in Table 2.1

We, therefore, focused on this region by preparing EGFP fusion constructs by amplifying shorter sequences using a polymerase chain reaction. In these experiments,
a construct prepared by the fusion of sequences within the nucleotides 2065-2259 again showed a large number of cells harboring the EGFP protein in nuclei, but there were also appreciable number of cells that only showed cytoplasmic fluorescence. A partial shift towards nuclear localization might mean that the NLS motif or motifs are unable to bind the importin proteins with high affinity and/or that the cargo is unable to interact with its nuclear retention anchor protein with high affinity. These results are summarized in Table 2.1

<table>
<thead>
<tr>
<th>eNOS Segment</th>
<th>Length and Nucleotide boundary</th>
<th>Observed subcellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI-BamHI</td>
<td>1170 bp(852-2024bp)</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>BamHI-BamHI fragment</td>
<td>956 bp(2024-2978bp)</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Bgl II-Kpn I</td>
<td>695 bp(1802-2497bp)</td>
<td>Nuclear and nucleolar localization</td>
</tr>
<tr>
<td>PCR amplified</td>
<td>2065-2259</td>
<td>Nuclear and nucleolar.</td>
</tr>
<tr>
<td>Kpn I-Kpn I fragment</td>
<td>1647 bp(2497-4144 bp)</td>
<td>Nuclear localization</td>
</tr>
</tbody>
</table>

Because, classical nuclear localization sequences comprise of a string of amino acids rich in basic amino acids such as arginine and lysine residues, an examination of amino acid sequence contained within nucleotides 2065-2259 region showed that there was a stretch of sequence containing five basic amino acids, 627Arg-Arg-Lys-Arg-Lys 631, which satisfies the requirements of a loose consensus sequence K (K/R)X(K/R).(326). Strikingly, this potential motif is flanked by a hydrophobic amino acid and two serine residues on its N-terminal side whereas; two serine residues surround it at its C-terminus. We, therefore, hypothesized that this short stretch of basic sequence may mediate NLS function. In order to test this hypothesis, we first generated an EGFP fusion expression construct that contained the residues Ser-Ser-Trp-Arg-Arg-Lys-Arg-Lys-Glu-Ser-Ser- Gln-Thr from this region (amino acids 624-636). EGF-eNOS 624-636 protein was found to be distributed within the nuclei in a majority of cells compared to another EGFP fusion construct that had the same amino acids in a scrambled order. These results indicated that the sequence within 624-636 was capable of effecting nuclear import and may serve as a functional NLS for eNOS.
protein. For this reason, we have designated this motif as eNOS-NLS1. One reason why the nuclear localization with EGFP-eNOS$^{624-636}$ was weak may derive from the fact that the basic motif is surrounded by serine residues, which are potential phosphorylation sites, and may make the surroundings of the basic motif highly charged after their phosphorylations. The four serine residues surrounding the motif are evolutionarily conserved in eNOS proteins across several genera, indicating that they may serve some important function. Of the four, the Serine located at the N-terminus (Serine 4 in our designation; see below) is the least conserved. It was possible that serine modifications at these sites may antagonize the interaction necessary between the cargo and a critical component of nuclear importing complex such as Importin $\alpha$. If such a model is true, then one prediction of this model would be that the replacement of the conserved serine residues would lead to a better nuclear trafficking. We generated and tested a series of EGFP fusion constructs that had these Serines replaced singly or in combinations with Alanine or Asparagine. The phenotype of cellular localization was affected in all these substitutions and the results indicated a complex regulation of nuclear and nucleolar trafficking. The data are summarized in Table 2.2.

<table>
<thead>
<tr>
<th>Construct used and mutation created</th>
<th>Size of the insert</th>
<th>Vector</th>
<th>Nucleolus</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV40 NLS (3X nuc) (PKKKRKV)$_3$</td>
<td>53 bp</td>
<td>pEGFPC</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS-NLS-1</td>
<td>54 bp</td>
<td>pEGFPC1</td>
<td>+++</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>PLVSS$^3$WRRKRKE'S$^2$N</td>
<td>54 bp</td>
<td>pEGFPC1</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>PLVSS$^3$WRRKRKE'A'S$^2$N</td>
<td>54 bp</td>
<td>pEGFPC1</td>
<td>+++</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>PLVSA$^3$WRRKRKE'A'S$^2$N</td>
<td>54 bp</td>
<td>pEGFPC1</td>
<td>+++</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>PLVSS$^3$WRRKRKE'A'N$^2$N</td>
<td>54 bp</td>
<td>pEGFPC1</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>PLVSA$^3$WRRKRKE'A'N$^2$N</td>
<td>54 bp</td>
<td>pEGFPC1</td>
<td>±</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Scrambled NLS-1</td>
<td>54 bp</td>
<td>pEGFPC1</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>
The localization patterns of the EGFP-fusion proteins encoded by the plasmid constructs that are summarized in the Table 2, are illustrated. MCF7 cells were transiently transfected with these plasmids and the images were captured on a Confocal laser microscope.

The results from this mutation analysis clarified that i) the short motif, SSWRRKRKRESS, is capable of mediating nuclear and nucleolar transport or retention, without the presence of three of its Serines (mutant of S1, S2 and S3). In some combinations, the protein was efficiently targeted to nuclei, but the nucleoli were empty. In other mutants, both nuclear and nucleolar GFP was seen, but a cytoplasmic leakage of the chimeric protein was noticeable. The individual or the grouped alterations of the three conserved Serine residues (S1, S2 and S3) together suggest that these three residues are the targets of post-translational modifications, most likely that of phosphorylation. These modifications on Serine residues are likely to play a complex role in mediating both nuclear and nucleolar targeting and retention of the cargo. The data are consistent with a simple model in which a modification on
S1 regulates the distribution of the protein between the nuclear and the cytoplasmic compartments, whereas modifications on S2 and S3 regulate the trafficking of the protein between nuclear and nucleolar sub-compartments. For example, a modification on S2 may favour the nucleolar targeting and/or retention, while a modification on S3 has the opposite effect: that is, it favours nuclear targeting and/or retention. The summary of this model is illustrated schematically in Figure 2.4.

![Figure 2.4](image)

**Figure 2.4** A model explaining the likely mechanism involved in the trafficking of the EGFP-NLS-1-fusion protein to various sub-cellular compartments, mediated by the modification of the serine residues flanking NLS-1.

The expression construct containing the fragment of eNOS (2024-2978) showed a more robust nuclear and nucleolar retention of the EGFP than the results seen with NLS1 containing constructs. Inspection of amino acids identified a second sequence PKRSWKRQRYR that satisfied the consensus NLS sequence criteria of K K/RXK/R (326). Since the expression of KpnI-KpnI (2497-4144 bp) fragment had also shown nuclear localization, we picked the sequence IHVHRRK as a likely candidate NLS. Because we observed that most of the nuclear eNOS was phosphorylated at its Threonine 495, we considered the possibility that it might have some role in mediating the nuclear localization of eNOS. We therefore generated by PCR, a fragment of eNOS sequence that contained the Threonine-495 phosphorylation sequence and its surrounding CaM binding motif, NLS1, PKRSWKRQRYR (NLS 2) and IHVHRRK (NLS 3). The motif IHVHRRK is an unusual NLS in that it contains two histidine residues that can behave as cationic centres at neutral pH due to protonation. It will be interesting to find if the nuclear localization of proteins containing this sequence are dependent upon cytoplasmic pH changes. Because this motif is only 14 amino acids apart from the NLS2, it is also possible that this motif cooperates with NLS2. When this PCR amplicon was expressed as a chimeric construct (designated as **T495-NLS**), EGFP fusion protein showed a dramatic
improvement in the nuclear and nucleolar retention of EGFP over other constructs examined before (Figure 2.5). The results indicated clearly that eNOS contained at least three distinct NLS sequences that cooperated with the Threonine-495 motif for an efficient nuclear and nucleolar targeting of EGFP.

