FUNCTIONAL ELUCIDATION OF HUMAN SOMATOSTATIN RECEPTOR-3: IMPLICATIONS IN BREAST TUMOR BIOLOGY

by

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Abstract

Somatostatin (SST) inhibits cell proliferation through five SST receptors (SSTR1-5). Amongst all SSTR subtypes, SSTR2 and SSTR3 are the prominent receptor subtypes which exert antiproliferative effects in cells of different origin. SSTR2-mediated inhibition of cell proliferation is largely cytostatic, whereas SSTR3 is cytotoxic. Whether SSTR2/SSTR3 display synergistic antiproliferation than single receptor is not well understood. To ascertain the role of SSTR3, the present study was first conducted in HEK-293 cells which lack endogenous SSTRs expression. Cells were stably transfected with *wt*-SSTR3, treated with agonist and studied for dimerization, cAMP, receptor trafficking and signaling molecules. Since receptor signaling properties are confined in C-tail, cells expressing C-tail deleted SSTR3 were also studied for comparative analysis. *wt*-SSTR3 exists as preformed homodimer at cell surface and displays agonist-mediated cytotoxic effects. The cell surface expression, homodimerization and agonist-induced internalization of SSTR3 were independent of C-tail, whereas agonist-mediated apoptosis was lost upon C-tail deletion.

Next, HEK-293 cells cotransfected with SSTR2/SSTR3 were examined for heterodimerization and signaling molecules governing cell proliferation. Pb-FRET/CO-IP analyses suggest SSTR2/SSTR3 heterodimerization. The decreased cAMP upon agonist activation of SSTR2/SSTR3 suggests that this heterodimer is functional. Agonist-mediated SSTR2/SSTR3 antiproliferation was G_i-dependent, and involved apoptosis and cell-cycle arrest.

To derive direct pathological significance of the observations from heterologous system, additional experiments were conducted in two breast cancer cell lines MCF-7 and MDA-MB-231, which differ in origin and biochemical features including presence or absence of ER_{α}. Breast tumor cell lines overexpressing SSTR3 were studied for cell proliferation and downstream signaling molecules. EGF served as an index of positive cell proliferation. SSTR3 overexpression in MCF-7 (R3-MCF-7) and MDA-MB-231 (R3-MB-231) cells displayed inhibition of EGF-induced proliferation and enhanced antiproliferative effect of SSTR3-specific agonist in comparison to non-transfected cells. SSTR3 overexpression in R3-MCF-7 cells constitutively enhanced TUNEL staining, PARP-1 and $p27^{Kip1}$ expression suggesting apoptosis and cell-cycle arrest. Conversely, in R3-MB-231 cells, SSTR3 overexpression exerted cytostatic but not cytotoxic effects.

These results provide compelling evidence for antiproliferative role of SSTR3 in breast cancer cell lines. The constitutive activation of cytotoxic signaling in R3-MCF-7 but not R3-MB-231 cells reveals a distinct cell-specific role for SSTR3 in breast tumor biology.

Preface

I performed the majority of the work mentioned in this dissertation which includes designing the study, performing the experiments, data analysis and manuscript preparation. Chapters 2 and 3 are published in peer-reviewed journals. Chapter 4 is currently unpublished. The contents of the above chapters are presented with slight modifications from the published version to maintain consistency in the dissertation. The individual contribution of authors to the chapters are outlined below.

Chapter 2. War, S.A., Somvanshi, R.K. and Kumar, U. (2011) Somatostatin receptor-3 mediated intracellular signaling and apoptosis is regulated by its cytoplasmic terminal. **Biochimica et Biophysica Acta. (1813): 390-402.** I designed the study, performed the experiments and wrote the manuscript. Dr. Somvanshi helped in transfection. Dr. Kumar edited the manuscript.

Chapter 3. War, S.A., and Kumar, U. (2012) Coexpression of human somatostatin receptor-2 (SSTR2) and SSTR3 modulates antiproliferative signaling and apoptosis. **Journal of Molecular Signaling. 7, 5**. I performed the experiments and wrote the manuscript. Dr. Kumar designed the study and edited the manuscript.

Chapter 4. War, S.A., and Kumar, U. Human somatostatin receptor-3 distinctively induces apoptosis in MCF-7 and cell cycle arrest in MDA-MB-231 breast cancer cells. I designed the study, performed the experiments and wrote the manuscript. Dr. Kumar edited the manuscript.

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List of abbreviations and symbols

A - Acceptor

aa - Amino acid

AC - Adenylyl cyclase

ACTH - Adrenocorticotropic hormone

AIDS - Acquired immunodeficiency syndrome

Ala - Alanine

ATP - Adenosine triphosphate

BAX - Bcl2 associated X protein

C - Celsius

Ca^{2+} - Calcium

cAMP - Cyclic adenosine mono phosphate

CDKs - Cyclin-dependent kinases

CDKIs - Cyclin-dependent kinase inhibitors

CHO-K1 - Chinese hamster ovary-K1 cells

CNS - Central nervous system

CO-IP - Coimmunoprecipitation

C-tail - Carboxyl terminus

Cys - Cysteine

D - Donor

DNA - Deoxyribonucleic acid

ECL - Extracellular loop

EGF - Epidermal growth factor

ER - Estrogen receptor

ErbBs - Epidermal growth factor receptors

ERK - Extracellular signal-regulated kinase

EST - 17-β Estradiol

FBS - Fetal bovine serum

FITC - Fluorescein isothiocyanate

fmol - Femtomole

FSHR - Follicle stimulating hormone receptor

FSK - Forskolin

g - Gram

GDP - Guanosine diphosphate

GH - Growth hormone

GIT - Gastrointestinal tract

GnRH - Gonadotropin releasing hormone

GPCRs - G-protein coupled receptors

G proteins - Guanine nucleotide binding proteins

GTP - Guanosine triphosphate

h - Hour

HEK-293 - Human embryonic kidney-293 cells

ICL - Intracellular loop

IGF-1 - Insulin-like growth factor-1

JNK - c-Jun N-terminal kinase

K^+ - Potassium

kDa - Kilo Dalton

MAPK - Mitogen activated protein kinases

mg - Milligram

min - Minute

ml - Milliliter

mRNA - Messenger ribonucleic acid

MTT - [(3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)]

NAD - Nicotinamide adenine dinucleotide

ng - Nanogram

nM - Nanomolar

N-terminal - Amino terminus

OCT - OCT

PARP-1 - Poly (ADP-Ribose) Polymerase-1

Pb-FRET - Photobleaching fluorescence resonance energy transfer

PCNA - Proliferating cell nuclear antigen

PBS - Phosphate buffered saline

PF - Paraformaldehyde

PIP_2 - Phosphatidylinositol 4,5-bisphosphate

PIP_3 - Phosphatidylinositol 3,4,5-triphosphate

PI3K - Phosphatidylinositol-3-protein kinase

PPSST - Preprosomatostatin

PR - Progesterone receptor

PSST - Prosomatostatin

PTEN - Phosphatase and tensin homolog

PTP - Phosphotyrosine phosphatases

PTX - Pertussis toxin

RIPA - Radio-immune precipitation assay

RT - Room temperature

RTKs - Receptor tyrosine kinases

SD - Standard deviation

SE - Standard error

Ser - Serine

SHP-1 - Src homology region 2 domain-containing phosphatase-1

SRIF - Somatotropin release-inhibiting factor

SST - Somatostatin

SSTRs - Somatostatin receptors

Thr - Threonine

TMDs - Transmembrane domains

TNFα - Tumor necrosis factor alpha

TRAIL - TNFα-related apoptosis-inducing ligand

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling

Tyr - Tyrosine

wt - Wildtype

α - Alpha

β - Beta

δ - Delta

µ - Mu

µg - Microgram

µl - Microliter

µM - Micromolar

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I would like to thank my supervisor Dr. Ujendra Kumar for guiding me throughout my Ph.D. program. I am grateful to him for his continued support, patience and belief in me. His thoughtful approach to research was incredibly inspirational.

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I deeply appreciate my family for their love and prayers. Last, but definitely not the least, I am indebted to my wife for her unconditional support, strength and sacrifices.

THIS THESIS IS DEDICATED

TO MY FAMILY

Chapter 1: Introduction

1.1 G protein-coupled receptors (GPCRs)

Signal transduction is one of the core elements for maintaining cell viability and physiological functions (Venkatakrishnan et al., 2013). Membrane-bound proteins play a vital role in transducing the extracellular environmental cues into a biological signal (Venkatakrishnan et al., 2013). GPCRs constitute the largest cell surface proteins superfamily in the human genome with more than 800 members (Fredriksson et al., 2003; Pierce et al., 2002; Venkatakrishnan et al., 2013). The fact that GPCRs are the leading therapeutic targets (>50%) in drug research attests the enormity of their physiological roles (Ali and Coombes, 2000; Flower, 1999; Fredriksson et al., 2003; Gudermann et al., 1995). GPCRs are broadly categorized into five families based on their amino acids (aa) sequence (Millar and Newton, 2010). The rhodopsin family (Family A) is the largest and consists of 672 members including odorant, rhodopsin and adrenergic receptors. The remaining GPCR families are relatively smaller and include Frizzled/Taste (36 members), Adhesion (33 members), Glutamate (22 members) and Secretin (15 members) families (Millar and Newton, 2010). As illustrated in Figure 1.1, the common molecular signatures in GPCRs include seven transmembrane domains (TMDs), an amino terminus (N-terminal) which is generally extracellular and an intracellular carboxyl terminus (C-tail). There are exceptions, e.g., gonadotropin releasing hormone (GnRH) receptor, member of GPCR family devoid of C-tail (Millar and Newton, 2010). GPCRs are activated by an array of extracellular signals which include light, taste, odorants, hormones, fatty acids, aa, intercellular messengers and neurotransmitters

(Ferguson, 2001; Millar and Newton, 2010). GPCRs regulate several physiological processes such as exocrine and endocrine secretions, contraction of cardiac and smooth muscles, nociception, fluid homeostasis, blood pressure and immune responses (Rozengurt, 2007). Also, GPCRs aberrant expression and signaling contribute to the pathogenesis of several diseases (Somvanshi et al., 2011a). In the central nervous system (CNS), GPCRs sense neurotransmitters to regulate satiety, body temperature, and behavioral responses (Allen et al., 2007; Graler and Goetzl, 2002; Premont and Gainetdinov, 2007; Rozengurt and Walsh, 2001; Sugimoto and Narumiya, 2007). GPCRs interact with heterotrimeric guanine nucleotide binding proteins (G proteins) comprised of G_{α}, G_{β}, and G_{γ} subunits. In the resting state, the G_{α} subunit is bound to guanosine diphosphate (GDP), and associated with $G_{\beta\gamma}$ subunits. Upon ligand binding, the receptor conformation is altered, which in turn, activates the G proteins resulting in GDP to guanosine triphosphate (GTP) exchange on G_{α} subunit. The GTP-bound G_{α} subunit then dissociates from the $G_{\beta\gamma}$ subunits to interact with effectors such as adenylyl cyclase (AC) resulting in the modulation of second messenger cyclic adenosine mono phosphate (cAMP) and the corresponding signaling events (Birnbaumer, 1992; Gilman, 1987; Neves et al., 2002; Patel, 2004). There is preponderance of evidence suggesting the role of GPCRs in abnormal biological processes such as tumorigenesis acting either independently or synergistically with cognate receptors (Rozengurt, 2007). There is an emerging notion that GPCRs also initiate a cascade of intracellular signaling events that play key roles in the inhibition of cell proliferation (Patel, 1999; Weckbecker et al., 2003).

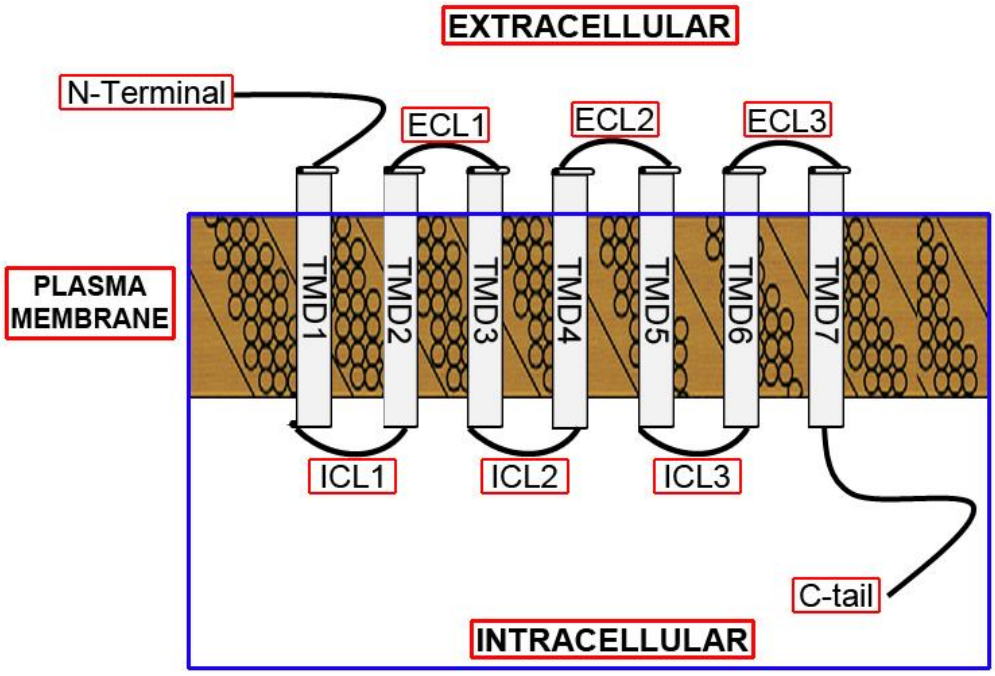

Figure 1.1 Illustration of GPCR structure. Schematic representation of GPCR with an extracellular N-terminal, intracellular C-tail, and seven transmembrane domains connected by three extracellular and three intracellular loops.

GPCR functioning is characterized by ligand binding which changes the receptor conformation to an active state and promotes stability (Luttrell and Gesty-Palmer, 2010). This is followed by a cascade of events such as modulation of second messenger cAMP and other downstream signaling molecules. Although the change in receptor conformation initiates signaling, it also acts as an active substrate for GPCR kinases, which phosphorylate the receptor and disrupt signaling (DeWire et al., 2007). This is followed by the recruitment of arrestins which play a major role in the signal termination by targeting the receptor to the endosomes via internalization (Luttrell and Gesty-Palmer, 2010). The internalized receptor is either targeted for lysosomal degradation (desensitization) or recycled back

(resensitization) to the plasma membrane (Calebiro et al., 2010). In case of GPCRs which recycle to plasma membrane, receptor internalization serves to restore agonist responsiveness.

1.2 GPCRs oligomerization

One of the core areas of interest surrounding the molecular events that regulate the functional dynamics of GPCRs is dimerization. GPCRs were earlier perceived to exist and act as monomeric entities. In the last 20 years, the evidence from a growing body of research has confirmed receptor-receptor interactions among GPCRs leading to the formation of dimers or higher order oligomers (Duran-Prado et al., 2008; Grant et al., 2004a; Grant et al., 2004b; Milligan, 2007; Patel et al., 2002a; Rocheville et al., 2000a; Rocheville et al., 2000b; Terrillon and Bouvier, 2004). If two identical proteins interact, they are called homodimers, whereas heterodimerization is a term used to define an interaction between two different proteins. There is emerging evidence suggesting the crucial role of dimerization in maintaining the receptor's activity profile (Bouvier, 2001; Milligan and White, 2001). GPCRs assemble as homo- and/or heterodimers in a receptor-specific manner to modulate receptor functions (Cvejic and Devi, 1997; Golebiewska et al., 2011; Grant et al., 2008; Grant et al., 2004a; Grant et al., 2004b; Jordan and Devi, 1999; Milligan, 2007; Rocheville et al., 2000a; Rocheville et al., 2000b). The dimerization domain among GPCRs is not restricted to any specific segment of the receptor, and can vary from N-terminus, TMDs or residues comprising the C-tail. For example, TMD6 region of β-adrenergic receptors has been described to play a critical role in

regulating receptor dimerization and signaling consequences (Hebert et al., 1996). Although, several studies have revealed the role of oligomerization in modulating the signaling features of the native receptors, the exact relevance of such interactions in biological functions is not yet fully understood. One of the first conclusive evidence of dimerization was provided for $GABA_B$ heteromer, which is a complex comprised of $GABA_BR1$ and $GABA_BR2$ subtypes (Marshall et al., 1999a; Marshall et al., 1999b). The protomers are inactive in their native state, but form a functionally active signaling unit upon association. In the heteromeric complex, $GABA_BR1$ confers the ligand binding ability, whereas $GABA_BR2$ provides the platform for effector coupling and downstream signaling events. The identification and quantification of dimerization varies based on the method used, e.g., Photobleaching Fluorescence Resonance Energy Transfer (Pb-FRET), coimmunoprecipitation (COIP) analysis, Bioluminescence Resonance Energy Transfer, etc.

1.3 Somatostatin (SST)

In 1973, Brazeau and colleagues from Roger Guillemin's laboratory at the Salk Institute reported potent inhibitory effects of ovine hypothalamic extracts on the release of GH from pituitary somatotrophs (Brazeau et al., 1973). These antisecretory properties were attributed to cyclic polypeptide SST or somatotropin release-inhibitory factor (SRIF). This remarkable discovery in neuroendocrinology attracted significant attention after Roger Guillemin shared the Nobel Prize in Medicine or Physiology in 1977. Consequently, several pioneering studies further uncovered the role of SST in many patho-/physiological conditions [*reviewed by*

(Patel, 1999; Weckbecker et al., 2003)]. SST biosynthesis is not limited to hypothalamus, but other CNS sites, gastrointestinal tract (GIT), peripheral neurons and the delta (δ) cells of pancreatic islets also contribute significantly to local SST production (Arimura et al., 1975; Dubois, 1975; Hokfelt et al., 1975; Luft et al., 1974; Orci et al., 1975; Patel and Reichlin, 1978; Pelletier et al., 1975; Polak et al., 1975). Altered status of SST expression has been correlated with several pathological conditions (Banki et al., 1992; Beal et al., 1985; Grouselle et al., 1998; Krantic et al., 1992; Kumar, 2005). SST-like immunoreactivity was prominent in stroma in the normal breast tissue, whereas, in malignant breast tumors it predominantly localized in epithelial cells (Watt et al., 2008). Due to widespread distribution and activity, SST is now recognized as a multifaceted peptide with two bioactive products; tetradecapeptide SST-14 which was first identified in the hypothalamus and SST-28, N-terminal extension of SST-14 containing 28 aa discovered later in GIT (Brazeau et al., 1973; Burgus et al., 1973; Pradayrol et al., 1980). SST, a phylogenetically ancient peptide is not only found in all vertebrates but also in invertebrates including protozoa as well as plant kingdom (Reichlin, 1983b).

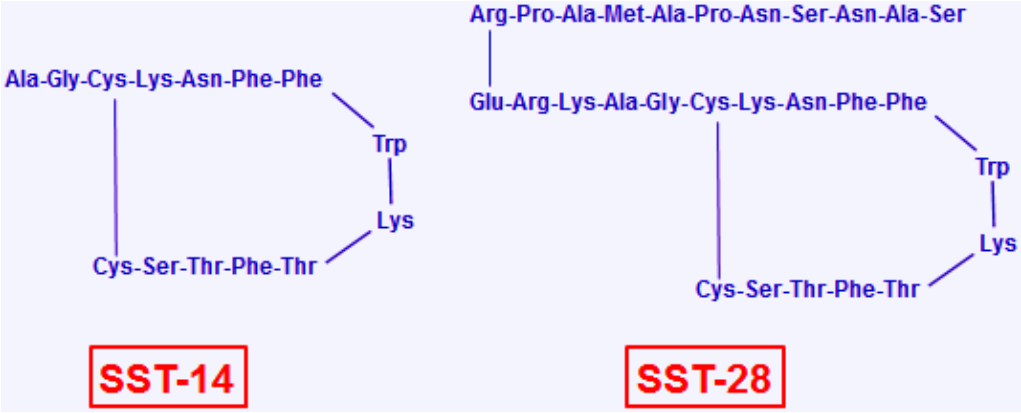

Figure 1.2 Schematic representation of SST-14 and SST-28. Illustration of cyclic structure and amino acid composition of SST-14 and SST-28.

1.3.1 Processing and distribution of somatostatin

In humans, SST is synthesized from a single precursor molecule, preprosomatostatin (PPSST) containing 116 aa, through a cascade of post-translational enzymatic cleavages to yield prosomatostatin (PSST) (Patel, 1999). PSST, a 92 aa protein is further processed at C-tail region by precursor convertases to generate SST-14 and SST-28. The cyclic structure of SST-14 includes a disulfide bond between the Cys^3 and Cys^{14} residues (Figure 1.2). Both peptides are produced in different proportions depending on the precursor processing. SST-14 is prominently expressed in pancreatic islets, stomach and neural tissues, whereas it is the only isoform identified in retina, peripheral nerves and enteric neurons (Patel et al., 1981). Approximately 20-30% of total SST-like immunoreactivity in the brain is attributed to SST-28 (Patel et al., 1981). The intestinal mucosal cells constitute the major peripheral pool of SST-28, which is produced as an end product of PSST processing (Patel, 1999).

1.3.2 Physiological actions of somatostatin

SST acts on numerous targets such as brain, pituitary, GIT, pancreas, adrenals, thyroid, kidneys and immune cells where it functions as neurotransmitter, autocrine, paracrine and endocrine regulator, as well as potent inhibitor of cell proliferation (Barnett, 2003; Patel, 1999; Reichlin, 1983a). SST plays an important role in neurotransmission to modulate cognitive, sensory, locomotor and autonomic

functions (Barnett, 2003; Epelbaum et al., 1994; Patel, 1999; Reichlin, 1983b). Its actions in midbrain include the inhibition of dopamine release, whereas in hypothalamus, not only it inhibits the release of norepinephrine, thyrotropin-releasing hormone and corticotrophin-releasing hormone, but also leads to its own endogenous suppression. SST actions in the pituitary result in the inhibition of GH, prolactin and thyrotropin (thyroid-stimulating hormone), whereas in the GIT, the secretion of majority of hormones including cholecystokinin, gastric-inhibitory peptide, gastrin, motilin, neurotensin and secretin is suppressed (Barinaga et al., 1985; Moller et al., 2003). The picomolar dose of SST paradoxically stimulated GH release from the pituitary cell culture model, most likely via cAMP pathway (Cordoba-Chacon et al., 2012). In addition, SST also counteracts the secretion of gastric acid, pepsin, pancreatic enzymes, bile and intestinal fluids (Weckbecker et al., 2003). Although, SST negatively impacts gastric emptying, gallbladder contraction and small intestine segmentation, conversely the splanchnic vasoconstriction response is stimulated (Patel, 1999). SST has been implicated in several neuropathological conditions such as Alzheimer's disease (Davies et al., 1980; Dournaud et al., 1994; Dournaud et al., 1995; Geci et al., 2007; Grouselle et al., 1998; Kumar, 2005; Mouradian et al., 1991), Huntington's disease (Aronin et al., 1983; Beal et al., 1988b; Kumar et al., 1997; Patel et al., 1991; Rajput et al., 2011), Parkinson's disease (Beal et al., 1988a; Epelbaum et al., 1994; Soghomonian and Chesselet, 1991; Strittmatter et al., 1996), AIDS encephalitis (Fox et al., 1997), excitotoxicity (Kumar, 2004, 2008; Rajput et al., 2012) and epilepsy (Strowbridge et al., 1992). Recent reports have also revealed that SST might act as an endogenous

antiepileptic in rats by maintaining an inhibitory homeostasis particularly in the hippocampus, suggesting an important therapeutic target in epilepsy (Tallent and Qiu, 2008). The multiple physiological roles of SST generated great interest as evident by numerous clinical studies investigating its potential in diseases ranging from diabetes, bleeding gastric ulcers and pancreatitis, to hypersecretory pituitary adenomas, gastrinomas, insulinomas, glucagonomas and vipomas (Reichlin, 1983a, b; Weckbecker et al., 2003). The ability to inhibit cell proliferation is one of the most studied functions of SST. The antitumor role of SST involves both direct and indirect actions. The most prominent direct actions of SST include the blockade of cell division and activation of programmed cell death (Kumar and Grant, 2010; Moller et al., 2003; Patel, 1999; Sharma et al., 1996; Susini and Buscail, 2006). SST-induced cytostatic and cytotoxic effects have been well appreciated with important therapeutic implications in tumor biology. The indirect antiproliferative actions of SST include suppression of essential growth factors and hormones for tumor growth, antiangiogenic role to restrict the blood supply to the growing tumors, and regulation of immune response to counter tumor progression and its metastasis (Susini and Buscail, 2006). SST analogs have shown promise in the clinical management of pituitary tumor, acromegaly (Ben-Shlomo and Melmed, 2008). SST system has also been suggested as a potential therapeutic target in many tumors such as hepatocellular, pancreatic and breast cancer, as well as medulloblastoma and medullary carcinoma of thyroid (Dimitroulopoulos et al., 2007; Guillermet et al., 2003; Hauser et al., 2009; Papotti et al., 2001; Reynaert et al., 2007; Rivera et al., 2005; Watt et al., 2008; Watt et al., 2009; Watt and Kumar, 2006).

1.4 Somatostatin receptors (SSTRs)

The diversity in physiological actions and receptor binding characteristics of SST-14 *vs.* SST-28 led to the concept of multiple SSTRs (Mandarino et al., 1981; Srikant and Patel, 1981). SST binds with nanomolar (nM) affinity to five distinct G_i-coupled Family A GPCRs namely SSTR1-5, which were first characterized by Schonbrunn and Tashjian in 1978 (Kumar and Grant, 2010; Schonbrunn and Tashjian, 1978). SSTRs have been studied in detail including their cloning, sequencing and tissue distribution (Bruno et al., 1992; Kluxen et al., 1992; O'Carroll et al., 1992; Viollet et al., 2008; Yamada et al., 1993a; Yamada et al., 1992a; Yamada et al., 1992b; Yamada et al., 1993b; Yasuda et al., 1992). The cloning of individual SSTRs had a significant impact on understanding the mechanisms of signal transduction pathways. The genes encoding all five SSTR subtypes with the exception of SSTR2 are intronless, and are located on different chromosomes (Patel, 1999; Patel et al., 1993; Vanetti et al., 1992). Based on their sensitivity to octapeptide analogs such as octreotide (OCT), SSTRs have been further classified into SRIF-1 and SRIF-2. The members of SRIF-1 include SSTR2, SSTR3 and SSTR5, and are activated by OCT, whereas SRIF-2 members, SSTR1 and SSTR4 fail to exhibit such property (Kumar and Grant, 2010; Reisine and Bell, 1995; Tran et al., 1985). SSTRs-like immunoreactivity has been reported in CNS, pituitary, pancreas, GIT, spleen, kidneys, lungs, thyroid, peripheral nervous system, blood vessels, immune cells etc. in a tissue-specific manner but often with overlapping pattern of distribution (Watt et al., 2008). The physiological SSTRs expression in rats corresponds to approximately 220-360 fmol/mg protein (Rocheville et al., 2000b).

Although, many tissues express multiple SSTRs, only one or two receptors are predominantly expressed in a tissue-specific manner**.** Importantly, tumors of pituitary, pancreas and breast, as well as insulinomas, neuroblastoma and glioma express SSTRs in a receptor-specific manner (Epelbaum et al., 1994; Ferone et al., 1999; Fukusumi et al., 1997; Hervieu and Emson, 1998; Patel, 1999). SST and SSTRs gene expression is modulated by several extracellular and intracellular mediators including 17β-estradiol (EST), which induces SST, SSTR2 and SSTR3, whereas exhibits an opposite effect on SSTR5 (Djordjijevic et al., 1998; Kimura et al., 1998; Patel, 1999; Visser-Wisselaar et al., 1997). The development and characterization of receptor specific antibodies for SSTRs has immensely contributed to the understanding of the receptor characteristics (Dournaud et al., 1996; Fischer et al., 2008; Handel et al., 1999; Helboe et al., 1997; Hukovic et al., 1996; Kumar et al., 1997; Kumar et al., 1999; Lupp et al., 2011; Lupp et al., 2012; Schulz et al., 1998).

SSTR subtypes exhibit great diversity in aa composition ranging from 363-418 with approximately 39-57% sequence resemblance (Kumar and Grant, 2010; Patel, 1999; Reisine and Bell, 1995). The highest sequence similarity of 55-70% is confined to TMDs across the SSTR family, whereas the maximum variability is frequently observed in the N-terminal and C-tail regions (Patel, 1999; Patel et al., 1995; Reisine and Bell, 1995). All human SSTR subtypes with the exception of SSTR3 exhibit a conserved Cys residue located 12 aa downstream of seventh TMD, which might serve as potential cysteine palmitoylation site for membrane anchorage (Patel, 1999). Among the five subtypes, SSTR3 possesses the longest C-tail

comprised of 100 aa (Patel, 1999). In terms of sequence conservation, SSTR1 shows approximately 94-99% similarity across human, rat and mouse species, whereas 93-96% of SSTR2 sequence is conserved between human, rat, mouse, porcine and bovine isoforms. While SSTR4 shows 88% sequence similarity across human and rat isoforms, SSTR3 and SSTR5 share least structural conservation as evident by approximately 82-83% sequence homology between receptors of human and rodent origin (Patel, 1999; Patel et al., 1995; Reisine and Bell, 1995). All SSTR subtypes possess multiple glycosylation sites which are generally confined to N-terminal and the second extracellular loop (ECL) (Kumar and Grant, 2010). In addition, several aa in the second and third intracellular loops (ICLs) as well as C-tail serve as potential phosphorylation sites (Ghosh and Schonbrunn, 2011; Hipkin et al., 2000; Kao et al., 2011; Kumar and Grant, 2010; Liu et al., 2009b; Liu et al., 2008). Moreover, the ligand binding pocket for all SSTRs is located within the residues forming ECL-II and TMDs3-7 (Greenwood et al., 1997; Patel, 1999).

SSTR1 (391 aa) was the first member in its family to be cloned from human pancreatic islets mRNA using reverse transcription polymerase chain reaction (RT-PCR) technique (Yamada et al., 1992a). This important finding had a tremendous impact on further understanding of SSTR functions. Human gene encoding for SSTR1 has been mapped to 14q13 region of chromosome 14 (Yamada et al., 1993b). SSTR1 possesses several glycosylation and phosphorylation sites, in addition to a conserved cysteine palmitoylation site for membrane anchorage. SSTR1 is expressed in several organs such as brain, pituitary, pancreas, stomach, liver, kidneys etc. In rat insulinoma cells, SSTR1-like immunoreactivity was also

found in sub-cellular compartments, in addition to plasma membrane suggesting a potential role for intracellular pool of receptors in specific biological functions (Ammon et al., 2002; Roosterman et al., 1997; Stroh et al., 2000). Human SSTR1 was resistant to agonist-induced internalization when expressed in chinese hamster ovary-K1 (CHO-K1) and human embryonic kidney-293 (HEK-293) cells, but was instead upregulated at the plasma membrane from the sub-cellular compartments (Hukovic et al., 1996; Hukovic et al., 1999). The authors initially speculated the potential contribution of C-tail residues in the failure of receptor internalization. Further studies revealed that C-tail deletion did not alter SSTR1 trafficking in response to agonist, therefore implicating a possible role for other receptor domains (Csaba and Dournaud, 2001; Hukovic et al., 1999). Conversely, agonist mediated upregulation of human SSTR1 was completely abrogated upon C-tail deletion (Ramirez et al., 2005). This study uncovered a crucial role of a C-tail Ser^{360} residue in regulating receptor upregulation. COS cells expressing human SSTR1, exhibited approximately 20% internalization in response to agonist, therefore suggesting internalization to be a cell-specific phenomenon (Nouel et al., 1997). Furthermore, SSTR1 of rat origin efficiently internalized upon agonist activation when expressed in rat pancreatic insulinoma and HEK-293 cells (Roosterman et al., 1997; Roth et al., 1997a).

In 1992, Yamada et al successfully cloned and characterized human SSTR2 along with SSTR1 (Yamada et al., 1992a). The location for the human gene encoding SSTR2 is confined to 17q24 region of chromosome 14 (Yamada et al., 1993b). Alternative splicing in SSTR2 mRNA generates two variants SSTR2A and

SSTR2B which differ in the length of C-tail. As a result SSTR2A contains 369 aa whereas SSTR2B is comprised of 346 aa (Moller et al., 2003; Reisine et al., 1993; Vanetti et al., 1992). There are several extracellular N-linked glycosylation sites as well as intracellular phosphorylation sites localized in the ICLs and C-tail of the human SSTR2. The difference in the number of potential phosphorylation sites between SSTR2A and SSTR2B plays a determinant role on receptor characteristics such as agonist-mediated desensitization (Moller et al., 2003). Unlike other SSTR subtypes, SSTR2 has the unique ability to induce the expression of SST (Delesque et al., 1997; Rauly et al., 1996). The tissue distribution sites for SSTR2 include brain, pituitary, pancreatic islets, stomach, kidneys etc. Agonist-stimulated desensitization and internalization of SSTR2 varies depending upon the receptor origin and the cell line studied (Csaba and Dournaud, 2001; Grant et al., 2008; Grant et al., 2004a; Hukovic et al., 1996). For example, CHO-K1 cells transfected with human SSTR2 displayed 25% internalization, whereas mouse SSTR2 internalization was 75% in COS-7 cells (Hukovic et al 1996; Nouel D et al 1997 pp296). Furthermore, mutational analysis has demonstrated that Thr and Ser phosphorylation in SSTR2 C-tail plays a major role in agonist-induced desensitization and internalization (Hipkin et al., 1997; Nouel et al., 1997; Roth et al., 1997b). SSTR2 displayed modest upregulation at the cell surface in response to prolonged agonist exposure (Hukovic et al., 1996). Previous studies have also implicated SSTR2 internalization in governing receptor downstream signaling (Sarret P et al 1999 pp19294; Boudin H et al 2000 pp5932).

Human SSTR3 was cloned and functionally characterized as 418 aa protein (Yamada et al., 1992b; Yamada et al., 1993b). On the other hand, the murine variant of SSTR3 contains 428 aa (Corness et al., 1993). SSTR3-like immunoreactivity has been reported in brain, pituitary, pancreas, stomach, liver and spleen (Kumar, 2007; Kumar et al., 1997; Kumar et al., 1999; Patel, 1999). SSTR3 is highly expressed in normal human thymus as well as in thymoma (Ferone et al., 2000; Ferone et al., 1999). In response to agonist, both rat and human SSTR3 undergo internalization in a cell-dependent manner (Hukovic et al., 1996; Roosterman et al., 1997; Roth et al., 1997a; Roth et al., 1997b). Agonist-mediated internalization of human SSTR3 was followed by lysosomal degradation, whereas rat SSTR3 recycled back to the plasma membrane after agonist-stimulated endocytosis (Hukovic et al., 1996; Roosterman et al., 1997; Roth et al., 1997a; Roth et al., 1997b). Although, rat SSTR3 internalization was independent of its coupling with pertussis toxin (PTX)-sensitive G proteins, four phosphorylation sites in C-tail were crucial for desensitization and internalization (Roosterman et al., 1997; Roth et al., 1997a). Also, the role for N-terminal region of rat SSTR3 in modulation of receptor transport to the plasma membrane has been described previously (Ammon et al., 2002). Most importantly, rat SSTR3 internalization in transfected rat insulinoma cells has been shown to be associated with β-arrestin in clathrin coated pits prior to its recruitment back to the plasma membrane (Kreuzer et al., 2001).

SSTR4 was cloned and characterized as a 42 kDa member comprised of 388 aa with gene location on 20p11.2 region of chromosome 20 (Demchyshyn et al., 1993; Patel, 1999; Rohrer et al., 1993; Yamada et al., 1993a; Yamada et al., 1993b).

Both rat and human isoforms of SSTR4 have comparable molecular weight (Helboe et al., 1997; Patel, 1999). Unlike other SSTRs, enzymatic N-linked glycosylation has not been reported for human SSTR4, despite a potential glycosylation site (Helboe et al., 1997). In addition, three potential phosphorylation sites have been identified in ICL-II and ICL-III regions of the receptor (Demchyshyn et al., 1993). The regions showing SSTR4-like immunoreactivity include brain, stomach, pancreatic islets, lungs and placenta (Patel, 1999). Human SSTR4 demonstrated rapid agonist-mediated internalization (29%) in CHO-K1 cells, whereas, the receptors were upregulated (22%) at the plasma membrane following 22 h of continued agonist exposure (Hukovic et al., 1996). Conversely, there was no evidence for agonist-induced internalization of rodent SSTR4 in rat insulinoma and HEK-293 cells (Kreienkamp et al., 1998; Roosterman et al., 1997; Roth et al., 1997b). Whether this represents a cell-specific phenomenon or the inherent nature of the receptor is not well understood. The inability to visualize the receptor in the cytoplasmic compartment upon agonist activation may well be attributed to the efficient recycling of the internalized receptor (Smalley et al., 2001). Interestingly, mutation of Thr^{331} residue to Ala in C-tail region of rat SSTR4 sensitized the receptor to agonist-induced internalization but not recycling to the cell surface (Kreienkamp et al., 1998). In addition to *in vitro* observations, SSTR4 failed to internalize *in vivo* following intracerebroventricular injection of SST in rats (Schreff et al., 2000). Recently, time-dependent internalization of human SSTR4 has been reported in HEK-293 cells (Somvanshi et al., 2009). Human SSTR4 internalized efficiently at 15 min post agonist activation, whereas the receptor-like immunoreactivity was comparable to

control upon agonist exposure for 30 min, which might provide an indirect explanation for the rapid receptor recycling.

Human SSTR5 is comprised of 363 aa with a molecular size of 58 kDa (Yamada et al., 1993a). SSTR5 gene is located on 16p13.3 region of the chromosome 16 (Patel, 1999; Yamada et al., 1993b). SSTR5 is mostly expressed in the brain, pituitary, pancreatic islets, stomach etc. (Patel, 1999). Because of the glycosylated nature of the receptor, SSTR5 exists in the size range of 52-66 kDa (Patel, 1999). There is evidence suggesting the presence of at least two glycosylation sites localized in the extracellular N-terminus region of human SSTR5. The maximum agonist-induced receptor internalization of approximately 66% was observed at 1 h post-treatment in CHO-K1 cells expressing human SSTR5 (Hukovic et al., 1996). Similarly, rat insulinoma, HEK-293 and COS-7 cells expressing rat SSTR5 demonstrated significant degree of internalization in response to SST-28, whereas no receptor internalization was observed upon treatment with SST-14 with the exception of COS-7 cells which exhibited moderate degree of internalization (Csaba and Dournaud, 2001; Hukovic et al., 1996; Roosterman et al., 1997; Roth et al., 1997b; Stroh et al., 2000). Interestingly, electron microscopy demonstrated clathrin-mediated intracellular trafficking of rat SSTR5 in COS-7 cells following SST-14 treatment (Stroh et al., 2000). Furthermore, the apparently unchanged receptor-like immunoreactivity at the plasma membrane was attributed to rapid recycling of the internalized receptor as well as cell surface recruitment of receptors from the intracellular pool (Csaba and Dournaud, 2001; Stroh et al., 2000). The diversity in the pattern of receptor endocytosis might be attributed to the different cell lines used

in these studies, or unique pharmacological properties of SSTR5 which preferentially binds to SST-28 than SST-14 (Csaba and Dournaud, 2001; O'Carroll et al., 1992; Patel, 1999; Raynor et al., 1993).