**A Strong nuclear and nucleolar retention of EGFP in T495-NLS chimeric protein**

*Figure 2.5*: Transfection of MCF7 cells with T495-NLS construct spanning Threonine-495 phosphorylation sequence and its surrounding CaM binding motif, NLS1, NLS 2 and NLS 3 sequences shows a strong nuclear and nucleolar localization without any cytoplasmic leakage.

Generation of a strong phenotype of nuclear and nucleolar retention in the T495-NLS construct provided an opportunity to examine the relative contribution of each one of the identified motifs. We set out to achieve this by a site-directed mutagenesis of these individual identified motifs. First, we modified the NLS1 sequence within T495-NLS, by changing the amino acid sequence from RKRRK to LRNRN, where three of the basic amino acids are substituted by leucine and asparagines, and the lysine residues are eliminated. With this mutation in NLS1, the protein was seen to (Figure 2.6) redistribute itself into both cytoplasm and nuclei, and the nucleoli were empty.

**Redistribution of fusion protein from nucleus to cytoplasm due to a mutation in NLS1**

*Figure 2.6*: Mutation in the NLS-1 motif of T495-NLS construct leads to a substantial cytoplasmic leakage and a non-nucleolar phenotype.
When the amino acid sequences KRSWKR were deleted from the second putative NLS sequence of T495-NLS sequence, the nuclear EGFP was retained, but the nucleoli were empty and there was a sufficient leakage of EGFP fluorescence to the cytoplasm (Figure 2.7)

**Deletion of the second putative NLS affects the nucleolar localization of EGFP**

![Figure 2.7](image)

*Figure 2.7* Deletion of NLS2 motif from T495-NLS construct affects the nucleolar localization of EGFP implicating its role in the nucleolar trafficking.

We also mutated the Threonine-495 to Alanine to determine if it affected the nuclear localization of the chimeric protein. This mutation had a very strong phenotype in that nearly all the protein was cytoplasmic (Figure 2.8).

**Nuclear localization abrogated due to mutation in T495 site in T495-NLS construct.**

![Figure 2.8](image)

*Figure 2.8:* Mutation of T495 residue to alanine in the T495-NLS construct completely abrogates its nuclear localization, indicating its critically important role in the process.

These results identified that NLS1, NLS2 and NLS3 may individually retain its autonomous ability to partition a protein into the nuclei or its sub-nuclear compartments, but when present together, in the context of other sequences present in eNOS, they work in a mutually interdependent, but cooperative, manner to regulate
the sub-cellular distribution of the protein. For example, the total disruption of nuclear and nucleolar localization with T495A mutation clearly suggest that this motif has an important role to modulate the anchoring of this protein to the cytoplasmic compartment in a phosphorylation-(or other modification)-dependent manner. Commensurate with this expectation, the T495-NLS fusion protein isolated from the nuclei of transfected cells was seen to be phosphorylated at a Threonine residue (Figure 2.9). Since T495A was pro-cytoplasmic, it is likely that either the phosphorylation of Threonine disrupts the interaction between the cytoplasmic anchor-protein for eNOS and allows its trafficking to the nucleus, or the Threonine-495 phosphorylation promotes a strong binding to its nuclear anchor enabling its longer retention.

**The fusion protein EGFP-T495-NLS gets phosphorylated in vivo**

![Figure 2.9](image)

**Figure 2.9** Immunoprecipitation of the nuclear lysate of MCF7/T495-NLS cells was carried out with an anti-GFP antibody and the blot was probed with an anti-phospho-threonine antibody. It is seen that the fusion protein gets Threonine-phosphorylated *in vivo*.

In order to have a confirmation of the model that three independent sequences of NLS and the T495 motif together constitute the necessary and sufficient elements to target a chimeric protein to nuclei and nucleoli, we generated a synthetic sequence encompassing all these motifs. When this artificial sequence was expressed as a chimera of EGFP, the sequence (termed here as “Syngene-NLS”) virtually recapitulated the properties of T495-NLS (Figure 2.10). The results, therefore, provide a formal proof that the three independent NLS sequences act in consort with modifications at Threonine-495 site to modulate nuclear entry and exit of eNOS protein.
Syngene-NLS shows strong nuclear and nucleolar retention of EGFP

**Figure 2.10:** Syngene-NLS that has been constructed with three independent sequences of NLS and the T495 motif, recapitulates the properties of T495-NLS construct.

Generation of a synthetic gene that recapitulated the nuclear targeting properties of the natural eNOS sequences faithfully provided an opportunity to test if phosphorylations other than T495 can alter the nuclear entry of NLS sequence. It has been reported that phosphorylation at Serine-114 is accompanied by nuclear localization of eNOS in mitotic cells. We, therefore, substituted the T495 motif with the Ser-114 motif into the Syngene to obtain a new synthetic chimera, where the three NLS sequences were combined with Ser-114 motif. As can be seen from Figure 2.11, the replacement of Threonine 495 motif by Ser-114 motif retains both nuclear and nucleolar entry of EGFP. However, a mutation of Ser-114A in this motif does not make the protein entirely cytoplasmic, as was seen with T495. The mutation however abrogates nucleolar retention and promotes cytoplasmic leakage (Figure 2.12).

**Ser-114 motif retains the nuclear and nucleolar localization of the fusion protein**

**Figure 2.11:** Replacement of the T495 motif with that containing a Serine-114 motif in the Syngene-NLS construct helps to retain the EGFP-fusion protein in both nucleus and nucleolus.
A mutation of Ser114 to alanine leads to a cytoplasmic leakage of GFP and also abrogates the nucleolar retention of EGFP

**Figure 2.12:** Mutation of Serine-114 to alanine in Syngene-Ser114-NLS construct partially abrogates the nuclear retention of EGFP-fusion protein and also abrogates its nucleolar localization.

eNOS association with the nucleus is dynamically regulated

Nuclear association of eNOS could be dynamic, that is the protein shuttles between cytoplasmic and nuclear compartments continuously. Availability of T495-NLS construct having a tight nuclear and nucleolar localization of GFP, allowed an opportunity to verify the hypothesis that eNOS may shuttle between nucleus and cytoplasm, by a heterokaryon assay. Figure 2.13 shows that the nuclear phenotypes of two different cells used in heterokaryon assay, namely mouse NIH3T3 and human MCF7 cells are distinguishable by staining the nuclear DNA of cells with the dye, 4’, 6-diamidino-2-phenylindole (DAPI).

**Figure 2.13** DAPI patterns of NIH3T3 and MCF7. NIH3T3 and MCF7 cells were stained with DAPI (300nM). NIH3T3 shows a speckled pattern of DAPI in the nucleus, whereas MCF7 displays a mattish pattern, enabling their identification in a heterokaryon assay.
T495-NLS protein shuttles between nucleus and cytoplasm

Figure 2.14 Heterokaryon assay was carried out to examine whether T495-NLS was a shuttling protein. The assay was done by fusing the MCF7/T495-NLS cells with NIH3T3 cells in the presence of a protein synthesis inhibitor, cycloheximide. The cells were fixed with chilled methanol, and were stained with DAPI. All images were viewed and captured by a Confocal laser microscope. A typical heterokaryon seen in the image clearly shows the migration of the EGFP-fusion protein from MCF7/T495-NLS cells to the nucleus of NIH3T3 cells, indicating that the protein actively shuttles between nucleus and cytoplasm.

MCF7 cells that stably expressed T495-NLS protein and exhibited a tight phenotype of nuclear and nucleolar localization of the EGFP-fusion protein, with no cytoplasmic leakage, were mixed with an equal number of mouse NIH 3T3 fibroblast cells and the mixture was treated with polyethylene glycol to effect cell-fusion to generate heterokaryons. Examination of heterokaryons revealed that the EGFP fluorescence could slowly migrate from the nuclei of MCF7 cells to nuclei and nucleoli of the NIH 3T3 cells (Figure 2.14) over a period of 6 hours in the presence of a protein synthesis inhibitor, cycloheximide. The data clearly shows that the T495-NLS is capable of trafficking between nucleus and cytoplasm; its association with nuclear and a nucleolar compartment is dynamic in nature.

eNOS is present in purified nucleoli isolated from epithelial cells.

Data obtained so far are consistent with the presence of three NLS motifs and two phosphorylation sites that cooperate to transport eNOS from cytoplasm to nucleus and even to the nucleolus. Several efforts made by indirect immunofluorescence assays did not detect (excepting on some rare occasions such as shown with data on B23/Nucleophosmin or on Nucleolin) the presence of eNOS within the nucleolar compartments. The lack of reactivity with antibodies in the nucleolar compartment may have been due to its presence within the structures that are impermeant to antibodies under the experimental conditions used, or the epitopes of the protein were masked. In order to confirm that eNOS was partitioned into nucleolar compartments;
we sought to approach the issue more directly. We isolated the nucleoli from purified nuclear preparations of MCF7 cells. Suitable amounts of proteins from the purified nucleoli were solubilized in Laemmli’s buffer and were resolved in 6% SDS-PAGE. The resolved proteins were processed for the detection of eNOS by western blots. The data are shown in Figure 2.15.

![Diagram of eNOS in purified nucleoli](image)

**Figure 2.15** Intact nucleoli isolated from MCF7, MCF7/T495-NLS and HBL100 were lysed in RIPA buffer and the proteins were subjected to a western blot analysis. The blots were sequentially probed with an anti-eNOS antibody (1:1000, N-20), an anti-fibrillarin antibody (1:1000) and an anti-nucleolin antibody (1:1000). It is evident that a substantial amount of eNOS is present in the nucleoli of these cells. The lower panel shows the data in a graphical form, after normalization with respect to Nucleolin.

Figure 2.15 shows clearly that a band of appropriate size and having reactivity with the specific antibody to eNOS is readily detectable in the purified nucleolar proteins after resolution in SDS-PAGE. The same blot had reactivity to fibrillarin and nucleolin; known to be specifically present in nucleoli. In addition to MCF7 and HBL-100 mammary epithelial cells, MCF7 cells having a stable and a high level expression of EGFP-T495-NLS fusion protein (MCF7/T495-NLS) show the presence of eNOS in their nucleoli. These results provide strong evidence that the over-expression, and the nuclear and the nucleolar retention of a chimeric EGFP-T495-NLS protein did not affect the distribution of natural eNOS in the nucleolar compartment. This observation was corroborated by the comparative assessments on
the \textit{in vitro} NO-generation capacity of a fixed amount of protein prepared from purified nuclei of MCF7, MCF7/T495-NLS and a clone of cells expressing the fusion protein encoded by EGFP-Syngene (designated as Syngene-NLS). Both T495-NLS and Syngene-NLS proteins are expressed to high levels in these cells, and both are targeted almost exclusively to nuclei and nucleoli. As shown in Figure 2.16, the \textit{in vitro} NO-generating capacity of a fixed amount of nuclear protein was almost similar in the two cases where the chimeric protein was being expressed. The MCF7/T495-NLS cells however showed a nearly 30\% higher capacity to generate NO from the same amount of nuclear protein. These data together show that the fusion protein Syngene-NLS is mildly inhibitory, while T495-NLS is slightly favourable to the eNOS mediated NO generation \textit{in vitro}; emphasizing that the basic aspects of native eNOS protein targeting into nuclear and nucleolar compartment remained largely unaltered in these cells. The results of the western blot experiment carried out with the nuclear lysates of MCF7/T495-NLS cells showed that a small amount of nuclear eNOS in these cells is in dimeric form (Figure 2.17). The dimer levels did not increase further in response to a thapsigargin-treatment, indicating that a mere increase in calcium levels were perhaps not sufficient for release of the monomer from its complexed form, to facilitate the formation of a dimer.

\textbf{The distribution of natural eNOS into nuclear compartments is not affected by the fusion protein-expression}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Figure2_16.png}
\caption{Proteins isolated from the nuclei of MCF7, MCF7/T495-NLS and MCF7/Syngene-NLS were assessed for their ability to produce nitric oxide, using DAF chemistry. It is seen that an active eNOS is present in all the lysates as indicated by enhanced cPTIO-sensitive DAF fluorescence. The data indicate that the fusion proteins do not alter the nuclear distribution of eNOS.}
\end{figure}
3. eNOS and intracellular reactive oxygen species (ROS) generation

**eNOS as a source of Reactive Oxygen Species**

eNOS has an oxygenase domain near its N-terminal segment and it can function independent of the reductase domain. Recombinant expressions of oxygenase and reductase domains of eNOS as recombinant proteins have shown that when they are mixed, both the protein segments can complement each other in vitro to produce NO (330). It is also known that under conditions such as a reduced bioavailability of BH4 and Arginine, the coordination between the oxygenase and reductase domains and the substrates may get uncoupled leading to a reduced NO generation, with a concomitant increase in superoxide anion formation by one electron transfer to the oxygen molecule in the oxygenase domain. Secondly, when Threonine-495 residue is phosphorylated, it is known that eNOS has a reduced ability to produce NO and an increased ability to produce superoxide anion (238) contributing to the production of reactive oxygen species (ROS) within the cells. Since ROS has been implicated in intracellular signaling mechanisms, this mode of eNOS producing ROS in the cellular compartments may serve some biological functions that still remain to be defined. However, a non-physiological or an excessive ROS generation may add to the stress and evoke pathological consequences. These considerations make it imperative and important to understand the process more fully, and define the factors that may influence the ROS generation from eNOS within the cells.

Superoxide anions have a single unpaired electron, but are relatively long-lived and less-reactive, compared to hydroxyl radicals. In view of their relatively less reactivity...
and longer life-time, these species can diffuse to several sub-cellular compartments from the site of their production to serve as possible signaling molecules. Superoxide anions are considered to be the major initiators of oxidative stress in the cells because they can: i) destroy iron-sulfur centres in proteins causing the release of Fe^{2+} ions, which can initiate the formation of very toxic hydroxyl radicals through Fenton chemistry; ii) react with unsaturated bonds in lipids to form lipid peroxides, iii) react very efficiently with NO to form peroxynitrite, which is known to cause oxidation of thiol groups and nitration of tyrosines and iv) react with CO_{2} to form nitroperoxycarbonic acid, which can oxidize deoxy adenosine and deoxy Guanosine moieties in free nucleotides and DNA to their oxo-derivatives. In addition, two superoxide anions can rapidly undergo a disproportion reaction in aqueous solutions (Equation 1) to produce hydrogen peroxide and oxygen.

\[
2 \text{O}_2^- + 2 \text{H}_2\text{O} \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 + 2 \text{OH}^- \quad (\text{Equation. 1})
\]

The reaction of Equation 1 is also catalyzed within the cells very efficiently by several isoforms of the enzyme, superoxide dismutase (SOD). The product, hydrogen peroxide formed in this reaction, being a relatively stable entity, can accumulate within the cells to serve its biological function, unless removed by the actions of catalase and peroxidase family of enzymes that convert it to water and oxygen. Genetic experiments in mice have shown that the deficiency in mitochondrial SOD makes the animal viable only for days (331), and a deficiency in the cytoplasmic SOD precipitates multiple diseases, including reduced life span, cataract, liver cancer and muscular degeneration (332,333). Moreover, damage to cells by reactive oxygen species is thought to underlie the mechanism of ageing processes (334).