1.5 Somatostatin analogs

Peptide-based drugs proficiently target most tissues without causing any serious adverse effects since they are physiological compounds (Watt et al., 2008). In addition, low potential for antigenicity, ease of synthesis/modifications and high affinity receptor binding make them suitable drug candidates. These compounds are susceptible to proteolytic degradation, and the hydrophilic nature potentially limits tissue permeability (Watt et al., 2008). SST has a very short half-life (2 min) attributed to rapid enzymatic degradation by tissue and blood peptidases (Ben-Shlomo and Melmed, 2010; Patel, 1999). Although, this prevents systemic side effects it also limits its therapeutic applications. The above pharmacokinetic issues led to further investigations in pursuit of drugs with improved stability and pharmacological activity. Several short synthetic analogs of SST such as OCT, lanreotide and vapreotide were developed in an attempt to specifically target SSTR subtypes, and studied extensively for biological processes such as hormone release as well as binding characteristics with SSTR subtypes (Bruns et al., 1996; Weckbecker et al., 2003). The development of SST analogs significantly improved the understanding of the previously unknown physiological roles associated with SSTRs (Weckbecker et al., 2003). Importantly, cloned SSTR subtypes have broadened the scope for characterization of these compounds in terms of biological

functions such as regulation of hormone release and cell growth (Weckbecker et al., 2003). OCT and lanreotide were among the first long-acting SST analogs to be introduced for the therapeutic management of hormone hypersecretory tumors of pituitary, GIT and pancreas (Bauer et al., 1982; Lamberts et al., 1991; Lamberts et al., 1996a, b). These peptides exhibit high affinity for SSTR2 followed by SSTR5 and SSTR3, whereas showing least binding properties for SSTR1 and SSTR4 (Patel, 1999; Weckbecker et al., 2003). Vapreotide, which became available later is more selective for SSTR5 and SSTR2, and has modest binding affinity for SSTR3 and SSTR4. This drug was approved for use in acute esophageal variceal bleeding secondary to portal hypertension (Patch and Burroughs, 2002; Patel, 1999). Seglitide, which displays highly selective binding for SSTR2 than SSTR3 or SSTR5 is structurally different from above mentioned congeners in having a cyclohexapeptide template without affecting the pharmacological and metabolic characteristics (Veber et al., 1981). The aforesaid SST analogs as discussed bind to SRIF-1 sub-family such as SSTR2, SSTR3 and SSTR5. CH275, a peptide comprising 11 aa is selective for SRIF-2 subfamily member SSTR1. It also exhibited modest binding to SSTR3 (Liapakis et al., 1996; Patel et al., 1996; Rivier et al., 2001). Novartis developed a cyclohexapeptide, pasireotide with remarkable binding properties for SSTR1, SSTR2, SSTR3 and SSTR5 (Bruns C et al 2001). Pasireotide exhibits significant inhibitory actions on GH/insulin-like growth factor-1 (IGF-1) axis in animals as well as in acromegaly and Cushing disease patients (Ben-Shlomo et al., 2007; Bruns et al., 2002; van der Hoek et al., 2004a; van der Hoek et al., 2004b; Weckbecker et al., 2002). Although the peptide analogs of SST show excellent

binding selectivity to at least one particular SRIF subfamily, no compound could achieve absolute receptor specificity. Merck Research Group revolutionized the field of SST biology with seminal contributions to the development and characterization of specific non-peptide agonists for SSTR subtypes using combinatorial chemistry (Rohrer and Schaeffer, 2000). These compounds exhibit selective binding profile for SSTRs with the exception of SSTR5 agonist L-817818, which also binds to SSTR1 albeit with a lesser affinity than SSTR5 (Rohrer and Schaeffer, 2000).

It is reasonable to speculate that subtype selective SSTR antagonists might provide a broader perspective of receptor characteristics. SSTR1 antagonist, SRA-880 enhanced social behavior and learning, whereas decreased the aggression, therefore making it a potential therapeutic tool in neurological disorders (Hoyer et al., 2004). SSTR2-specific antagonists have potential in the therapeutic management of pituitary disorders such as insufficient GH secretion (Ben-Shlomo and Melmed, 2010; Weckbecker et al., 2003). Recently, SSTR2 antagonist PRL-2903 was shown to reverse hypoglycemia by stimulating glucagon release in diabetic rats (Karimian et al., 2013). Another SSTR2 antagonist BIM-23627, attenuated rat GH/IGF-1 axis suppression upon chronic dexamethasone treatment (Tulipano et al., 2005). Several strategies were employed such as using octapeptide template to design CYN-154806, a peptide-based antagonist with high affinity for SSTR2 and modest binding profile for SSTR5 (Bass et al., 1996; Feniuk et al., 2000). It was later discovered that this compound behaved as an agonist as evident by cAMP inhibition comparable to SST (Nunn et al., 2003). SSTR3 antagonists BN-81674 and SST3-ODN-8 abolished SST-mediated cAMP inhibition (Poitout et al., 2001; Reubi et al., 2000). Several

other antagonists such as BIM 23627 and BIM 23056 were developed against SSTR2 and SSTR5, respectively, but their overall utility was limited by lack of receptor specificity and stability (Weckbecker et al., 2003).

1.6 Somatostatin receptors signaling

The biological outcomes in response to SST are governed by multiple factors including the nature of the SSTR subtypes, internalization properties, signaling partners as well as the cell type (Kumar and Grant, 2010). Activation of SSTR subtypes by agonist triggers diverse cellular processes via modulation of several intracellular signal transduction molecules, also called second messengers in a PTX-dependent or independent manner (Csaba and Dournaud, 2001; Florio and Schettini, 1996; Patel, 1999; Reisine and Bell, 1995). SSTRs in a receptor-specific manner regulate signaling molecules such as AC, phosphotyrosine phosphatases (PTPs), mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K)/AKT, phospholipase C, phospholipase A2, in addition to Ca^{2+} and K^{+} channels, as well as Na^{+}/H^{+} antiporter (Csaba and Dournaud, 2001; Kumar and Grant, 2010; Moller et al., 2003; Patel, 1999; Patel et al., 1996; Patel et al., 1995; Reisine and Bell, 1995).

1.6.1 Cyclic adenosine mono phosphate

The signaling events initiated by cAMP were described for the first time in 1958 by Earl Sutherland's group during their studies on epinephrine (Blumenthal, 2012; Rall and Sutherland, 1958). Subsequently, Earl Sutherland was honored with the

Nobel Prize in Physiology or Medicine in 1971 for the discovery and advancement of cAMP. At the cellular level, adenosine triphosphate (ATP) is converted to cAMP by the enzyme AC. This discovery actually uncovered the concept of first messenger (e.g. epinephrine) transducing an effect via second messenger (e.g. cAMP). The physiological response of a cell to extracellular signals is significantly influenced by GPCR associated AC/cAMP pathway resulting in signal amplification (Moller et al., 2003; Somvanshi and Kumar, 2012). cAMP has been identified as an inhibitor of cell proliferation in some, whereas a potent mitogenic signal for other cell types (Rozengurt, 2007). Although the precise mechanism by which cAMP inhibits proliferation in some cell types and stimulates mitogenesis in others remains incompletely understood, it is likely that these effects are mediated by distinct pathways (Dumaz and Marais, 2005). SSTR subtypes regulate both the basal and forskolin (FSK)-stimulated cAMP via G_i proteins, which couple to AC to modulate its activity (Patel, 1999). The degree of cAMP inhibition by SST is concentration-dependent as evident by a bell-shaped curve in response to very low to very high concentration range of SST (Moller et al., 2003; Schindler et al., 1998). Remarkably, 10^{-15} M SST increased cAMP, which was associated with stimulation of GH release from cultured pituitary somatotropes of porcine and primate origin (Cordoba-Chacon et al., 2012; Ramirez et al., 2002). It seems plausible that the discrete activation of G proteins (G_i to G_s switching or vice versa) upon receptor activation might account for the unusual response on cAMP signaling. Also, previous studies have identified ligand independent constitutive SSTR signaling in pituitary, evident by high basal levels of cAMP and ACTH in AtT20 mouse pituitary cells using SSTR knockdown

strategy (Ben-Shlomo et al., 2010). Although, PTX abolished the inhibitory role of SSTRs on cAMP accumulation, the antisecretory effect was only partially blocked (Moller et al., 2003). These findings suggest that SSTRs mediated functions are mediated at least in part via G_i-independent pathways. Furthermore, several lines of evidence have also described G protein switching in GPCRs such as β-adrenergic and opioid receptors with distinct functional consequences (Cruciani et al., 1993; Daaka et al., 1997; Martin et al., 2004; Zhu et al., 2001).

1.6.2 Mitogen-activated protein kinases

MAPKs are implicated in several physiological and developmental processes such as cell growth, proliferation, differentiation and apoptosis (Caunt et al., 2006; Nishimoto and Nishida, 2006). Three major MAPKs including extracellular signal-regulated kinase1/2 (ERK1/2), ERK5, p38 and c-Jun N-terminal kinase (JNK) have been extensively characterized, and are known to elicit important biological roles in mammalian cells (Caunt et al., 2006; Kato et al., 1998; Luttrell, 2002; Nishimoto et al., 2005; Nishimoto and Nishida, 2006; Pierce et al., 2001). ERK cascade plays an important role in translating the extracellular signals to appropriate biological response (Chambard et al., 2007). Insulin or epidermal growth factor (EGF) induced cell proliferation was associated with transient ERK activation (Chambard et al., 2007). Previous studies on neuronal and kidney cells have revealed that deregulated ERK signaling leads to cell death (Cagnol et al., 2006; Stanciu and DeFranco, 2002). Furthermore, ERK activation has been attributed to apoptosis induced by several cytotoxic drugs (Calcabrini et al., 2006; Chambard et al., 2007; Hsu et al.,

2005; Tang et al., 2002; Xiao and Singh, 2002). MAPK signaling has also been associated with cell proliferation; therefore inhibition of MAPKs in response to SST and its analogs correlates with the antiproliferative effects of SSTR subtypes (Csaba and Dournaud, 2001; Seger and Krebs, 1995). Several previous studies have reported the modulation of MAPK signaling cascade upon activation of SSTR subtypes in a receptor-specific manner (Bousquet et al., 2001; Csaba and Dournaud, 2001; Kumar and Grant, 2010; Moller et al., 2003; Patel, 1999). SST mediated activation of MAPK in CHO-K1 cells expressing SSTR1 was associated with inhibition of cell proliferation and concomitant increase in the expression of cell cycle inhibitor p21 (Florio et al., 1999; Moller et al., 2003).

1.6.3 Phosphotyrosine phosphatases

Protein tyrosine kinases (PTKs) and PTPs are important signaling molecules for maintaining cellular equilibrium in many biological processes (Wu et al., 2003). PTKs stimulate tyrosine phosphorylation, whereas PTPs play a key role in dephosphorylation of phosphotyrosine residues and regulating aberrant cell proliferation and tumor progression (Tonks, 1996; Tonks and Neel, 1996; Wu et al., 2003). The regulation of phosphorylation by PTPs leads to amplification or termination of corresponding signaling events (Ostman and Bohmer, 2001; Ostman et al., 2006). Many GPCRs including dopamine-2, angiotensin-II, and α- and β-adrenergic receptors are known to control cell proliferation via PTP regulation (Patel, 1999). Human genome encodes more than 100 members of PTP family including SHP-1 or PTP-1C, SHP-2 and phosphatase and tensin homolog (PTEN), a well-

known tumor suppressor protein (Alonso et al., 2004; Ostman et al., 2006). Because of its ability to inhibit cell proliferation signaling cascades, PTP-1C is regarded as a potential tumor suppressor in many cancers (Wu et al., 2003). PTP-1C is substantially down-regulated and/or inactivated by mutations in the majority of cancers (Oka et al., 2001; Ostman et al., 2006; Wu et al., 2003). Re-expression of PTP-1C gene in PTP-1C deficient cancer cell lines resulted in tumor suppression (Bruecher-Encke et al., 2001; Zapata et al., 2004; Zapata et al., 2002). PTPs negatively impact receptor tyrosine kinases such as EGF receptors (ErbBs), insulin and platelet derived growth factor receptors leading to dephosphorylation and inhibition of signaling (Elchebly et al., 1999a; Elchebly et al., 1999b; Haj et al., 2003; Ostman et al., 2006).

PTP-1C is coexpressed with membrane SSTRs, and is activated by all five SSTR subtypes in a PTX-sensitive manner to play a major role in antiproliferation (Buscail et al., 1994; Florio, 2008a, b; Patel, 1999; Sharma et al., 1996; Weckbecker et al., 2003; Zatelli et al., 2005; Zeggari et al., 1994). PTP plays a crucial role in transducing SST actions on downstream signaling molecules including ERKs and cell-cycle inhibitors such as p21 and $p27^{Kip1}$ (Florio, 2008b). PTP-1C translocation from cytosol to the cell membrane in MCF-7 breast cancer cells is a key event in SST analog OCT induced antiproliferation (Srikant and Shen, 1996). SSTR induced apoptosis in AtT-20, MCF-7 and CHO-K1 cells is PTP dependent, as demonstrated by blockade of antiproliferative effects upon pre-treatment with orthovanadate which is an inhibitor of PTP (Sharma and Srikant, 1998b; Srikant, 1995). SSTR mediated activation of PTP does not appear to be universal across all species as rat SSTR5

was devoid of any effects on PTP regulation. It is possible that the intrinsic nature of the associated G proteins might account for this difference (Patel, 1999).

1.6.4 Poly (ADP-ribose) polymerase-1 (PARP-1)

Human PARPs belong to the family of enzymes comprised of 17 members. Of note, the catalytic domain among all the PARP members is highly conserved (Krishnakumar and Kraus, 2010a, b). In cells, PARPs play a fundamental role in the transfer of ADP-ribose moiety of NAD^+ to target proteins leading to the formation of PAR polymers. PARP-1, a 113 kDa protein, is the most abundant isoform and contributes to approximately 85-90% of the total cellular PARP activity, whereas PARP-2 predominates the rest of the PARP activity (Bai and Canto, 2012). It forms polymers of ADP-ribose and attaches them to acceptor proteins, including histones, DNA repair proteins and transcription factors. PARP-1 is one of the key players known for its definitive role in facilitating DNA repair process following single strand DNA breaks. Although PARP-1 and PARP-2 have been mostly attributed to DNA damage repair, recent evidence based on transgenic models suggest that these enzymes might also play a determinant role in modulating several pathological conditions such as tumorigenesis, inflammation and cellular differentiation (Krishnakumar and Kraus, 2010b). Using knockout mice, it was also demonstrated that PARP-1 and PARP-2 are not integral for conferring DNA stability in conditions devoid of genotoxic stress (Bai and Canto, 2012; Bai et al., 2011a; Bai et al., 2011b). Interestingly, PARP knockout studies have revealed new insights in understanding the metabolic aspects following PARP activation (Bai and Canto, 2012). Under DNA

damage, there is a robust reduction (80-90%) in PARP-1 mediated NAD^+ when compared to the normal enzyme levels (Houtkooper et al., 2010). In response, the cell machinery adopts alternative mechanisms to maintain the depleting NAD^+ levels and cell survival. These compensatory pathways are energy dependent which consume the cellular ATP stores and ultimately predispose the cell to apoptosis (Bai and Canto, 2012; Houtkooper et al., 2010). Also, PARP-1 mediated decline in NAD^+ severely interferes with glycolysis, a biochemical pathway which plays a key role in ATP generation needed for NAD^+ production following genotoxic stress (Houtkooper et al., 2010). PARP-1 undergoes enzymatic hydrolysis by pro-apoptotic caspases into two major cleaved products; 24 kDa DNA binding fragment, and 89 kDa apoptosis promoting fragment (Casiano and Tan, 1996; D'Amours et al., 2001; Kaufmann et al., 1993; Wang et al., 2012). With the current notion that PARP-1 is a major player in restoring genome stability after DNA insult, recent work has demonstrated the potential role of PARP-1 inhibitors in the treatment of several tumors including breast cancer (Mangerich and Burkle, 2011; O'Shaughnessy et al., 2011; Wang et al., 2012).

1.6.5 Proliferating cell nuclear antigen (PCNA)

PCNA as the name suggests was identified as a nuclear protein in dividing cells, with high levels specifically observed during the DNA synthesis phase (S phase) of the cell cycle (Miyachi et al., 1978; Warbrick, 2000). PCNA exists as a homotrimer formed by interactions between three monomeric units (Stoimenov and Helleday, 2009). PCNA plays a central role in DNA synthesis, repair, cell

proliferation and preservation of chromatin integrity (Mailand et al., 2013; Moldovan et al., 2007; Prelich et al., 1987a; Prelich et al., 1987b). It is well appreciated that deregulated DNA replication as a result of genetic alterations drives a cell to become oncogenic (Stoimenov and Helleday, 2009). There is accumulating evidence to support the notion that alterations in PCNA governs the cell fate (Stoimenov and Helleday, 2009). PCNA levels are significantly enhanced in tumoral when compared to normal cells, and therefore are regarded as one of the important prognostic markers in cancer (Naryzhny, 2008; Naryzhny and Lee, 2007). Further investigations into the functional aspects of this protein demonstrated that PCNA acts as a molecular platform for DNA polymerases and other proteins associated with cell proliferation, DNA synthesis, repair and damage avoidance (Bowman et al., 2004; Gulbis et al., 1996; Naryzhny, 2008; Stoimenov and Helleday, 2009).

1.6.6 Cyclin-dependent kinase inhibitors (CDKIs)

Several lines of evidence have revealed that neoplastic cell proliferation is associated with a loss of regulatory control on cell cycle progression (Grant and Roberts, 2003; Roberts et al., 1994; Sherr, 2000; Sherr and Roberts, 1999). Cyclin-dependent kinases (CDKs) mediate diverse biological functions including proliferation, neurogenesis, myogenesis, differentiation, apoptosis, insulin exocytosis and post-natal pancreatic β-cell proliferation (Fischer and Gianella-Borradori, 2005; Knockaert et al., 2002; Marzo et al., 2004). CDKs are characterized by their unique ability to act as Ser/Thr kinases, and exhibit full functionality when coupled with cyclins or other related proteins (Dai and Grant, 2003). It is now widely accepted that

cell proliferation pathways are regulated by well-orchestrated events including the activation of CDKs and CDKIs (Chu et al., 2007; Sherr, 2000; Sherr and Roberts, 1999). The biochemical functions of CDKs are under the inhibitory influence of CDKIs, a group of specialized proteins regulating cell-cycle. The prominent proteins which negatively impact the functions of most CDKs are p21, $p27^{Kip1}$ and $p57^{Kip2}$ (Dai and Grant, 2003). There is enough evidence to suggest that CDKs/CDKIs mutations lead to tumor cell proliferation and hyperactive oncogenes (Dai and Grant, 2003). Functional elucidation of this potential anti-neoplastic target will lead to a novel therapeutic approach in the treatment of several cancers.

Until recently, p21, a 164 aa protein was known to modulate cell cycle progression and tumor proliferation (Chen et al., 1996; Romanov et al., 2012). There is plethora of evidence suggesting that this protein might be associated with other biological functions such as differentiation, migration, senescence and apoptosis (Romanov et al., 2012). p21 mediates its cytostatic functions by interfering with the Cyclin-CDK complexes at different stages of cell cycle including G_1, G_2, S and M phases. It also inhibits Cyclin-CDK complex by suppressing CDK phosphorylation at Thr^{160}, which eventually results in the blockade of complex activation. In addition, p21 negatively impacts DNA synthesis by its direct association with DNA polymerases and PCNA at C-terminus end (Chen et al., 1996; Li et al., 1994; Romanov et al., 2012; Shivji et al., 1994). It has been previously shown that cancer cells undergo cytostasis in response to p21 overexpression, therefore attesting its significance as a potential tumor target (Chang et al., 1999; Kagawa et al., 1999; Romanov et al., 2012). Interestingly, the cytoplasmic pool of p21 has been linked to

cell cycle progression (Cheng et al., 1999; Romanov et al., 2012). Cyclin-CDK complex was almost absent in p21/p27^{Kip1} double knockout, which was reinstated upon expression of p21 and p27^{Kip1}. The authors speculated that the nuclear pool of p21 contributed to tumor suppression and cell cycle inhibition, whereas the cytoplasmic p21 was oncogenic (Romanov et al., 2012). In human primary fibroblasts, ERK2 mediated phosphorylation of p21 triggers its translocation from nucleus to the cytoplasm, a process that is directly correlated with evading the apoptotic insults following mild DNA damage (Heo et al., 2011; Hwang and Kwon, 2009; Hwang et al., 2009; Romanov et al., 2012). It is therefore conceivable that the contrasting functional consequences of p21 depend on its precise location inside the cell.

In humans, p27^{Kip1} is most abundantly expressed CDKI and serves important cell cycle regulatory functions. p27^{Kip1} was cloned and characterized as 198 aa protein derived from its gene CDKN1B which is localized on chromosome 12 (Polyak et al., 1994). The most widely recognized role for p27^{Kip1} is the regulation of G_1 phase of cell cycle in normal as well as tumoral cells, thereby creating a growth checkpoint (Chu et al., 2007). There is a clear consensus on the existing notion that perturbed p27^{Kip1} activity is a strong driving force in leading a cell towards tumorigenesis. The importance of p27^{Kip1} in the maintenance of cell cycle dynamics is highlighted by the observations in p27^{Kip1} knockout mice which exhibit abnormal enlargement of body and the organs, deregulated cellular differentiation, as well as tumor of the pituitary (Fero et al., 1996; Hershko, 2010; Nakayama et al., 1996). Low expression levels of p27^{Kip1} have been observed in many precancerous as well as

active breast, ovarian, colorectal and oral squamous carcinomas, which strongly support its utility as a predictive prognostic marker in the therapeutic management of tumors (Hershko, 2010; Hirano and Minamoto, 2000; Massarelli et al., 2005; Moriya et al., 2000; Sui et al., 2001).

1.6.7 Phosphoinositide 3-kinase pathway

PI3K is an essential effector molecule that coordinates key signaling events mediated by several receptor classes, and is mostly activated in human cancers (Liu et al., 2009a). PI3K belongs to lipid kinase family and its biochemical role is to catalyze the phosphorylation of 3-hydroxyl residues of phosphoinositides (Baselga, 2011; Cantley, 2002). PI3K in association with its downstream effectors such as AKT (protein kinase B) are key components of the signaling cascade that regulate cell growth, motility, survival and metabolism (Bartholomeusz et al., 2011). PI3K exists in a heteromeric complex which is comprised of regulatory (p85) and catalytic (p110) subunits. Upon activation, the catalytic subunit of PI3K contributes to the conversion of PIP_2 to PIP_3 at the plasma membrane. This initiates a cascade of multiple downstream signaling pathways, including the recruitment and activation of Ser/Thr kinase AKT to the plasma membrane. Over activation of PI3K pathway is frequently associated with breast, bladder and prostate tumors, and contributes to the development of resistance to anti-cancer therapies (Aksamitiene et al., 2012; Bartholomeusz et al., 2011; Folgiero et al., 2012). In addition, several mutations in PI3K pathway have also been reported in breast cancer leading to resistance against Her-2 and hormone targeted therapies (Baselga J 2011). The agonist

binding to receptor tyrosine kinases (RTKs) such as ErbBs and insulin-like growth factor-1 (IGF-1) receptors initiates a cascade of events including the activation of PI3K. The heteromeric assembly of PI3K binds to the ligand activated receptor through p85 subunit. This step is essential in releasing the p85 inhibitory barrier on the catalytic p110 subunit. The role of tumor suppressor phosphatase and tensin homolog (PTEN) in the inhibition of PI3K functional activity, as evidenced by the conversion of PIP3 to PIP2, is well established (Bartholomeusz et al., 2011).

1.7 Somatostatin receptors mediated control of cell proliferation

SSTRs expression have been observed in endocrine as well as non-endocrine tumors including breast cancer (Barbieri et al., 2013). In the majority of cases, SSTR2 is the predominant subtype, whereas SSTR3 and SSTR4 exhibit comparatively modest expression in several tumors. Although, SSTRs are present in an overlapping manner at different expression levels in tumor cells, only one or two receptor subtypes predominate in a receptor-specific manner (Barbieri et al., 2013). Previously, antitumor drugs were conjugated with SST analogs to target tumors expressing SSTRs (Mier et al., 2000; Sun and Coy, 2011; Sun et al., 2008; Taylor et al., 1994). The receptor-specific distribution of SSTRs tumors facilitated the correct delivery of drug-SST analog to the tumor site (Reubi et al., 2000; Schaer et al., 1997; Sun and Coy, 2011; Volante et al., 2008). Activation of SSTRs triggers antiproliferative effects in normal cells including intestinal mucosal cells, activated lymphocytes as well as cells of tumor origin (Patel, 1999; Weckbecker et al., 2003). There is also evidence for the growth promoting role of SST via SSTR4 (Sellers et

al., 2000). The authors demonstrated that the sustained activation of ERK by protein kinase C contributed to the proliferative function of SSTR4. SST inhibition of tumorigenesis precisely depends upon the location of its cognate receptors on the cancer cell or in the endothelial cells of the blood vessels surrounding the tumor. Angiogenesis (which refers to the formation of new blood vessels from the pool of pre-existing vessels) is a fundamental determinant for the accelerated growth and metastatic ability of the growing tumors (Folkman, 1995). Several previous studies have reported the inhibition of angiogenesis as a critical factor in regulating cancer progression and metastasis (Folkman, 1995; O'Reilly et al., 1997; O'Reilly et al., 1996). SST inhibits angiogenesis by altering the expression and activity of signaling molecules governing vascularization (Barbieri et al., 2013; Florio, 2008a; Florio et al., 2003). Moreover, in a previous study on Kaposi's sarcoma, which is a SSTRs-negative tumor characterized by strong meshwork of newly formed blood vessels, SST/SSTR3 has been demonstrated to prevent/inhibit the tumor growth and angiogenic pathways by modulating key signal transduction molecules such as endothelial nitric oxide synthase, PTP and MAPKs (Albini et al., 1999; Florio et al., 2003). The potential roles for SST/SSTRs in modulation of angiogenesis include direct inhibition of proliferation, migration and invasion of endothelial cells to the tumor. SST might also modulate the secretion and release of vascular endothelial growth factor and basic fibroblast growth factor. SST might potentially modulate the activation and migration of monocytes in the peritumoral region, resulting in inhibition of neovascularization (Florio, 2008a). SST/SSTRs mitigate tumor migration and invasiveness by blocking PI3K/AKT cell survival pathway, in addition to modulating

special functionality proteins Rac and Rho, which are essential for the maintenance of actin filament (Pyronnet et al., 2008). These findings suggest that SST/SSTRs could impart efficient antiproliferative roles even in tumors devoid of SSTR expression.

Many fundamental biological processes such as proliferation, differentiation and apoptosis are regulated by a series of phosphorylation and dephosphorylation events. The key signaling molecules triggering these processes include protein tyrosine kinases and PTPs (Denu et al., 1996; Hunter, 2000; Tonks, 1996; Tonks and Neel, 1996). SST/SSTRs inhibit cell proliferation via modulation of an array of intracellular effector molecules such as tyrosine kinases (JAK, c-src), PTPs, MAPKs, and PI3K/AKT, and as a result lead to increased levels of cell cycle inhibitors p21 and $p27^{Kip1}$ (Florio et al., 2001; Florio et al., 1999; Grant et al., 2008; Lahlou et al., 2003; Pages et al., 1999; Somvanshi et al., 2009; Theodoropoulou et al., 2006). The presence of specific SSTR subtype/s as well as target cells/tissues plays a critical role in the precise regulation of these intracellular signaling molecules (Pyronnet et al., 2008). Of all the aforementioned signaling molecules, PTPs play major role in SST mediated antiproliferation, and act by dephosphorylating mitogenic signaling molecules (Arena et al., 2005; Barbieri et al., 2013; Lopez et al., 1996; Lopez et al., 2001; Patel, 1999; Srikant and Shen, 1996; Theodoropoulou et al., 2006). Previous biochemical evidence has demonstrated PTP-1C activation via SSTR2, followed by a robust dissociation of activated PTP-1C from SSTR2-PTP-1C complex, and subsequent binding with insulin receptor, causing the dephosphorylation of the insulin receptor and its mitogenic substrates (Barbieri et al., 2013; Bousquet et al.,

1998). This complex chain of events accounts for the inhibition of insulin growth promoting signaling pathways. SSTRs have been reported to induce cell cycle arrest in pituitary, breast, pancreas and thyroid tumors (Barbieri et al., 2013). In case of SSTR2, the antiproliferative signal in response to receptor activation is intimately associated with elevated levels of $p27^{Kip1}$, resulting in cell cycle arrest (Pages et al., 1999). SST via SSTR1 has been demonstrated to trigger cell cycle arrest by modulating the activity of another PTP isoform, PTP-1D also known as SHP2 (Florio et al., 1999; Reardon et al., 1997; Reardon et al., 1996). The activated PTP-1D targets the receptor tyrosine kinases mediated ras/Raf/ERK1/2 signaling cascade, and eventually leads to inhibition of mitogenic effects of growth factors including EGF, insulin and platelet derived growth factor (Barbieri et al., 2013; Pan et al., 1992).

1.8 Somatostatin receptors mediated apoptosis

SST by activating SSTR subtypes triggers apoptosis in both normal and cancerous cells (He et al., 2009; Liu et al., 2000; Liu et al., 2004; Sharma et al., 1996; Thangaraju et al., 1999a; Thangaraju et al., 1999b). Although, the molecular determinants of SST/SSTRs induced cytostatic effects have been well characterized, the pro-apoptotic mode of SST mediated antiproliferation is not well understood. The first report describing the cytotoxic role for subtype-specific SSTRs in CHO-K1 cells was published in 1996, where the authors demonstrated the involvement of SSTR3 in promoting OCT induced apoptosis (Sharma et al., 1996). SSTR3 was reported to promote apoptosis by activating the tumor suppressor p53

and pro-apoptotic Bcl2 associated X protein (Bax). So far, the available evidence suggests that SST mediates majority of its cytotoxic functions by activating SSTR2 and SSTR3 in both normally dividing and tumoral cells (He et al., 2009; Liu et al., 2004; Sharma et al., 1996; Weckbecker et al., 2003). In MCF-7 breast adenocarcinoma cells, OCT was shown to inhibit cell growth by accelerating the cytotoxic activity (Pagliacci et al., 1991). SST triggers apoptosis in several cell lines by activating PTP-1C. SSTR2 also exhibits apoptosis in HL-60 acute promyelocytic leukemia cells in p53 independent manner. In MCF-7 breast cancer cells which lack estrogen receptor-α (ER_{α}), the overexpression of SSTR2 induced apoptosis and cell cycle arrest (He et al., 2009). The authors also demonstrated the loss of ErbB1 expression and EGF stimulated cell proliferation in MCF-7 cells transfected with SSTR2 when compared to non-transfected cells. These findings have significant therapeutic implications, keeping in mind the potential role in suppressing the growth promoting effects of EGF. SSTR2 expression potentiated tumor necrosis factor α (TNFα) mediated cytotoxicity in nontransformed murine fibroblastic NIH3T3 cells (Guillermet-Guibert et al., 2007; Guillermet et al., 2003). SSTR2 expression transduced apoptotic signaling through PTP-1C mediated activation of nuclear factor kappa B and abrogation of JNK. These observations provided evidence for the functional cross-talk between SSTRs and death receptors in modulating the pro-apoptotic signaling. Approximately 90% of all human pancreatic cancers exhibit selective ablation of SSTR2 expression (Buscail et al., 1996). SSTR2 transfection in these previously SSTR2-negative tumors was associated with the activation of apoptosis (Guillermet et al., 2003). SSTR2 also potentiated the cytotoxic ability of

TNFα and TNFα-related apoptosis-inducing ligand (TRAIL) as evident by the cleavage of executioner caspase-3 and DNA repair protein PARP-1. In addition, there was upregulation of TNFα and TRAIL receptors, activation of caspase-8 and post-transcriptional down-regulation of antiapoptotic protein Bcl-2. Importantly, PTP-1C was found to be the principal mediator of SSTR2-dependent apoptosis in these cells. These findings strongly support the role of SSTR2 as tumor suppressor in pancreatic cancer cells. OCT treatment triggered apoptosis in MCF-7 human breast cancer cells (Sharma and Srikant, 1998b). The cytotoxic signaling events included the robust activation of p53 time-dependently, and elevated levels of Bax. The authors demonstrated that OCT selectively activated cation-insensitive acidic endonuclease to promote DNA fragmentation. Moreover, using SSTR2-selective analog BIM23120 in human pituitary tumors, the cytotoxic potential of SSTR2 was further illustrated. The pro-apoptotic effects were PTP dependent and correlated with the increased activity of caspase-3. There was no evidence for the involvement of other signaling molecules governing apoptosis e.g. p53, Bcl-2 and Bax (Ferrante et al., 2006). Moreover, in hepatocellular carcinoma cells, OCT was demonstrated to elicit cytotoxicity, an effect attributed to SSTR3 (Liu et al., 2004). The presence of SST and SSTRs on lymphoid tissues suggests a potential role in modulating immune functions (Lattuada et al., 2002). OCT has been shown to bind activated human lymphocytes resulting in apoptosis. The biological consequences of OCT action include the activation of caspase-3 and cleaved PARP-1, which provides evidence for the pro-apoptotic role of SSTRs in non-tumoral lymphocytes.

1.9 Photo-bleaching fluorescence resonance energy transfer

In the past, different strategies such as CO-IP or Western blot analyses were employed to confirm whether GPCRs form dimers or higher order oligomers. These experimental approaches were often challenged because of the fact that the model systems used overexpressed receptors in an artificial environment (Patel et al., 2002b). This led to a general skepticism to the concept of GPCR oligomerization and its functional significance. Microscopic Pb-FRET analysis was introduced to address the problems faced in the above mentioned approaches. Pb-FRET analysis can be performed in selected regions of interest in intact cells (Kaczor and Selent, 2011; Patel et al., 2002b; Rocheville et al., 2000b). Moreover, the kinetic nature of Pb-FRET confers a major advantage over other methodologies. Pb-FRET analysis is a powerful technique which is highly sensitive and can be used to examine protein-protein interactions at physiological levels of expression (Kaczor and Selent, 2011; Patel et al., 2002b).

The basic principle of FRET (Figure 1.3) is based on the distance-dependent non-radiative energy transfer from an excited fluorophore (donor) to another fluorophore (acceptor) through dipole-dipole coupling (Bouvier, 2001; Patel et al., 2002a; Patel et al., 2002b; Rocheville et al., 2000a; Rocheville et al., 2000b). The efficiency of the energy transfer between the donor and acceptor is inversely proportional to the sixth power of their distance, thus making FRET extremely sensitive to small distances (Patel et al., 2002b). FRET is measured as the quantum yield commonly referred to as FRET efficiency (E), according to the following formula.

$$E = [1-(D-A/D+A)] \times 100$$

D-A and **D+A** denote time constants of the photobleaching decay of the donor in the absence or presence of an acceptor, respectively.

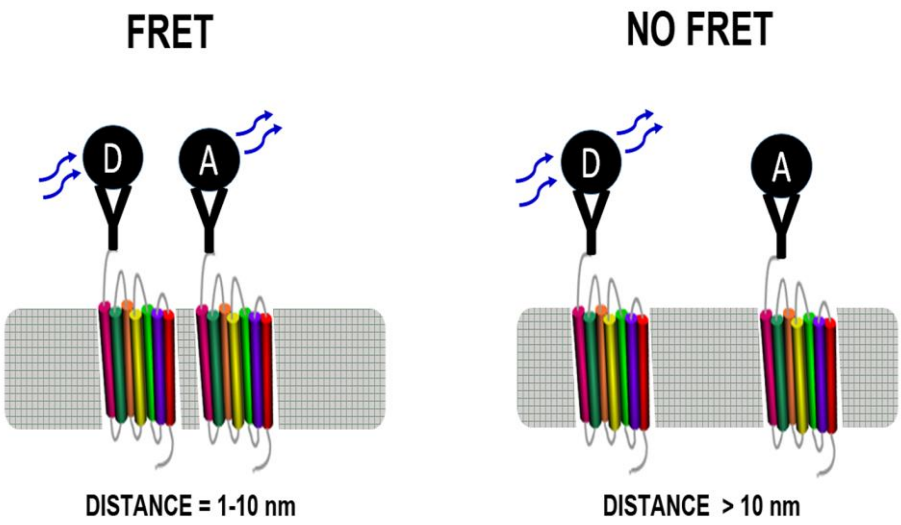

Figure 1.3 GPCR oligomerization by FRET. Illustration of GPCRs dimerization as a function of distance using FRET approach.

There are three basic criteria that must be met for a successful FRET signal, and include the following: (i) Donor and acceptor molecules must be in close proximity (1-10 nm). (ii) The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor. (iii) Donor and acceptor molecules must be in approximately parallel orientation (Iqbal et al., 2008a; Iqbal et al., 2008b; Patel et al., 2002b; VanBeek et al., 2007). FRET has numerous applications which include study of protein-protein interactions such as association/dissociation, conformational states as well as enzyme activity (Patel et al., 2002b). Pb-FRET is based on the irreversible loss of donor fluorophore's ability to fluoresce, and measured through the photobleaching dynamics of the donor molecule. The receptor

is targeted by antibody which is covalently conjugated with either a donor [fluorescence isothiocyanate (FITC)] or an acceptor (cy3/rhodamine) molecule. Following the extended exposure to excitation light, the attenuated donor fluorescence signal due to photobleaching is calculated in the absence and presence of acceptor. The photobleaching time lag observed upon addition of acceptor-labeled antibody would suggest that the interacting molecules are in close proximity for energy transfer. Although, a highly sensitive technique, the reliability of Pb-FRET analysis is limited by the expression level of the target receptors in the cell. Therefore, the positive evidence of FRET signal can also be an artifact of the receptor aggregation due to overexpression, as observed in case of human SSTR5 (Patel et al., 2002b).

1.10 Homo- and heterodimerization of somatostatin receptors

SSTRs have been reported to form oligomers within family, or with other GPCRs such as dopamine, opioid and adrenergic receptors (Baragli et al., 2007; Grant et al., 2008; Grant et al., 2004a; Grant et al., 2004b; Patel et al., 2002a; Pfeiffer et al., 2001; Pfeiffer et al., 2002; Rocheville et al., 2000a; Rocheville et al., 2000b; Somvanshi et al., 2009; Somvanshi et al., 2011a; Somvanshi et al., 2011b). The ability of SSTRs to dimerize or remain in monomeric form is dependent upon the receptor-subtype. Using biophysical (Pb-FRET analysis) and biochemical (Western blot analysis) approaches, the first evidence supporting SSTRs dimerization was provided for human SSTR5 subtype (Rocheville et al., 2000b). This study showed that the monomeric sub-population of SSTR5 was predominant at the

cell surface in absence of agonist exposure, when expressed at comparable physiological expression levels in CHO-K1 cells. A dose-dependent increase in SSTR5 homodimers upon SST treatment suggested that dimerization was a necessary step for SSTR5 mediated signaling following agonist activation. In the same study, the authors also reported SSTR5 heterodimerization with SSTR1 but not SSTR4, when the receptors were co-expressed in CHO-K1 cells, an indication that SSTRs heterodimerization was limited to specific receptor combinations. Importantly, SSTRs oligomerization is not only governed by the specificity of receptor combinations, but also depends on the cell-type. In contrast to the data reported in CHO-K1 cells by Rocheville et al., recently Somvanshi et al., described constitutive heterodimerization of human SSTR4/SSTR5 in HEK-293 cells (Rocheville et al., 2000b; Somvanshi et al., 2009). The observed discrepancy between the two findings was attributed to the lack of specific G proteins in CHO-K1 cells when compared to HEK-293 cells. In the same study, SSTRs heterodimerization was found to be selective, as cells coexpressing SSTR1 and SSTR4 showed no evidence of physical or functional interaction. The data from Somvanshi et al., also provided the first evidence for constitutive homodimerization of human SSTR4, which was enhanced upon SST treatment. SSTR2, the most extensively investigated SSTR is a preformed homodimer at plasma membrane, and undergoes agonist-mediated dissociation to monomers, a pre-requisite for receptor internalization (Grant et al., 2004a). This conclusion was drawn based on the observation that blocking the dissociation of homodimer complex by using cross-linking agent resulted in impaired receptor internalization. This is not just an isolated

case among GPCRs, because the agonist-induced dissociation of δ-opioid receptor dimers to monomers has also been demonstrated to be the limiting factor for receptor internalization (Cvejic and Devi, 1997). Recently, Grant et al., described heterodimerization between SSTR2 and SSTR5 which was only dependent upon selective activation of SSTR2, whereas the receptors failed to heterodimerize upon selective activation of SSTR5 or co-activation of SSTR2/SSTR5 (Grant et al., 2008). It is conceivable that SSTR2-specific agonist caused the dissociation of SSTR2 homodimers to monomers, thereby increasing the monomeric entities available to physically interact with SSTR5. In this context, SSTR2/SSTR5 heterodimer displayed enhanced signaling profile in terms of cAMP, ERK1/2 and $p27^{Kip1}$. Most importantly, the heteromeric complex of SSTR2/SSTR5 exhibited sustained antiproliferative signal. Studies with SSTRs of non-human origin expressed in different cell types yielded contrasting results in terms of receptor trafficking and functions. For example, SSTR2 and SSTR3 of rat origin displayed homodimerization with or without agonist exposure when expressed in HEK-293 cells (Pfeiffer et al., 2001). In cotransfected HEK-293 cells, rat SSTR2/SSTR3 heterodimerization was evident in basal state, with the preservation of full characteristics of SSTR2, whereas, SSTR3-related functionality such as ligand binding and cAMP inhibition was abolished. Furthermore, agonist treatment resulted in the disruption of the heteromeric complex, and was followed by endocytosis of SSTR2, whereas SSTR3 was retained at the cell surface. This study challenged the earlier notion that heterodimerization enhances the functional profile of the interacting protomers. In this context, SSTR3 inactivation in SSTR2/SSTR3 heteromeric complex was

speculated as an explanation for the lack of SSTR3 binding sites in rat cerebellum exhibiting high mRNA expression for SSTR3 (Viollet et al., 1997). Whether or not the same scenario fits in experimental conditions using human SSTR2/SSTR3 isoforms remains a point of debate and needed to be addressed in detail. Interestingly, SSTR1 is not only devoid of homodimerization, but is also internalization resistant (Hukovic et al., 1996; Hukovic et al., 1999; Patel et al., 2002a). It is plausible that dimerization and internalization are not the absolute determinants of SSTRs functions, and the receptors could also function as monomers. Whether the lack of homodimerization and internalization in SSTR1 are interconnected and warrants further investigation. Moreover, the role of specific domains as well as ligand-induced receptor conformational dynamics that govern homo- vs. heterodimerization in SSTRs need further characterization. Grant et al., also discovered a crucial role for G proteins in homo- and heterodimerization of SSTR2/SSTR5 (Grant and Kumar, 2010). The authors demonstrated the dissociation of preformed SSTR2 homodimers in response to G proteins inactivation by PTX. Furthermore, cotransfected cells elicited SSTR2/SSTR5 heterodimerization upon PTX treatment, independent of agonist activation. These findings might provide a possible mechanistic role for G proteins in regulating constitutive oligomerization of SSTRs. Recently, Duran-Prado et al., reported mutations in TMD regions of SSTR5 (SST5TMD4) in poorly differentiated breast tumor tissues as well as in MCF-7 breast adenocarcinoma cells (Duran-Prado et al., 2012). The presence of this mutant receptor dramatically potentiated the markers for tumor proliferation, migration and invasion, in addition to altering the tumor cell morphology. The authors correlated the intracellular signaling

molecules such as ERK1/2, PI3K-AKT and cyclins etc. with the aberrant tumorigenic properties of SSTR5 mutant. Moreover, mutant SST5TMD4 physically and functionally interacted with SSTR2 to modulate receptor localization and downstream signaling including antiproliferative effects. This important discovery might explain the reported failure of SST analogs in clinical cases of breast cancer.