Accordingly, superoxide dismutase and peroxidases are considered to be the major defensive enzymes capable of buffering the cellular components against damage inflicted by the oxidative stress.

Interestingly, NO can be considered as an anti-oxidant molecule because it can react with superoxide anions extremely rapidly to generate peroxynitrates, which is a reactive intermediate that can nitrosylate protein side chains and oxidize proteins, lipids and DNA. Peroxynitrates also react with thiol moieties of proteins and amino acids to form nitroso thiols that have been implicated in signal transduction.
Since we found in our experiments that the nuclear eNOS of MCF7 cells was predominantly phosphorylated at its Threonine-495 residue, and because we had over-expressed the chimeric proteins containing T495 motif (such as in T495-NLS and Syngene-NLS) in the same cells, it was of interest to examine if the presence of excess amounts of phosphorylated Threonine-495 motif in the cells affected the ROS generation capacity of cells.

3.1 Over expression of T495NLS and Syngene-NLS increases the ROS content of cells.

We examined the ROS content of MCF7 cells and compared it to that of MCF7 cells over-expressing the T495-NLS proteins by a standard procedure. When the cells were loaded with 2’, 7’-dichlorodihydrofluorescein diacetate (H2DCFDA) and were examined by flow cytometry, higher percentage of fluorescent cells were seen with MCF7/T495-NLS cells (75.47%) compared to the host MCF7 cells (56.39%). The detection and quantitation of fluorescein in some cells became difficult on account of high levels of EGFP expression. Nonetheless, the results clearly indicated that the overall ROS levels in the T495-NLS cells were higher as compared to the MCF7 cells (Figure 3.1). Since the T495-NLS protein was unlikely to produce ROS within the cells by itself, it became clear that the elevated ROS levels must originate due to a deregulation of endogenous mechanisms that can produce ROS.

![Figure 3.1 Flow cytometric analysis of H2DCF-DA-stained cells showing an increased fluorescence in MCF7/T495-NLS cells when compared to MCF7. Corresponding % positive population are depicted above each graph.](image-url)
3.2 T495NLS and SyngeneNLS proteins induce an increase in ROS production in specific sub-cellular compartments.

Since the levels of expression and localization of both T495-NLS and Syngene-NLS fusion proteins were similar – both were largely confined to nuclear and nucleolar compartments – it was of interest to examine if the increases seen in ROS also mapped to these sub-cellular compartments. In order to achieve this goal, it was required that the ROS species were detected at the cellular sites of their origin, where the local concentrations of these ROS are expected to be the highest. In order to assess the origin of superoxide anions in sub-cellular compartments of nuclei or nucleoli, we used a specific probe for superoxide, Hydroethidine (307,335,336). Hydroethidine-treated MCF7 cells or the stable clones harbouring NLS-1, T495-NLS or Syngene-NLS, together with a normal mammary epithelial cell MCF10A were examined by confocal microscopy. The results displayed in Figure 3.2 show that both MCF10A and MCF7 cells had detectable levels of superoxide anions within their nuclear compartments, but strikingly, the nucleolar compartments were more intensely stained with ethidium. Since the regions of nucleoli are enriched with the segments of chromosomal DNA that contain highly repetitive units of ribosomal genes, the nucleoli were expected to efficiently sequester ethidium and hydroxyl ethidium ions formed from the oxidation of dihydroethidium.
Superoxide anion production in focal areas of nuclei and nucleoli in cells detected by dihydroethidium (DHE)

Figure 3.2 Fixed and permeabilized cells were stained with 1μM DHE and were imaged by a confocal laser microscope using an excitation wavelength of 480nm and emission wavelength of 567 nm. The signal was very distinctly seen in the nucleolus of the cells. Both MCF7/T495-NLS and MCF7/Syngene-NLS cells showed a higher content of superoxide in the nucleoli as compared to MCF7 cells; MCF7/Syngene-NLS cells have the highest superoxide content.

The data therefore imply strongly that the more intense staining within the nucleolar sites might derive due to a greater conversion rate of hydroethidine to ethidium or 2-Hydroxy ethidium by superoxide anions present in the nucleolar environment. It was further seen in several experiments that MCF10A cells had marginally less intense ethidium staining in their nuclei and nucleoli as compared to MCF7 cells. An increase in the ROS has been noted earlier in cancer cells (189). Although a distinct increase in ROS levels was seen in MCF7 cells with flow cytometry, the corresponding increase did not appear to be highly significant in hydroethidine based assays (Figure 3.2). A higher rate of production of superoxide anions within the nucleoli was a common feature for both normal and transformed cells. This clearly indicates that the mechanism generating superoxide anions in this compartment may be inherent to normal cells themselves and it may be upregulated further in cancer cells.
Figure 3.2 shows that the targeting of EGFP by tethering it to a short nuclear localization sequence (NLS1), comprising of the amino acid sequence motif SSWRRKRSWKEN had caused no significant change on the rate of superoxide generation in nuclei or nucleoli. By contrast, the over expression and the specific targeting of T495-NLS and Syngene-NLS sequences elevated the rate of superoxide generation, in the nuclei as well as in the nucleoli, significantly (Figures 3.2). Thus, a mere expression of EGFP or its nuclear targeting by NLS1 did not appear to enhance the production of superoxide anions in the cells; excluding non-specific mechanisms. On the other hand, the data are consistent with the idea that an over-expression of T495 and Syngene motifs coupled with their nuclear and nucleolar targeting, probably plays a fundamental role in this process. Availability of mutant constructs that enable only nuclear, but not the nucleolar, targeting of these motifs, will allow further tests on this possibility.

3.3 Cells harboring T495-NLS and Syngene-NLS produce more peroxide

Superoxide anions produced within cells are quickly converted to hydrogen peroxide by the enzyme superoxide dismutase (SOD) according to the Equation 3.

\[
\text{SOD} \quad \text{O}_2^- + 2 \text{H}_2\text{O} \quad \rightarrow \quad 2\text{H}_2\text{O}_2 \quad (\text{Equation 3}).
\]

Hydrogen peroxide is a relatively stable entity, and is relatively less reactive, compared to superoxide. Cells tolerate reasonable amounts of \(\text{H}_2\text{O}_2\) without showing any adverse reactions. Hence SOD has been thought to be the first line of defense in containing the damage by superoxide anions. Since there was an increase in superoxide production in cells harboring T495-NLS and Syngene-NLS, we examined if the increase in superoxide would get translated into an increased peroxide levels in the same compartments. In order to trap the peroxide within the compartments, we took advantage of the sensitive Jenfluor px red, a substrate for hydrogen peroxide. It forms a red fluorescent product that can very rapidly bind covalently to the peroxide soon after its generation, and thereby gets focally immobilized. We hypothesized that since Jenfluor px Red has a \(K_m\) for \(\text{H}_2\text{O}_2\) in the range of 6 millimolar, a sufficient
build up of $\text{H}_2\text{O}_2$ concentration to show reactivity to Jenfluor px red is more likely to be in those locations in the cell, where it is being actively produced. Thus, reactivity to Jenfluor px red to a first approximation is expected to reveal the subcellular compartments, where superoxide ions are being generated and are being actively converted to peroxide.

Increased peroxides in nuclei and nucleoli of cells harbouring T495-NLS and Syngene-NLS

Figure 3.3 Fixed and permeabilised cells were stained with Jenfluor dye for 1 hour as per the manufacturer's instructions to detect endogenous peroxide. The red signal of peroxide is distinctly seen both in nucleus and nucleolus of MCF7/T495-NLS and MCF7/Syngene-NLS cells. MCF7/T495-NLS cells have the maximum amount of peroxide levels.

The results obtained in these experiments are displayed in Figure 3.3. It is evident from the figure that MCF7, MCF10A cells showed very little staining with Jenfluor whereas, T495-NLS and Syngene-NLS cells showed an elevated staining with this reagent, indicating an apparent increase in their peroxide content.
3.4 Jenfluor detects endogenous peroxide

We also carried out two sets of control experiments by exposing T495-NLS cells to catalase, and MCF7 cells to a catalase inhibitor, 3-Amino triazole. As can be seen from the Figure 3.4 A, a treatment of T495-NLS cells with catalase (100µg/ml for 30 minutes) attenuated the Jenfluor signals; whereas the treatment of MCF7 cells with the catalase inhibitor (20mM for 6hrs) enhanced the Jenfluor signals to the extent of 9.6 fold (Figure 3.4 B). These results together confirmed that indeed, intracellular peroxide was being measured by the Jenfluor staining.

**Figure 3.4 A** The MCF7/T495-NLS cells, with or without the treatment with catalase, were stained with Jenfluor to assess the peroxide levels. The peroxide levels are less in the catalase-treated cells with respect to the control, showing the specificity of the detection method.

**Catalase inhibitor induces stronger Jenfluor staining in MCF7 cells**
Figure 3.4 B MCF7 cells were treated with 3-aminotriazole, a catalase inhibitor, for a 20hrs duration and were stained with Jenfluor dye. A distinct signal of peroxide is seen in the treated cells, indicating that in MCF7 cells the peroxides are rapidly degraded by catalase. The data underscore the specificity of the detection system used. Quantitation of the peroxide levels in the nuclei of MCF7 cells after their exposure to a catalase inhibitor clearly shows a significant increase in their peroxide contents. ***=p<0.001.

3.5 MCF7/Syngene-NLS cells harbor higher levels of superoxide, but lower levels of peroxide, as compared to MCF7/T495-NLS cells.

It was noteworthy that the peroxide contents within nuclei and nucleoli of Syngene-NLS were relatively lower than seen with T495-NLS, in contrast to their relatively higher superoxide levels in nucleoli. The results are consistent with a model that in Syngene-NLS cells, the superoxides produced within the nucleoli are less efficiently utilized by dismutation machinery. Unlike focal localizations of superoxide-rich sites in the nucleoli, the distribution of peroxide-rich sites appeared to be relatively diffuse and was spread across nucleoli, nuclei and cytoplasm. Although the increases in superoxide were modest, the corresponding increases in peroxide levels in the cells were more substantial. This may be because peroxides in these cells originate from multiple pathways other than from superoxides generated in the nucleolus or they accumulate to high levels within the cells in view of their relative stability. Relatively higher amounts of peroxides detected in the cytoplasmic compartment might also derive from the more efficient conversion of superoxides in the cytoplasm due to contributions from the mitochondrial SOD.

Figure 3.5 Mean values of superoxide (A) and peroxide (B) levels in various cells as determined by the pixel analysis of the nucleolar and nuclear signals respectively are depicted. It is clearly seen that
MCF7/T495-NLS cells and MCF7/Syngene-NLS cells have higher levels of both species as compared to MCF7 and MCF10A cells. MCF7/Syngene-NLS cells have a higher level of superoxide, but a lower level of peroxide, as compared to MCF7/T495-NLS cells. *** p<0.001.

Quantitation of peroxide levels in the nucleoli of cells.

It can be seen in the Figure 3.5 A and B, that the superoxide as well as the peroxide content of both MCF7 and MCF10A cells were low. There was no appreciable increase in the nuclear superoxide or peroxide contents of MCF7-NLS1 cells despite high levels of expression and nuclear targeting of EGFP-NLS1 protein (Figure 3.2 panel E, and Figure 3.3 panel E, respectively). By contrast, sharp increases in the nuclear superoxide as well as peroxide contents were noted for T495-NLS (1.7 and 20.7 fold respectively) and Syngene-NLS (2.4 and 9.7 fold respectively) cells. A similar increase in peroxide was also manifest in their nucleoli, higher by 8.1 fold and 5.3 fold respectively as compared to MCF7 cells (Figure 3.5 C). It was also clear that there were 2.5 and 1.8 fold excess peroxide in the nuclei over nucleoli in T495-NLS and Syngene-NLS cells respectively.

Taken together, the superoxide and the peroxide measurements confirm that a major fraction of ROS in T495-NLS and Syngene-NLS cells originate from their nuclear and nucleolar compartments.
3.6 NOS inhibitors impair ROS generation in MCF7/T495-NLS cells

In order to assess to what extent the nuclear eNOS contributes to the peroxide generation in nuclei, we measured the intra-nuclear peroxide levels in the presence of specific pharmacological inhibitors to eNOS (N, N’, dimethyl L-Arginine, L-NMMA and 7-Nitro Indazole, 7-NI) (Figure 3.6A).

**Peroxide generation is sensitive to NOS inhibitors.**

![Image of fluorophore staining](image)

**Figure 3.6 A** MCF7/T495-NLS cells were treated with L-NMMA or 7-Nitroindazole for 20 hours in a complete medium and were then stained with Jenfluor to detect peroxides. The peroxide levels decreased in the treated cells, clearly implicating eNOS in the generation of ROS in these cells.

The peroxide content of T495-NLS cells were reduced substantially by two NOS inhibitors (N, N’-dimethyl L-Arginine and 7-Nitro Indazole) in independent experiments. The results are shown in Figure 3.6A and the quantitation of data is depicted in Figure 3.6B.
3.7 Increased ROS in MCF7/T495-NLS cells can be suppressed by exogenous BH4.

The results with NOS inhibitors implicated that eNOS was involved in the generation of a major amount of ROS in nuclei. Because the ROS generation capacity of eNOS gets augmented due to uncoupling resulting from a limiting supply of the oxidation-sensitive cofactor BH4, it was possible that the excess amounts of ROS generated in T495-NLS cells were due to uncoupling induced by depletions of BH4. This possibility was verified to be true in our experiments, because an addition of 1µM BH4 to the culture medium could reduce the nuclear ROS generation (Figure 3.7 A) by more than 80% in the treated cells (Figure 3.7 B).

Supplementation of exogenous BH4 attenuates ROS production in MCF7/T495-NLS cells
Figure 3.7A MCF7/T495-NLS cells were treated with 1 µM BH4 in a complete medium for an overnight-duration, and were then subjected to Jenfluor staining. The treated cells showed an attenuation in peroxide levels as seen by the 5.42 folds decrease in Jenfluor staining (Figure 3.7B).