Considerable interest has also been focused on the cross-talk between SSTR subtypes and members of other GPCR families in the modulation of key signaling pathways. Recent studies have established several lines of evidence for the possible functional interaction between SSTRs and dopamine, opioid and β-adrenergic receptors (Baragli et al., 2007; Pfeiffer et al., 2002; Rocheville et al., 2000a; Somvanshi et al., 2011a; Somvanshi et al., 2011b). These findings have significant pathophysiological implications especially in several endocrine, neurological, cardiovascular and pain disorders. SSTRs might also functionally interact with non-GPCR families such as the members of receptor tyrosine kinases. One such example is the heteromeric complex between SSTRs and ErbBs (Kharmate et al., 2011a, b; Watt et al., 2009). Such an association has revealed a new therapeutic target with significant implications, specifically in many cancers.

1.11 Somatostatin receptors functioning; role of C-tail

Several lines of evidence have provided insights that C-tail plays a determinant role in regulating GPCR characteristics such as receptor oligomerization, cell surface expression, phosphorylation, internalization, down-regulation and signal transduction (Cvejic and Devi, 1997; Liu et al., 2008; Trapaidze et al., 1996). In case

of GABA-B receptor, C-tail interactions facilitate receptor heterodimerization in the endoplasmic reticulum regardless of agonist exposure prior to being targeted to the cell surface. This remarkable finding provided one of the first evidence for the molecular determinants of oligomerization present in the receptor's C-tail (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; Marshall et al., 1999a; Marshall et al., 1999b). Studies on metabotropic glutamate receptor 5 and calcium-sensing receptor have shown that receptor dimerization occurs via interactions between extracellular N-terminal regions (Bai et al., 1998; Romano et al., 1996). In terms to the role of C-tail on SSTRs oligomerization, it has been shown earlier in HEK-293 cells that C-tail deleted mutant of rat SSTR3 displayed comparable degree of homodimerization in comparison to the wild type (Pfeiffer et al., 2001). In contrast, C-tail deleted mutant of human SSTR4 failed to dimerize attributed in part to the diminished plasma membrane targeting of the mutant receptor (Somvanshi et al., 2009). Using chimeric receptors, the molecular determinants in the C-tail have been suggested to play a decisive role in promoting homodimerization of human SSTR1 and SSTR5, as well as SSTR1/SSTR5 heterodimerization (Grant et al., 2004b). The role of C-tail on cell surface expression is receptor-specific. For example, C-tail deletion did not alter the plasma membrane expression of rat SSTR3 and human SSTR5 in transfected cells (Hukovic et al., 1998; Pfeiffer et al., 2001). Conversely, C-tail deleted mutant of human SSTR4 was devoid of cell surface expression, and was largely localized intracellularly in the Golgi apparatus (Somvanshi et al., 2009). The authors concluded that SSTR4 C-tail was crucial for receptor targeting to the plasma membrane.

Previous studies have shown that the C-tail of many other GPCRs, including angiotensin II, β_2-adrenergic, and neurotensin receptors plays a critical role in receptor internalization (Chabry et al., 1995; Jockers et al., 1996; Thomas et al., 1995a; Thomas et al., 1995b). C-tail truncation of chemokine receptor 5 resulted in the progressive loss of membrane expression of the receptor (Venkatesan et al., 2001). Furthermore, deletion of 15 residues from the C-tail of δ-opioid receptor has been shown to impair cell surface expression (Cvejic and Devi, 1997). Phosphorylation of C-tail residues in rat SSTR3 governs receptor desensitization, followed by internalization and subsequent recycling to the plasma membrane (Roth et al., 1997a). Mutations in the C-tail residues at Ser^{341}, Ser^{346}, Ser^{351} and Thr^{357} negatively impacted the kinetics of receptor phosphorylation, desensitization and internalization, suggesting an important role for C-tail in receptor functions. Later, it was also discovered that phosphorylation of Ser and Thr residues in SSTR2 C-tail regulate receptor endocytosis (Liu et al., 2008). Furthermore, the C-tail truncated mutants of human SSTR5 display distinct response to agonist-induced endocytosis when compared to the wild type, suggesting the role of region-specific C-tail domains in mediating positive or negative internalization response (Hukovic et al., 1998). In another study, the ICL-3 region of SSTR5 was demonstrated to play a key role in receptor internalization, whereas the aa residues (328-347) located in the C-tail were shown to impede internalization (Peverelli et al., 2009; Peverelli et al., 2008). It has also been previously described that C-tail governs up-regulation and/or internalization of human SSTR1 and SSTR5 subtypes (Grant et al., 2004b; Hukovic et al., 1999). Several lines of evidence have highlighted the role of C-tail in

governing GPCR dependent intracellular signaling events. The functionality of SSTRs is assessed by their ability to inhibit cAMP in response to agonist. C-tail truncated mutants of human SSTR4 and SSTR5 displayed complete loss of cAMP inhibition (Hukovic et al., 1998; Somvanshi et al., 2009). In case of human SSTR1, the ability to inhibit cAMP was partially lost upon C-tail deletion (Hukovic et al., 1999).

1.12 Breast cancer

According to 2012 statistics published by Canadian Cancer Society, 2 out of 5 Canadians are at risk of having a cancer in their lifetime, with a mortality rate of approximately 1 out of 4. Breast cancer has the highest incidence (25.6%) in women, and is also the second most commonly diagnosed cancer overall (Canadian Cancer Society, 2012). Barring lung cancer with a mortality rate of 25.9%, breast cancer (14.2%) is the second leading cause of cancer-related deaths in women (Canadian Cancer Society, 2012). Many hormones including estrogens, progesterone, GH, prolactin, insulin, EGF, IGF-1 and transforming growth factor which are essential for the normal growth and development of breast, also play a critical role in tumor progression (Clarke et al., 1992; Dickson and Lippman, 1992). Although uncommon (<1%), breast cancer has also been reported in men, with 90% of tumors being ER-positive (Fentiman et al., 2006; Weiss et al., 2005). A positive family history has been attributed as the major risk factor in male breast cancer (Ottini et al., 2010).

Of all the breast cancer cases, almost 5-10% are inherited as a result of mutations, whereas the majority of breast tumors are sporadic and correlate with hormonal exposure (Dickson and Lippman, 1992; Vogelstein and Kinzler, 1994). The onset and progression of breast cancer is intimately associated with the age at menarche and menopause, pregnancy history, environment, diet, obesity and exposure to radiation (Brinton et al., 1988; McPherson et al., 1997; McPherson et al., 2000). Approximately one-third of hereditary breast tumor cases exhibit mutations in breast cancer 1 and 2-susceptibility genes (Easton et al., 1995; Rosen et al., 2003; Wooster et al., 1995; Wooster and Stratton, 1995). Although, generally not detected in normal breast tissue, c-Myc gene is overactive in about 20-30% of breast cancers and corresponds to highly aggressive metastatic grade tumor (Deming et al., 2000; Spaventi et al., 1994). The implementation of diagnostic screening procedures such as mammography, advanced biopsy techniques, as well as surgery and pharmacotherapy has considerably declined the death rates due to breast cancer. The statistics are likely to change over the next few decades, taken into consideration an aging population and extended life expectancy.

Breast cancer is classified into three major categories; ER-positive (ER^+), ER-negative (ER^-) and ER/progesterone receptor (PR)/ErbB2-negative (triple negative). Amongst them, the therapeutic management of triple negative is most challenging due to the aggressive proliferative nature and the absence of conventional tumor targets. For the clinical management of post-menopausal women diagnosed with ER^+ breast cancer, aromatase inhibitors are among the most commonly used adjuvant treatments. They act by inhibiting estrogen biosynthesis in peripheral

tissues. In pre-menopausal women, tamoxifen which belongs to the class of drugs known as selective ER modulators, has been widely regarded as a gold standard for breast cancer treatment, since aromatase inhibitors are avoided in women with functional ovaries (Rao and Cobleigh, 2012). Tamoxifen has estrogenic activity in bones, brain and liver, whereas in breast and endometrium, it acts as an antiestrogen. It was recently shown that endoxifen, which is a metabolite of tamoxifen, functions as a potent antiestrogen by initiating proteasome mediated $ER\alpha$ degradation in breast cancer cells. In addition, the presence of $ER\beta$ enhances the sensitivity of breast cancer cells to the anti-estrogenic effects of endoxifen, possibly via $ER\alpha/ER\beta$ heterodimers (Wu et al., 2009; Wu et al., 2011b). Unfortunately, hormone targeted therapy using tamoxifen has been limited by development of native and acquired drug resistance. The role of PR in breast cancer therapeutics is gaining importance, as evident from the clinical research findings which have suggested that the response of ER^+/PR^- breast cancers to tamoxifen therapy is inferior to ER^+/PR^+ tumors (Arpino et al., 2005; Bardou et al., 2003). In tumors exhibiting tamoxifen resistance, PR expression is completely lost, leading to an aggressive disease phenotype with poor survival (Balleine et al., 1999; Gross et al., 1984). Also, tamoxifen resistant ER^+/PR^- breast tumors exhibit aberrant expression of ErbB1 and ErbB2, along with low ER levels (Arpino et al., 2005). Therefore, the status of hormone receptors might prove crucial in the success of breast cancer therapeutics. In addition, ovarian ablation or suppression has also demonstrated benefit in reducing the risk of death and breast cancer recurrence (Rao and Cobleigh, 2012).

Among all the endogenous estrogens, EST is most abundant, whereas the levels of other estrogens such as estrone and estriol are modest (Ascenzi et al., 2006). Estrogens elicit their physiological actions by activating ERs, members of the nuclear receptor superfamily (Ascenzi et al., 2006). Emerging evidence also suggests the presence of ERs at the cell surface to mediate robust signaling events such as modulation of MAPKs and AKT (Renoir et al., 2013). There are two major ER subtypes namely ER_{α} and ER_{β}, both encoded by separate genes ESR1 and ESR2, respectively (Burns, 2003; Burns and Korach, 2012). Both receptor subtypes exhibit typical tissue- and receptor-specific distribution (Burns and Korach, 2012). The growth promoting actions of EST in breast, uterus and ovaries have been attributed to ER_{α} (Ali and Coombes, 2000; Renoir et al., 2013). Although, low ERs expression in breast tissues has been observed under normal physiological conditions, ER_{α} is the predominant isoform found in the majority of breast cancers (Renoir et al., 2013). In contrast, ER_{β} expression is lost in breast tissues as the tumor progresses suggesting a potential antitumor role (Murphy and Watson, 2006; Roger et al., 2001; Skliris et al., 2003). On the other hand, ER_{β} has been described to modulate ER_{α}-mediated transcriptional activity and growth promoting signaling in several cancer cells (Gougelet et al., 2005; Paruthiyil et al., 2004; Strom et al., 2004). The exact role of ER_{β} in breast cancer is still not well defined. The deregulation of ER_{α}/ER_{β} ratio has been postulated as one of the contributing factors in breast cancer progression (Grober et al., 2011). In agreement with this notion, EST-induced proliferation was attenuated in MCF-7 and T47D breast cancer cell lines transfected with ER_{β} (Omoto et al., 2003; Strom et al., 2004). These studies

provided future directions in the development of drugs with antagonistic properties on ER_{α}, whereas acting as agonist on ER_{β} as a viable treatment option in breast tumors expressing ER_{α}/ER_{β} (Saji et al., 2005). The fact that ERs expression is evident in most invasive breast tumors makes it a key prognostic marker and an attractive target for endocrine-based treatments (Ali and Coombes, 2002; Murphy and Watson, 2006).

EGF is an essential growth factor known to regulate cell proliferation, differentiation, migration and survival in normal and malignant cells (Henson and Gibson, 2006; Kumar, 2011). The role of EGF in activating oncogenes like c-Fos and c-Myc has been previously reported (Muller et al., 1984). In conditions of EGF deprivation, cells exhibited an increased susceptibility to cytotoxic triggers (Jost et al., 2001). EGF actions are transduced via ErbBs (ErbB1, ErbB2, ErbB3 and ErbB4), subclass I members of RTKs superfamily (Olayioye et al., 2000). ErbBs share common structural features such as; extracellular ligand binding domain, a single TMD and an intracellular tyrosine kinase domain (Olayioye et al., 2000; Savage et al., 1972). ErbBs are widely distributed in different tissues to govern essential biological functions (Olayioye et al., 2000). The fact that ErbBs form homo-and heterodimers within the family signifies their role in diverse signaling events including tumor progression (Olayioye et al., 2000). The fundamental role of ErbBs in the development and proliferation of mammary glands is well established (Schroeder and Lee, 1998). Also, the majority of breast cancers express multiple ErbBs (Renoir et al., 2013). The importance of ErbBs for survival is evident by the mortality observed in ErbBs knockout mice (Olayioye et al., 2000). All ErbBs bind

their endogenous ligands with the exception of ErbB2, for which no ligand has been reported so far. From the available evidence, it appears that ErbB2 functions as a partner receptor in heteromeric complex with other ErbBs, where it plays a critical role in amplification of mitogenic signal in response to ErbBs ligands (Beerli et al., 1995; Graus-Porta et al., 1997; Graus-Porta et al., 1995; Tzahar and Yarden, 1998). The heteromeric complex of ErbBs is associated with high ligand binding affinity, reduced functional desensitization and sustained intracellular signaling events including cell proliferation and tumorigenesis (Beerli et al., 1995; Graus-Porta et al., 1997; Graus-Porta et al., 1995; Karunagaran et al., 1996; Spencer et al., 2000). Moreover, ErbB2 overexpression in tumors of breast and ovarian origin has also been shown to induce constitutive homodimerization. As a result, the tyrosine kinase activity of the homodimer is potentiated even in the absence of ligand, leading to hyperproliferative signaling cascades (Olayioye et al., 2000; Slamon et al., 1989). ErbBs activation modulates several downstream signaling pathways including MAPKs, PI3K-AKT, and Janus kinase/signal transducers and activators of transcription pathways (Citri and Yarden, 2006; Jorissen et al., 2003; Saxena and Dwivedi, 2012; Zhong et al., 1994). Activation of the aforesaid signaling cascades induces proliferation, migration, survival and invasion, as well as inhibition of apoptosis (Saxena and Dwivedi, 2012).

1.13 Breast cancer cell lines

The studies on the molecular mechanisms of breast cancer pathology have been mostly performed in breast cancer cell lines (Burdall et al., 2003). Amongst

them, MCF-7 ($ER\alpha$-positive) is the most commonly used *in vitro* breast cancer cell line model (Burdall et al., 2003). This cell line was established in 1973 from Michigan Cancer Foundation, and was obtained from a pleural effusion (Soule et al., 1973). The other commonly used breast cancer cell lines include MDA-MB-231 ($ER\alpha$-negative), MDA-MB-435 ($ER\alpha$-negative) MDA-MB-468 ($ER\alpha$-negative), SkBr3 ($ER\alpha$/$ER\beta$-negative), T47D ($ER\alpha$/PR-positive), ZR-75-71 ($ER\alpha$-positive) (Cailleau et al., 1978; Cailleau et al., 1974; Engel et al., 1978; Keydar et al., 1979). Most of these commonly used cell lines are obtained from pleural effusions of metastatic tumors, and not from primary breast tumors. The presence or absence of $ER\alpha$ as well as estrogen requirement is the major distinguishing feature between these experimental breast cancer cell lines.

1.14 Somatostatin receptors and breast cancer

The evidence from protein expression studies, have revealed that SSTR3 and SSTR5 are the least expressed receptor subtypes in MCF-7 and MDA-MB-231 cells, whereas SSTR4 is most abundant subtype (Watt and Kumar, 2006). Moreover, breast tumor tissues and different cell-based models exhibit SST-like immunoreactivity (Ciocca et al., 1990; Nelson et al., 1989; Prevost et al., 1994). Previous studies have also demonstrated SSTRs expression in primary human breast tumors (Kumar, 2005; Orlando et al., 2004; Prevost et al., 1994; Weckbecker et al., 1994). Several lines of evidence have described SSTR2 as the predominant receptor subtype expressed in human normal breast epithelium as well as breast cancer (Evans et al., 1997; Kumar, 2005; Pilichowska et al., 2000; Schaer et al.,

1997; Schulz et al., 1998). Furthermore, the role of EST and tamoxifen in the regulation of SSTRs expression has been described previously (Rivera et al., 2005; Xu et al., 1996).

The inhibitory role of OCT on EST and GH mediated proliferation in MCF-7 cells suggested its potential anti-proliferative actions on human breast cancer cells (Setyono-Han et al., 1987). Long-term treatment with vapreotide in metastatic breast cancer patients are devoid of side effects, and correlated with reduced IGF-1 levels (O'Byrne et al., 1999). Also, lanreotide treatment demonstrated remarkably low levels of IGF-1 in postmenopausal breast cancer patients (Canobbio et al., 1995). Earlier studies on breast cancer cell lines including MCF-7, T47D and ZR-75-71 have demonstrated an antiproliferative role for SST analogs via cytostatic and cytotoxic mechanisms (Pagliacci et al., 1991; Prevost et al., 1991; Sharma and Srikant, 1998a, b). OCT was shown to potentiate the antitumor effects of tamoxifen and ovariectomy in an *in vivo* model of breast tumor (Weckbecker et al., 1994). SSTRs/ErbBs oligomerization governed EGF-mediated proliferative downstream signaling cascades in MCF-7 and MDA-MB-231 cells, suggesting a potential functional cross-talk between GPCRs (SSTRs) and RTKs (ErbBs) in breast cancer (Kharmate et al., 2011a, b; Watt et al., 2009). Consistent with this notion, He et al., showed that overexpression of SSTR2 in MCF-7 cells resulted in apoptosis and cell-cycle arrest, as well as suppression of ErbB1 expression and EGF-mediated mitogenic effects (He et al., 2009). It seems plausible that SST/SSTRs represent a potential target in breast cancer. Most importantly, the role of cytotoxic SSTR3 in breast cancer is not well understood. Therefore, overexpression of SSTR3 in breast

cancers lacking this receptor subtype might constitute a novel therapeutic approach in tumor biology, and warrants further investigation.

1.15 Human embryonic kidney cells as experimental model

HEK-293 cells were generated more than 35 years ago by incorporation of adenovirus type 5 DNA in human embryonic kidney cells (Graham FL et al 1977; Thomas P and Smart TG 2004). These cells constitute an important expression tool to study recombinant protein and their signaling pathways. The advantage of studying receptor signal transduction in heterologous system such as HEK-293 cells is the absence of interference from receptors belonging to the same or closely related families. The other advantages include; easy to grow, maintain, and transfect (Thomas P and Smart TG 2004). HEK-293 cells characterization by microarray detection revealed mRNA expression for 28 GPCRs (Shaw G et al 2002). So far, the available evidence suggests very low expression of SSTR2 (18 fmol/mg protein), whereas other SSTR subtypes were not detected, therefore making this cell line as an ideal cell-based model to study SSTRs characteristics (Law SF et al 1993).

1.16 Summary of background

Several lines of evidence have recognized the potential antitumor role for SST/SSTRs through multiple converging mechanisms. The majority of those studies were carried out on SSTR2, the most abundant receptor subtype in normal and tumoral tissues. In contrast, clinical studies in breast cancer patients using SST analogs showed disappointing results. Strikingly, in those clinical trials, patient tumor

samples were not screened for SSTRs expression (Bajetta et al., 2002; Ingle et al., 1999a; Ingle et al., 1999b; Orlando et al., 2004). Whether SSTR3 mediated cytotoxic signaling might regulate breast tumors of different origin is not well understood. It is conceivable that functional characterization of SSTR3 might lead to a novel therapeutic approach in breast cancer.

1.17 Research hypothesis

From the preceding discussion, the emerging hypothesis is ***"Understanding the molecular mechanisms involved in SSTR3 mediated signaling and cytotoxic effects can serve to develop strategies to effectively employ these pathways in cancer therapy".***

1.18 Specific objectives

1.18.1 Aim 1 To characterize human SSTR3 in HEK-293 cells for surface expression, trafficking, homodimerization and signaling upon deletion of aa from its C-tail, and to identify a role of C-tail in regulating the apoptotic pathways mediated by SSTR3.

1.18.2 Aim 2 To explore human SSTR2 and SSTR3 heterodimerization in HEK-293 cells and determine if coexpression of SSTR2 and SSTR3 modulates the antiproliferative effects mediated by the native receptors.

1.18.3 Aim 3 To investigate whether overexpression of human SSTR3 can regulate the proliferation of human breast cancer cell lines, MCF-7 and MDA-MB-231.

Chapter 2: Somatostatin receptor-3 mediated intracellular signaling and apoptosis is regulated by its cytoplasmic terminal

2.1 Background

GPCRs represent one of the largest and most diverse families of cell surface proteins which are known to play important roles in a variety of pathological conditions. Most of the current therapeutic drugs target GPCRs, implicating their clinical importance in the pathogenesis of various diseases (Blois and Bowie, 2009; Milligan, 1998; Milligan and McGrath, 2009; Pierce et al., 2002). SST is a multifunctional regulatory peptide secreted by endocrine, neuronal and immune cells and acts to regulate cell secretion, neurotransmission and cell proliferation (Patel, 1999). These distinct and diverse biological effects of SST are mediated via five different receptor subtypes namely SSTR1-5, which belong to GPCR family and are widely distributed and highly expressed in normal as well as in several pathological conditions (Reubi et al., 2001). Structure-function studies have shown great diversity among SSTR subtypes (Baragli et al., 2007; Grant et al., 2004a; Grant and Kumar, 2009; Grant et al., 2004b; Patel, 1999; Pfeiffer et al., 2001; Rocheville et al., 2000a; Rocheville et al., 2000b; Somvanshi et al., 2009). All five SSTRs are known to inhibit AC, a key intracellular signal transducer through $G\alpha_{i/o}$ subunits (Grant et al., 2004b; Patel, 1999). C-tail of SSTRs has been shown to play a critical role in cAMP inhibition. Previous studies have shown that C-tail deleted mutants of human SSTR5 and SSTR4 failed to inhibit FSK-stimulated cAMP (Rocheville et al., 2000a; Rocheville et al., 2000b; Somvanshi et al., 2009).

Several previous studies have emphasized the importance of GPCR dimerization in receptor-biogenesis, regulation and pharmacology (Grant et al., 2008; Prinster et al., 2005; Terrillon and Bouvier, 2004). SSTRs form homo- and heterodimers within the same family as well as with other related GPCRs and members of receptor tyrosine kinase family (Grant et al., 2008; Grant et al., 2004a; Grant and Kumar, 2009; Grant et al., 2004b; Pfeiffer et al., 2001; Rocheville et al., 2000a; Rocheville et al., 2000b; Somvanshi et al., 2009; Watt et al., 2009). It has been shown previously that rat SSTR3 exists as homodimer and also heterodimerize with SSTR2 with the functional activity of SSTR2 whereas SSTR3-like properties were abrogated (Pfeiffer et al., 2001).

Despite detailed pharmacological characterization of homo-and heterodimerization, very little is known about the role of GPCRs in the regulation of physiological response of the cells and MAPK signaling modulating cell proliferation, differentiation and migration (Nishimoto and Nishida, 2006). The phosphorylation of ERK1/2 is known to be involved in cell proliferation. ERK1/2 has been also shown to regulate the cell proliferation during G_1/G_S cell-cycle transition induced by the EGF (Nishimoto and Nishida, 2006; Watt et al., 2009). SSTRs are known to differentially regulate the ERKs in a receptor-specific manner (Grant et al., 2008; Lahlou et al., 2003; Patel, 1999; Somvanshi et al., 2009; Weckbecker et al., 2003). SSTR1, SSTR2 and SSTR4 stimulate ERK phosphorylation whereas SSTR5 inhibits it (Grant et al., 2008; Lahlou et al., 2004; Sellers, 1999; Smalley et al., 1999; Somvanshi et al., 2009). SSTR3 upon activation may stimulate or inhibit the ERK phosphorylation

depending upon the cell type (Lahlou et al., 2004; Lahlou et al., 2003; Patel, 1999; Pfeiffer et al., 2001).

SST is widely recognized for its antiproliferative role in PTP-dependent manner and the antiproliferative mechanism may be cytostatic or cytotoxic (Patel, 1999; Sharma et al., 1996, 1999; Sharma and Srikant, 1998a; Weckbecker et al., 2003; Weiss et al., 1981). In particular, SSTR3 mediated pro-apoptotic effects involve translocation of PTP to cell membrane, and p53 activation (Liu et al., 2000; Sharma et al., 1996). These unique antiproliferative characteristics of SST have been exploited in the treatment of various tumors. Previously, it was shown that C-tail of SSTR5 is critical in mediating the SST-analogue-induced cytostatic activity and there was loss of antiproliferative effect proportional to aa deleted from the C-tail (Sharma et al., 1999). A recent study has described the role of SSTR4 C-tail in modulating various downstream signaling cascades (Somvanshi et al., 2009). The exact role of C-tail in regulating SSTR3 mediated downstream signaling and antiproliferative effects is not well understood. Taking advantage of the morphological, biochemical and biophysical techniques, the present study describes the role of C-tail of human SSTR3 on receptor expression, coupling to AC, downstream signaling pathways and cell proliferation in HEK-293 cells transfected with *wt*-SSTR3 as well as four C-tail deleted SSTR3 mutants. This study demonstrated for the first time that human SSTR3 signaling and antiproliferative effects are dependent on aa residues present in the C-tail.

2.2 Materials and methods

2.2.1 Materials

SST and Leu^{8}-D-Trp^{22}-Tyr^{25} SST-28 were purchased from Bachem, Torrance, CA. The SSTR3 selective non-peptide agonist L-796778 was kindly provided by Dr. S.P. Rohrer from Merck and Co (Rohrer and Schaeffer, 2000). Rabbit polyclonal anti-SSTR3 antibody was purchased from Santa Cruz Biotechnology Inc. Santa Cruz, CA. Fluorescein/rhodamine-conjugated mouse monoclonal antibodies against hemagglutinin (HA) and TUNEL kit were purchased from La Roche Applied Science, Mannheim, Germany. Mouse monoclonal anti-PARP-1 and PTP-1C antibodies were purchased from BD-Biosciences, Mississauga, ON. Rabbit polyclonal antibodies for total and pERK1/2 were purchased from Cell Signaling Technology, Danvers, MA. Fluorescein and rhodamine or peroxidase conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA. Mouse monoclonal anti-PCNA and β-actin antibodies were purchased from Sigma-Aldrich Inc., St. Louis, MO. cAMP kit was purchased from BioVision, Inc. CA, USA. ECL Western blotting detection kit and nitrocellulose Hy-Bond ECL membrane was purchased from Amersham Ltd. Oakdale, ON. Dulbecco's modified eagle medium (DMEM), trypsin-EDTA, phosphate buffered saline (PBS) were purchased from Invitrogen, Burlington, ON, Canada. Reagents for electrophoresis were purchased from BIO-RAD Laboratories Mississauga ON. Other reagents were of AR grade and were purchased from various sources.

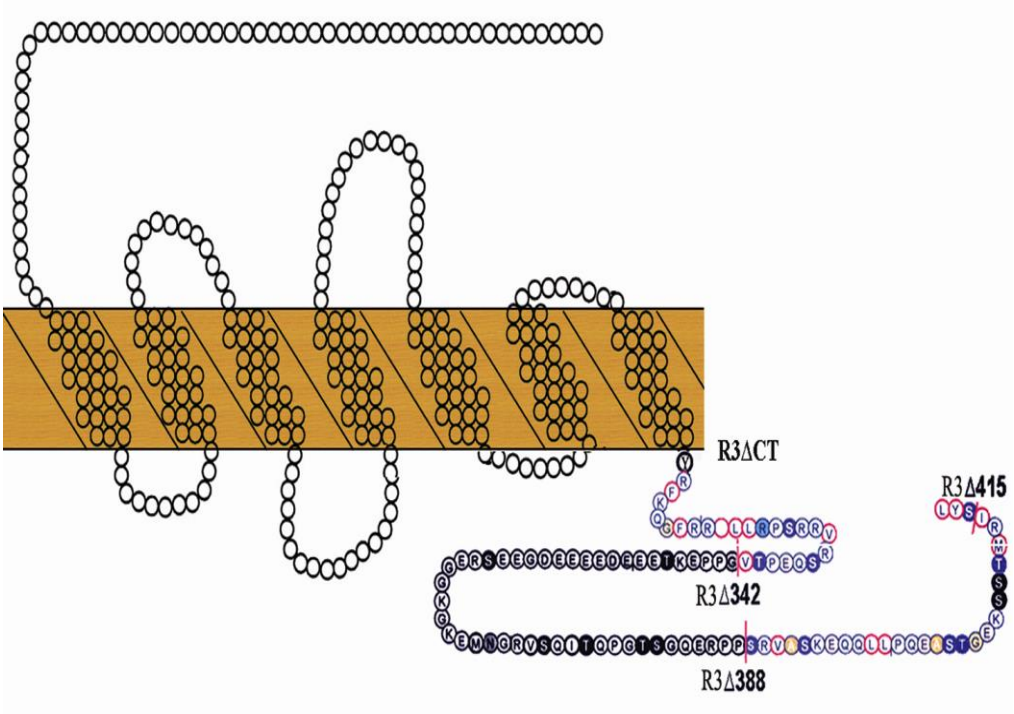

Figure 2.1 Illustration of human SSTR3 structure. Schematic representation of 418 aa ***wt*-**SSTR3 and the aa sequence in its C-tail, and the sites for truncation of C-tail aa [3 aa (R3 Δ415), 30 aa (R3 Δ388), 76 aa (R3 Δ342) and 100 aa (R3ΔCT)].

2.2.2 SSTR3 constructs and transfection

Constructs of *wt*-SSTR3, N-terminal HA-tagged SSTR3 (HA-SSTR3) and four different mutants of SSTR3 (Figure 2.1) with aa deletions in the C-tail; 3 aa (R3 Δ415), 30 aa (R3 Δ388), 76 aa (R3 Δ342) and 100 aa (complete C-tail deletion or R3ΔCT) were made using pCDNA3.1/Neo (neomycin resistance) as previously described (Grant et al., 2004b; Somvanshi et al., 2009). HEK-293 cells expressing *wt*-SSTR3, HA-SSTR3 and C-tail mutants were stably transfected using Lipofectamine transfection reagent as described earlier (Grant et al., 2004b). Clones

were selected and maintained in DMEM with 10% fetal bovine serum (FBS) and 700 µg/ml neomycin.

2.2.3 Binding analysis

HEK-293 cells expressing *wt*-SSTR3 and mutants were grown to 70-80% confluency, and membrane was prepared and processed for binding studies. Briefly, 20-40 µg of membrane protein was used and incubated with radiolabelled SST-28 for 30 min at 37ºC as previously described (Grant et al., 2008). Saturation binding experiments were performed with membranes using increasing ligand concentrations under equilibrium binding conditions. Incubations were terminated by the addition of 1 ml ice-cold PBS containing 0.2% bovine serum albumin, rapid centrifugation, and washing. Radioactivity was measured with a LKB gamma counter (LKB-Wallac, Turku, Finland). The dissociation constant (K_d) and the number of SST-28 binding sites (B_{max}) were calculated and analyzed with Graph Pad Software, San Diego, CA.

2.2.4 Receptor coupling to adenylyl cyclase

To determine the receptor coupling to AC, HEK-293 cells stably expressing *wt*-SSTR3 and different C-tail mutants were grown in 6-well plates and used at 70% cell confluency for cAMP assay as described previously (Somvanshi et al., 2009). Briefly, cells were treated for 30 min with FSK (20 µM) and 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX) in the presence or absence of SST (1 µM) or L-796778 (50 nM) at 37°C. Cells were then scraped in HCl (0.1 N) and FSK-stimulated cAMP was

determined by immunoassay using a cAMP Kit following the manufacturer's guidelines.

2.2.5 SSTR3 membrane expression and agonist-induced internalization

To study SSTR3 membrane expression and receptor internalization, HEK-293 cells expressing *wt*-SSTR3 and C-tail mutants were grown on poly-D-Lysine coated glass coverslips to 60-70% confluency and then treated with SST (1 μM) or L-796778 (50 nM) for 15 min at 37ºC. Treatment was terminated with ice cold PBS and the cells were fixed with 4% paraformaldehyde (PF) for 15 min at room temperature (RT). For membrane and cytosolic expression, cells were treated without or with 0.3% Triton X-100 for 15 min at RT, washed in PBS and then processed for indirect immunofluorescence immunocytochemistry using rabbit polyclonal anti-SSTR3 antibodies. Receptor expression in non-permeabilized and permeabilized cells was analyzed under Leica TCS SPE Confocal Microscope. Adobe Photoshop was used for making composites.

2.2.6 Pb-FRET analysis

To determine SSTR3 homodimerization, microscopic Pb-FRET analysis was performed in HEK-293 cells expressing HA-SSTR3 (B_{max}, 285 $\pm$ 10 fmol/mg protein; K_d, 1.2 $\pm$ 0.21 nM) as previously described (Grant et al., 2004b). Briefly, cells were grown on poly-D-Lysine coated glass coverslips to 60-70% confluency and treated with SST (1 μM) or L-796778 (50 nM) for 10 min at 37ºC, and fixed with 4% PF for 15 min at RT. Following three subsequent washes in PBS, cells were incubated with

FITC conjugated anti-HA antibody and rhodamine-conjugated anti-HA antibody for donor and acceptor pair respectively. The plasma membrane region was used to analyze the photobleaching decay on a pixel-by-pixel basis. The FRET efficiency (E) was calculated as a percent based upon the photo bleaching time constants of the donor taken in the absence (D-A) and presence (D+A) of acceptor. The effective FRET efficiency (E) was calculated according to the formula E = [1-(D-A/D+A)] ×100 (Somvanshi et al., 2009). Adobe Photoshop and Image J software, NIH (Collins, 2007) were used make composites.

2.2.7 Western blot analyses

To determine homodimerization, cells expressing *wt*-SSTR3 and C-tail mutants were treated with SST (1 μM) and L-796778 (50 nM) for 15 min at 37°C. Cell lysate was prepared and processed for Western blot analysis using SSTR3 antibody (1:500 dilution) as previously described (Grant et al., 2004b; Somvanshi et al., 2009). For other procedures like membrane blocking, primary and secondary antibody incubation, and chemiluminescence detection, the manufacturer's instructions were followed. Images were captured with an Alpha Innotech FluorChem 8800 gel box imager (Protein Simple, Santa Clara, CA, USA). To standardize for protein loading, all membranes were reprobed for beta-actin.

Immunoblotting for ERK1/2 and PARP-1 was performed using specific antibodies. Cells stably expressing *wt*-SSTR3 and C-tail mutants were treated with SST (1 μM) or L-796778 (50 nM) at 37°C for 5 or 15 min. For PARP-1 expression, cells were treated with L-796778 (50 nM) for 24 h at 37°C. Reaction was terminated

by using ice-cold PBS and cells were collected and homogenized in radioimmune precipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris–HCL, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, protease and phosphatase inhibitors 1:100, pH 8.0). Cell lysate was prepared and probed for total and pERK1/2 using specific antibodies (1:1000 dilution). For PARP-1 expression, antibody dilution was 1:5000.

To estimate the membrane and cytosolic expression of PTP-1C, cells expressing *wt*-SSTR3 and C-tail mutants were treated with SST (1 μM) or L-796778 (50 nM) for 15 min at 37°C. Cell lysate was centrifuged at 10000 g for 1 h at 4°C. The membrane and cytosolic fractions were fractionated on 10% SDS-PAGE and processed for PTP-1C expression using monoclonal anti-PTP-1C antibody (1:500 dilution). β-actin was used as loading control. Densitometric analysis on all the bands was performed by using FluorChem software (Protein Simple, Santa Clara, CA, USA).

2.2.8 Cell proliferation assay

To characterize the antiproliferative role of SSTR3, non-transfected HEK-293 cells or cells expressing *wt*-SSTR3 and C-tail mutants were seeded at a density of 5000 cells/well in 96-well plates and allowed to grow for 24 h. Cells were then serum deprived (without FBS) for 24 h followed by treatment with SST (1 μM) and L-796778 (50 nM) in the presence of 5% FBS for 24 h. As a control, cells were supplemented only with FBS to compare for the normal cell growth. Conventional MTT [(3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay protocol

was used to measure the cell viability. Briefly, 20 µl of 5 mg/ml MTT solution was added and incubated for 2 h at 37ºC in a humidified atmosphere containing 5% CO_2. The formazan precipitate formed was dissolved in 200 µl of isopropanol. The absorbance was measured in a microplate spectrophotometer at 550 nm.

2.2.9 PCNA immunofluorescence and TUNEL staining

To characterize the cells susceptible to apoptosis, cells expressing *wt*-SSTR3 and C-tail mutants were grown on poly-D-Lysine coated glass coverslips to 60-70% confluency followed by treatment with SST (1 µM) or L-796778 (50 nM) for 24 h. Cells were permeabilized using 0.3% Triton X-100 and then incubated overnight at 4ºC with monoclonal anti-PCNA primary antibody followed by Cy3-conjugated secondary antibody for 1 h at RT. The coverslips were washed in PBS and were further incubated with TUNEL reaction mixture for 1 h at 37ºC in a humidified atmosphere in dark. Following subsequent washes in PBS, the coverslips were mounted on glass slides and analyzed under Leica fluorescence microscope. Adobe Photoshop was utilized for making composites and merged images illustrating colocalization were generated by using Image J software, NIH (Collins, 2007).