Figure 3.7B Tetrahydrobiopterin treatment significantly reduces peroxide levels in nuclei of MCF7/T495-NLS cells *** p<0.001.

3.8 Ca\(^{++}\) mobilization increases ROS in MCF7/T495-NLS cells

eNOS function is regulated by intracellular Ca\(^{++}\) concentrations through Ca\(^{++}\)-CaM and an increased binding of CaM to eNOS leads to a more efficient generation of NO. Therefore, it was of interest to determine the effects of an increase in the intracellular Ca\(^{++}\) ions on the ROS levels. When we treated the T495-NLS cells with 1 µM of a Ca\(^{++}\) mobilization agent, thapsigargin, the nuclear peroxide levels increased. The data therefore provides evidence that an increased intracellular Ca\(^{++}\), under conditions of BH4 deficiency can generate substantially large amounts of ROS (Figure 3.8).

Intracellular Ca\(^{2+}\) mobilization contributes to increased nuclear peroxide
Fig 3.8 MCF7/ T495-NLS cells were treated with a calcium mobilization agent, thapsigargin. A striking accentuation in the peroxide levels was seen (lower panel) as compared to that seen in the control cells (upper panel).

4. Some in *vitro* consequences of augmented ROS generation by MCF7/T495-NLS and MCF7/Syngene-NLS cells

**ROS can be a modulator of physiological functions**

ROS can have multiple effects on cells. At low levels, ROS may participate in a variety of cellular processes (337), including gene expression (338), tumor metastasis, gene regulation, cytokine signaling etc, but at higher levels they can be destructive to cellular constituents, especially to metal-sulphur clusters present in proteins, can induce damage to the organelles such as mitochondria, can cause gene mutations and can even provoke apoptosis (339). The physiological role of ROS seems to be conserved through evolution, because there is evidence that simple microbes can sense ROS to activate redox-sensitive transcription. In yeast, H2O2 can induce transcription of genes such as gpx1, ctt1 through an elaborate pathway of signaling. In mammalian cells, several protein functions are known to be redox sensitive. For example, enzymes like tyrosine and ser/thr phosphatases, xanthine dehydrogenase, transcription factors like c-Myc, members of FOXO family, and tumor suppressors like p53 are known to be affected by ROS. Since MCF7/T495-NLS and MCF7/Syngene-NLS cells had elevated levels of ROS in them, it was of interest to determine if their tumor related properties were altered.

4.1 MCF7/T495-NLS cells have compromised colony forming ability in soft agar

MCF7 cells and the three clones of MCF7 cells having a stable expression of T495-NLS were found to grow in soft agar medium, forming colonies of heterogeneous sizes. For practical purposes, these were categorized as large and medium colonies. Representative colonies are shown in Figure 4.1a.

Number of colonies formed in each category by the parental MCF7 cells and three different clones of MCF7/T495-NLS are compared in Figure 4.1b. The two T495-NLS clones had a considerable decrease in their colony forming ability. This difference was seen in large and medium sized colonies of clone #5 and clone #1.
Although the criterion of colony size was not a tight phenotype, a clear trend of compromised colony forming ability of the MCF7/T495-NLS cells is indicated.

**Soft agar colony formation ability of MCF7/ T495-NLS cells is impaired**

![Image of colonies](image)

*Figure 4.1* Colony-formation in soft agar: $2 \times 10^4$ cells were plated in 0.3% agarose in a 35 mm dish. After 1 month of incubation, the colonies were counted and were photo-micrographed. Two types of colonies were seen (a) and were scored independently (b). A significant decrease in colony formation was seen in the clones as compared to the parental MCF7 cells *** $p < 0.001$.

A similar compromised growth was even clearer in two of the Syngene-NLS clones (Figure 4.2). In both the Syngene clones tested, both large and medium-sized colonies had reduced in number significantly. Because ROS has been implicated in cell adhesions through upregulation of integrin receptors (339), it is possible that the adhesive nature of T495-NLS and Syngene-NLS clones has been modified due to an elevated expression of ROS. Taken together, the data obtained on T495-NLS and Syngene-NLS cells have both indicated that anchorage-independence of these cells was significantly reduced most likely due to an increased ROS content.
MCF7/Syngene-NLS cells have a compromised ability to grow in soft agar

**Figure 4.2** Two clones of MCF7 cells generated using Syngene-NLS were subjected to soft agar colony formation assay. It is seen that these clones have lost their ability to grow in an anchorage-independent way. ***p<0.001

### 4.2 Increased ROS in tumor cells decrease their invasiveness through matrigel.

Alterations in the cell surface properties may affect tumor functions related to invasion and metastasis. Indeed in hepatoma cells, ROS has been found to promote tumor cell-invasion, which can be blocked by N-acetyl L-cysteine (340). In the case of T495-NLS cells, however, contrary to this expectation, it was found that the invasiveness of cells had greatly decreased compared to the parental MCF7 cells (Figure 4.2A). Furthermore, cells of two Syngene–NLS clones also showed much reduced percentage of input cells to invade through matrigel. (Figure 4.2B).
Matrigel invasion is impaired in MCF7/ T495-NLS cells

**Fig 4.2A** Matrigel invasion assay of MCF7 cells and its clone generated with T495-NLS. The clone has lost its ability to invade the matrigel * p< 0.05.

Syngene-NLS cells are compromised in invasion through matrigel

**Figure 4.2B** Matrigel assay shows that MCF7/Syngene-NLS clones are far less invasive as compared to the parental MCF7 cells.

4.3 Increase in ROS in T495-NLS and Syngene-NLS cells leads to increased 8-Oxo 2' -deoxygen Guanine formation.

When pyrimidine and purine bases are exposed to higher levels of ROS (H₂O₂, Superoxide, nitrosoperoxyxcarbonate ions etc), oxidation products of bases similar to those found *in vivo* are obtained *in vitro*, suggesting that the bases present in DNA, RNA and free nucleotide pools are susceptible to oxidative damage. Although both
pyrimidine and purine bases are susceptible to oxidation, purines, particularly Guanosine, seems to be more sensitive. A specific oxidation product, 8-Hydroxy-2′-deoxyribo Guanosine, and its tautomer, 8-oxo-2′deoxyribo Guanosine (8-Oxo-dG), have been fairly well characterized with oxidized DNA of cells. In addition to 8-Oxo-dG, the cellular DNA might have additional lesions in their dA, dC and dT residues as well as in the back bone of deoxy pentose sugar. Similarly, the cellular RNA may get damaged due to ROS-induced lesions in their guanosine, adenosine, cytidine and uracil residues. The 8-Oxy-dG has a structure very similar to d-Biotin and can even bind to avidin or streptavidin with sufficient affinity (341). Therefore, one can use labeled avidin or streptavidin to locate 8-Oxo-dG or 8-Oxo-rG moieties within the cells, by using protocols similar to those used in immunocytochemistry. In order to find out if the increased ROS content in T495-NLS and Syngene-NLS cells gave rise to an increased burden of 8-Oxo-dG and 8-Oxo-rG contents within the cells, we used a Cy5-labeled streptavidin (Cy5-SA) to locate such lesions in these cells and compared the results obtained with the parental MCF7 cells. The results from these experiments are shown in Figure 4.