2.2.10 Statistical analysis

Results are presented as mean ± S.E unless otherwise stated. Statistical analysis was carried out using Graph Pad Prism 4.0 as indicated and statistical differences were taken at p values < 0.05.

2.3 Results

2.3.1 Binding analysis of *wt*-SSTR3 and mutants

The membrane expression of *wt*-SSTR3 and C-tail mutants was determined by binding analysis using radiolabelled SST-28 as described in sub-section 2.2.3. As shown in Table 2.1, saturation analysis indicates high expression of *wt*-SSTR3 ($B_{max,}$ 285 ± 10 fmol/mg of protein; $K_{d,}$ 1.2 ± 0.21 nM) at the plasma membrane. As compared to the *wt*-SSTR3, the C-tail mutants displayed marginal changes in receptor density at the cell surface and the differences were statistically insignificant. These results suggest that C-tail deletion had no significant effect on SSTR3 cell surface expression.

Table 2.1 Binding characteristics of *wt*-SSTR3 and C-tail mutants

Receptor	K_d (nM)	B_{max} (fmol/mg of protein)
wt-SSTR3	1.2 ± 0.21	285 ± 10
R3 Δ415	1.4 ± 0.28	292 ± 19
R3 Δ388	1.5 ± 0.31	315 ± 11
R3 Δ342	1.8 ± 0.38	260 ± 17
R3ΔCT	1.5 ± 0.36	255 ± 13

Data shown in the table represents mean ± S.E. of three independent experiments. Data analysis was done by using one-way ANOVA and *post hoc* Dunnett's test to compare against wt-SSTR3.

Table 2.2 Semi quantitative analysis of receptor-like immunoreactivity in HEK-293 cells transfected with *wt*-SSTR3 and C-tail mutants, and treated with SST and L-796778 for 15 min at 37ºC

Condition	*wt*-SSTR3		R3 Δ415		R3 Δ388		R3 Δ342		R3ΔCT	
	NP	P	NP	P	NP	P	NP	P	NP	P
Control	+++	++	+++	++	+++	+++	++	+++	++	+++
SST (1 µM)	++	+++	++	+++	++	+++	++	+++	++	+++
L-796778 (50 nM)	++	+++	++	+++	++	+++	++	+++	++	+++

NP = non-permeabilized cells. P = permeabilized cells. Values are ranked as follows: +++ strongly positive and ++ moderately positive. Immunoreactivity ranged on the basis of intensity of receptor like immunoreactivity determined by using NIH Image J (Collins, 2007).

2.3.2 SSTR3 membrane expression and agonist-induced internalization

Previous studies have shown that amongst all SSTR subtypes, SSTR3 internalized the most in a time and temperature-dependent manner and does not recycle back to the cell membrane, rather targeted for degradation (Hukovic et al., 1996). As shown in Figure 2.2 and Table 2.2, the receptor-like immunoreactivity was observed at both cell membrane and intracellularly in cells expressing *wt*-SSTR3 whereas upon treatment with SST and L-796778, the receptor specific agonist, there was loss of receptor expression at the cell surface. In C-tail mutants (R3 Δ415, R3 Δ388, R3 Δ342 and R3ΔCT), the receptor expression at the cell surface was not significantly different when compared with *wt*-SSTR3. Importantly, cells expressing

C-tail mutants displayed agonist-mediated receptor internalization. These results suggest that C-tail does not play determinant role on receptor internalization.

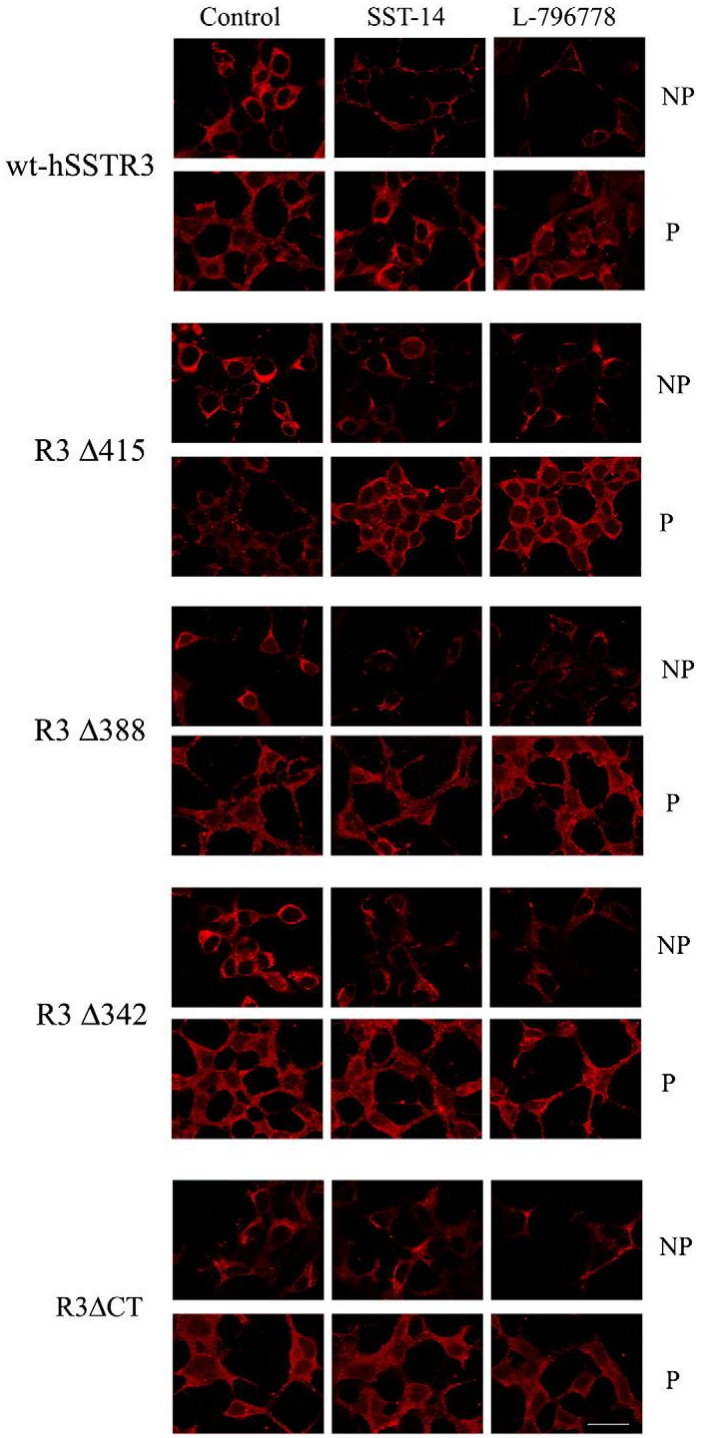

Figure 2.2 Representative photomicrographs illustrating the expression and internalization of SSTR3. Cells transfected with *wt*-SSTR3 and C-tail mutants were treated with SST (1 μM) and L-796778 (50 nM) for 15 min at 37ºC, and processed for membrane and intracellular expression in non-permeabilized (NP) and permeabilized (P) cells using indirect immunofluorescence immunocytochemistry. Note the comparable receptor expression at the cell surface between *wt*-SSTR3 and C-tail mutants. In cells expressing *wt*-SSTR3 and C-tail mutants, the receptor-like immunoreactivity was decreased at plasma membrane upon agonist treatment. Data are presented as Mean ± S.E. of three independent experiments. Scale bar = 10 μm.

2.3.3 Receptor coupling to adenylyl cyclase is disrupted upon SSTR3 C-tail deletions

All SSTRs are known to inhibit AC, whether SSTR3 coupling to AC is impaired in C-tail mutants is not known. Accordingly, the role of SST and SSTR-specific agonist on inhibition of FSK-stimulated cAMP was examined in cells expressing *wt*-SSTR3 and different mutants. The inhibition of cAMP by SSTRs is a direct measure of receptor functions. Cells were treated with SST (1 μM) and L-796778 (50 nM) for 30 min at 37ºC. As shown in Figure 2.3, FSK-stimulated cAMP in cells expressing *wt*-SSTR3 was inhibited by 52% and 51% upon treatment with SST (1 μM) and L-796778 (50 nM) respectively. In C-tail mutants, there was a gradual loss of agonist mediated inhibition of cAMP which was proportional to the aa deleted. Consistent with previous studies, these results implicate the importance of C-tail in mediating agonist induced cAMP inhibition.

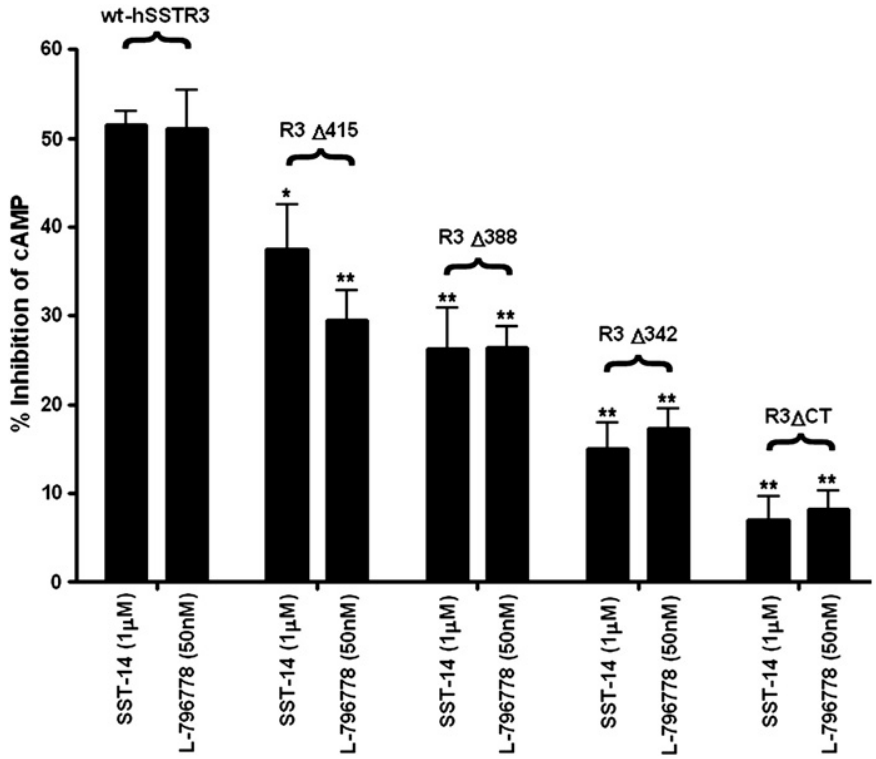

Figure 2.3 Receptor coupling to adenylyl cyclase. Cells expressing *wt*-SSTR3 and mutants were treated with FSK (20 μM) in the presence or absence of SST (1 μM) and L-796778 (50 nM) at 37°C for 30 min and processed for cAMP assay. Note a progressive loss in the ability of SST or L-796778 to inhibit the FSK stimulated cAMP in cells transfected with C-tail mutants in comparison to *wt*-SSTR3, which indicates that C-tail plays an important role in receptor coupling to AC. Data are presented as Mean ± S.E. of three independent experiments. Data analysis was done by using one-way ANOVA and *post hoc* Dunnett's test to compare against wt-SSTR3 (*, $p < 0.05$; **, $p < 0.01$).

2.3.4 Human SSTR3 exists as a preformed homodimer

To determine whether human SSTR3 exists as monomer and/or dimer, microscopic Pb-FRET analysis was performed at cell surface. Cells expressing HA-SSTR3 were treated with SST (1 μM) and L-796778 (50 nM) and processed for Pb-FRET analysis as previously described (Grant et al., 2008; Somvanshi et al., 2009).

As illustrated in the Figure 2.4A, B and C and Table 2.3, HA-SSTR3 is a preformed homodimer in basal conditions and displayed a relative FRET efficiency of 17 ± 1% which was reduced to 12.2 ± 0.9% and 13.3 ± 1% upon treatment with SST and L-796778 respectively. The decreased effective FRET efficiency indicates that HA-SSTR3 homodimers were decreased upon agonist treatment.

Table 2.3 Microscopic Pb-FRET analysis in HEK-293 cells expressing HA-SSTR3 and treated with SST-14 and L-796778 for 10 min at 37ºC

Cell line	Treatment	τ_{avg} (s)		n	E (%)
HA-SSTR3	Control	D-A	24.18 ± 0.9	48	17 ± 1
		D+A	29.12 ± 1	48	
	SST-14 (1 µM)	D-A	24.16 ± 0.6	54	12.2 ± 0.9**
		D+A	27.52 ± 0.3	54	
	L-796778 (50 nM)	D-A	24.8 ± 1	42	13.3 ± 1**
		D+A	28.6 ± 0.8	42	

D-A and D+A indicate donor without and with acceptor respectively; τ_{avg} denotes average photobleaching time constants; n is the total number of cells analyzed; E (%) refers to mean effective FRET efficiency. Mean ± S.E. are representative of three independent experiments. Data analysis was performed by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (**, $p < 0.01$).

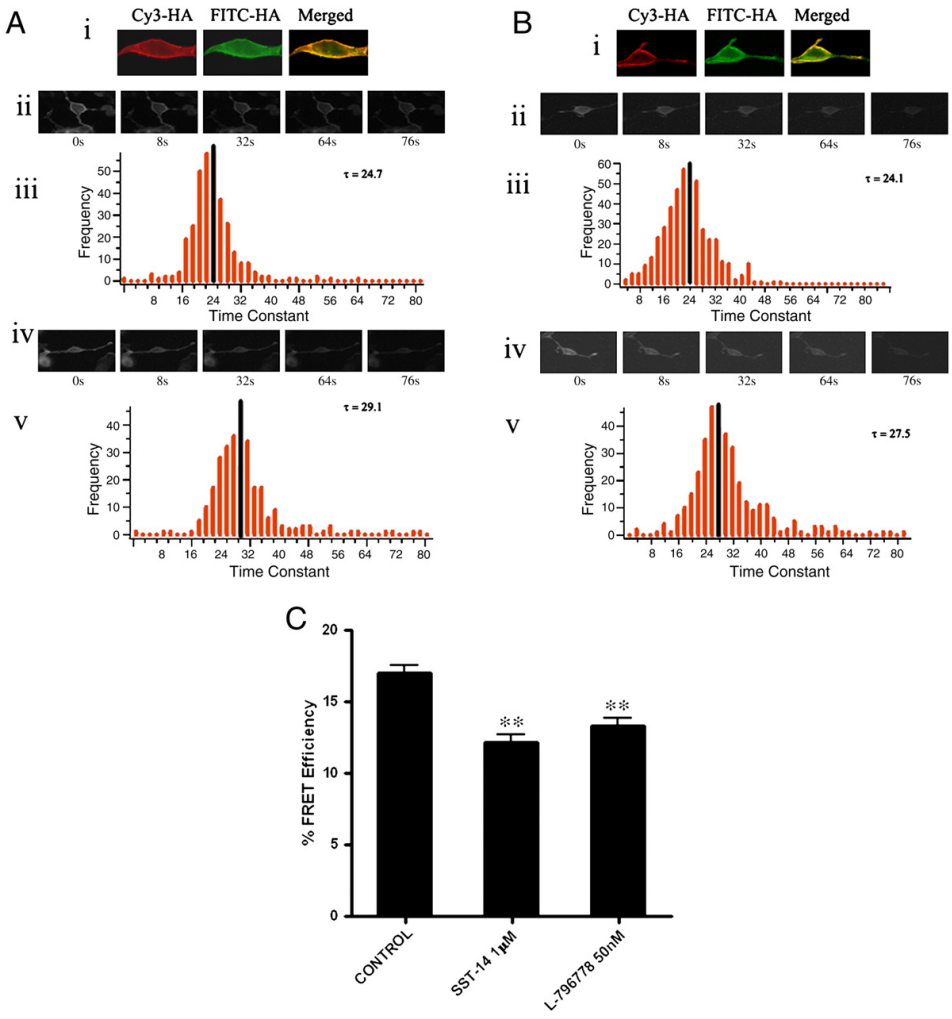

Figure 2.4 Microscopic Pb-FRET illustrating HA-SSTR3 homodimerization. HEK-293 cells expressing HA-SSTR3 were processed for microscopic Pb-FRET analysis without **(A)** or with SST (1 μM) treatment **(B)** for 10 min at 37ºC. Representative photomicrographs illustrating Cy3-HA-SSTR3 (red), FITC-HA-SSTR3 (green) and colocalization (yellow) in HEK-293 cells in the absence **A (i)** and presence **B (i)** of SST treatment. A selection of photomicrographs illustrating photobleaching profile of the cells either expressing donor alone **A (ii) and B (ii)** or donor + acceptor **A (iv) and B (iv)**. Histograms representing pixel by pixel analysis of time constant of donor alone **A (iii) and B (iii)** and donor + acceptor **A (v) and B (v)**. **(C)** Histogram illustrating a decrease in FRET efficiency upon SST and L-796778 treatment suggests decrease of homodimers in response to agonist. Mean ± S.E. is representative of three independent experiments. Data analysis in **(C)** was performed by one-way ANOVA and *post hoc* Dunnett's test to compare against control (**, $p < 0.01$).

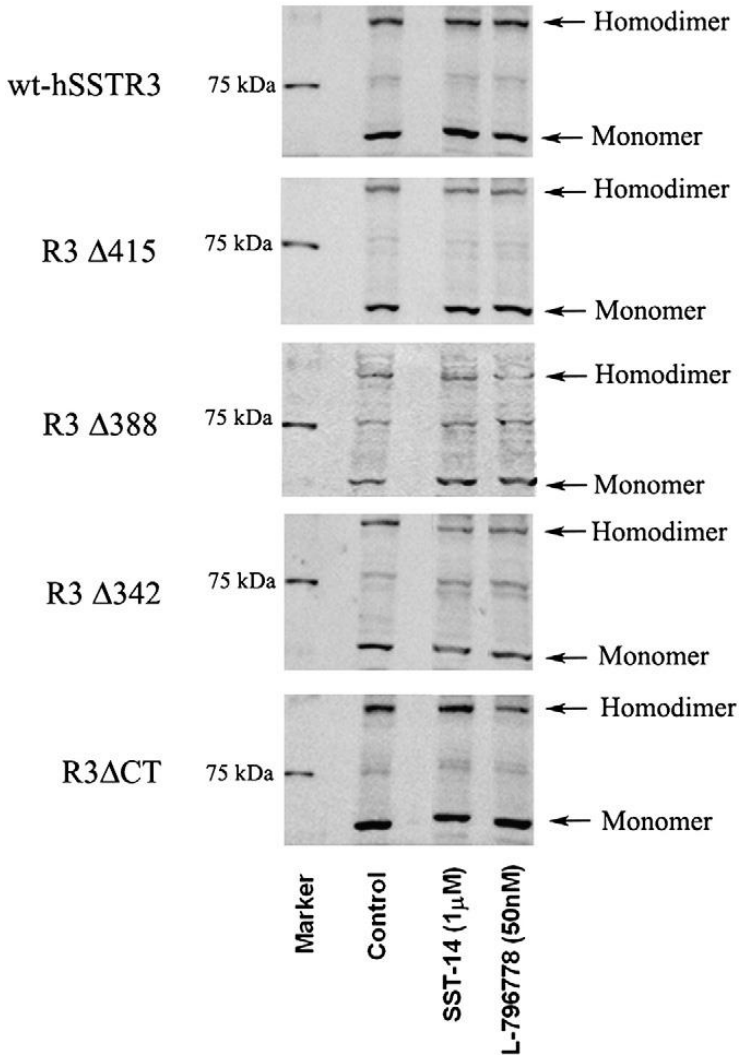

Figure 2.5 Homodimerization of wt-SSTR3 and C-tail mutants by Western blot analysis. HEK-293 cells transfected with *wt*-SSTR3 and C-tail mutants were treated with SST (1 μM) and L-796778 (50 nM) for 15 min and the membrane extract was prepared and processed for Western blot analysis as described in sub-section 2.2.7. The receptor-specific band for monomer and homodimer was detected at the expected size of ~55 kDa and ~110 kDa, respectively. *wt*-SSTR3 and C-tail mutants exist as preformed homodimers at cell membrane. Note the decrease in dimers in *wt*-SSTR3 and mutants upon agonist treatment. These results indicate that agonist dependent effect on dimerization is not altered upon C-tail deletion. Densitometry on the bands was performed and data represent Mean ± S.E. of three independent experiments. Data analysis was done by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$) (Data not shown).

The important role of C-tail in SSTR homodimerization has been previously demonstrated (Grant et al., 2004b; Somvanshi et al., 2009). To ascertain the role of C-tail deletion in SSTR3 on homodimerization, Western blot analysis was performed in membrane preparation from cells expressing *wt*-SSTR3 and C-tail mutants with or without agonist. As shown in Figure 2.5, *wt*-SSTR3 exists as a preformed dimer and the results are consistent with Pb-FRET analysis. C-tail mutants also displayed homodimerization in basal conditions in a comparable manner to wt-SSTR3. Receptor dimerization was decreased in the presence of SST and L-796778 without any significant changes in the status of monomers possibly suggesting the internalization of the receptor as a homodimer as previously described for rat SSTR3 (Pfeiffer et al., 2001).

2.3.5 Influence of SSTR3 C-tail on ERK1/2 signaling

Having seen the significant changes in cAMP inhibition between *wt*-SSTR3 and C-tail mutants, the next step was to determine whether C-tail deletions in SSTR3 would affect ERK1/2 phosphorylation. As illustrated in Figure 2.6, pERK1/2 expression was decreased upon L-796778 treatment in cells expressing *wt*-SSTR3 and mutants R3 Δ388, R3 Δ342 and R3ΔCT. On the other hand, SST inhibited pERK1/2 expression only in case of R3 Δ342, and conversely, increased pERK1/2 in R3 Δ415. Interestingly, the basal pERK1/2 in *wt*-SSTR3 was less when compared to C-tail mutants suggesting the inhibitory effect of *wt*-SSTR3 on ERK1/2 phosphorylation.

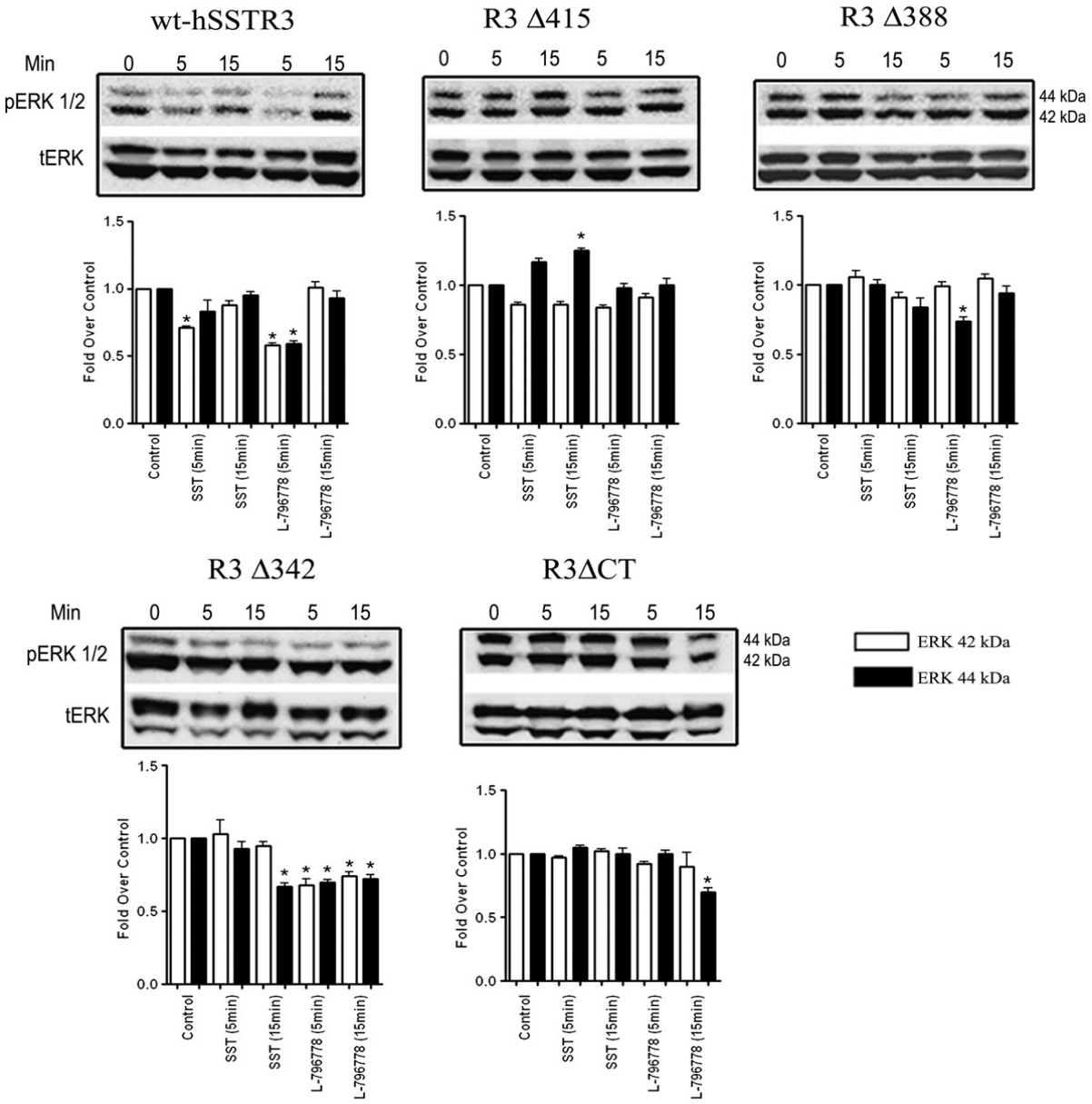

Figure 2.6 Western blots illustrating ERK1/2 phosphorylation. Cells stably transfected with *wt*-SSTR3 and C-tail mutants were treated with SST (1 μM) and L-796778 (50 nM), and were processed for total and pERK1/2 using western blot analysis as described in sub-section 2.2.7. *wt*-SSTR3 inhibits ERK1/2 phosphorylation as shown by low pERK1/2 levels when compared to C-tail mutants which exhibit high pERK1/2. Densitometry on western blots for individual ERK1/2 was performed. Data are presented as Mean ± S.E. of three independent experiments. Data analysis was done by using one-way ANOVA and post hoc Dunnett's test to compare against control (*, $p < 0.05$).

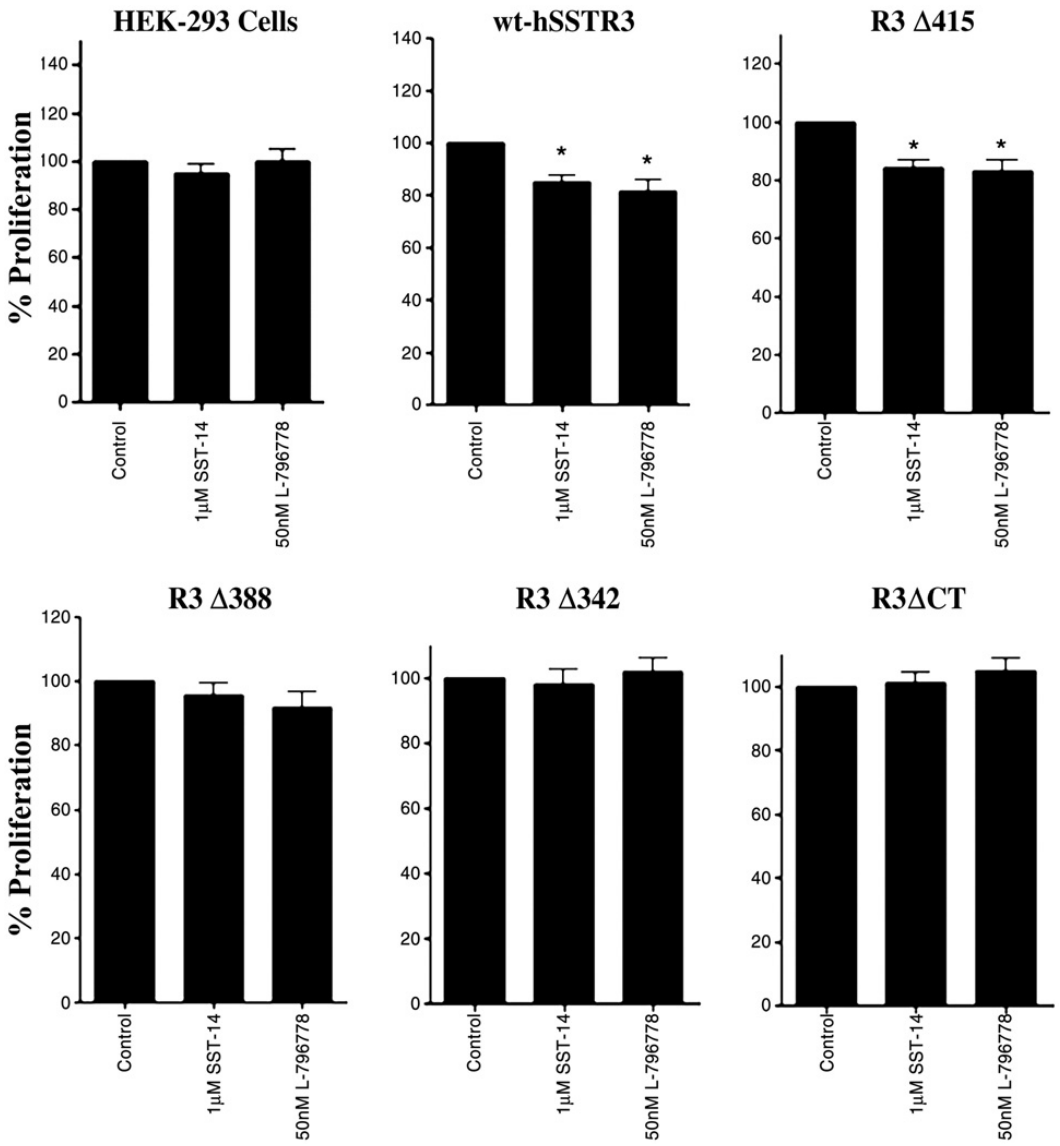

Figure 2.7 The role of C-tail deletion on cell proliferation. HEK-293 cells expressing *wt*-SSTR3 and C-tail mutants were treated with SST (1 μM) and L-796778 (50 nM) for 24 h before processing for MTT assay as described in sub-section 2.2.8. SST and L-796778 inhibited cell proliferation in *wt*-SSTR3 and R3 Δ415, while this effect was lost in R3 Δ388, R3 Δ342 and R3ΔCT, indicating that C-tail plays a decisive role on cell proliferation. Mean ± SE representative of three independent experiments. Data analysis was done by using one-way ANOVA and post hoc Dunnett's test to compare against control (*, $p < 0.05$; **, $p < 0.01$).

2.3.6 The role of C-tail in regulation of SSTR3 mediated inhibition of cell proliferation

SSTR3 is known to exert antiproliferative effects (Lahlou et al., 2004; Sharma et al., 1996; Sharma and Srikant, 1998a). Next, the effect of SST and L-796778 on cell proliferation in non-transfected HEK-293 cells or cells expressing *wt*-SSTR3 and C-tail mutants was determined by MTT assay as described in sub-section 2.2.8. As shown in Figure 2.7, in *wt*-SSTR3 cells, SST and L-796778 inhibited the cell proliferation by 15 ± 5% and 18.5 ± 8% respectively when compared to control. Comparable results were obtained in R3 ∆415, as the inhibition of cell proliferation by SST and L-796778 was 16 ± 5% and 17 ± 7% respectively. Conversely in C-tail mutants, R3 ∆388, R3 ∆342 and R3∆CT, the antiproliferative effect of SST and L-796778 was lost. No significant changes in cell proliferation were observed in non-transfected cells when treated with SST or L-796778.

2.3.7 PARP-1 expression is increased in the presence of SSTR3 selective agonist

As previously described, SSTR3 mediated antiproliferative effects are uniquely triggered by apoptosis (Sharma et al., 1996). Accordingly, in this experiment the expression of PARP-1 was determined as an index of apoptosis. Cells transfected with *wt*-SSTR3 and C-tail mutants were treated with L-796778 (50nM) for 24 h at 37ºC and processed for western blot analysis for PARP-1 expression. As depicted in Figure 2.8, cells expressing *wt*-SSTR3, R3 ∆415 and R3 ∆388 displayed increased expression of PARP-1 upon treatment with L-796778 when compared to control.

With subsequent deletions in the C-tail (R3 Δ342 and R3ΔCT), the agonist-induced expression of PARP-1 was completely abolished, suggesting the failure of agonist mediated apoptosis.

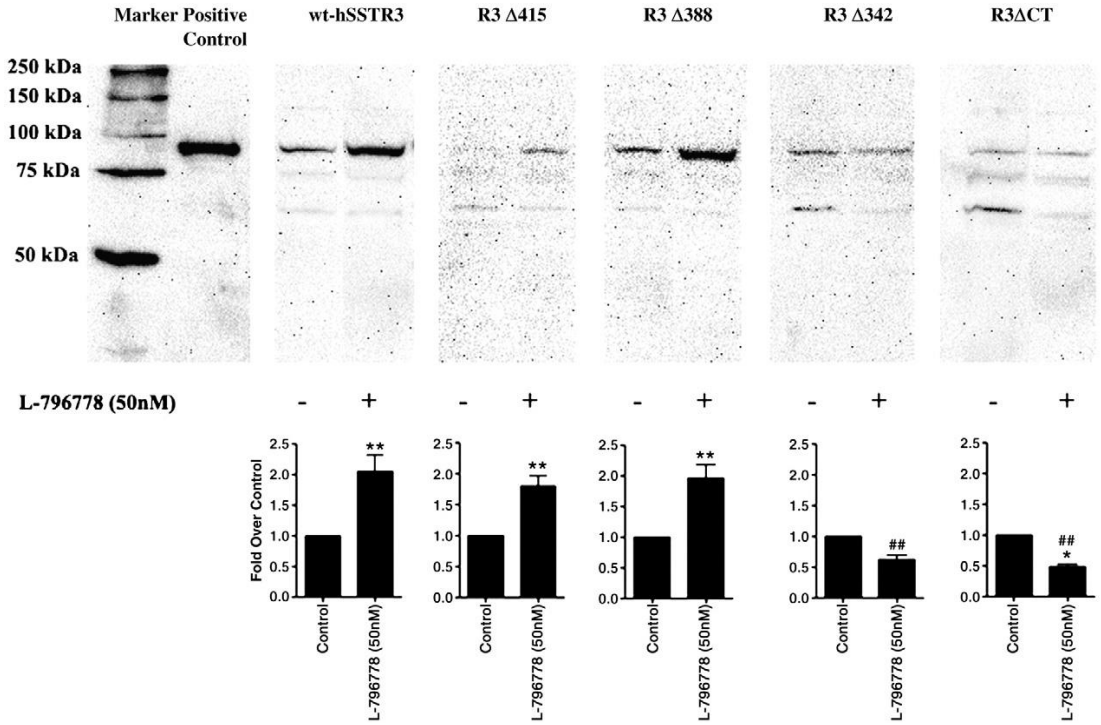

Figure 2.8 Immunoblots illustrating the expression of PARP-1. Stable transfectants of *wt*-SSTR3 and C-tail mutants were treated with L-796778 (50 nM) for 24 h and processed for PARP-1 expression as described in sub-section 2.2.7. The receptor specific agonist mediated activation of PARP-1 in *wt*-SSTR3, R3 Δ415 and R3 Δ388 confirms apoptosis and this effect was completely lost in R3 Δ342 and R3ΔCT. Camptothecin-treated Jurkat cell lysate was used as positive control for PARP-1. β-actin was used as a control for protein loading. Histograms illustrate the changes in PARP-1 activation. Data are presented as Mean ± S.E. of three independent experiments. Data analysis was done using Student's *t*-test (unpaired) to compare treatment with control (*, $p < 0.05$; **, $p < 0.01$). Statistical analysis was performed by one-way ANOVA and *post hoc* Dunnett's test to compare against *wt*-SSTR3 ($^{\#\#}$, $p < 0.001$).

2.3.8 Increased translocation of PTP-1C to the cell membrane is required for SSTR3-mediated apoptosis

Evidence from previous studies have suggested the involvement of PTP in mediating the antiproliferative actions by SSTRs and this effect is attributed to its translocation to the cell membrane (Lopez et al., 1996; Patel, 1999; Sharma et al., 1996). Next, the role of C-tail of SSTR3 in modulating the agonist-dependent membrane translocation of PTP-1C was determined. Cells expressing *wt*-SSTR3 and mutants were treated with SST (1 μM) and L-796778 (50 nM) for 15 min at 37ºC. Membrane and cytosolic fractions were prepared and processed for PTP-1C expression. As shown in Figure 2.9, cells expressing *wt*-SSTR3 displayed an increased membrane expression of PTP-1C in response to SST and L-796778 when compared with control. On the other hand, C-tail mutants R3 Δ415, R3 Δ388 and R3 Δ342 displayed no significant changes in membrane expression of PTP-1C in response to agonist treatment. Conversely, agonist treatment in R3ΔCT decreased PTP-1C expression at the membrane in comparison with control. Furthermore, comparable changes in cytoplasmic expression of PTP-1C were observed in *wt*-SSTR3 and mutants. These results provide evidence that membrane translocation of PTP-1C by SSTR3 is abrogated upon C-tail deletion.

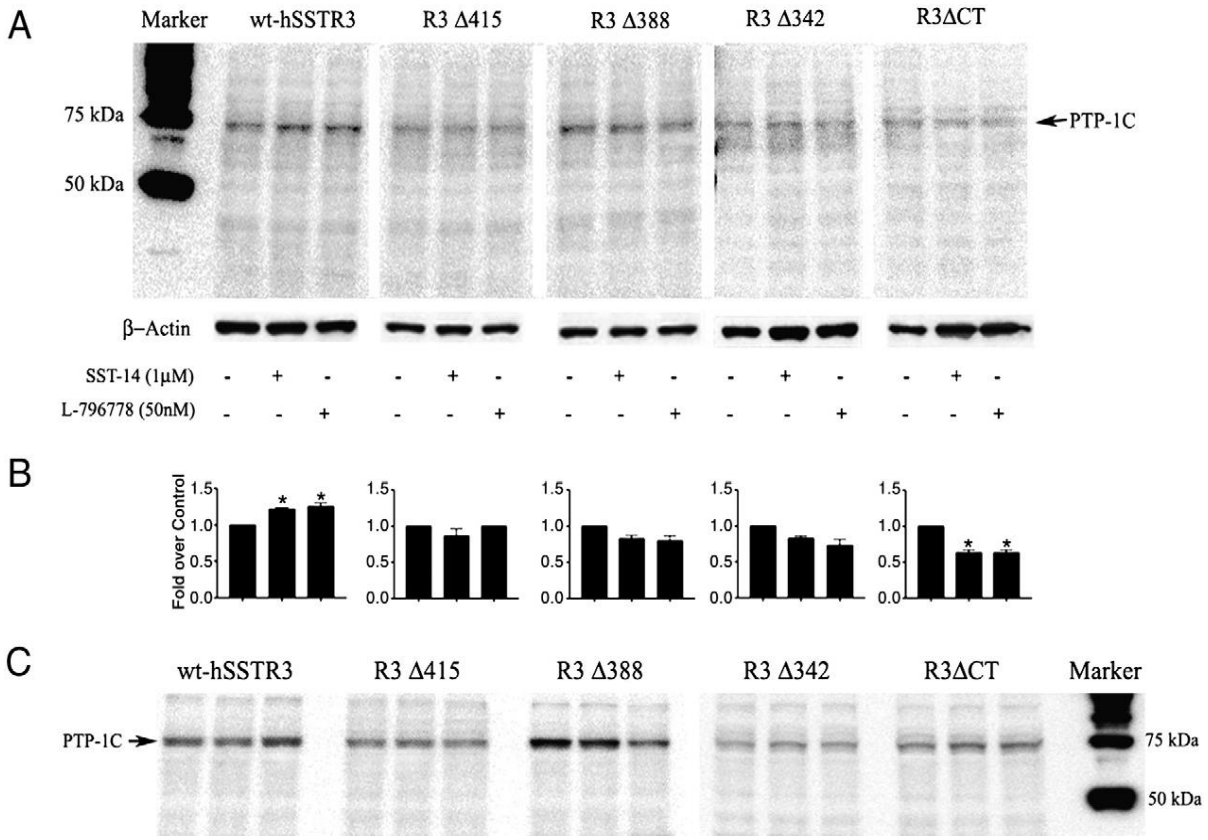

Figure 2.9 Western blots showing membrane and cytosolic expression of PTP-1C. HEK-293 cells expressing *wt*-SSTR3 and C-tail mutants were treated with SST (1 μM) and L-796778 (50 nM) for 15 min. Membrane and cytosolic extract was processed for PTP-1C expression as described in sub-section 2.2.7. Panels **(A)** and **(C)** represent immunoblots displaying PTP-1C expression in the membrane and cytosol respectively. Note the increased membrane expression of PTP-1C in response to SST and L-796778 treatment in *wt*-SSTR3, and conversely, in C-tail mutants this effect is significantly lost. These results suggest that C-tail is essential for membrane translocation of PTP-1C. Densitometric analysis performed on the bands shown in **(A)** and **(C)** to determine changes in PTP-1C expression are illustrated in panels **(B)** and **(D)** respectively. Results represent Mean ± SE of three independent experiments. Data analysis was done by using one-way ANOVA and post hoc Dunnett's test to compare against control (*, $p < 0.05$).

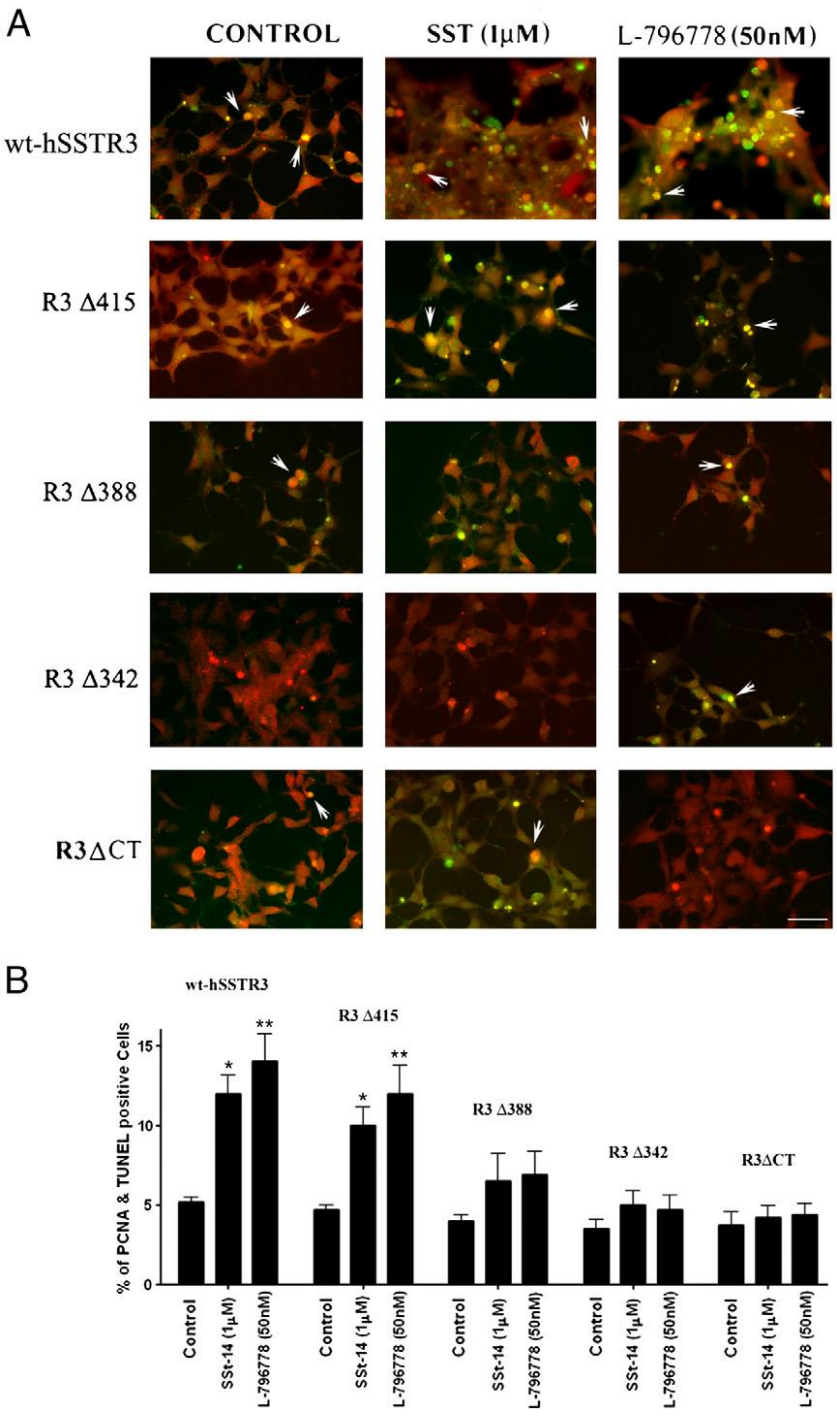

Figure 2.10 Representative photomicrographs showing colocalization of PCNA and TUNEL positive HEK-293 cells. (A). HEK-293 cells transfected with *wt*-SSTR3 and C-tail mutants were treated with SST (1 μM) and L-796778 (50 nM) for 24 h, and were processed for PCNA

immunofluorescence and TUNEL staining (Please see sub-section 2.2.9 for details). *wt*-SSTR3 cells exhibiting PCNA immunoreactivity undergo agonist-induced apoptosis as evident by increased TUNEL-positive reaction. Cells expressing C-tail mutants display significant loss in TUNEL reactivity which suggests decreased pro-apoptotic activity. These findings implicate a key role for SSTR3 C-tail in mediating agonist dependent apoptosis. Arrows indicate colocalization of PCNA and TUNEL positive cells. Scale bar = 10 µm. **(B).** Histograms represent percent of cells showing positive PCNA and TUNEL reaction. A total of 500-800 cells were counted for each treatment condition. Results are expressed as Mean ± SE of three independent experiments. Data analysis was performed by one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$; **, $p < 0.01$).

2.3.9 C-tail is the fundamental regulator of SSTR3 dependent pro-apoptotic pathways

To further investigate the role of C-tail of SSTR3 in apoptosis, in this experiment the immunostaining of PCNA, a marker for actively proliferating cells was combined with TUNEL assay for apoptosis. Cells expressing *wt*-SSTR3 and mutants were treated with SST (1 µM) or L-796778 (50 nM) for 24 h at 37ºC followed by PCNA immunoreactivity and TUNEL staining as described in sub-section 2.2.9. As shown in Figure 2.10, cells expressing *wt*-SSTR3 displayed increased TUNEL reaction upon agonist treatment in comparison to non-treated cells. Interestingly, it was observed that most TUNEL-positive cells were also PCNA-positive indicating that actively proliferating cells were susceptible to SSTR3-mediated apoptosis. With the increasing aa deletions from the C-tail of SSTR3 (R3 Δ415, R3 Δ388, R3 Δ342 and R3ΔCT), there was a proportionate loss of TUNEL-positive cells in response to agonist treatment. These observations strongly indicate that the apoptotic activity of SSTR3 is conserved in the aa residues present in the C-tail.

2.4 Discussion

The aim of the present study was to determine the role of SSTR3 C-tail on the physiological response of cell in presence of ligand. To ascertain this, the experiment was designed in HEK-293 cells stably transfected with *wt*-SSTR3 and C-tail mutants of SSTR3 and studied for receptor internalization, coupling to AC and modulation of downstream signaling pathways regulating cell proliferation and apoptosis. In addition, SSTR3 dimerization was also described with or without agonist. To the best of knowledge this is the first study describing a comprehensive characterization of SSTR3.

Receptor binding analysis and immunocytochemistry indicate no significant differences in the membrane expression between *wt*-SSTR3 and C-tail mutants. The receptor expression in C-tail mutants was sufficient to demonstrate comparable signaling properties, as described earlier for SSTR5 mutants (Cordoba-Chacon et al., 2010; Sharma et al., 1999). Furthermore, the receptor density in *wt*-SSTR3 and mutants is within the range of physiological SSTR expression (Rocheville et al., 2000b; Srikant, 1987). It was previously shown that C-tail deletions of SSTR5 did not affect the ligand binding (Hukovic et al., 1998). Several previous studies have also described that the ligand binding domain in GPCRs is confined in the ECL and/or TMDs (Gether et al., 1993; Greenwood et al., 1997; Schwartz and Rosenkilde, 1996; Strader et al., 1995). In accordance, the data demonstrate no significant changes in ligand binding upon C-tail deletions in SSTR3, suggesting that the absence of C-tail fails to exert any significant effect on binding. SSTR subtypes respond to agonist treatment and internalize in time, temperature and receptor specific manner. The

data from this study demonstrate no significant differences in receptor internalization between *wt*-SSTR3 and C-tail mutants in response to SST or the receptor specific agonist, which suggests that SSTR3 C-tail does not play any key role in receptor internalization. These results are in contrast to a previous study on rat SSTR3 where mutations in the C-tail significantly altered the rate and extent of receptor internalization (Roth et al., 1997a). Interestingly, human and rat SSTRs exhibit distinct and diverse response to agonist in modulation of receptor internalization and signaling pathways and this discrepancy is likely due to species difference (Cescato et al., 2006; Liu et al., 2005; Liu et al., 2008; Pfeiffer et al., 2001; Sharif et al., 2007).

Previous studies have shown distinct receptor-specific homo-and heterodimerization of human SSTR subtypes (Baragli et al., 2007; Grant et al., 2004a; Grant and Kumar, 2009; Grant et al., 2004b; Rocheville et al., 2000a; Rocheville et al., 2000b; Somvanshi et al., 2009). Most importantly, all five human SSTRs with the exception of SSTR1 exhibit receptor-specific homodimerization which is also attributed to the functional consequences (Grant et al., 2008; Grant et al., 2004a; Grant et al., 2004b; Pfeiffer et al., 2001; Rocheville et al., 2000a; Rocheville et al., 2000b; Somvanshi et al., 2009). As shown earlier, rat SSTR3 is a constitutive homodimer at cell surface and C-tail of rat SSTR3 was not essential for homodimerization (Pfeiffer et al., 2001). Previous studies have shown that metabotropic glutamate receptor-5 and Ca^{2+} sensing receptor undergo dimerization via extracellular N-terminal domain (Bai et al., 1998; Romano et al., 1996). Cumulatively, these data suggest that C-tail is not essential requirement for GPCR dimerization. This notion is supported by the data showing homodimerization in *wt*-

SSTR3 and C-tail mutants. The molecular mechanism of SSTR3 homodimerization still remains unclear and needs to be further elucidated. The decreased relative FRET efficiency observed in *wt*-SSTR3 in presence of SST and SSTR3 specific agonist might be attributed to the receptor internalization as dimer. FRET efficiency is directly related to the distance and receptor orientation at the cell surface. The loss in FRET efficiency in presence of agonist might also be due to conformational changes as well as receptor orientation at the plasma membrane.

There are mounting evidence showing critical role of C-tail in GPCR functioning (Chabry et al., 1995; Hipkin et al., 1997; Hukovic et al., 1998; Jockers et al., 1996; Roth et al., 1997a; Somvanshi et al., 2009; Thomas et al., 1995a; Venkatesan et al., 2001). Previous studies have shown concentration dependent effect of SST on the inhibition of FSK stimulated cAMP and SST in range of 0.1-1μM was found to exert maximal effect (Grant et al., 2004b; Hukovic et al., 1998; Hukovic et al., 1999). Consistent with these observations, FSK-stimulated cAMP was significantly inhibited in cells expressing *wt*-SSTR3 and the results are further supported by several previous studies (Barbieri et al., 2008; Pfeiffer et al., 2001; Yasuda et al., 1992). The ability of SST or SSTR3 agonist to inhibit cAMP was proportionally lost with aa deleted from the C-tail. This is in agreement with previous studies demonstrating the loss of receptor coupling to AC in C-tail deleted human SSTR5 and SSTR4 (Hukovic et al., 1998; Rocheville et al., 2000b; Somvanshi et al., 2009).

Several previous studies have shown that activation of GPCRs by agonist plays a determinant role on phosphorylation of ERK1/2 and the changes in status of ERK phosphorylation are directly implicated in cell proliferation and activation of apoptotic

pathways (Berra et al., 1998; Hubina et al., 2006; Nishida and Gotoh, 1993; Nishimoto and Nishida, 2006). The inhibition of basal ERK activity has been shown to trigger apoptosis in HeLa cells (Berra et al., 1998). A recent study has shown increased ERK1/2 phosphorylation upon C-tail deletion in human SSTR4 (Somvanshi et al., 2009). The results from the present study demonstrate reduced pERK1/2 in cells expressing *wt*-SSTR3, R3 Δ388, R3 Δ342 and R3ΔCT in response to receptor specific agonist. On the other hand, SST increased ERK1/2 in R3 Δ415 expressing cells, while inhibiting pERK1/2 only in cells transfected with R3 Δ342. These diverse effects on ERK1/2 phosphorylation between *wt*-SSTR3 and mutants might be attributed to distinct role in signaling played by aa in the C-tail as previously described for follicle stimulating hormone receptor (FSHR) (Hipkin et al., 1995). Most significantly, cells expressing *wt*-SSTR3 exhibited decreased pERK1/2 when compared to C-tail mutants, indicating that *wt*-SSTR3 has an inhibitory influence on ERK1/2 phosphorylation, an effect that was not evident in C-tail mutants. The high basal pERK1/2 in C-tail mutants may be due to activation of signaling pathways which are G-protein independent, as demonstrated previously for β_2-adrenergic receptor (Sun et al., 2007). Accordingly, the distinct regulation of ERK1/2 in the presence of SST and SSTR3 specific agonist might be due to SST mediated activation in PTX-insensitive manner and cannot be excluded from discussion.

The antiproliferative effect mediated by SSTRs is receptor specific phenomenon either associated with blocking cell growth or activating cytotoxic pathways (Liu et al., 2000; Patel, 1999; Sharma et al., 1996, 1999; Weckbecker et al., 2003). In the present study, MTT assay provides direct evidence for agonist

mediated inhibition of cell proliferation in *wt*-SSTR3 and R3 Δ415, an effect which was lost in other C-tail mutants. It is unclear whether SSTR3 mediated antiproliferative effects are dependent upon pERK1/2 inhibition. Importantly, MTT assay was accomplished after 24 h treatment whereas ERK1/2 was studied only after 5 or 15 min treatment. A diverse cellular response to transient or sustained ERK activation could possibly lead to this inconsistency. Moreover, the biological effects of ERK1/2 activation are not only sensitive to cell types, extracellular factors and the receptors involved, but also on the amplitude and duration of ERK1/2 activation (Lahlou et al., 2004). In case of human SSTR mediated cytotoxic effect, the translocation of PTP from cytosol to the cell membrane and increased expression of PARP-1 are considered to be essential steps (Lasfer et al., 2005; Lattuada et al., 2002; Thangaraju et al., 1999a). The data from this study demonstrate an increase in agonist-induced PTP-1C translocation to the cell surface in *wt*-SSTR3 with a parallel increase in PARP-1 expression. All these events directly support the antiproliferative effects of SSTR3 through cytotoxic mode of cell death. The changes in PTP-1C and PARP-1 activation were further correlated by TUNEL assay for apoptosis. The result demonstrate that in the presence of SST or L-796778, a SSTR3 specific agonist, only cells positive to PCNA exhibit TUNEL staining indicating that actively proliferating cells are more vulnerable to apoptosis; an observation not described previously. Most importantly, membrane expression of PTP-1C is completely lost in cells transfected with C-tail mutants which is accompanied by an increase in pERK1/2. These findings suggest that PTP-1C regulates ERK1/2 phosphorylation as also previously reported (Weckbecker et al.,

2003). PARP-1 activation is lost in cells expressing R3 Δ342 and R3ΔCT. In R3 Δ388 expressing cells, despite PARP-1 expression there were no significant changes in cell proliferation most likely due to high pERK1/2 levels opposing the antiproliferative activity by agonist in this mutant.

The molecular mechanism and the specific motif in the C-tail that are responsible for receptor trafficking, coupling to AC and most importantly, the pro-apoptotic response of SSTR3 are still elusive and further studies are in progress in this direction. Moreover, the role of G proteins functioning independent of agonist stimulation cannot be ruled out as recently demonstrated (Grant and Kumar, 2009). A detailed analysis of SSTR3 revealed that there was a gradual increase in the proportion of acidic aa upon deletions in the C-tail. Previous studies have implicated the critical role of basic aa present in the C-tail of GPCRs on receptor function. Comparative studies on the human class 1 GPCRs have indicated the presence of the conserved positively charged aa sequences in the C-tail adjacent to the TMD7 region (Saito et al., 2005). In addition, mutations in the Arginine and Lysine residues in the C-tail of melanocorticotropin hormone receptor-1 (MCHR-1) resulted in the variable changes in the receptor expression and function (Saito et al., 2005). Consistent with these studies, C-tail of *wt*-SSTR3 contains 18 basic aa and their gradual loss in the truncated mutants might be responsible for the loss of receptor function. In accordance, the loss of 4 and 10 basic aa from SSTR3 mutants R3 Δ388 and R3 Δ342 respectively might directly correlate with the loss of receptor signaling.

C-tail of SSTR3 constitutes approximately 100 aa containing 11 Ser and 6 Thr residues other than 37 charged aa residues. Loss of one Ser residue in the R3 Δ415

resulted in decreased inhibition of FSK-stimulated cAMP upon agonist activation by >15% in comparison to the *wt*-SSTR3 suggesting the loss of potential phosphorylation site. Recent study on *wt*-FSHR has demonstrated that truncation at 635th residue in the C-tail removes all potential phosphorylation sites except one and the mutant FSHR behaves more efficiently than the *wt*-FSHR. The authors claimed that this unexpected behavior was due to the exposure of potential phosphorylation sites in the mutant receptor which were not readily accessible in the *wt*-FSHR (Hipkin et al., 1995). Taken into consideration, this might as well be true for SSTR3 mutants, in which the non-accessible potential phosphorylation sites were exposed upon deletion possibly leading to distinct response of the mutants on ERK1/2 phosphorylation and PARP-1 activation.

In summary, the deletion of aa from the C-tail of SSTR3 appears to alter the physiological response of cells to agonist. C-tail deletions in SSTR3 inhibit the normal receptor function in cells. There are many questions yet to be answered with the results emerging from this study, like why only PCNA positive cells are susceptible to SST and SSTR3 specific agonist-induced apoptosis, and to address such a complicated scenario, further studies are required in time to come. Most importantly, data presented in this study describe some previously unnoticed role of SSTR3 which was not explored earlier.

Chapter 3: Coexpression of human SSTR2 and SSTR3 modulates antiproliferative signaling and apoptosis

3.1 Background

GPCRs assemble as oligomers with distinct pharmacological, biochemical and physiological properties (Grant et al., 2008; Prinster et al., 2005; Terrillon and Bouvier, 2004). The concept of oligomerization with efficacious changes in downstream signaling pathways have broadened the therapeutic potential of drugs targeting GPCRs. SST is a pleiotropic inhibitory peptide and regulates endocrine and exocrine secretions, neurotransmission and cell proliferation through five different receptor subtypes coupled to G_i proteins namely SSTR1-5 (Patel, 1999; Reubi et al., 2001). SSTR subtypes display receptor-specific homo- and/or heterodimerization within the family and other GPCRs with unique signaling characteristics (Grant et al., 2008; Grant et al., 2004a; Grant and Kumar, 2010; Somvanshi et al., 2009; War et al., 2011). This notion is further supported by previous studies demonstrating enhanced signaling properties in the heteromeric complex of human SSTR2 or SSTR5 with dopamine receptor-2 (Baragli et al., 2007; Rocheville et al., 2000a). Interestingly, the heterodimer of SSTR2/SSTR3 of rat origin has been reported to abrogate SSTR3 functions (Pfeiffer et al., 2001). Indeed, rat SSTRs show distinct response to agonist in comparison to human SSTRs, (Pfeiffer et al., 2001; Roosterman et al., 2008; Roth et al., 1997a; War et al., 2011).

All SSTR subtypes upon activation couple to G_i proteins and inhibit AC in a PTX-sensitive manner (Patel, 1999). Importantly, in cells coexpressing human SSTR2 and SSTR5, cAMP inhibition was enhanced upon activation of SSTR2

(Grant et al., 2008). Conversely, cAMP levels remained unchanged in response to SSTR3-specific agonist in cells cotransfected with rat SSTR2 and SSTR3, whereas the activation of SSTR2 caused significant inhibition of cAMP (Pfeiffer et al., 2001). Several studies have shown the role of SSTRs in regulating intracellular signaling molecules including MAPKs which are implicated in cell proliferation, differentiation, migration and apoptosis (Berra et al., 1998; Grant et al., 2008; Hubina et al., 2006; Kharmate et al., 2011a, b; Nishida and Gotoh, 1993; Nishimoto and Nishida, 2006; War et al., 2011). The antiproliferative response of SSTRs is associated with the phosphorylation of selective downstream cascades including ERKs depending upon the receptor subtype, cell environment and extracellular factors (Grant et al., 2008; Lahlou et al., 2003; Patel, 1999; Somvanshi et al., 2009; War et al., 2011; Watt et al., 2009; Weckbecker et al., 2003). ERK is activated by SSTR1 and SSTR4, whereas inhibited upon activation of SSTR5 (Lahlou et al., 2004; Sellers, 1999; Smalley et al., 1999; Somvanshi et al., 2009). On the other hand, SSTR2 and SSTR3 exhibit dual effect on ERK phosphorylation in a cell-specific manner (Alderton et al., 2001; Lahlou et al., 2004; Lahlou et al., 2003; Patel, 1999; Pfeiffer et al., 2001; War et al., 2011). In the oligomeric complex of human SSTR2/SSTR5 or rat SSTR2/SSTR3, ERK1/2 phosphorylation has been attributed to SSTR2 activation (Grant et al., 2008; Pfeiffer et al., 2001). Alterations in stress-related p38 MAPK have been frequently observed in human tumors and various other cell lines of tumor origin (Chuang et al., 2000; Wagner and Nebreda, 2009). p38 signaling has diverse biological consequences including pro-/anti-apoptotic effects in a cell-dependent manner (Alderton et al., 2001; Chuang et al., 2000; Wagner and

Nebreda, 2009). Importantly, the antiproliferative response mediated by SSTR2 but not SSTR3 has been associated with the activation of p38 MAPK (Alderton et al., 2001). SSTR subtypes inhibit cell proliferation by multiple mechanisms. SSTR2 and SSTR3 are specifically linked to cell cycle arrest and apoptosis, respectively (Ferrante et al., 2006; Florio, 2008a; Florio et al., 2003; Grant et al., 2008; Patel, 1999; Sharma et al., 1996; War et al., 2011; Zhou et al., 2009; Zou et al., 2009).

Agonist-induced internalization of SSTRs is time, temperature, and receptor-specific (Hukovic et al., 1996; Pfeiffer et al., 2002). SSTRs oligomerization plays a determinant role in receptor trafficking. Importantly, agonist-mediated internalization of SSTRs varies significantly between receptors of rat and human origin (Hukovic et al., 1996; Pfeiffer et al., 2001; Roth et al., 1997a; War et al., 2011). SSTR1 of rat origin internalized in response to agonist, whereas human SSTR1 rather up-regulated at the cell surface (Hukovic et al., 1996; Roosterman et al., 2007). The constitutive homodimer of human SSTR2 dissociated into monomers at the plasma membrane prior to agonist-stimulated internalization, whereas rat SSTR2 internalized as homodimer (Grant et al., 2004a; Pfeiffer et al., 2001). On the other hand, human and rat SSTR3 internalized as homodimers upon agonist treatment (Pfeiffer et al., 2001; War et al., 2011). Strikingly, rat SSTR3 internalized and subsequently recycled to the cell surface in response to agonist, whereas human SSTR3 was targeted to degradation (Hukovic et al., 1996; Roosterman et al., 2008; Roth et al., 1997a). More importantly, C-tail of human SSTR3 was not essential for receptor trafficking, conversely, mutations in C-tail of rat SSTR3 abrogated the agonist-mediated internalization (Roth et al., 1997a; War et al., 2011).

SSTR3 shares the least aa homology between rat and human species as compared to other SSTR subtypes (Patel, 1999). Although, rat SSTR2/SSTR3 heterodimerization abolished the functions of SSTR3 (Pfeiffer et al., 2001), the same may not be speculated for human SSTR2 and SSTR3 as receptors of different origin have diverse signaling properties and need to be elucidated in detail. Using morphological, biochemical and biophysical techniques, this study provides first evidence for human SSTR2/SSTR3 heterodimerization in HEK-293 cells and its implications in signaling and antiproliferation.

3.2 Materials and methods

3.2.1 Materials

Non-peptide agonists for SSTR2 (L-779976) and SSTR3 (L-796778) were kindly provided by Dr. S.P. Rohrer from Merck and Co (Rohrer and Schaeffer, 2000). SST was purchased from Bachem Inc., Torrance, CA, USA. HEK-293 cells were obtained from ATCC, Manassas, VA, USA. Rabbit polyclonal antibodies against SSTR2 were generated and characterized as described previously (Kumar et al., 1999). Mouse monoclonal antibodies against p21 and PARP-1 were purchased from BD-Biosciences, Mississauga, ON, Canada. Rabbit polyclonal antibodies for total and phospho-ERK1/2 and p38 were obtained from Cell Signaling Technology, Danvers, MA, USA. Fluorescein and rhodamine or peroxidase conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Mouse monoclonal antibodies for $p27^{Kip1}$ were obtained from Santa Cruz Biotechnology Inc., Santa

Cruz, CA, USA. Mouse monoclonal antibodies against HA and β-Tubulin were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. cAMP kit was obtained from BioVision Inc., Milpitas, CA, USA. TUNEL kit was purchased from La Roche Applied Science, Mannheim, Germany. ECL Western blotting detection kit and nitrocellulose Hy-Bond ECL membrane were purchased from GE Healthcare, Piscataway, NJ, USA. Protein A/G-Agarose beads were obtained from Calbiochem, EMD Biosciences, Darmstadt, Germany. DMEM, trypsin-EDTA and PBS were purchased from Invitrogen, Burlington, ON, Canada. Reagents for electrophoresis were obtained from Bio-Rad Laboratories, Mississauga, ON, Canada. Other reagents were of AR grade and were purchased from various sources.

3.2.2 Human SSTR2 and SSTR3 constructs and transfection

Human SSTR2 construct was prepared using pCDNA3.1/Hyg vector (hygromycin resistance). Constructs expressing human SSTR3 and N-terminal HA-tagged human SSTR3 (HA-SSTR3) were prepared using pCDNA3.1/Neo vector (neomycin resistance), as previously described (Grant et al., 2004b; Somvanshi et al., 2009). HEK-293 cells which lack endogenous SSTRs were stably transfected with SSTR2 and SSTR3 using Lipofectamine transfection reagent as previously described (Grant et al., 2004b). Clones were selected and maintained in DMEM containing 10% FBS with 400 µg/ml hygromycin or 700 µg/ml neomycin or both in an incubator containing 5% CO_2 at 37°C.

3.2.3 Cell treatments

The concentrations for SST and receptor-specific agonists used in this study are as follows: SST (1, 50 nM and 1 μM); L-779976 (10 and 100 nM); L-796778 (25 and 50 nM). The binding affinity of SST for SSTR2 and SSTR3 is 0.2-1.3 nM and 0.3-1.6 nM, respectively. Furthermore, the binding affinity for L-779976 (SSTR2 agonist) and L-796778 (SSTR3 agonist) for their respective receptors is 0.05 nM and 24 nM, respectively (Patel, 1999). Previous studies demonstrated significant changes in dimerization and signaling molecules when SSTR subtypes were activated by different concentrations of SST and receptor-specific agonists (Grant et al., 2008; Grant et al., 2004b; Rocheville et al., 2000b). Accordingly in the present study, the different concentrations of SST and receptor-specific agonists were used to understand the signaling aspects of SSTR2 and SSTR3.

3.2.4 Co-immunoprecipitation analysis

To determine the formation of heteromeric complex between SSTR2 and SSTR3, CO-IP was performed in mono- and/or cotransfectants expressing SSTR2 and SSTR3, and *wt*-HEK-293 cells. Briefly, 250 μg of total membrane protein prepared from untreated cells was solubilized in 1 ml RIPA buffer for 1 h at 4°C. Samples from cotransfected cells were then incubated overnight at 4°C with anti-HA antibody to immunoprecipitate SSTR3. To rule out detection of non-specific band, membrane fractions from monotransfectants expressing SSTR2 or SSTR3 were immunoprecipitated with anti-HA and anti-SSTR2 antibodies, respectively; whereas *wt*-HEK-293 were incubated with anti-SSTR2 antibody. On the following day, protein

A/G-agarose beads (25 µl) were added to the samples for 2 h at 4°C to immunoprecipitate the antibody. The immunoprecipitate was fractionated on 7% SDS-PAGE and processed for Western blot analysis to detect SSTR2/SSTR3 heteromeric complex as previously described (Somvanshi et al., 2011b).

3.2.5 Microscopic Pb-FRET analysis for heterodimerization

To explore SSTR2 and SSTR3 heterodimerization, microscopic Pb-FRET analysis was performed in cotransfected cells as previously described (Grant et al., 2004b). Cells grown on poly-D-lysine coated glass coverslips were treated with SST (1, 50 nM and 1 µM), L-779976 (10 and 100 nM) and L-796778 (25 and 50 nM) or in combination for 10 min at 37ºC. Post-treatment, cells were fixed in 4% PF for 15 min on ice. The coverslips were incubated at 4ºC overnight with rabbit polyclonal anti-SSTR2 and mouse monoclonal anti-HA (for SSTR3) primary antibodies, followed by the addition of rhodamine-goat anti-rabbit (for SSTR2) and FITC-goat anti-mouse (for SSTR3) secondary antibodies to create donor-acceptor pair. Finally, the coverslips were processed for Pb-FRET analysis as previously described (Somvanshi et al., 2009; War et al., 2011). The FRET efficiency (E) was measured as a percent based upon photo bleaching time constants of the donor taken in the absence (D-A) and presence (D+A) of acceptor according to the formula

$$E = [1-(D-A/D+A)] \times 100$$

3.2.6 Immunocytochemistry for receptor colocalization and internalization

To analyze receptor colocalization and internalization, cotransfected cells were grown on poly-D-lysine coated glass coverslips and treated with different concentrations of SST and receptor-specific agonists for 15 min at 37ºC as described in the sub-section 3.2.5. Following fixation in 4% PF on ice, the cover slips were washed three times in PBS. Cells were permeabilized for intracellular expression in 0.3% Triton X-100 for 15 min at RT, whereas non-permeabilized cells were used for membrane expression. The incubation with primary and secondary antibodies was done as described in sub-section 3.2.5. After three washes in PBS, the coverslips were mounted on glass slides and analyzed under Leica fluorescence microscope as previously described (Somvanshi et al., 2011a). Adobe Photoshop and Image J software, NIH (Collins, 2007) were used for making the composites.

3.2.7 cAMP assay

Mono- and/or cotransfected cells expressing SSTR2 and SSTR3 were seeded in 6-well plates and grown to 70% confluency, and cAMP assay was performed as previously described (Somvanshi et al., 2009). Briefly, cells were pre-treated for 30 min with IBMX (0.5 mM) to prevent cAMP degradation. Cells were then treated for 30 min with SST and receptor-specific agonists in the presence of FSK (20 µM). DMEM was used as control. Cells were scraped in 0.1 N HCl, normalized for protein amount and cAMP was determined by immunoassay using a cAMP kit following the manufacturer's instructions.

3.2.8 Western blot analyses for signaling

For each experiment, 10000 cells were seeded per culture flask and grown to 80% confluency. For ERK1/2 and p38, cells were treated with SST and receptor-specific agonists for 30 min and 24 h, respectively with or without 24 h serum starvation (no FBS in culture media). To determine the expression of PARP-1, p21 and $p27^{Kip1}$, cells were treated with SST and receptor-specific agonists for 24 h with or without serum deprivation. Post-treatment, cells were lysed in RIPA buffer and the cell lysate was fractionated on SDS-PAGE and probed for total and phospho ERK1/2 and p38 (1:1000), PARP-1 (1:5000), p21 and $p27^{Kip1}$ (1:750) using specific antibodies as previously described. β-Tubulin (1:10000) was used as loading control. All other procedures like membrane blocking, primary and secondary antibody incubation, and chemiluminescence detection were performed as previously described (War et al., 2011). Immunoblots were visualized with an Alpha Innotech FluorChem 8800 gel box imager (Protein Simple, Santa Clara, CA, USA) and densitometric analysis was performed by using FluorChem software (Protein Simple, Santa Clara, CA, USA).

3.2.9 Cell proliferation assay

Cell proliferation was determined by MTT assay as previously described (Grant et al., 2008; War et al., 2011). Mono- and/or cotransfected cells expressing SSTR2 and SSTR3 were serum starved for 24 h. Cells were either subjected to pre-incubation with PTX (100 ng/ml) for 18 h, or directly treated with different concentrations of SST and receptor-specific agonists for 24 h in DMEM containing

FBS before processing for MTT assay. Briefly, 20 µl of 5 mg/ml MTT solution in DMEM was added and incubated for 2 h at 37ºC. The formazan crystals formed were dissolved in 200 µl of isopropanol and the absorbance was measured in a microplate spectrophotometer at 550 nm.

3.2.10 TUNEL staining to detect apoptosis

Cells coexpressing human SSTR2 and SSTR3 were grown on poly-D-lysine coated glass coverslips and treated with different concentrations of SST and receptor-specific agonists for 24 h. Cells were fixed in 4% PF and rinsed three times with PBS. After permeabilization in solution containing 0.1% Triton X-100 and 0.1% sodium citrate, cells were washed in PBS followed by incubation with TUNEL reaction mixture for 1 h at 37ºC in dark. Finally, the coverslips were washed in PBS and mounted on glass slides for analysis under Leica fluorescence microscope. Adobe Photoshop was utilized for making composites.

3.2.11 Statistical analysis

Results are expressed as mean ± S.D of three independent experiments. Statistical analysis was done by one- or two-way ANOVA and *post hoc* Dunnett's or Bonferroni's tests, as applicable. GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for performing data analysis and p value < 0.05 was considered statistically significant.

3.3 Results

3.3.1 Human SSTR2 and SSTR3 exist as constitutive heterodimer at cell surface

To investigate whether human SSTR2 and SSTR3 exist in a heteromeric complex, CO-IP was performed in cotransfected cells expressing SSTR2 (279 ± 28 fmol/mg protein) and SSTR3 (285 ± 31 fmol/mg protein). As illustrated in Figure 3.1A (i), SSTR2 is expressed in the SSTR3 immunoprecipitate at the expected molecular size of ~117 kDa. The specificity of the oligomeric complex was confirmed by the absence of heterodimer band in monotransfectants and *wt*-HEK-293 cells under the same experimental conditions [Figure 3.1A (iv-vi)]. Upon probing SSTR3 immunoprecipitate with anti-HA antibody, a band corresponding to SSTR3 monomers was observed at ~60 kDa [Figure 3.1A (ii)]. In addition, SSTR2 monomers were detected at ~57 kDa after SSTR2 immunoprecipitate was probed with anti-SSTR2 antibody [Figure 3.1A (iii)]. To further validate the data from CO-IP, microscopic Pb-FRET analysis was performed in cotransfected cells following treatment with different concentrations of SST and receptor-specific agonists. As depicted in Table 3.1 and Figure 3.1B, SSTR2 and SSTR3 assembled as heterodimers at the cell surface in basal conditions and displayed a high relative FRET efficiency of 14.9 ± 1.2%. Moreover, upon treatment with SST (1 nM and 1 μM), SSTR2-specific agonist (10 nM), and SSTR3-specific agonist (50 nM) or in combination, the effective FRET efficiency was significantly decreased when compared to control (*, $p < 0.05$). Importantly, the combination of receptor-specific agonists displayed comparable FRET efficiencies with SST which activates SSTR2

and SSTR3 equally. Taken together, these results confirm that SSTR2/SSTR3 exist in a heteromeric complex with the relative FRET efficiency sufficient to support heterodimerization even upon receptors activation.

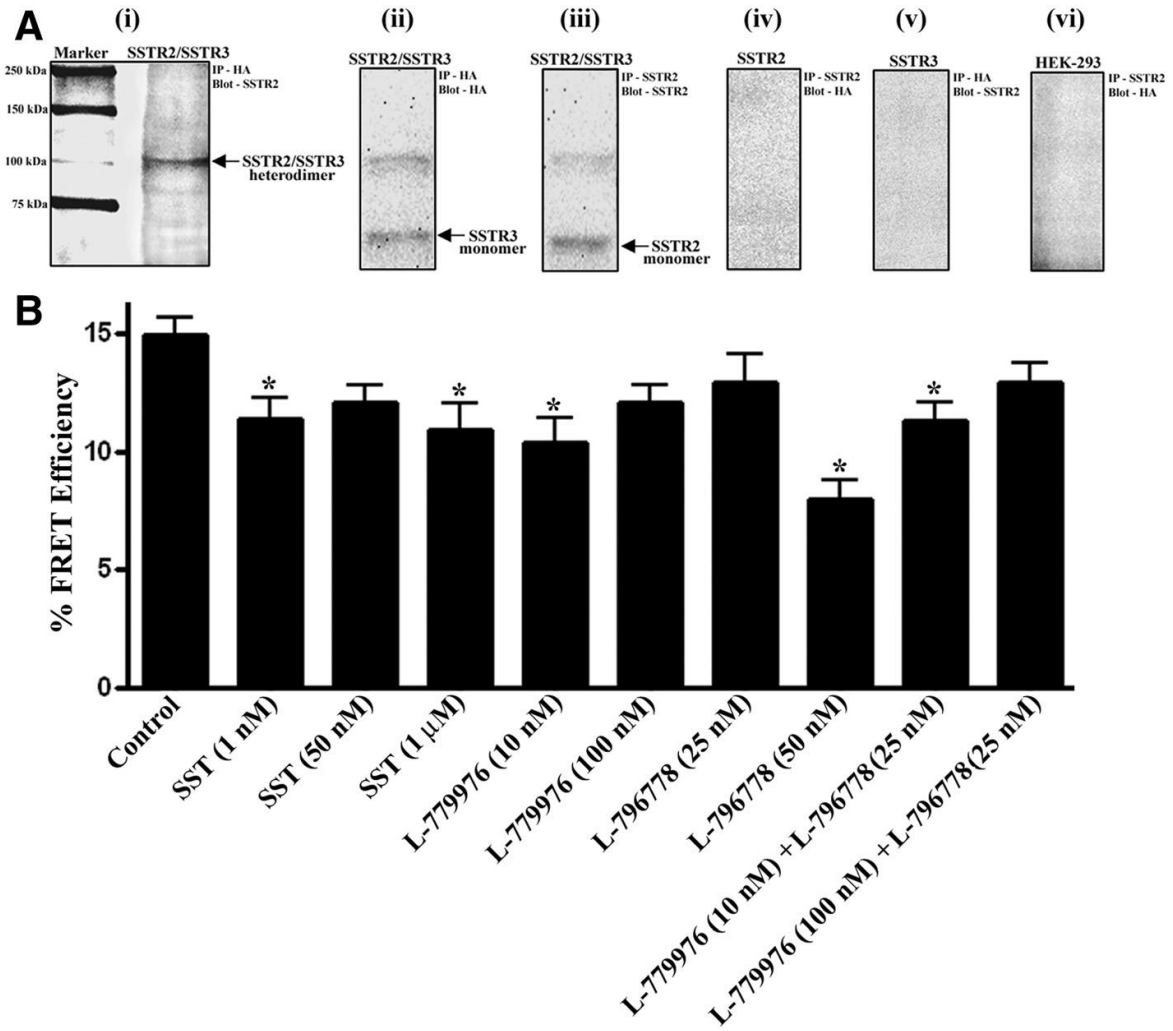

Figure 3.1 SSTR2/SSTR3 exhibit heterodimerization at cell surface. A (i). Membrane extracts prepared from cotransfected cells were immunoprecipitated with HA antibody (SSTR3) and probed for SSTR2 as described in sub-section 3.2.3. The receptor-specific band for the heteromeric complex of SSTR2/SSTR3 was detected at the expected size of ~117 kDa. **A (ii-iii).** The membranes were reprobed to detect SSTR3 and SSTR2 monomers, respectively. **A (iv-vi).** The specificity of the heterodimer was confirmed in HEK-293 cells and monotransfectants expressing SSTR2 and SSTR3

B. Cotransfected cells were treated with SST (1, 50 nM and 1 μM), L-779976 (10 and 100 nM) and L-796778 (25 and 50 nM) or in combination for 10 min at 37ºC and subjected to microscopic Pb-FRET analysis. Histogram represents relative FRET efficiency of 14.9% in control suggesting the presence of SSTR2/SSTR3 heterodimers. The heterodimer is stable upon treatment with SST and receptor-specific agonists, albeit with decreased relative FRET efficiency. Results are expressed as mean ± S.D of three independent experiments.