**Detection of putative 8-oxo derivatives of Guanine in DNA, RNA and free nucleotides by Cy-5 labeled streptavidin**

*Figure 4.3A* Fixed and permeabilized MCF7 and MCF7/T495-NLS cells were stained with Streptavidin- Cy5 for 2 hrs at 37°C. The streptavidin-reactive 8-oxo derivatives were seen to be present in more amounts in the nucleoli of MCF7/T495-NLS cells (indicated by white arrows) as compared to the MCF7 nucleoli indicating an increased damage to guanosine residues inflicted by the ROS. Pixel density analysis of the fluorescent signals at nucleolar regions are depicted graphically on the right hand side. *** p<0.001.
The data shown in Figure 4.3 makes it clear that the staining patterns of Cy5-SA in MCF7 and MCF7/T495-NLS cells were broadly similar, but with quantitative differences. First, the general level of reactivity with Cy5-SA had increased in MCF7/T495-NLS cells, >2 folds, compared to MCF7 cells. Second, the pattern of staining within nucleoli was reminiscent of the results obtained with superoxide stain, dihydroethidine. Particularly, the nucleolar bodies (white arrows) and cytoplasm (yellow arrows) in many MCF7/T495-NLS cells had stained more brightly with Cy5-SA, than those in the MCF7 cells. These results are consistent with a greater extent of damage to Guanosine residues being inflicted by the generated ROS in MCF7/T495-NLS cells, and particularly, the damage seemed to be more pronounced within the nucleoli, rather than in the nuclei. An increased extent of damage seen in the cytoplasm was again consistent with data obtained from the use of inhibitors to NADPH-oxidase and mitochondrial respiration in MCF7/T495-NLS cells (please see Figure 6 – General Discussion), indicating that these two surrogate modes of ROS generation can substantially affect the cytoplasmic damage to RNA, free nucleotides and probably to mitochondrial DNA.

Formation of 8-Oxo-dG or other oxidized bases in DNA lead to their detachment from the DNA deoxyribose sugar chain leaving the abasic sites behind. The abasic sites then isomerize to reactive entities with aldehyde functions. Occurrence of such aldehyde functions in DNA, in a quantitative sense, is related to the extent of damage inflicted to DNA by ROS. We examined the reactivity of MCF7 cells and T495-NLS cells to an aldehyde specific probe which takes place according to reaction in Equation 4.

We therefore carried out experiments to examine if such abasic sites, giving rise to reactivity to an aldehyde reactive probe, are also increased in T495-NLS cells. The results are shown in Figure 4.
Aldehyde reactive probe detects more of abasic sites in nucleoli

Figure 4.3B Both MCF7 and MCF7/T495-NLS cells were treated with 1µM ARP for 20 hrs. The cells were then fixed, permeabilised and were stained with Streptavidin-Cy5. The ARP reactive abasic sites were observed only in the nucleoli of MCF7/T495-NLS cells. The lower panel shows magnified images and the white arrows indicate the ARP-specific signal.

Figure 4.3B shows clearly that the ARP was able to label the cytoplasm of both MCF7 and MCF7/T495-NLS cells, but the nucleolar labeling was seen to a distinctly greater extent in T495-NLS cells than was seen with MCF7 cells (Figure 4.3 B,lower panel), confirming that the abasic sites were more prevalent in their nucleoli. The strong reactivity seen in the cytoplasm of both the cell types may stem from the reactivity of the probe to aldehyde and keto-functions produced by glycolytic intermediates as well as by the oxidation of primary hydroxyl groups present in cytoplasmic macromolecules by the ROS generated.

Because the detection of 8-Oxo-dG residue by Cy5-SA was an indirect method, we attempted an independent and more direct experiment by using a specific antibody to
8-oxo-deoxy guanosine. As shown in Figure 4.3C, MCF10A cells were practically unreactive to the antibody in their nuclei and had only a weak staining in their cytoplasm (upper-most panel). When MCF10A cells were briefly treated with H2O2 (5µM, 30 minutes), the reactivity to antibody increased considerably (middle panel) indicating that the 8-oxo-dG was being generated in the cells in an ROS-dependent manner. Of note, there were antibody reactive spots within the nucleoli (yellow arrows) of cells treated with H2O2, which were absent before the treatment. It was possible that the antibody had cross reactivity to Guanosine residues that were present in RNA and part of the signals originated because of this reactivity. When the cells were treated with DNase free RNase A (100µg/ml, 15 minutes) the signal on these spots apparently diminished, but were not eliminated completely. These results support the idea that within nucleoli, the apparent anti-8-oxo-dG signals were distributed within RNA as well as within DNA. The results obtained with MCF10A may be contrasted with MCF7/ T495-NLS cells. It is seen that these cells brightly stained with anti-8-Oxo-dG antibody even without being treated with H2O2 (Fig 4.3C, b, middle panel). The strong cytoplasmic fluorescence seen due to 8-Oxo-dG antibody in the untreated MCF7/T495-NLS cells (Fig 4.3C,b, middle panel) may arise from several sources, such as the oxidation of mitochondrial DNA, from purine rich RNA sequences, which were resistant to RNase A and residual free dG and rG mono, di- and triphosphates, which were incompletely washed out after RNase treatments. MCF7 cells did not show any reactivity except a faint cytoplasmic fluorescence (Fig 4.3C, b, left panel).
Direct detection of 8-oxo-G derivatives by using the Anti-8-oxo-G antibody

Figure 4.3C. a. MCF10A cells were immunostained with an antibody to 8-Oxo-G. The nucleolar signal is clearly seen in response to H2O2 treatment (middle panel). The treatment of cells with RNAse leads to a partial loss of their nucleolar signal (lower panel). b. MCF7 and MCF7 T495-NLS cells were methanol fixed and were immunostained with anti 8-oxo-G antibody. MCF7/T495 cells show presence of a high level of 8-oxo-G residues, especially in the nucleolar compartment (middle panel) and the nucleolar signal gets reduced significantly after RNAse treatment (right panel).

Since there was a strong concordance among Cy5-SA, ARP and 8-oxo-dG antibody labeling experiments, it is reasonable to conclude that the MCF7/T495-
NLS cells harbor higher amounts of oxidized nucleotide bases and abasic sites in their DNA, as a consequence of increased ROS content.

5. Some in vivo consequences of augmented ROS generation by MCF7/T495- NLS and MCF7/Syngene-NLS cells.

Reactive oxygen species has been postulated to modulate several tumor cell properties, including their invasiveness, metastasis, and angiogenesis. Since MCF7/T495-NLS and MCF7/Syngene-NLS cells were found to have increased contents of endogenous ROS, it was of interest to compare tumor related properties between them and with the parental MCF7 cells. We used female nude/nude mice pre-implanted with 17-β-Estradiol pellets (4mg pellet; 60 days release; from Innovative Research of America, USA).