Table 3.1 Photobleaching time-constants and relative-FRET efficiencies in HEK-293 cells coexpressing SSTR2 and SSTR3

Treatment		τ_{avg}	n	E (%)
Control	D - A	18.3 ± 0.5	55	14.9 ± 1.2
	D + A	21.5 ± 0.7	54	
SST (1 nM)	D - A	18.6 ± 0.4	58	11.4 ± 1.4*
	D + A	21.0 ± 0.5	56	
SST (50 nM)	D - A	18.8 ± 0.4	59	12.1 ± 1.1
	D + A	21.4 ± 0.4	60	
SST (1 μM)	D - A	19.6 ± 0.6	57	10.9 ± 1.6*
	D + A	22.0 ± 0.2	55	
L-779976 (10 nM)	D - A	20.7 ± 0.3	56	10.4 ± 1.5*
	D + A	23.1 ± 0.3	59	
L-779976 (100 nM)	D - A	20.3 ± 0.4	58	12.1 ± 1.2
	D + A	23.1 ± 0.4	54	
L-796778 (25 nM)	D - A	19.5 ± 0.9	59	12.9 ± 1.7
	D + A	22.4 ± 0.7	54	
L-796778 (50 nM)	D - A	20.8 ± 0.5	60	8.0 ± 1.2*
	D + A	22.6 ± 0.6	57	
L-779976 (10 nM) + L-796778 (25 nM)	D - A	19.6 ± 0.5	55	11.3 ± 0.9*
	D + A	22.1 ± 0.2	54	
L-779976 (100 nM) + L-796778 (25 nM)	D - A	19.6 ± 0.5	57	12.9 ± 1.4
	D + A	22.5 ± 0.5	54	

D−A and D+A correspond to donor without and with acceptor, respectively; τ_{avg} refers to average of *n* photobleaching time constants; *n* is the total number of cells analyzed; *E (%)* is mean relative FRET efficiency. Mean ± SD is representative of three independent experiments (*, $p < 0.05$).

3.3.2 SSTR2 and SSTR3 colocalize at the cell surface

As previously described, rat SSTR2/SSTR3 complex dissociated upon treatment with SST leading to SSTR2 internalization whereas the endocytosis of SSTR3 was impaired (Pfeiffer et al., 2001). To ascertain whether the same holds true for human SSTR2 and SSTR3, immunocytochemistry was performed in cotransfected cells. As shown in Figure 3.2, SSTR2 and SSTR3-like immunoreactivity and colocalization at the cell surface was decreased in response to SST, accompanied by a parallel increase in cytosolic expression. Most importantly, the independent activation of SSTR2 or SSTR3 resulted in the loss of immunoreactivity for both receptors at the plasma membrane, suggesting a potential role for heterodimerization in regulating receptor trafficking.

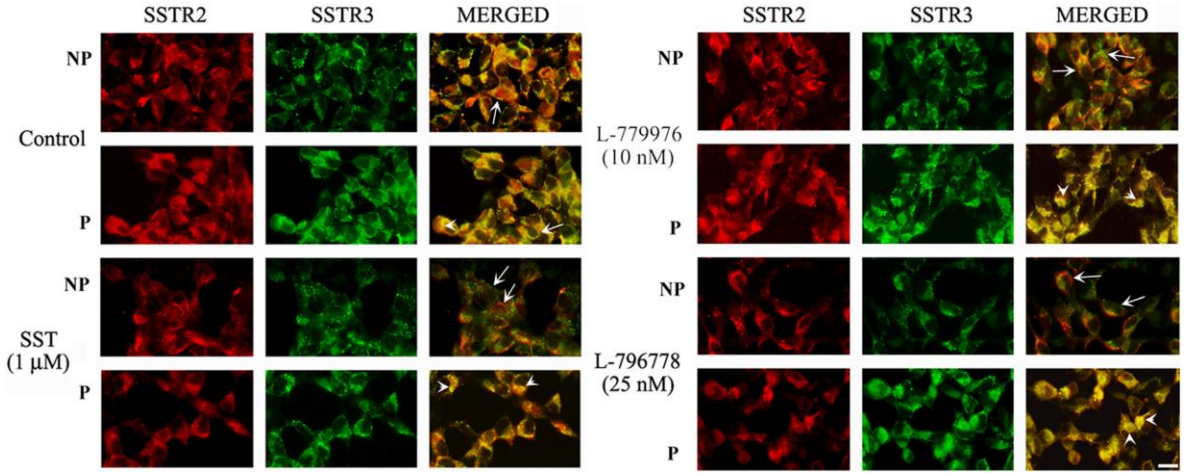

Figure 3.2 Representative photomicrographs illustrating fluorescence analysis and co-internalization of SSTR2 and SSTR3 upon agonist treatment. Cells were treated with SST (1 µM), L-779976 (10 nM) and L-796778 (25 nM) for 15 min at 37ºC, and processed for immunocytochemistry as described in sub-section 3.2.5. Fluorescent microscopic images show receptor distribution of SSTR2 (red), SSTR3 (green) and colocalization (yellow) at the cell surface and intracellularly. SSTR2 and SSTR3 display colocalization at the cell surface and internalize upon treatment with SST and

specific agonists. Note that activation of one receptor caused a significant down-regulation of both receptors at cell surface. Arrows and arrowheads in the merged panel represent membrane and cytosolic expression, respectively. Scale bar = 10 μm. Data are representative of three independent experiments.

3.3.3 Agonist-mediated cAMP inhibition by SST2/SSTR3

The decreased cAMP accumulation via negative regulation of AC upon SSTRs activation gives an unswerving estimate of receptor functionality (Patel, 1999). Next cAMP levels were examined in mono- and/or cotransfected cells expressing SSTR2 and SSTR3. As illustrated in Figure 3.3, SST and the receptor-specific agonists significantly inhibited cAMP in cotransfected cells when compared to control (*, $p < 0.05$). As expected, cAMP levels upon treatment with SST were comparable with the combination of receptor-specific agonists. In monotransfected cells, SST and receptor-specific agonists significantly inhibited cAMP (**, $p < 0.01$; *, $p < 0.05$). Although, the levels of cAMP inhibition upon treatment with receptor-specific agonists were significantly decreased in cotransfected *vs.* monotransfected cells (#, $p < 0.05$), the receptor complex still demonstrated significant G_i coupling to AC.

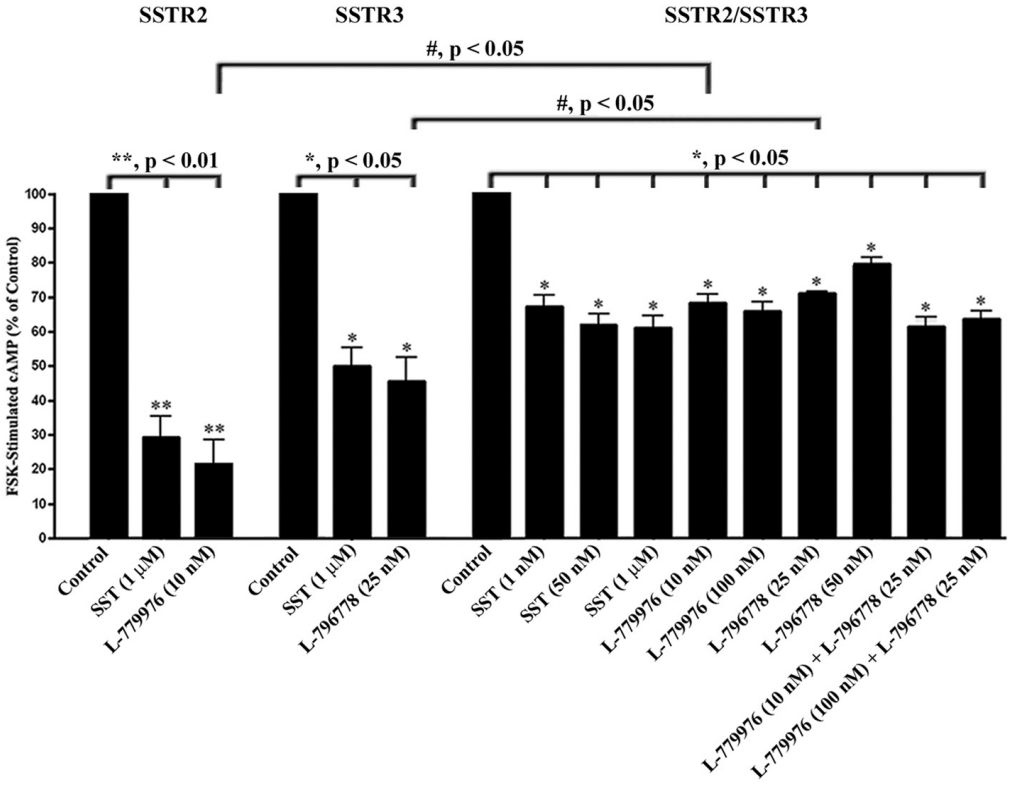

Figure 3.3 cAMP inhibition upon activation of SSTR2/SSTR3. Mono- and/or cotransfected expressing SSTR2 and SSTR3 were pre-treated with IBMX (0.5 mM) for 30 min followed by addition of FSK (20 μM) in the presence or absence of SST (1, 50 nM and 1 μM), and receptor-specific agonists L-779976 (10 and 100 nM) and L-796778 (25 and 50 nM), or as indicated for 30 min at 37ºC and processed for cAMP assay. Note the significant inhibition of FSK-stimulated cAMP in the presence of SST and receptor-specific agonists; an indication that SSTR2/SSTR3 complex in cotransfected cells is functionally active, albeit at a lesser degree than the monotransfectants. Data represent mean ± S.D of three independent experiments

3.3.4 Time and receptor-dependent regulation of MAPK signaling by SSTR2/SSTR3

Recent studies have suggested agonist and time-dependent modulation of pERK1/2 in cells expressing SSTR2, SSTR3 or SSTR2/SSTR5 (Grant et al., 2008; War et al., 2011). Using time-course experiments (30 min and 24 h), the effect of

heterodimerization on ERK1/2 phosphorylation was examined in cotransfected cells with or without FBS. As depicted in Figure 3.4, the levels of pERK1/2 were significantly increased at lower and higher concentrations of SST for 30 min; whereas in response to receptor-specific agonists, ERK1/2 phosphorylation decreased at lower concentration and increased at higher concentration when compared to control (*, $p < 0.05$; **, $p < 0.01$). In the absence of FBS, pERK1/2 was significantly decreased by SST and receptor-specific agonists in comparison to control (*, $p < 0.05$). SST treatment for 24 h increased pERK1/2 when compared to non-treated cells (*, $p < 0.05$). No significant changes were observed with receptor-specific agonists alone suggesting that co-activation of both receptors is a prerequisite for sustained ERK1/2 signaling. In FBS-deficient conditions, SST and receptor-specific agonists significantly enhanced pERK1/2 in comparison to control (*, $p < 0.05$).

As illustrated in Figure 3.5, p38 phosphorylation was enhanced in cotransfected cells treated with SST and receptor-specific agonists for 30 min when compared to control (*, $p < 0.05$). SST significantly increased p38 phosphorylation in serum-deprived cells, whereas the independent receptor activation had no effect in comparison to control (*, $p < 0.05$). Upon prolonged treatment with SST and receptor-selective agonists, p38 signaling was enhanced when compared to control (*, $p < 0.05$). No discernable changes in p-p38 levels were observed between treated and untreated cells under serum deprived conditions. Collectively, these results demonstrate distinct pattern of MAPK modulation upon agonist treatment in cotransfected cells.

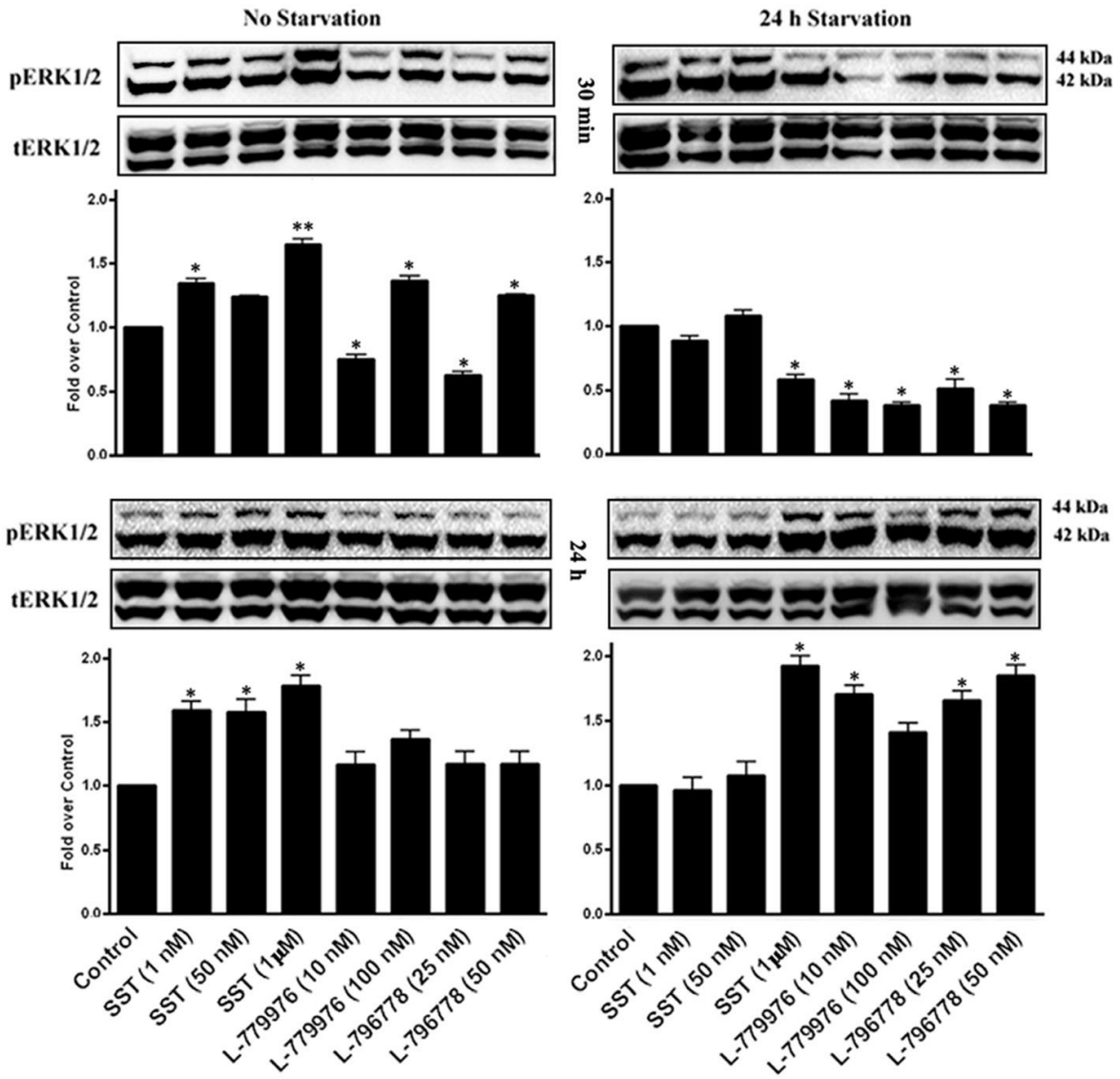

Figure 3.4 Concentration and time-dependent modulation of ERK1/2 phosphorylation by SSTR2/SSTR3. Cells stably cotransfected with SSTR2 and SSTR3 were treated with SST (1, 50 nM and 1 μM), L-779976 (10 and 100 nM) and L-796778 (25 and 50 nM) for 30 min (*top panel*) or 24 h (*bottom panel*) with or without FBS, and processed for total and pERK1/2 using Western blot analysis. Note a distinct pattern of ERK1/2 phosphorylation in response to SST and receptor-specific agonists in a concentration and time-dependent manner. Histograms represent the densitometric analysis and the results are presented as the ratio of phospho- and total-ERK1/2 expressed as fold over control. Mean ± S.D is representative of three independent experiments.

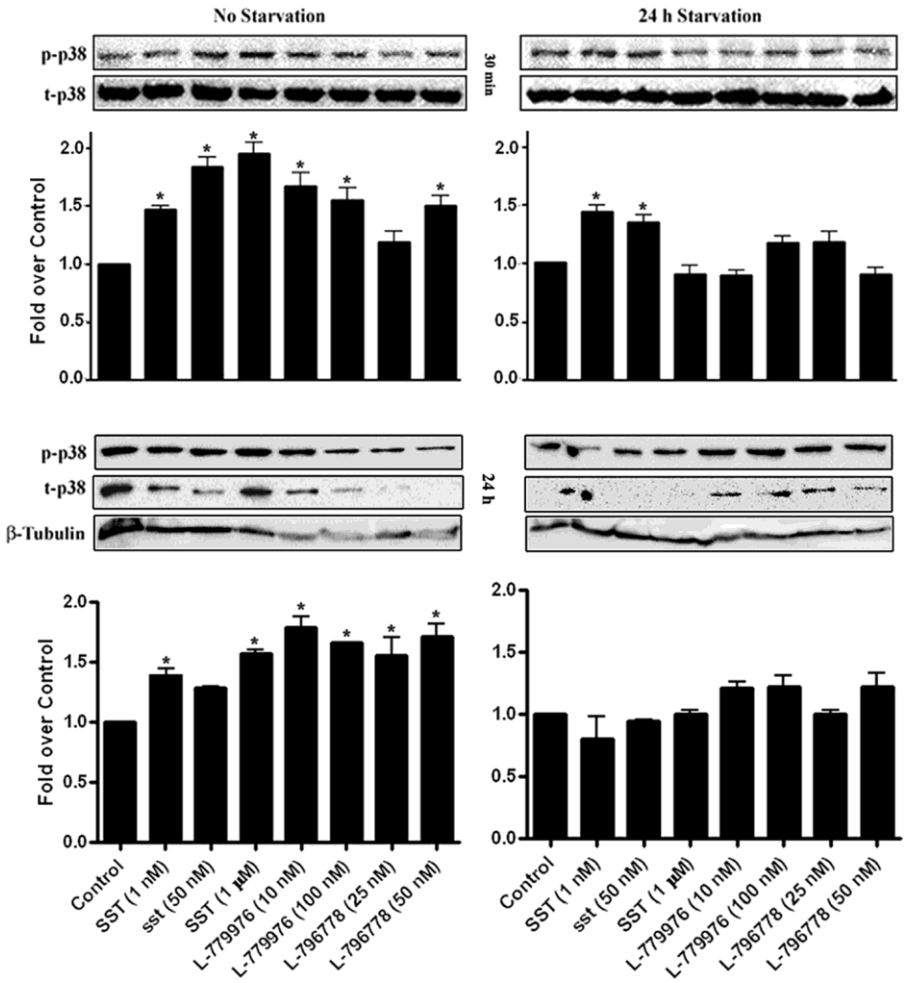

Figure 3.5 Modulation of p38 phosphorylation in response to SSTR2/SSTR3 activation. Cotransfected cells were treated with SST (1, 50 nM and 1 μM), L-779976 (10 and 100 nM) and L-796778 (25 and 50 nM) for 30 min (*top panel*) or 24 h (*bottom panel*) in the presence or absence of FBS, and processed for total and p-p38 using Western blot analysis. p38 phosphorylation increased upon treatment with SST and receptor-specific agonists for 30 min. In serum-deprived conditions, SST significantly increased p38 activation, whereas the effects of independent receptor activation were comparable to control. Prolonged treatment with SST and receptor-specific agonists significantly increased p-p38 levels, whereas in serum-deficient conditions, no significant changes were observed between treatment and control. Histograms represent the densitometric analysis and the results are presented as the ratio of phospho- and total-p38 (*top panel*) or phospho-p38 and β-Tubulin (*bottom panel*) expressed as fold over control. Mean ± S.D is representative of three independent experiments.

3.3.5 Inhibition of cell proliferation by SSTR2/SSTR3

To determine the antiproliferative effects upon activation of SSTR2/SSTR3 by SST and receptor-specific agonists, MTT assay was accomplished in monotransfectants expressing SSTR2 or SSTR3 and cotransfected cells. Importantly, cells were treated in the presence or absence of PTX pre-treatment to elucidate a functional relationship between G_i coupling and antiproliferation. As depicted in Figure 3.6 (*top panel*), SST and the receptor-specific agonists resulted in significant inhibition of cell proliferation in cotransfected cells (*, $p < 0.05$). Furthermore, the combination of receptor-specific agonists exhibited a similar antiproliferative response to SST. In addition, monotransfectants expressing SSTR2 displayed a comparable inhibition of cell proliferation with cotransfected cells upon treatment with SSTR2-specific agonist. Strikingly, the effect of SSTR3-specific agonist was significantly higher in cotransfected cells in comparison to monotransfectants (28.6 ± 4.3% *vs.* 18.9 ± 1.6%; #, $p<0.05$), suggesting the enhanced antiproliferative functions of SSTR3 in the heteromeric complex. Moreover, SSTR2 and SSTR3 mediated antiproliferative effects in mono- and cotransfected cells were G_i-dependent as evident by no significant changes in cell proliferation upon receptor activation in cells pre-treated with PTX (Figure 3.6, *bottom panel*). Taken together, the data from MTT assay suggest a role played at least in part by receptor heterodimerization in mediating the antiproliferative effects of SST and receptor-specific agonists in cotransfected cells.

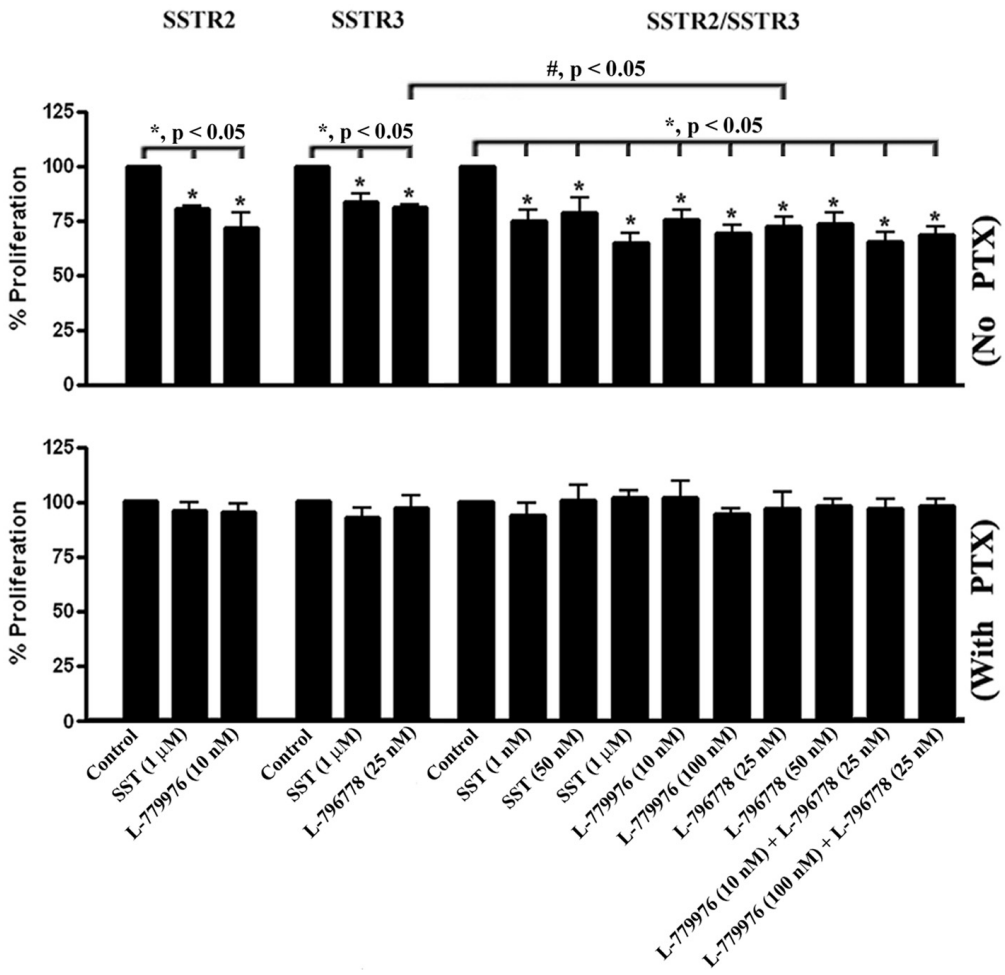

Figure 3.6 The antiproliferative activity of SSTR2/SSTR3. Mono- and cotransfected cells expressing SSTR2 and SSTR3 in the presence (*top panel*) and absence (*bottom panel*) of PTX were subjected to treatment with SST (1, 50 nM and 1 μM), L-779976 (10 and 100 nM) and L-796778 (25 and 50 nM) or as indicated for 24 h and processed for MTT assay. SST and receptor-specific agonists significantly inhibited cell proliferation in cotransfected cells in a PTX-sensitive manner. Note an increased antiproliferative effect of SSTR3-specific agonist in cotransfected cells when compared to monotransfectants expressing SSTR3. Data are presented as mean ± S.D of three independent experiments.

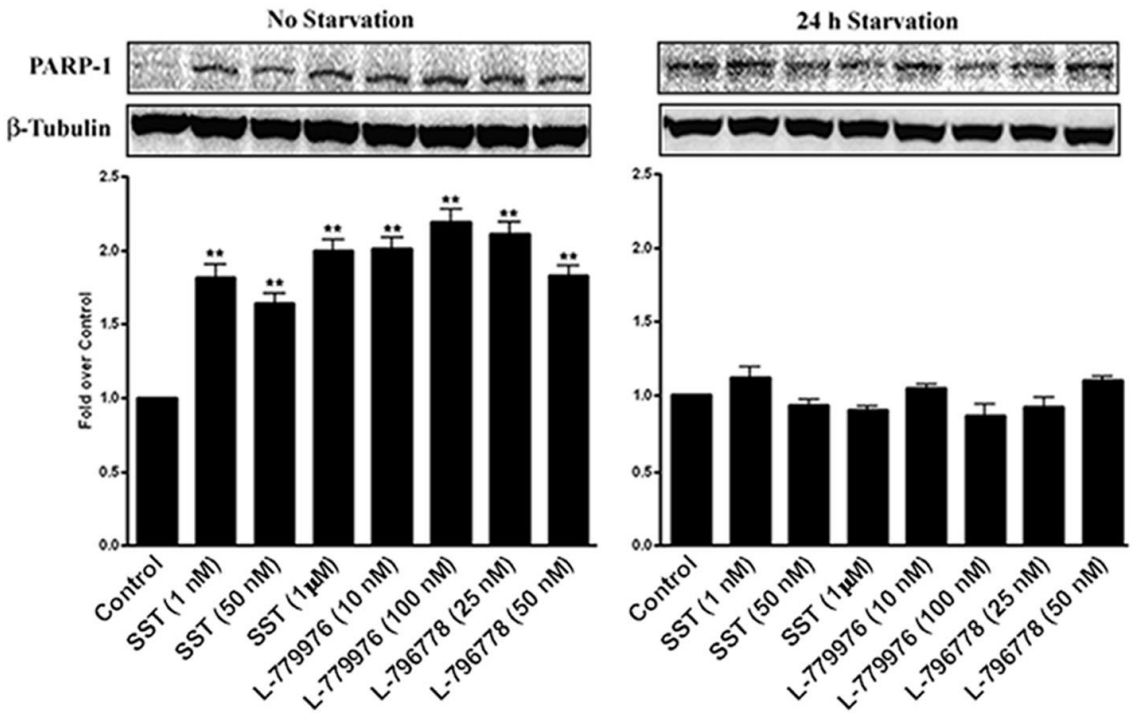

Figure 3.7 The cytotoxic role for SSTR2/SSTR3. Stable cotransfectants of SSTR2 and SSTR3 were subjected to 24 h treatment with SST (1, 50 nM and 1 μM), L-779976 (10 and 100 nM) and L-796778 (25 and 50 nM) in the presence or absence of serum-starvation and processed for PARP-1 expression by Western blot analysis. Cells treated with SST and receptor-specific agonists displayed increased expression of PARP-1. Note a high basal PARP-1 expression in serum-deprived conditions with no significant changes in response to SST and receptor specific agonists. β-Tubulin was used as a loading control. Data represent mean ± S.D of three independent experiments.

3.3.6 Pro-apoptotic role of SSTR2 and SSTR3 in cotransfected cells

To further identify the putative mechanisms for the antiproliferative signal in cotransfectants, the expression of PARP-1 was determined for apoptosis. As illustrated in Figure 3.7, SST and the receptor-specific agonists significantly increased the expression of PARP-1 in comparison to control suggesting a cytotoxic effect (**, $p < 0.01$). High basal PARP-1 expression was observed in cells deprived of FBS with or without treatment with SST and receptor-specific agonists. These

observations correlate with p38 signaling and might suggest a role for p38 MAPK in SSTR2/SSTR3 mediated apoptosis.

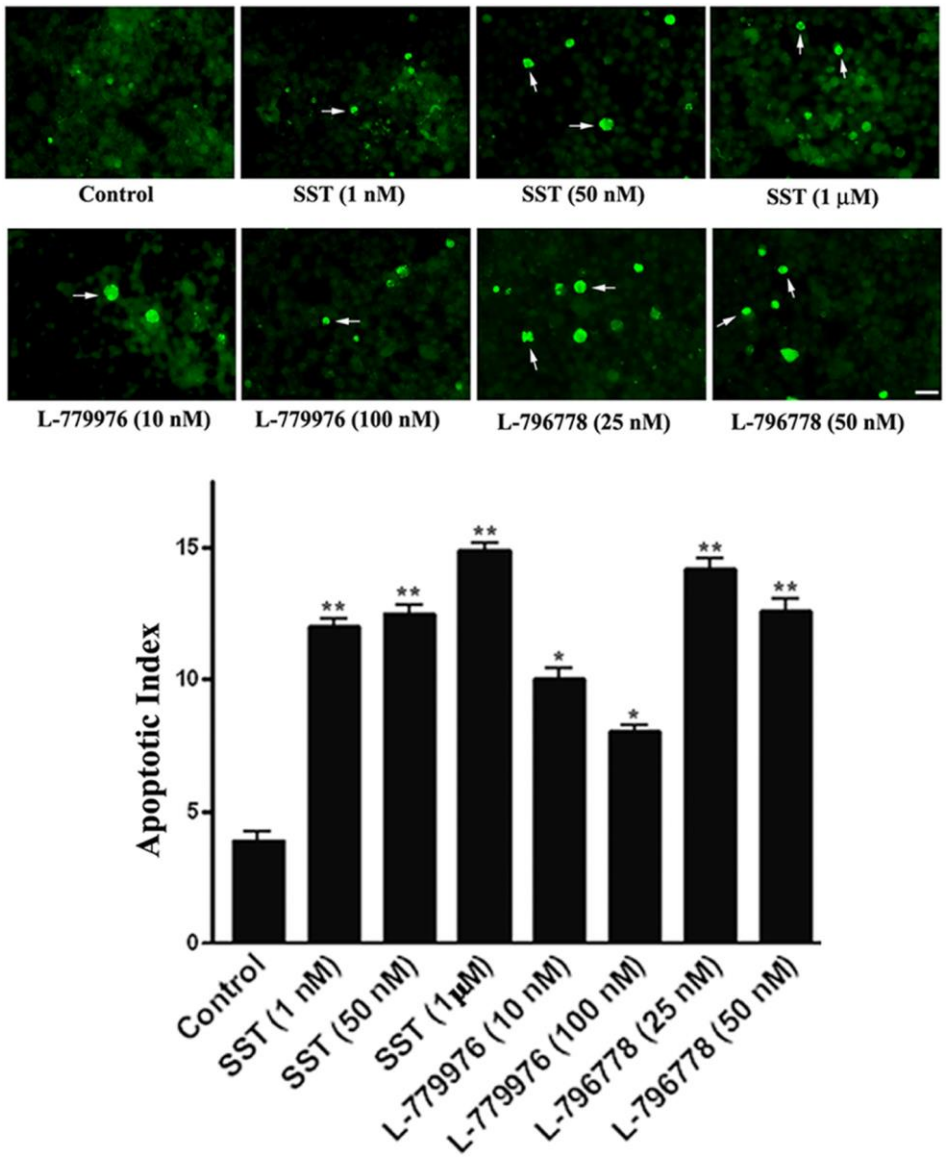

Figure 3.8 Representative photomicrographs illustrating TUNEL positive cells as an index of apoptosis. HEK-293 cells cotransfected with SSTR2/SSTR3 were treated with SST (1, 50 nM and 1 μM), L-779976 (10 and 100 nM) and L-796778 (25 and 50 nM) for 24 h, and processed for TUNEL staining. SST and receptor-specific agonists increased TUNEL labeling indicating cytotoxic role for SSTR2 and SSTR3. Histogram represents quantitative analysis of apoptotic cells. A total of 600-750

cells were counted for each treatment. Arrows in representative panels indicate apoptotic cells. Scale bar = 10 µm. Mean ± S.D is representative of three independent experiments.

To support the enhanced PARP-1 expression, *in situ* TUNEL assay was performed to quantify apoptosis in cotransfected cells expressing SSTR2/SSTR3. As depicted in Figure 3.8, SST and the receptor-specific agonists significantly increased the number of TUNEL-positive cells in comparison to control (*, $p < 0.05$; **, $p < 0.01$). Taken together, these findings show the cytotoxic role for SSTR2/SSTR3 upon activation.

3.3.7 Induction of cell cycle arrest upon activation of SSTR2 and SSTR3

Earlier studies have revealed that SSTR2 but not SSTR3 mediated induction of cyclin-dependent kinase inhibitors p21 and $p27^{Kip1}$ leads to cell cycle arrest (Alderton et al., 2001; Grant et al., 2008; Pages et al., 1999; Sharma et al., 1996). To confirm whether SSTR2/SSTR3 complex alters the antiproliferative nature of native receptors, p21 and $p27^{Kip1}$ expression was determined to attest the role of receptors for cytostatic activity. As illustrated in Figure 3.9 (*top panel*), SST and receptor-specific agonists significantly increased p21 expression upto ~2 fold in comparison to control, suggesting a cytostatic effect exerted by SSTR2 and SSTR3 (*, $p < 0.05$). In the absence of FBS, the high basal expression of p21 was maintained upon receptors activation. Furthermore, $p27^{Kip1}$ expression increased upto ~1.5 fold upon treatment with SST and receptor-specific agonists when compared to control.

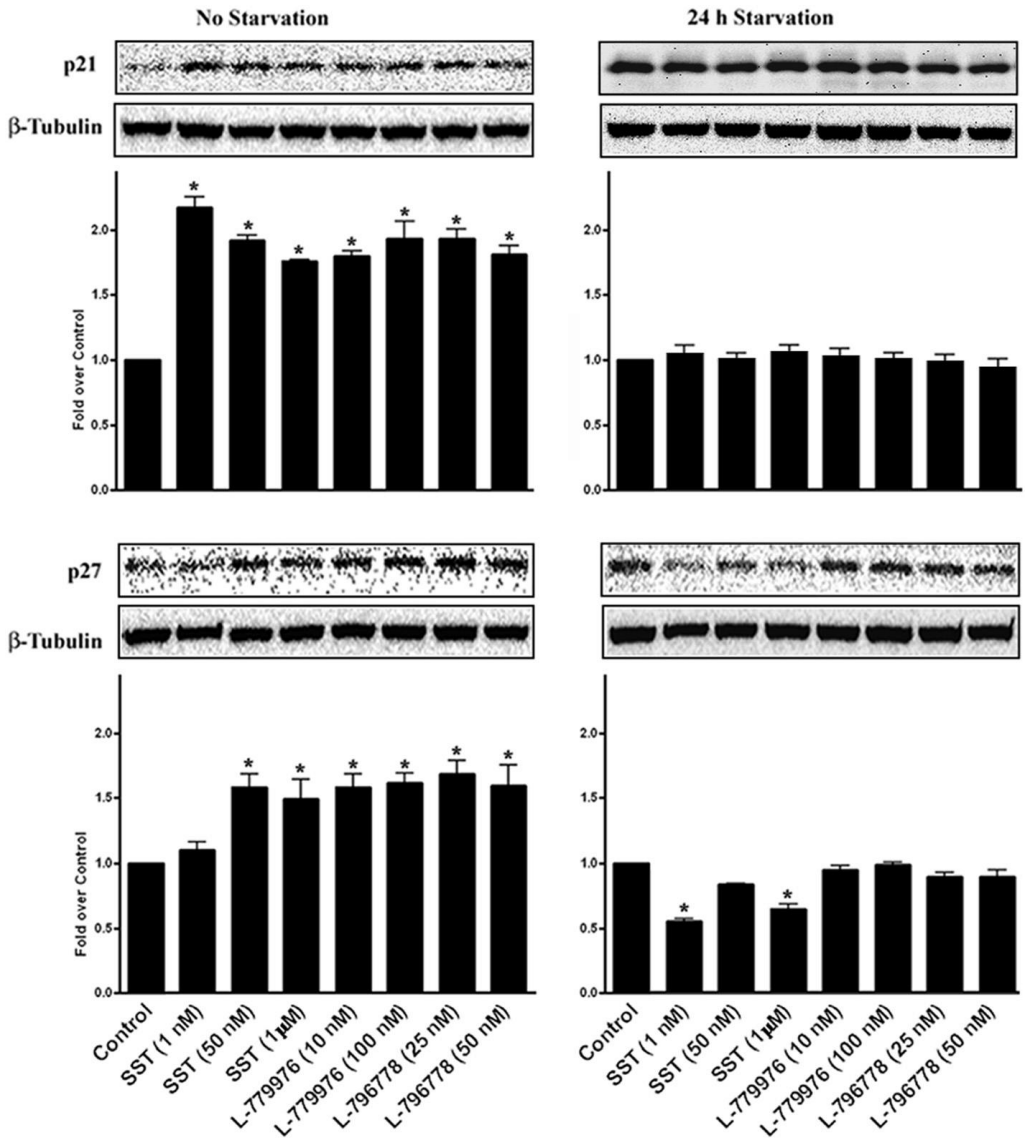

Figure 3.9 p21 and p27^{Kip1} mediated cytostatic effects upon activation of SSTR2/SSTR3. Cotransfected cells were treated with SST (1, 50 nM and 1 μM), L-779976 (10 and 100 nM) and L-796778 (25 and 50 nM) for 24 h with or without serum starvation and processed for p21 (*top panel*) and p27^{Kip1} (*bottom panel*) expression using Western blot analysis. Induction of p21 and p27^{Kip1} in response to SST and receptor-selective agonists suggests a cytostatic role for both receptors. The high basal levels of p21 observed in serum-deprived cells were maintained upon treatment with SST and specific agonists. Conversely, SST decreased p27^{Kip1} expression in cotransfected cells by ~50% in FBS-deficient conditions. β-Tubulin was used as a loading control. Results are expressed as mean ± S.D of three independent experiments.

3.4 Discussion

Several lines of evidence have described the role of GPCR oligomerization in the modulation of intracellular signaling cascades. The underlying molecular mechanisms are complex and unclear, and have only been partly elucidated to date. The present study provides detailed description of functional analysis of human SSTR2 and SSTR3 heterodimerization in HEK-293 cells including receptor surface expression, internalization, MAPK signaling, cell proliferation and apoptosis in response to SST and receptor-specific agonists. To the best of knowledge, this is the first comprehensive demonstration of a functional crosstalk between human SSTR2 and SSTR3.

Human SSTR2 and SSTR3 were previously reported to exist as preformed homodimers in monotransfected cells and respond to agonist treatment in receptor-specific manner (Grant et al., 2004a; War et al., 2011). Earlier studies also showed that homodimers of SSTR5 and heterodimers of SSTR4/SSTR5 are stable upon agonist activation (Rocheville et al., 2000b; Somvanshi et al., 2009). SST induced dissociation of human SSTR2 homodimer and rat SSTR2/SSTR3 heterodimer at the cell surface has been described previously (Grant et al., 2004a; Pfeiffer et al., 2001). Using CO-IP and microscopic Pb-FRET analysis, this study reports that human SSTR2 and SSTR3 exist in a constitutive heteromeric complex at the plasma membrane. The receptor complex tends to remain stable upon treatment with SST and receptor-specific agonists with FRET efficiency sufficient to support heterodimerization. Nonetheless, the decrease in relative FRET efficiency might be

attributed to the changes in receptor conformation and orientation at the cell surface upon agonist activation which cannot be excluded from the discussion.

Agonist-induced dissociation of human SSTR2 homodimers into monomers at plasma membrane is a prerequisite for receptor internalization, whereas human SSTR3 internalized as homodimer (Grant et al., 2004a; War et al., 2011). In this study, the immunocytochemistry data illustrate decreased cell surface distribution of SSTR2 and SSTR3 upon receptors activation. Strikingly, the independent activation of SSTR2 or SSTR3 resulted in a dramatic down-regulation of both receptors and decreased colocalization at plasma membrane. It is likely that the receptors internalize as heterodimers, which in part might be a plausible explanation for an increased colocalization in cytosol. These data contradict an earlier report where rat SSTR2/SSTR3 heterodimer, while promoting SSTR2 internalization, was shown to abrogate agonist-mediated endocytosis of SSTR3 (Pfeiffer et al., 2001). A follow-up study from the same group revealed that the activation of SSTR2 in the heteromeric complex of SSTR2/μ-opioid receptor of rat origin promoted co-internalization of both receptors (Pfeiffer et al., 2002). Accordingly, the results from this study for human SSTR2/SSTR3 reinforce the concept that heterodimerization confers unique species-selective properties to native receptors.

SSTR subtypes elicit their cellular actions by inhibiting second messenger cAMP through PTX-sensitive G_i proteins, and oligomerization plays a key role in modulating such effects (Baragli et al., 2007; Grant et al., 2004b; Patel, 1999; Pfeiffer et al., 2001; Rocheville et al., 2000a). The present study demonstrates significant cAMP inhibition in response to SST and receptor-specific agonists. In

cotransfectants, G_i coupling was attenuated when compared to monotransfected cells expressing SSTR2 or SSTR3. It is not clear whether the blunted G_i coupling in cotransfected cells is linked to the decreased FRET efficiencies in response to receptors activation. Moreover, it remains elusive whether the determinant role on cAMP inhibition in cotransfected cells is mediated by monomeric, homodimeric or heteromeric sub-populations. Whether the loss of G_i coupling in cotransfectants is linked to downstream signaling pathways is not well understood and future studies are warranted to delineate the molecular mechanisms involved.

Several previous studies have shown a significant role for MAPKs in cell survival, proliferation and apoptosis (Berra et al., 1998; Hubina et al., 2006; Nishida and Gotoh, 1993; Nishimoto and Nishida, 2006). The cytostatic role for SSTR2 has been intimately associated with the modulation of ERK1/2 signaling in a cell-dependent manner (Cattaneo et al., 2000; Dent et al., 1997; Lahlou et al., 2003). Previous study showed a robust increase in ERK1/2 phosphorylation upon transient activation of SSTR2 in monotransfected cells (Grant et al., 2008). Interestingly, ERK1/2 remained in phosphorylated form upon prolonged activation of SSTR2 in cells coexpressing SSTR2/SSTR5, an effect attributed to receptor heterodimerization. The activation of SSTR3 leads to cell cycle arrest or apoptosis depending upon the cell-type (Florio et al., 2003; War et al., 2011). Upon short-term agonist exposure, the cytotoxic role of SSTR3 was associated with pERK1/2 inhibition (War et al., 2011). Serum and growth factors have also been shown to stimulate ERK activation and cell proliferation in astrocytes and CHO-K1 cells expressing SSTR1 (Florio et al., 1999; Thellung et al., 2007). In the present study,

although ERK1/2 modulation by SST and receptor-specific agonists was concentration-dependent when cells were treated for 30 min, prolonged stimulation of both receptors was essential to maintain a sustained ERK1/2 activation. More importantly, the agonist functions in modulating ERK1/2 phosphorylation in cotransfected cells were enhanced in serum-deficient conditions. Several reports have implicated a role for p38 MAPK pathway in different types of tumors (Alderton et al., 2001; Chuang et al., 2000; Wagner and Nebreda, 2009). The antiproliferative function of SSTR2 has been associated with increased p38 signaling, while in contrast, SSTR3 was devoid of such property (Alderton et al., 2001). In the heteromeric complex of human SSTR2/SSTR3, the activation of p38 in response to SSTR3-specific agonist uncovered a previously unnoticed role of SSTR3 on p38 MAPK. Taken together, the diverse functional response on MAPKs might be attributed to different duration of activities among various cell types, and SSTR-independent pathways (Lahlou et al., 2004; Murphy et al., 2002; War et al., 2011).

Recently, the pronounced effect of oligomerization on the agonist mediated inhibition of cell proliferation was described for cotransfected cells expressing SSTR2/SSTR5 or SSTR4/SSTR5 when compared to monotransfectants (Grant et al., 2008; Somvanshi et al., 2009). The data from this study demonstrate significant antiproliferative effects upon activation of SSTR2/SSTR3 in cotransfected cells. Importantly, the activation of SSTR3 in cotransfectants displayed significant antiproliferative effect in comparison to monotransfected cells despite decreased cAMP inhibition. The antiproliferative functions of SSTR2/SSTR3 in cotransfectants

were G_i-dependent and might be exerted via modulation of ERK1/2 and p38 MAPK pathway which cannot be ruled out from the discussion.

In the current study, the activation of PARP-1 in the presence of SST and receptor-specific agonists in a pattern similar to p38 MAPK suggests a pro-apoptotic role for SSTR2/SSTR3. In agreement, TUNEL assay demonstrated a similar degree of cytotoxic response. Low expression of p21 and $p27^{Kip1}$ has been often reported in tumor of various origins, and their up-regulation upon activation of SSTRs plays an important role in cell cycle arrest (Bamberger et al., 1999a; Bamberger et al., 1999b; Grant et al., 2008; Lahlou et al., 2003; Lidhar et al., 1999; Patel, 1999; Romics et al., 2008; Sharma et al., 1996; Wu et al., 2011a). In line with this notion, an increased expression of p21 and $p27^{Kip1}$ described here in response to SST and receptor-specific agonists suggests a cytostatic function for SSTR2/SSTR3. An earlier study attributed the sustained activation of ERK1/2 and p38 MAPKs to p21-mediated antiproliferation via SSTR2, whereas SSTR3 exhibited no effect on p21 and p38 signaling (Alderton et al., 2001). Also, SSTR2 mediated ERK1/2 activation has been linked to $p27^{Kip1}$ mediated inhibition of cell proliferation (Lahlou et al., 2003). In agreement with these observations, the sustained pERK1/2 and p38 signaling, together with induction of p21 and $p27^{Kip1}$ might account for SSTR2/SSTR3 mediated antiproliferation in response to receptors activation.

In conclusion, this study show the modulation of intracellular signaling and antiproliferative functions by SSTR2 and SSTR3 attributed at least in part to receptor heterodimerization. These findings are of interest and might lead to identification of a

novel therapeutic target in tumors expressing these receptor subtypes. To better understand the complexities of receptor functions, further studies are in progress.

Chapter 4: Human somatostatin receptor-3 distinctively induces apoptosis in MCF-7 and cell cycle arrest in MDA-MB-231 breast cancer cells

4.1 Background

Breast cancer is the most frequent malignancy and second leading cause of cancer-related deaths in women (Ferlay et al., 2010). Breast cancer is characterized by distinct biological changes in the mammary epithelial cells leading to aggressive cell proliferation (Dickson and Lippman, 1992; Ferlay et al., 2010; Harris et al., 1992; Jemal et al., 2010; Watt et al., 2008). Several hormones which are essential for normal breast development also become tumorigenic once overexpressed. Furthermore, the aberrant local production of growth factors such as EGF, IGF-1 and transforming growth factor have also been implicated in progression of breast tumor (Dickson and Lippman, 1992; Harris et al., 1992; Stampfer and Yaswen, 1993). The management of breast cancer includes surgical resection and radiation, as well as adjuvant chemo/hormone based therapeutics (Hsu et al., 2005). Most advanced stage breast tumors specifically hormone-independent cancers develop resistance to chemotherapy. This results in limited effectiveness of pharmacotherapy and increased mortality. SST is a widely expressed endogenous peptide and known to regulate diverse cellular processes such as neurotransmission, hormone secretion and cell proliferation (Lahlou et al., 2003; Patel, 1999). The biological actions of SST are mediated by five GPCRs namely SSTR1-5. SSTRs mediated inhibition of cell proliferation is attributed either to induction of cell cycle arrest and/or apoptosis, or suppressing the hormones and growth factors associated with tumor

promoting pathways (Grant et al., 2008; He et al., 2009; Thangaraju et al., 1999a; Thangaraju et al., 1999b; War and Kumar, 2012; War et al., 2011; Watt et al., 2009). Importantly, SST induced activation of cytotoxic signaling is predominantly mediated via SSTR2 and SSTR3 (He et al., 2009; Sharma et al., 1996; War and Kumar, 2012; War et al., 2011).

In contrast to other SSTR subtypes, SSTR3 was targeted for degradation instead of recycling back to the cell surface following agonist-induced internalization (Hukovic et al., 1996; War et al., 2011). The appropriate density of membrane bound SSTRs contributes to the sensitivity and effectiveness of *in vivo* peptide-targeted therapy for somatotroph adenomas (Acunzo et al., 2008; Daly et al., 2006). Also, a longer relapse-free survival was observed in breast cancer patients with positive SSTRs expression (Foekens et al., 1989). Human pancreatic cancers display loss of SSTR expression. Importantly, these tumors exhibited enhanced apoptosis and antiproliferation upon reinstating SSTRs expression (Bousquet et al., 2006; Guillermet et al., 2003; Kumar et al., 2004; Reubi et al., 1988a; Vernejoul et al., 2002). Importantly, MCF-7 cells transfected with SSTR2 exhibit increased cytotoxicity and cell cycle arrest, with subsequent decreased expression of ErbB1 (He et al., 2009). There is a growing body of evidence suggesting a functional cross-talk between ER_{α} and SSTRs (Djordjijevic et al., 1998; Pilichowska et al., 2000; Rivera et al., 2005; Van Den Bossche et al., 2004; Xu et al., 1996). For example, in primary cultures of female rat pituitary cells, EST treatment increased the ability of SST and SST analogs to inhibit cAMP (Djordjijevic et al., 1998). EST also increased the expression of SSTR2 and SSTR3 at the mRNA level in these cells. Furthermore,

EST regulated SSTR2 mRNA expression of ER_{α}-positive human breast cancer cell lines in a time and dose-dependent manner (Van Den Bossche et al., 2004). Recently, SSTRs/ErbBs heterodimerization was also demonstrated in breast cancer cells MCF-7 (ER_{α}-positive) and MDA-MB-231 (ER_{α}-negative) in a cell-specific manner (Watt et al., 2009). Such an association resulted in the modulation of key signaling molecules governing ErbBs mediated cell proliferation.

In breast cancer, activated PI3K is associated with tumor growth and treatment failure. Previous studies have shown that downregulation of PI3K represents a key step in the development of potential therapeutic targets in tumor of different origin (Altomare and Testa, 2005; Berns et al., 2007; Hynes and Dey, 2009; Kumar, 2011). Correspondingly, recent studies have demonstrated a role for SSTRs in the inhibition of PI3K pathway in insulinoma, pituitary and pancreatic tumors (Bousquet et al., 2006; Grozinsky-Glasberg et al., 2008; Kharmate et al., 2011a, b; Theodoropoulou et al., 2006). SSTRs modulate PTPs, cell-cycle inhibitor $p27^{Kip1}$ and MAPKs such as ERK1/2 and p38 in a cell/receptor-specific (Grant et al., 2008; Sharma et al., 1999; Somvanshi et al., 2009; Thangaraju et al., 1999a; Thangaraju et al., 1999b; War and Kumar, 2012; War et al., 2011). In monotransfected cells, SSTR3 activation had no effect on p38 signaling whereas ERK1/2 inhibition as well as membrane translocation of PTP correlated with SSTR3 cytotoxic effects (Alderton et al., 2001; War et al., 2011). Furthermore, cells coexpressing SSTR2/SSTR3 show increased expression of phospho-p38 and $p27^{Kip1}$ upon SSTR3 activation (War and Kumar, 2012).

Although, previous studies attest the role of SSTR3 in apoptosis and cell cycle arrest, the underlying mechanisms are not well understood. The clinical studies focusing on SSTR3 tumor suppressive role have been limited due to modest expression in many tumors such as breast cancer. Accordingly in the present study, SSTR3 was overexpressed in MCF-7 and MDA-MB-231 cells to investigate the mechanisms associated with cell proliferation and apoptosis in ER dependent manner.

4.2 Material and methods

4.2.1 Materials

Non-peptide agonist for SSTR3 (L-796778) was a kind gift from Dr. S.P. Rohrer from Merck and Co (Rohrer and Schaeffer, 2000). SST was purchased from Bachem Inc., Torrance, CA, USA. EGF, EST and mouse monoclonal antibody against HA were obtained from Sigma-Aldrich Inc., St. Louis, MO, USA. Rabbit polyclonal antibody against SSTR3 was generated and characterized as described previously (Kumar et al., 1999). Mouse monoclonal antibodies against PARP-1 and PTP-1C were purchased from BD-Biosciences, Mississauga, ON, Canada. Rabbit polyclonal antibodies for total and phospho-ERK1/2, p38 and PI3K were obtained from Cell Signaling Technology, Danvers, MA, USA. Fluorescein, rhodamine or peroxidase conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Mouse monoclonal anti-$p27^{Kip1}$ and HRP conjugated anti-β-Actin (C4) antibodies were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA,

USA. TUNEL kit was purchased from La Roche Applied Science, Mannheim, Germany. ECL Western blotting detection kit and nitrocellulose Hy-Bond ECL membrane were purchased from GE Healthcare, Piscataway, NJ, USA. Trypsin-EDTA, PBS, and cell culture media including RPMI 1640 and L-15 were purchased from Invitrogen, Burlington, ON, Canada. Reagents for electrophoresis were obtained from Bio-Rad Laboratories, Mississauga, ON, Canada. Other reagents were of AR grade and were purchased from various sources.

4.2.2 Constructs of human SSTR3 and transfection

N-terminal HA-tagged human SSTR3 (HA-SSTR3) construct was made using pCDNA3.1/Neo (neomycin resistance), as previously described (War et al., 2011). Stable transfection of HA-SSTR3 in MCF-7 (R3-MCF-7) and MDA-MB-231 (R3-MB-231) was done using Lipofectamine 2000 transfection reagent as previously described (Grant et al., 2004b; Somvanshi et al., 2009). Clones were selected and maintained in RPMI (R3-MCF-7) or L-15 (R3-MB-231) containing 10% FBS and 700 µg/ml neomycin. Non-transfected MCF-7 and MDA-MB-231 cells were maintained in RPMI and L-15 media, respectively containing 10% FBS.

4.2.3 Cell treatments

Unless specified otherwise, the drug concentrations used in this study are: SST (1 nM and 1 µM); L-796778 (25 nM); EGF (10 nM); EGF (10 nM) + L-796778 (25 nM); EST (1 µM); and EST (1 µM) + L-796778 (25 nM).

4.2.4 Immunocytochemistry

To study receptor trafficking, R3-MCF-7 and R3-MB-231 cells were grown on poly-D-Lysine coated glass coverslips, and treated with SST (1 nM and 1 μM) and L-796778 (25 nM) for 15 min at 37ºC. Cells were fixed in 4% PF on ice followed by permeabilization in 0.3% Triton X-100 for 15 min at RT. After three washes in PBS, cells were blocked in 5% normal goat serum for 1 h at RT, followed by overnight incubation with mouse monoclonal anti-HA antibody at 4ºC. Non-transfected MCF-7 and MDA-MB-231 cells were treated under identical experimental conditions and incubated overnight using polyclonal anti-SSTR3 antibody at 4ºC. The cover slips were washed in PBS and further incubated with FITC-conjugated goat anti-mouse or rhodamine-conjugated goat anti-rabbit secondary antibodies for 1 h at RT. The coverslips were mounted on glass slides and analyzed under Leica fluorescence microscope. Adobe Photoshop and Image J software, NIH (Collins, 2007) were used for making the composites.

4.2.5 Cell proliferation assay

Cell proliferation was determined by MTT assay as previously described (War et al., 2011). R3-MCF-7 and R3-MB-231 cells overexpressing SSTR3, and non-transfected cells were grown on 96-well culture plates. Cells were first serum deprived for 24 h and then treated for 24 h, as indicated before processing for the cell proliferation assay. Briefly, 20 μl of 5 mg/ml MTT solution was added and the cells were incubated for 2 h at 37ºC. The formazan crystals formed were dissolved in

200 µl of isopropanol and the absorbance was measured in a microplate spectrophotometer at 550 nm.

4.2.6 TUNEL staining for apoptosis

Non-transfected MCF-7 and MDA-MB-231 and cells overexpressing SSTR3 were grown on poly-D-Lysine coated glass coverslips in 24-well culture plates and treated for 24 h, as indicated. Cells were fixed in 4% PF and rinsed three times with PBS. A solution containing 0.1% Triton X-100 and 0.1% sodium citrate was added for 15 min at RT to permeabilize the cells. After washes in PBS, cells were incubated with TUNEL reaction mixture for 1 h at 37ºC in dark. Finally, the coverslips were washed in PBS and mounted on glass slides for analysis under Leica fluorescence microscope. Adobe Photoshop was utilized for making composites. For quantification of apoptotic cells, a total of 600-800 cells were counted for each treatment condition. Apoptotic cells exhibit intense nuclear staining in comparison to diffused staining in non-apoptotic cells. Apoptotic index was calculated as % of TUNEL-positive cells divided by total number of cells analyzed.

4.2.7 Western blot analyses

For SSTR3 and PTP-1C expression, cells were treated for 15 and 30 min, respectively, and collected in homogenization buffer (20 mM Tris-HCl, 2.5 mM dithiothreitol, 11% sucrose and protease inhibitor 1:100). Briefly, membrane and/or cytosolic extracts were fractionated on 10% SDS-PAGE and probed for SSTR3 and PTP-1C using specific antibodies as previously described (War et al., 2011; Watt et

al., 2009). For PARP-1 and $p27^{Kip1}$, cells were treated for 24 h, whereas for PI3K, ERK1/2 and p38 experiments, cells were treated for 30 min as indicated. Following treatment, cells were lysed in RIPA buffer, and the cell lysate was fractionated on 10% SDS-PAGE and probed using specific antibodies as previously described (War and Kumar, 2012). Samples were normalized by Bradford assay and equal protein amount (20 µg) was loaded in all Western blot experiments. β-Actin was used as loading control.

All other procedures such as membrane blocking, primary and secondary antibody incubation, and chemiluminescence detection were performed as previously described (War et al., 2011). Immunoblots were visualized with an Alpha Innotech FluorChem 8800 gel box imager (Protein Simple, Santa Clara, CA, USA), and subjected to densitometric analysis using FluorChem software (Protein Simple, Santa Clara, CA, USA).

4.2.8 Statistical analysis

Results are expressed as mean ± S.D of three independent experiments. Statistical analysis was done by one-way ANOVA and *post hoc* Dunnett's test to compare against control as applicable. Student's *t*-test (unpaired) was used to compare basal levels between non-transfected (MCF-7 and MDA-MB-231) and transfected (R3-MCF-7 and R3-M-231) cells. GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for performing data analysis and p value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Cell-specific receptor distribution upon SSTR3 overexpression in breast cancer cells

The transfection efficiency was examined in non-transfected (MCF-7 and MDA-MB-231) and cells overexpressing SSTR3 (R3-MCF-7 and R3-MB-231) using immunocytochemistry as described in sub-section 4.2.4. As illustrated in Figure 4.1A and C, SSTR3-like immunoreactivity was observed in MCF-7 and MDA-MB-231 cells. Furthermore, R3-MCF-7 and R3-MB-231 cells exhibited increased SSTR3-like immunoreactivity when compared to non-transfected cells (Figure 4.1B and D).

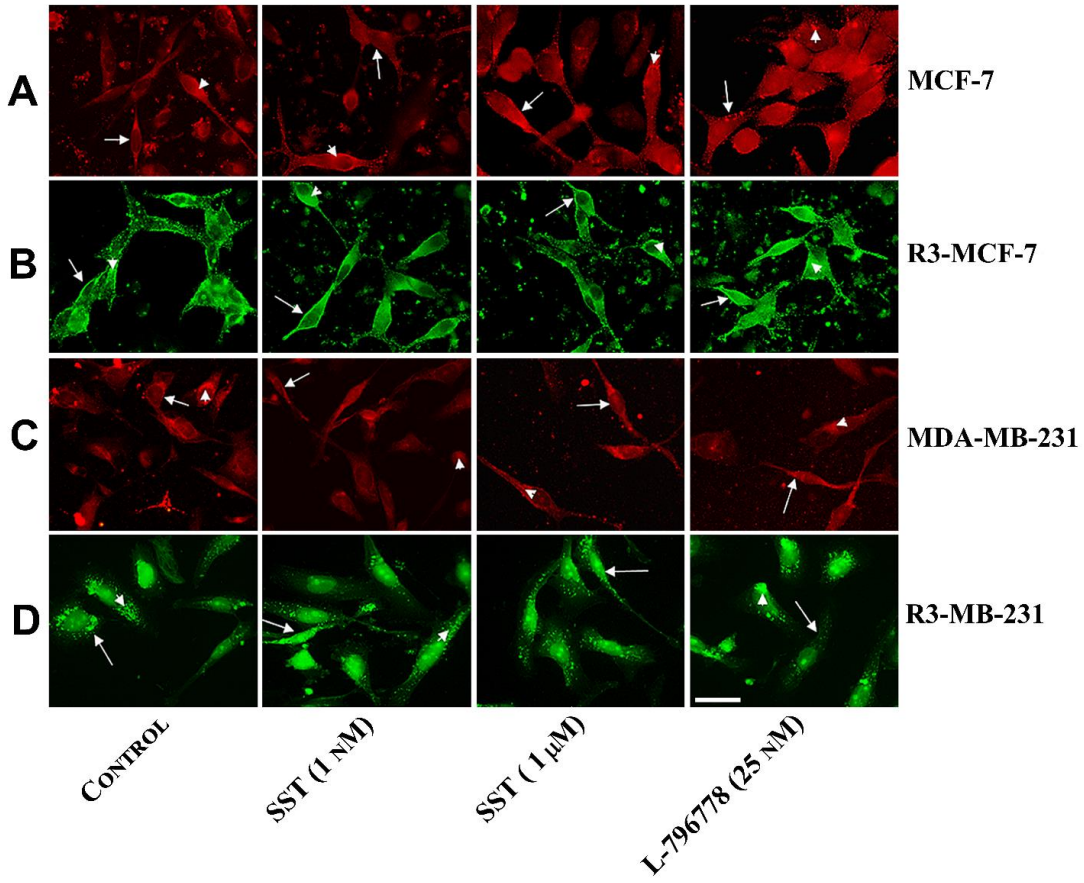

Figure 4.1 Representative photomicrographs illustrating SSTR3-like immunoreactivity in breast cancer cells. Non-transfected MCF-7 and MDA-MB-231, and cells overexpressing SSTR3 (R3-MCF-7 and R3-MB-231) were treated as indicated for 15 min at 37°C and processed for immunocytochemistry. SSTR3-like immunoreactivity was observed in MCF-7 and MDA-MB-231 cells in basal conditions (A and C). Note SSTR3-like immunoreactivity largely confined to the cell surface in R3-MCF-7 cells, whereas it appears predominantly cytoplasmic in R3-MB-231 cells (B and D). Results are representative of three independent experiments. Arrows and arrowheads indicate cell surface and intracellular receptor localization, respectively. Scale bar = 10 μm.

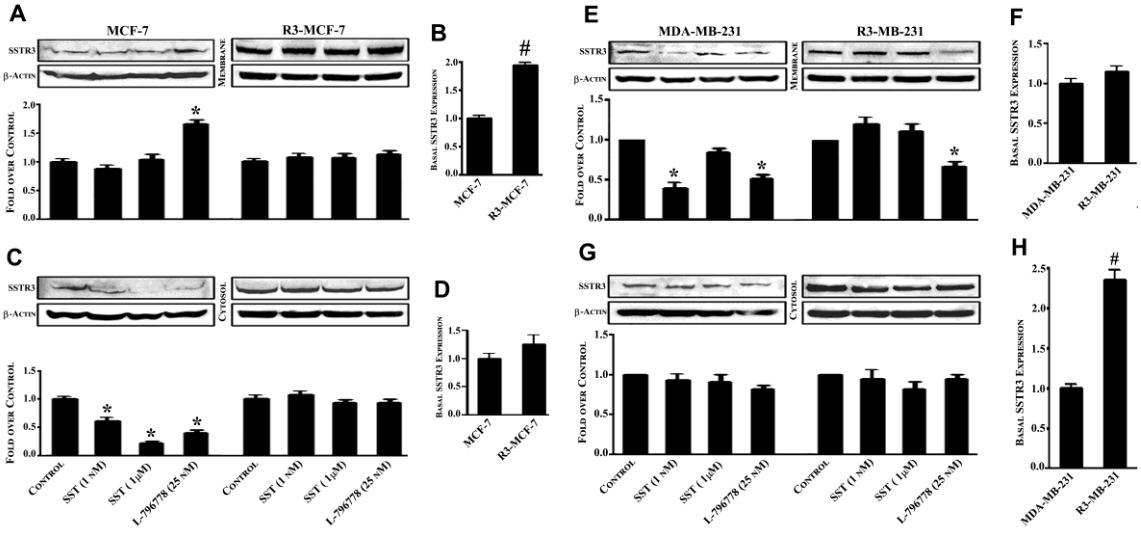

Figure 4.2 Western blot analysis illustrating SSTR3 expression in breast cancer cells. Non-transfected MCF-7 and MDA-MB-231, and cells overexpressing SSTR3 (R3-MCF-7 and R3-MB-231) were treated as indicated for 15 min at 37°C. Membrane and cytosolic fractions were then processed for Western blot analysis. SSTR3 expression significantly increased in the membrane fraction of MCF-7 cells upon treatment with L-796778 when compared to control (A). Note a significant (~ 2-fold) increase in basal SSTR3 expression in the membrane fraction of R3-MCF-7 when compared to MCF-7 cells (B). In MDA-MB-231 cells, the cell surface expression significantly decreased upon treatment with SST (1 nM) and L-796778 (E). SSTR3 expression in R3-MB-231 cells was predominantly intracellular (F and H). In R3-MB-231 cells, the basal SSTR3 expression in the cytosolic fraction was significantly increased (>2-fold) when compared to MDA-MB-231 cells (H). L-796778 significantly

decreased receptor membrane expression in comparison to control (E). Results are representative of three independent experiments. The status of SSTR3 expression was determined by densitometric analysis of SSTR3 vs. β-Actin. Results represent Mean ± S.D. of three independent experiments. Data analysis was done by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$). The basal SSTR3 expression between non-transfected and cells overexpressing SSTR3 was compared using Student's *t*-test ($^{\#}$, $p < 0.05$).

Next, Western blot analysis was performed to further support immunocytochemistry findings. As depicted in Figure 4.2A and B, SSTR3 expression significantly increased in the membrane fraction of R3-MCF-7 in comparison to non-transfected MCF-7 cells. The membrane fraction prepared from MCF-7 cells exhibited significantly increased receptor expression upon treatment with SSTR3-specific agonist L-796778, whereas no discernable changes were observed in R3-MCF-7 cells. The receptor expression was significantly higher in cytosolic fraction prepared from R3-MB-231 when compared to MDA-MB-231 cells (Figure 4.2G and H). In comparison to control, the membrane extract from MDA-MB-231 cells displayed significantly decreased SSTR3 expression following treatment with SST and L-796778 (Figure 4.2E). In R3-MB-231 cells the receptor membrane expression was significantly decreased only upon treatment with L-796778 when compared to control. These results suggest that SSTR3 expression and trafficking in these different breast cancer cells is cell-line specific.

4.3.2 SSTR3 overexpression inhibits cell proliferation in MCF-7 and MDA-MB-231 breast cancer cells

To delineate the physiological significance of SSTR3 overexpression, cell proliferation was analyzed using MTT assay. EGF was used as positive inducer of cell proliferation. As depicted in Figure 4.3, SST exerted significant antiproliferation in MCF-7 cells. In addition, SSTR3-specific agonist L-796778 decreased the proliferative activity of EGF and EST comparable to basal levels. In R3-MCF-7 cells, EGF mediated proliferation was significantly blunted; whereas EST induced proliferation was unaffected when compared to MCF-7 cells. In addition, the combined treatment of L-796778 with EGF or EST significantly inhibited the cell proliferation in comparison to control. Furthermore, MDA-MB-231 cells displayed inhibition of cell proliferation in response to SST but not L-796778 under similar experimental conditions. EGF mediated proliferation was significantly attenuated by L-796778, without any discernable effect of EST on cell proliferation. Since these cells are devoid of endogenous $ER\alpha$ expression, the negative effect of EST effect is not surprising. In R3-MB-231 cells, concomitant with L-796778 mediated antiproliferative effect, EGF stimulated proliferation was completely abolished. These data indicate that SSTR3 overexpression in breast cancer cells negatively regulate the EGF mediated proliferation, and provide evidence for the tumor suppressive function for SSTR3. In addition, SSTR3 overexpression significantly decreased the basal proliferation in R3-MCF-7 and R3-MB-231 cells when compared to MCF-7 and MDA-MB-231 cells, respectively. These data indicate that SSTR3 overexpression constitutively regulates cell proliferation of breast cancer cells.

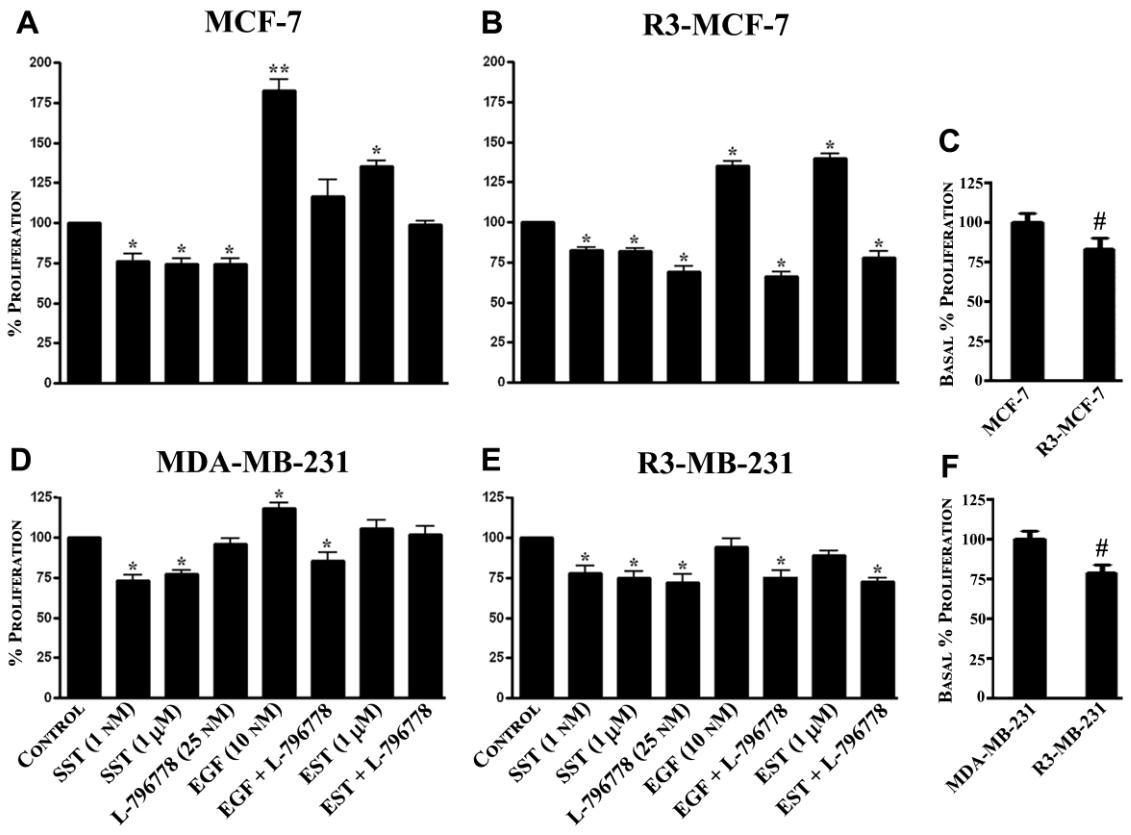

Figure 4.3 Regulation of cell proliferation upon SSTR3 overexpression in breast cancer cells. Non-transfected MCF-7 and MDA-MB-231, and cells overexpressing SSTR3 (R3-MCF-7 and R3-MB-231) were first serum-deprived for 24 h, and then treated as indicated for 24 h before processing for MTT assay. MCF-7 cells display inhibition of cell proliferation in response to SST and L-796778 (A). EGF and EST increased cell proliferation which was abolished upon co-treatment with L-796778 (A). In R3-MCF-7 cells, EGF but not EST induced proliferation was less pronounced in comparison to non-transfected cells (A and B). In MDA-MB-231 cells, SST but not L-796778 inhibited proliferation; whereas EGF effect was significantly attenuated by L-796778 (D). Note the blunted proliferation in R3-MB-231 cells with EGF (E). Of note, SSTR3 overexpression significantly decreased the basal proliferation in R3-MCF-7 and R3-MB-231 cells when compared to MCF-7 and MDA-MB-231 cells, respectively (C and F). Results represent mean ± SD of three independent experiments. Data analysis was done by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$). The basal cell proliferation between non-transfected and cells overexpressing SSTR3 was compared using Student's *t*-test ($^{\#}$, $p < 0.05$).

4.3.3 SSTR3 overexpression promotes apoptosis in breast cancer cells

The activation of SSTR3 induces cytotoxic effects in a cell-specific manner (Sharma et al., 1996; War and Kumar, 2012; War et al., 2011). Whether increased inhibition of cell proliferation in cells overexpressing SSTR3 was due to apoptosis, two different approaches were used. First, the expression of PARP-1 was determined as a marker for apoptosis; secondly, TUNEL assay was performed. In MCF-7 cells, SST and L-796778 significantly increased PARP-1 expression when compared to control (Figure 4.4). In contrast, EST with or without L-796778 significantly decreased PARP-1 expression in comparison to control. Notably, R3-MCF-7 cells exhibited significantly higher (> 2-fold) basal PARP-1 expression when compared to MCF-7 cells, indicating the induction of apoptosis upon SSTR3 overexpression in ER_{α}-positive breast cancer cells. Conversely, no significant difference in basal PARP-1 expression between MDA-MB-231 and R3-MB-231 cells suggests no cytotoxic role for SSTR3 in ER_{α}-negative breast cancer cells. Next, TUNEL assay was performed to further support the above results. As depicted in Figure 4.5, the number of TUNEL-positive MCF-7 cells significantly increased upon treatment with SST (1 μM), L-796778 and EGF + L-796778 when compared to control. R3-MCF-7 cells displayed significantly increased (>3-fold) basal apoptotic index in comparison to MCF-7 cells. In response to treatment, no significant change in number of TUNEL-positive R3-MCF-7 cells was observed when compared to control. Consistent with PARP-1 data, MDA-MB-231 and R3-MB-231 cells exhibited low apoptotic index and no significant changes were observed upon treatment with SST or L-796778 (Figure 4.5). These experiments confirm that SSTR3 induced

apoptosis is cell-specific; whether there is any involvement of ER_{α} remains to be established.

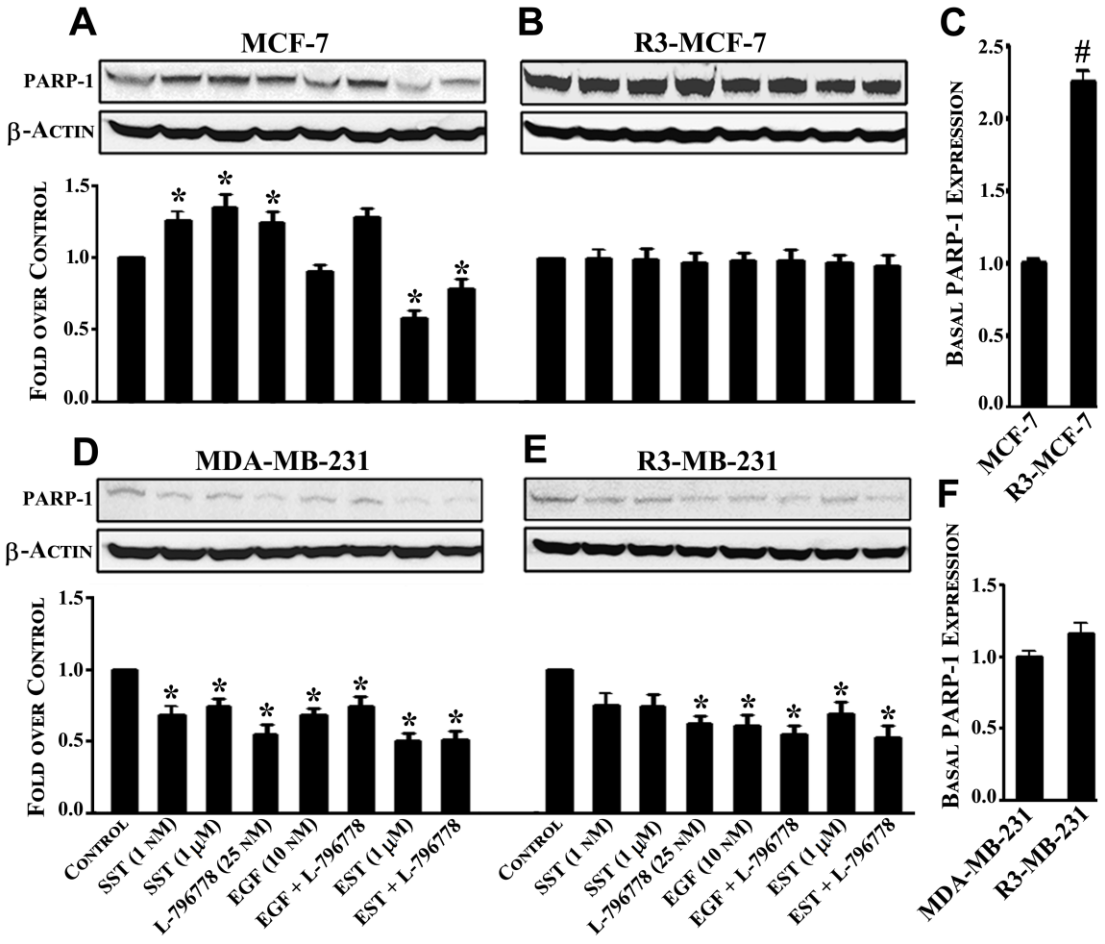

Figure 4.4 The cytotoxic effects upon SSTR3 overexpression in breast cancer cells. Non-transfected MCF-7 and MDA-MB-231, and cells overexpressing SSTR3 (R3-MCF-7 and R3-MB-231) were treated as indicated for 24 h at 37°C. Cell lysate was processed for Western blot analysis using monoclonal anti-PARP-1 antibody. In MCF-7 cells, SST and L-796778 significantly increased PARP-1 expression as compared to control (A). Conversely, EST and EST + L-796778 significantly decreased PARP-1 expression. Note significantly increased basal PARP-1 levels, characteristic of apoptosis in R3-MCF-7 when compared to MCF-7 cells (C). In contrast, no significant difference in PARP-1 expression was observed between MDA-MB-231 and R3-MB-231 cells (D, E and F). In MDA-MB-231

cells, PARP-1 expression was significantly decreased upon treatment with SST, L-796778, EGF, and EST or in combination when compared to control (D). R3-MB-231 cells exhibited significantly lower PARP-1 expression in response to L-796778, EGF and EST or in combination in comparison to control (E). Results are representative of three independent experiments. The status of PARP-1 expression was determined by densitometric analysis of PARP-1 vs. β-Actin. Data analysis was performed by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$). Student's *t*-test was used to compare the basal PARP-1 expression between non-transfected and cells overexpressing SSTR3 ($^{\#}$, $p < 0.05$).