5.1 The tumor growth properties of MCF7/T495-NLS and MCF7/Syngene-NLS cells are different in vivo.

2.5 X 10^6 cells of MCF7, MCF7/T495-NLS or MCF7/Syngene-NLS tumor cells in 100μl of PBS were placed carefully under the skin of the mice using a 0.1ml Soloshot syringes (BD), and the tumor growth was monitored subsequently at frequent intervals. It was seen in three independent experiments that the MCF7/T495-NLS cells were the slowest to grow into tumors, and in one experiment, even failed to grow any tumor. The parental MCF7 cells grew into tumors of appreciable size in about 2-3 weeks in all the three experiments carried out independently. By contrast, MCF7/Syngene-NLS cells showed a more aggressive growth and formed larger tumors compared to MCF7 cells. These results from a representative experiment are shown in Figure 5.1.
Disparate *in vivo* behaviour of the T495-NLS and Syngene-NLS clones

A)

![Image of nude mice with tumors](image1)

Site of injection showing tumor

B)

![Image of excised tumors](image2)

C)

![Graph showing tumor weights](image3)

Figure 5.1 Tumorigenesis of MCF7 and its clones in nude mice

A) The *in vivo* tumorigenic ability was examined by a sub-cutaneous injection of 2.5 X10⁶ cells in the flank of nude mice. The tumor formation was evaluated after 4 weeks. Nude mice injected with the respective cells, showing tumor formation only in MCF7 and MCF7/Syngene-NLS. MCF7/T495-NLS cells did not give rise to a tumor while MCF7/Syngene-NLS gave rise to a larger one. B) Comparative sizes of the excised tumors. C) The weights of the excised-tumors are shown graphically.
Since the *in vitro* growth characteristics of MCF7/T495-NLS and MCF7/Sygene-NLS were comparable, the results obtained *in vivo*, were paradoxical. The results, however, underscored that changes in cellular ROS content need not be related to monotonic changes in the growth properties of tumor cells *in vivo*.

### 5.2 Tissue histology of MCF7, MCF7/T495-NLS and MCF7/Sygene-NLS Tumors are distinct.

The tissue histology of the tumors of MCF7, MCF7/T495-NLS and MCF7/Sygene-NLS appeared to be distinct in hematoxylin and eosin stained sections (Figure 5.2A). The MCF7/Sygene-NLS tumors had cells that were relatively less densely packed, were of larger size than MCF7 tumor cells and had apparently increased elaborations of micro-vessels in them, compared to MCF7 or MCF7/T495-NLS tumors.

**Tumor histology of MCF7/Sygene-NLS is different from MCF7 and MCF7/T495-NLS tumors**

![Figure 5.2A](image)

In keeping with their ability for rapid growth in the animal body, MCF7/Sygene-NLS tumors showed signatures of angiogenesis such as cells staining for endothelial cell-specific markers. For example, it was possible to demonstrate that tumor cells of Sygene-NLS expressed more amounts of von Willebrand factor (vWF) and PECAM-1 (Figure 5.2B) than MCF7 cells.
Immunohistochemical analysis of tumor sections of MCF7 and MCF7/Syngene-NLS

Figure 5.2B The sections of MCF7 and Syngene-NLS tumors were immunostained with antibodies to GFP (upper panel), vWF (middle panel) and PECAM-1 (lower panel). As expected, nuclear staining of GFP is seen only in MCF7/Syngene-NLS tumor, but not in MCF7 tumor. MCF7/Syngene-NLS tumor shows the angiogenic activity as evidenced by the presence of vWF and PECAM-1 positive cells.

In a double labeling experiment, nuclear GFP expression was seen in most cells and the presence of patches of PECAM-1 positive cells indicated that an active angiogenic programme was underway (Figure 5.2C).
Angiogenic activity seen in the tumor formed by MCF7/Syngene-NLS

![Image](image.png)

**Figure 5.2C.** The tumor section was stained with anti-GFP and anti-PECAM-1 antibodies. GFP positive cells were seen as violet nuclei. The patches of PECAM-1 positive cells (brown) seen indicate activation of an angiogenic process.

These results are consistent with tumor histology that Syngene tumors had a higher degree of angiogenesis.

### 5.3 T495-NLS tumor cells show signature of Epithelial Mesenchyme cell Transition (EMT).

Epithelial to mesenchymal transition is a critical step during organogenesis. EMT and its reverse process, mesenchyme to epithelial transition (MET), have been linked to the regulation of oncogenesis, tumor invasiveness and metastasis. In the mammary tissue, EMT is regulated through a complex mechanism involving several signaling molecules that includes, beta-catenin, Twist, Snail 1, Snail2, EGFR, c-Met, FOXC2 and TGFβ1. The receptor for hepatocyte growth factor c-Met, a well known inducer of EMT, has been proposed to cooperate with ErbB2/Neu for malignant transformation of mammary epithelial cells. We examined if MCF7/T495-NLS and MCF7/Syngene-NLS tumor cells to see if they expressed any markers of EMT. The data shown in Figure 5.3 show that, while MCF7 and MCF7/Syngene-NLS cells expressed relatively low levels of Vimentin, the MCF7/T495-NLS cells expressed copious amounts of this mesenchymal marker.
MCF7/T495-NLS and MCF7/Syngene-NLS show signature of EMT

Figure 5.3. Fixed and permeabilized cells were immunostained with an anti-vimentin antibody. MCF7/T495-NLS (lowest panel) express very high levels of vimentin, a mesenchymal marker.

Since induction of mesenchymal characteristic is associated with migration, invasion and metastasis of tumor cells, it is paradoxical that despite a high vimentin expression, T495-NLS cells showed poor invasive properties in an *in vitro* cell invasion assay. Because several studies have documented the existence of “hybrid cells” that show characteristics of both epithelial and mesenchymal cells, such cells have been recently proposed to be in a “metastable” state (342). Further, co-expression of mixed lineage markers within a cell has been regarded akin to a stem-cell gene-expression profile (343). Vimentin has also been regarded as a mammary stem-cell marker. It was, therefore, reasonable to examine if T495-NLS and Syngene-NLS cells expressed other stem-cell characteristics.
5.4 MCF7/T495-NLS and MCF7/Syngene-NLS cells have Stem cell characteristics

MCF7/T495-NLS and MCF7/Syngene-NLS have higher percentage of SP cells

Figure 5.4 A  Side population (SP) analysis of the clones in comparison to MCF7. The cells were labeled with Hoechst33342 in the presence or absence of verapamil, and the cells were analyzed by flow cytometry. The SP-population disappeared in the presence of verapamil. Similar results were obtained in three independent experiments.

Stem cells generally express ATP-dependent, membrane associated pumps to expel xenotoxic compounds from the cells’ interior. This phenomenon can be experimentally studied with a suitable dye that will accumulate in cells, but not in stem cells. The phenomenon of dye efflux, and, therefore, the “side population”-phenotype detectable with flow cytometry, provides an estimate of the fraction of stem-like cells. Dye efflux experiments done with MCF7 cells showed that a barely 0.7% of the population exhibited this phenotype. However, 3.5% of MCF7/Syngene-NLS cells and 4.1% of MCF7/T495-NLS cells, showed the same phenotype, representing 5 and 6 fold increase in the stem cell-like fraction in these populations respectively. Similarly, cytokeratin 19 has been regarded as a mammary stem cell
marker. Both MCF7/T495-NLS and MCF7/Syngene-NLS cells showed a higher expression level of Cytokeratin-19 compared to MCF7 cells (Figure 5.4B).

**MCF/T495-NLS and MCF7/Syngene-NLS express Cytokeratin-19**

Figure 5.4B Cells were examined for yet another marker of mammary stem cells namely, cytokeratin-19, by immunofluorescence. It is clearly seen that the clones, especially MCF7/T495-NLS, show a higher level expression of this marker.

The association of stem cell markers with MCF7/T495-NLS and MCF7/Syngene-NLS may provide a possible explanation to the observed differences seen in the *in vivo* tumor growth. Stem cells may remain quiescent or may proliferate rapidly to give rise to progenitors in response to cues from the micro-environment. On the basis of our data we propose that MCF7/T495-NLS cells progress to a more quiescent state whereas MCF7/T495-NLS cells adopt a more proliferative state.