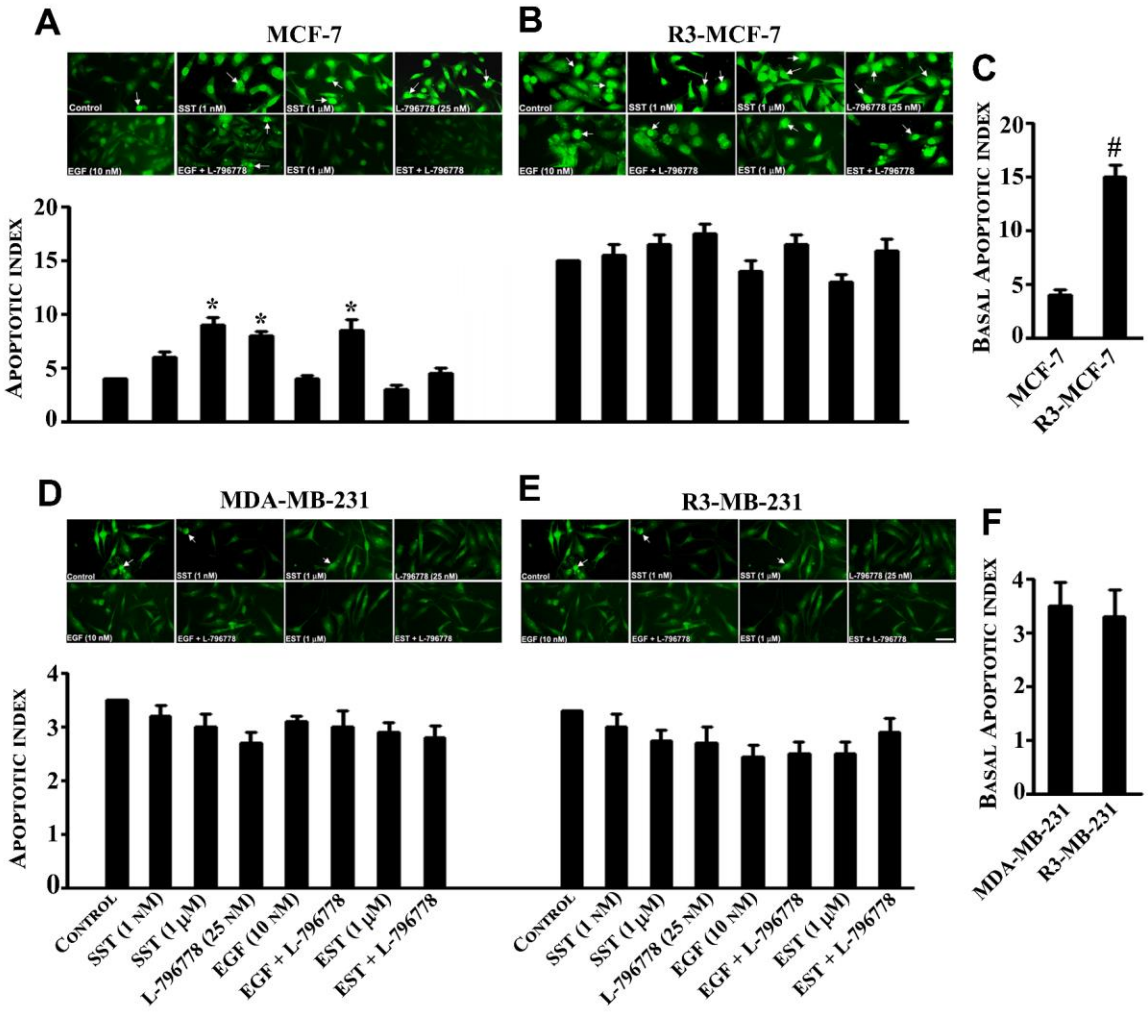

Figure 4.5 Representative photomicrographs showing TUNEL staining. Non-transfected MCF-7 and MDA-MB-231, and cells overexpressing SSTR3 (R3-MCF-7 and R3-MB-231) were treated as

indicated for 24 h at 37°C. Following treatment, cells were processed for TUNEL staining to examine the extent of DNA fragmentation. MCF-7 cells show an increased TUNEL reaction with SST (1 μM) and L-796778 (A). It is noteworthy that R3-MCF-7 cells displayed increased apoptosis even in the absence of receptor activation (B). Conversely, MDA-MB-231 and R3-MB-231 cells did not reveal any pro-apoptotic activity (D and E). Histograms represent apoptotic index (total number of TUNEL-positive cells/total number of cells analyzed × 100) for untreated and treated cells as indicated. Results are representative of three independent experiments. Data analysis was performed by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$). Student's *t*-test was used to compare the basal apoptotic index between non-transfected and cells overexpressing SSTR3 ($^{\#}$, $p < 0.05$). Arrows represent apoptotic cells. Scale bar = 10 μm.

4.3.4 SSTR3 overexpression in breast cancer cells is associated with $p27^{Kip1}$ mediated cell-cycle arrest

Previous studies have attributed the cytostatic functions of SSTRs to $p27^{Kip1}$, a CDKI (Grant et al., 2008; Hubina et al., 2006). The expression of $p27^{Kip1}$ was determined using Western blot analysis to further clarify the putative mechanisms for SSTR3 antiproliferation in breast cancer cells. As illustrated in Figure 4.6, SST, L-796778, EGF and EST or in combination significantly increased $p27^{Kip1}$ expression in MCF-7 cells when compared to control. SSTR3 overexpression significantly increased (> 4-fold) the basal $p27^{Kip1}$ expression in R3-MCF-7 when compared to MCF-7 cells. MDA-MB-231 cells revealed increased $p27^{Kip1}$ expression upon treatment with SST (1 nM) and L-796778. The combined treatment with EGF and L-796778 but not EGF alone significantly increased $p27^{Kip1}$ expression in comparison to control. Furthermore, $p27^{Kip1}$ expression was significantly decreased upon treatment with EST + L-796778 in comparison to control. Notably, R3-MB-231 cells

demonstrated significantly higher (>3-fold) $p27^{Kip1}$ expression when compared to MDA-MB-231 cells, suggesting that SSTR3 overexpression is able to switch antiproliferative machinery and trigger cell cycle arrest in $ER\alpha$-negative breast cancer cells.

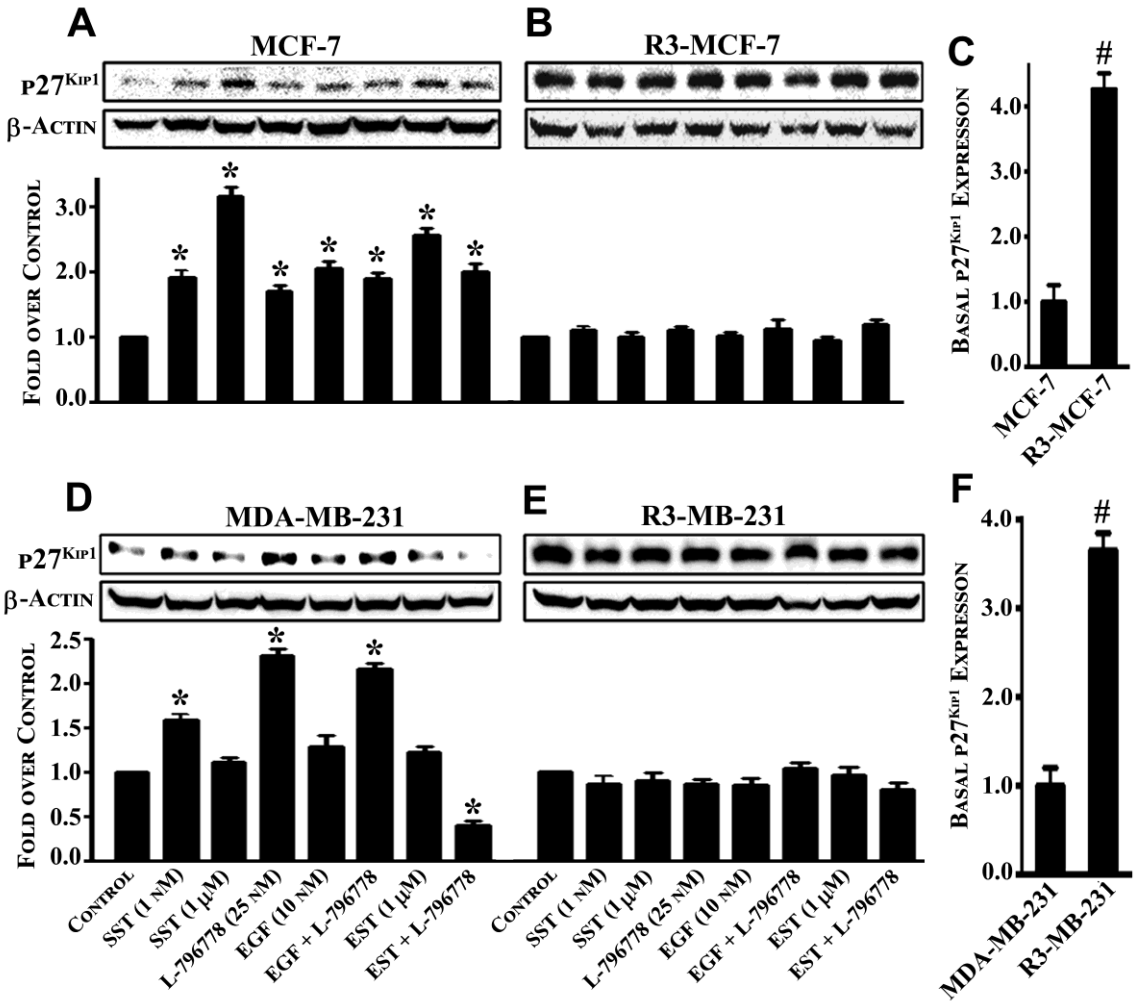

Figure 4.6 The cytostatic role for SSTR3 in breast cancer cells is mediated by $p27^{Kip1}$. Non-transfected MCF-7 and MDA-MB-231, and cells overexpressing SSTR3 (R3-MCF-7 and R3-MB-231) were treated as indicated for 24 h at 37°C. Cell lysate was processed for $p27^{Kip1}$ expression using Western blot analysis. MCF-7 cells treated with SST, L-796778, EGF and EST or in combination exhibited significantly increased $p27^{Kip1}$expression in comparison to control (A). Note the significant

upregulation of basal p27^{Kip1} in R3-MCF-7 when compared to MCF-7 cells (C). MDA-MB-231 cells display significantly increased p27^{Kip1} levels with SST (1 nM), L-796778 and EGF + L-796778 in comparison to control (D). Conversely, EST + L-796778 resulted in significant decrease in p27^{Kip1} expression when compared to control (E). R3-MB-231 cells exhibit significantly high basal p27^{Kip1} expression when compared to MDA-MB-231 cells (F). Results are representative of three independent experiments. The status of p27^{Kip1} expression was determined by densitometric analysis of p27^{Kip1} vs. β-Actin. Data analysis was done by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$). Student's *t*-test was used to compare the basal p27^{Kip1} expression between non-transfected and cells overexpressing SSTR3 ($^{\#}$, $p < 0.05$).

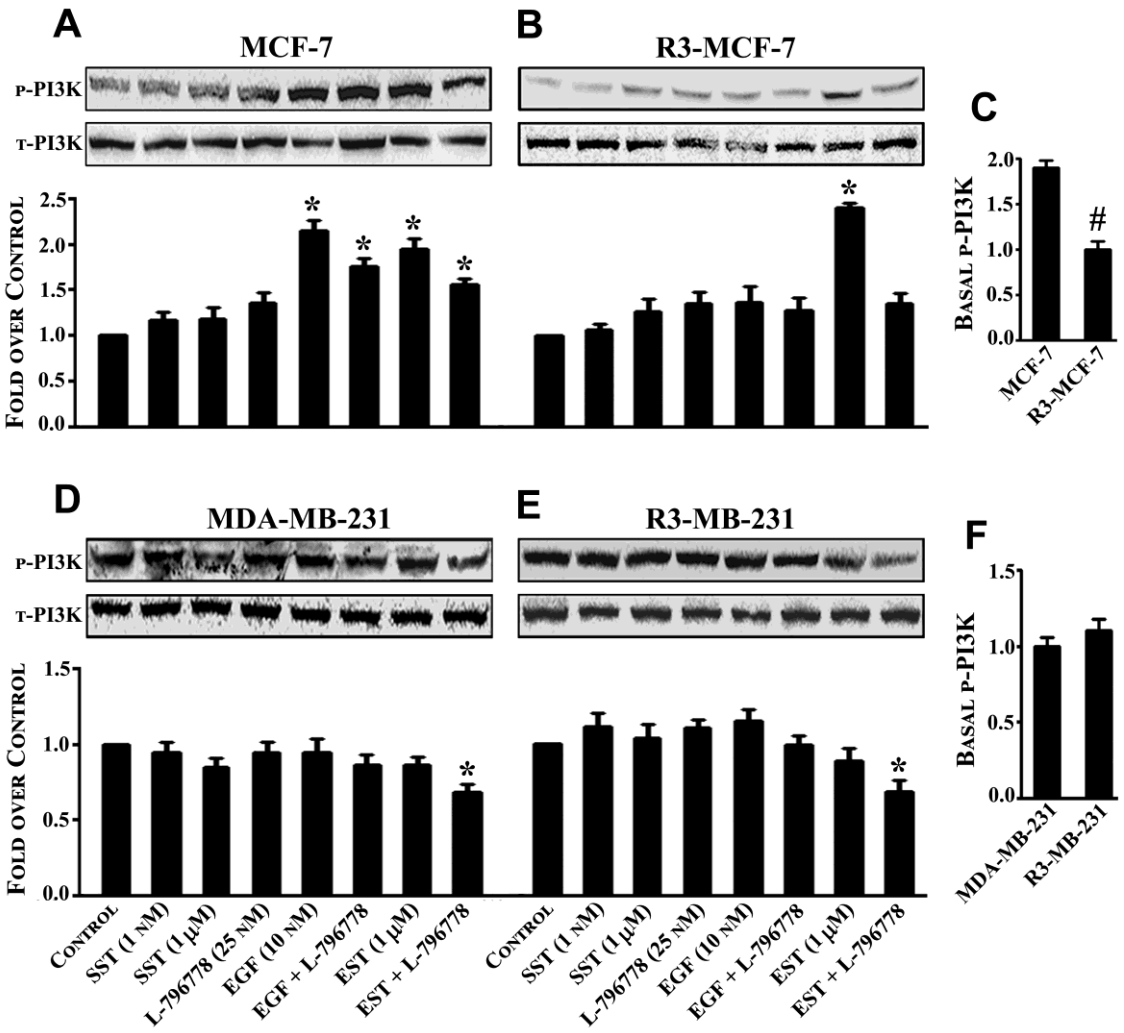

Figure 4.7 Role of elevated SSTR3 expression on PI3K phosphorylation in MCF-7 and MDA-MB-231 cells. Non-transfected MCF-7 and MDA-MB-231, and cells overexpressing SSTR3 (R3-MCF-7 and R3-MB-231) were treated as indicated for 30 min at 37°C. Cell lysate was processed for total and phospho-PI3K using Western blot analysis. MCF-7 cells show significant PI3K phosphorylation in response to EGF and EST with or without L-796778 (A). SSTR3 overexpression in R3-MCF-7 abolished PI3K phosphorylation in response to EGF but not EST treatment (B). Note a significant decrease in basal PI3K phosphorylation in R3-MCF-7 when compared to MCF-7 cells (C). In MDA-MB-231 and R3-MB-231 cells, EST + L-796778 significantly decreased phosphorylated PI3K levels in comparison to control (D and E). MDA-MB-231 and R3-MB-231 cells show comparable basal PI3K phosphorylation status (F). Results represent three experiments performed independently. The phosphorylation status was determined by densitometric analysis of phosphorylated vs. total PI3K. Data analysis was done by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$). The basal PI3K phosphorylation between non-transfected and cells overexpressing SSTR3 was compared by using Student's *t*-test ($^{\#}$, $p < 0.05$).

4.3.5 Negative regulation of PI3K cell survival pathway upon SSTR3 overexpression in breast cancer cells

The significant inhibition of cell proliferation in cells overexpressing SSTR3 led to query whether this effect is due to the inhibition of PI3K signaling. To address this question, breast cancer cells with normal- and overexpression of SSTR3 were treated with SST as well as with EGF and EST either alone or in combination with L-796778 and processed for PI3K phosphorylation. As shown in Figure 4.7, phosphorylated PI3K was significantly increased in MCF-7 cells upon treatment with EGF and EST, both independently and in combination with L-796778. In comparison to non-transfected cells, the status of basal PI3K phosphorylation was abolished in R3-MCF-7 cells. Conversely, in R3-MB-231 cells, PI3K phosphorylation was

comparable to MDA-MB-231 cells. Treatment with EST + L-796778 significantly decreased phosphorylated PI3K when compared to control. These results suggest that SSTR3 mediated regulation of PI3K pathway is dependent on cell type and intensity of receptor expression at the cell surface.

4.3.6 Cell-specific regulation of PTP-1C upon SSTR3 overexpression in breast cancer cells

OCT induced antiproliferative effects in MCF-7 cells are associated with increased cell surface expression of PTP-1C (Srikant and Shen, 1996). The next step in this study was to determine whether PTP-1C plays a part in SSTR3 antiproliferation in MCF-7, MDA-MB-231, and cells overexpressing SSTR3. As shown in Figure 4.8, the membrane expression of PTP-1C was significantly increased in MCF-7 cells upon treatment with SST (1 μM), L-796778 and EST + L-796778 when compared to control. In R3-MCF-7 cells, PTP-1C expression was significantly increased in response to EGF and EST + L-796778. MDA-MB-231 cells displayed significantly lower PTP-1C expression upon treatment with EGF + L-796778, EST and EST + L-796778 in comparison to control. In R3-MB-231 cells, the expression of PTP-1C was significantly decreased in response to L-796778 when compared to control. SSTR3 overexpression in R3-MCF-7 demonstrated significantly higher (>3-fold) basal PTP-1C levels when compared to MCF-7 cells. Conversely, PTP-1C levels were comparable between MDA-MB-231 and R3-MB-231 cells despite SSTR3 overexpression. These data are consistent with PARP-1

and TUNEL experiments and suggest the cell-specific role of PTP-1C in SSTR3-induced apoptosis in breast cancer cells.

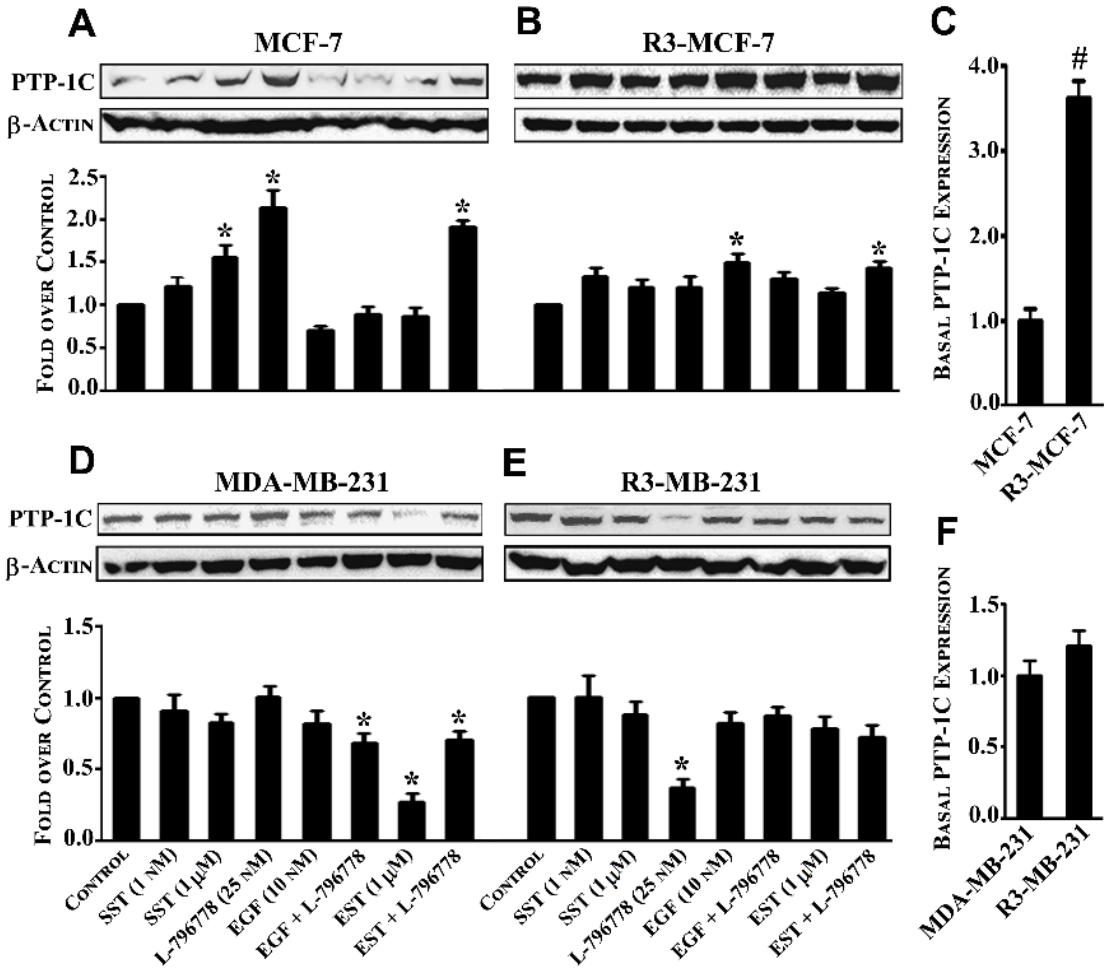

Figure 4.8 Role of PTP-1C in SSTR3 induced apoptosis in breast cancer cells. Non-transfected MCF-7 and MDA-MB-231, and cells overexpressing SSTR3 were treated as indicated for 30 min at 37°C. Membrane extracts were processed for PTP-1C expression using Western blot analysis. MCF-7 cells show significantly increased PTP-1C membrane expression with SST (1 μM), L-796778 and EST + L-796778 (A). R3-MCF-7 cells exhibit significantly increased PTP-1C expression with EGF and EST + L-796778 (B). Note a significant (>3-fold) upregulation of PTP-1C in R3-MCF-7 when compared to MCF-7 cells (C). MDA-MB-231 cells show significantly lower PTP-1C expression with EGF + L-796778, EST and EST + L-796778 when compared to control (D). In R3-MB-231 cells, PTP-

1C expression was significantly decreased in response to L-796778 when compared to control (E). The basal expression level of PTP-1C was comparable between MDA-MB-231 and R3-MB-231 cells (F). Results represent three independent experiments. The status of PTP-1C expression was determined by densitometric analysis of PTP-1C vs. β-Actin. Data analysis was done by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$). Student's *t*-test was used to compare the basal PTP-1C expression between non-transfected and cells overexpressing SSTR3 ($^{\#}$, $p < 0.05$).

4.3.7 SSTR3 overexpression modulates ERK1/2 phosphorylation in MCF-7 and MDA-MB-231 cells

The status of ERK1/2 phosphorylation was determined in MCF-7 and MDA-MB-231, with or without SSTR3 overexpression. As shown in Figure 4.9, MCF-7 cells displayed significant increase in phosphorylated ERK2 (44 kDa) isoform upon treatment with SST (1 μM), L-796778, EGF and EST or in combination when compared to basal levels. Importantly, EGF and EST induced ERK2 phosphorylation was approximately 4-fold over the basal levels. Conversely, no significant changes in ERK1 (44 kDa) phosphorylation were determined between control and treated groups. In R3-MCF-7 cells, the status of ERK1/2 was unchanged between control and treatment. The basal levels of phosphorylated ERK2 was significantly higher (>3-fold) in R3-MCF-7 when compared to MCF-7 cells. In contrast, the status of basal ERK1 phosphorylation was significantly higher in MCF-7 cells when compared to R3-MCF-7 cells. In MDA-MB-231 cells, ERK2 phosphorylation was significantly increased upon treatment with SST (1 nM), EGF, EGF + L-796778 and EST. The levels of phosphorylated ERK1 were significantly increased upon treatment with SST, EGF and EST or in combination. In R3-MB-231, the status of ERK2 was

significantly increased upon treatment with SST, L-796778, EGF, EST and EST + L-796778 when compared to control. ERK1 phosphorylation was significantly higher with EST and EST + L-796778 in comparison to control. Of note, the basal ERK1/2 phosphorylation levels were significantly higher in MCF-7 than R3-MCF-7 cells. These results indicate cell and isoform-specific modulation of ERK1/2 phosphorylation. Whether the distinct regulation of ERK1/2 phosphorylation in these different breast cancer cell lines is associated with the diverse antiproliferative role of SSTR3 remains to be investigated in future studies.

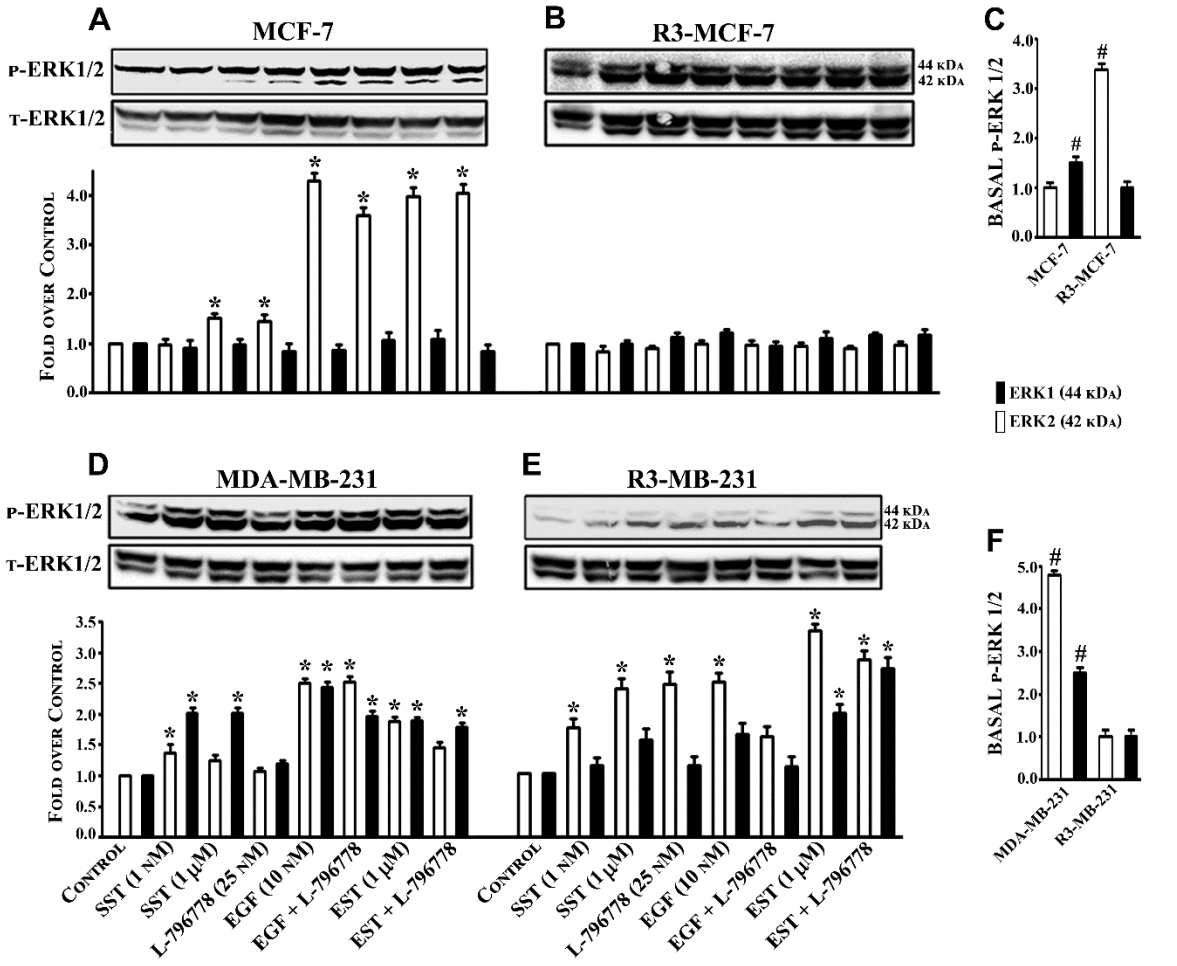

Figure 4.9 Effect of SSTR3 overexpression on ERK1/2 cascade in MCF-7 and MDA-MB-231 breast cancer cells. Non-transfected MCF-7 and MDA-MB-231, and cells overexpressing SSTR3 were treated as indicated for 30 min at 37°C. Cell lysate was processed for total and phosphorylated ERK1/2 using Western blot analysis. It is noteworthy that the basal P-ERK1 was significantly increased whereas basal p-ERK2 was significantly decreased in MCF-7 when compared to R3-MCF-7 cells (C). On the other hand, the status of basal pERK1/2 phosphorylation was significantly increased in MDA-MB-231 when compared to R3-MB-231 cells (F). Results are representative of three independent experiments. The phosphorylation status was determined by densitometric analysis of phosphorylated vs. total ERK1/2. Data analysis was done by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$). The basal ERK1/2 phosphorylation status between non-transfected and cells overexpressing SSTR3 was compared by using Student's *t*-test ($^{\#}$, $p < 0.05$).

4.3.8 MCF-7, but not MDA-MB-231 cells display p38 phosphorylation upon SSTR3 overexpression

As illustrated in Figure 4.10, MCF-7 cells exhibited significantly increased phosphorylated p38 levels upon treatment with SST when compared to basal levels. No discernable changes in p38 phosphorylation were observed in response to L-796778 or EGF alone. Cotreatment with EGF + L-796778 significantly increased phospho-p38 in comparison to control. In contrast, the status of p38 phosphorylation was completely abolished with EST with or without L-796778 in comparison to control. Importantly, R3-MCF-7 cells exhibited significantly high levels (approx. 2-fold) of basal p38 phosphorylation when compared to MCF-7 cells. Nonetheless, no significant changes were observed between control and treated groups. Remarkably, p38 phosphorylation was not detected in MDA-MB-231 and R3-MB-231

cells. These results suggest the regulation of p38 MAPK in a cell-specific manner. Whether the loss of p38 phosphorylation in MDA-MB-231 and R3-MB-231 cells might be associated with the lack of SSTR3 mediated cytotoxic effects in these cell lines warrants further insight.

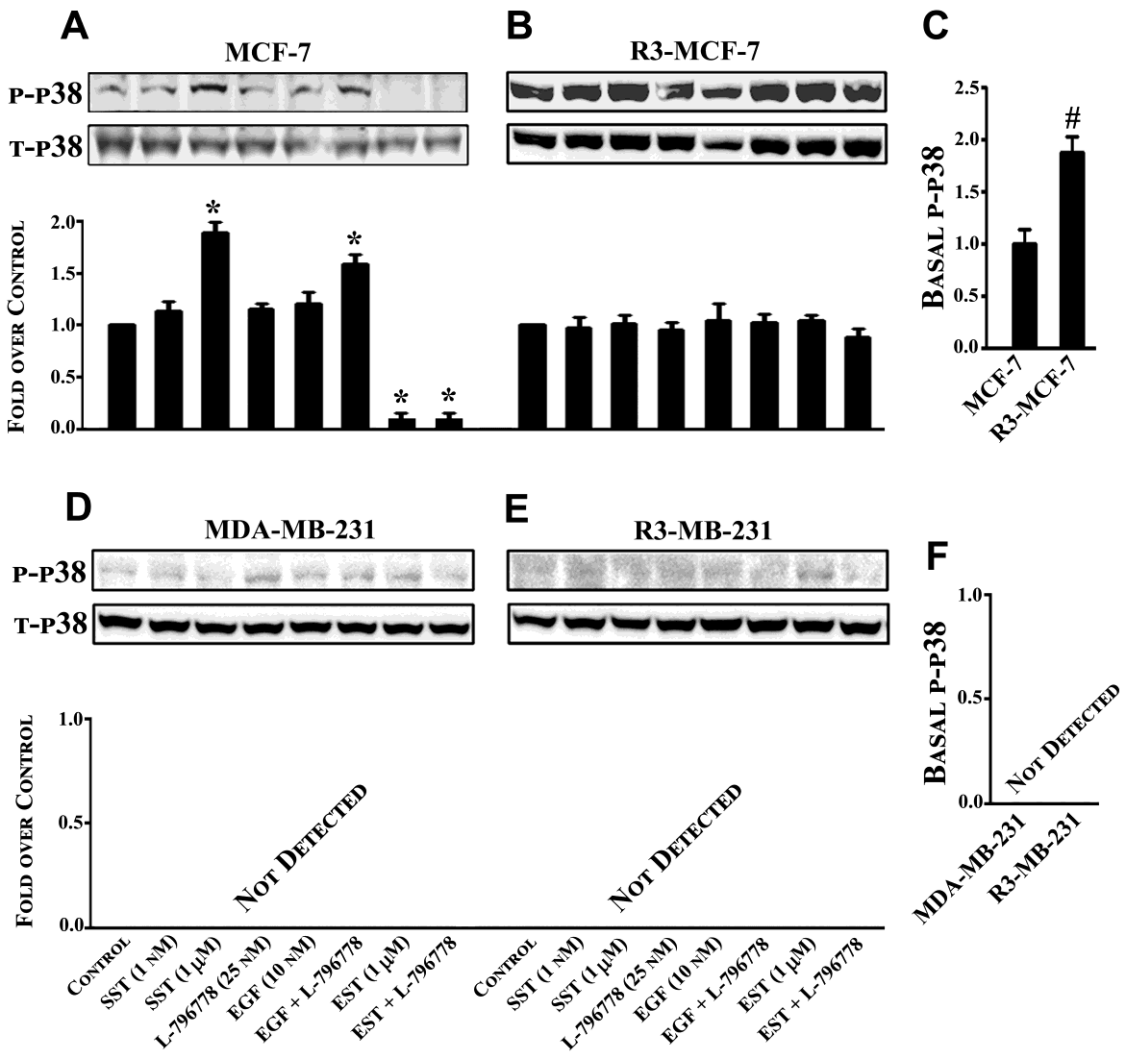

Figure 4.10 Cell-specific modulation of p38 MAPK by SSTR3. Non-transfected MCF-7 and MDA-MB-231 and cells overexpressing SSTR3 were treated as indicated for 30 min at 37°C. Cell lysate was processed for total and phospho-p38 using Western blot analysis. MCF-7 cells show significantly

enhanced p38 phosphorylation upon treatment with SST (1 μM) and EGF + L-796778 when compared to control (A). The status of p38 phosphorylation was completely abolished by EST and EST + L-796778 in comparison to control. Note the significant increase in status of basal p38 phosphorylation in R3-MCF-7 when compared to MCF-7 cells (C). Strikingly, the status of p38 phosphorylation was not detected in MDA-MB-231 and R3-MB-231 cells (D, E and F). Results represent three experiments performed independently. The phosphorylation status was determined by densitometric analysis of phosphorylated vs. total p38. Data analysis was done by using one-way ANOVA and *post hoc* Dunnett's test to compare against control (*, $p < 0.05$). The basal ERK1/2 phosphorylation status between non-transfected and cells overexpressing SSTR3 was compared by using Student's *t*-test ($^{\#}$, $p < 0.05$).

4.4 Discussion

The aggressive and poorly differentiated tumors such as breast cancer pose a huge therapeutic challenge. These malignancies show unrestrained growth characteristics and evade the tumor inhibitory barrier by downregulating SSTRs, which are known to inhibit cell proliferation (Berns et al., 2007; Kumar, 2011). Lack of membrane expression of SSTRs indicates the possible failure of SST effect in tumors. Therefore, delineating the role of SSTR overexpression to overcome low endogenous receptor levels in breast cancer cells is important for the development of novel therapeutic strategies. The present study demonstrates the role for SSTR3 overexpression in exerting pronounced inhibition of cell proliferation in MCF-7 and MDA-MB-231 breast cancer cells by distinctly activating cytotoxic and cytostatic pathways. These results underscore the failure of SST analogs in cancer therapeutics.

The cell surface expression and trafficking of SSTRs are highly dynamic processes, and the cellular responses are intimately associated with the relative surface abundance of the receptor subtype (Ben-Shlomo et al., 2007; Thodou et al., 2006). The physiological relevance of SSTR3 expression in breast cancer has remained elusive so far. R3-MCF-7 cells show significantly increased expression levels of SSTR3 at the cell surface than MCF-7 cells. Conversely, the intracellular SSTR3 expression in R3-MB-231 cells was significantly higher than MDA-MB-231 cells. No discernible changes in the cell surface expression of SSTR3 were observed between MDA-MB-231 and R3-MB-231 cells. The agonist-mediated upregulation of SSTR3 in MCF-7 cells, in part, contradicts a previous report demonstrating SSTR3 internalization and its failure to recycle back to the cell surface in heterologous system (Hukovic et al., 1996). These observations led us to propose that SSTR3 trafficking in cells expressing this receptor endogenously is different than transfected cells. Whether the presence or absence of ER_{α} in MCF-7 and MDA-MB-231 breast cancer cells plays a determinant role in the cell-specific expression pattern of SSTR3 in these cell lines is not well understood. The possibility that transient expression of ER_{α} in MDA-MB-231 cells would retain SSTR3 at the cell surface remains open and warrants additional studies.

Over secretion of growth hormones is often associated with uncontrolled tumor proliferation and treatment failure. There is an inverse relationship between growth factor receptors and SSTR subtypes, which is characterized by gradual loss of SSTRs upon tumor progression and consequent failure of SST analogs mediated antiproliferation (Kumar, 2011). This study describe that SSTR3 overexpression in

breast cancer cells leads to significant inhibition of EGF induced cell proliferation in the presence of SSTR3-specific agonist. The next step was to dissect the mechanisms by which SSTR3 inhibits cell proliferation. The activation of PARP-1 and increased TUNEL staining in a cell-specific manner strengthens the concept that SSTR3 overexpression in breast cancer might serve as a potential therapeutic approach. These observations are in agreement with previous study reporting apoptosis upon SSTR2 overexpression in MCF-7 breast cancer cells (He et al., 2009). SST is reported to display cytostatic functions via multiple mechanisms including $p27^{Kip1}$ induction (Grant et al., 2008; Hubina et al., 2006). Consistent with these studies, tumor cells with overexpression of SSTR3 exhibit constitutive upregulation of $p27^{Kip1}$ in comparison to non-transfected cells. Importantly, the possibility that SST induced significant upregulation of $p27^{Kip1}$ in MCF-7 cells might be attributed to multiple SSTR subtypes cannot be ruled out from the discussion. Taken in consideration, these results suggest that SSTR3 overexpression in R3-MCF-7 is associated with apoptosis and cell-cycle inhibition, whereas display predominantly cytostatic signaling in R3-MB-231 cells.

PI3K signaling cascade promotes cell survival signals and plays an anti-apoptotic role. The inhibition of this pathway triggers cell death and has emerged as potential therapeutic target in breast cancer treatment (Hubina et al., 2006; Kauffmann-Zeh et al., 1997; Kulik et al., 1997). SST analog OCT has been previously shown to mediate antiproliferation via inhibition of PI3K cascade in pituitary tumor cells (Theodoropoulou et al., 2006). In agreement with these observations, SSTR3 overexpression in R3-MCF-7 cells is associated with PI3K

inhibition. Interestingly, the data from the present study show correlation between PI3K inhibition and SSTR3 induced apoptosis. Although, the rapid modulation of PI3K phosphorylation upon EST treatment in breast cancer cells supports the emerging notion for the non-genomic actions of EST, very little is known about the underlying mechanisms. Remarkably, PI3K was constitutively phosphorylated in MDA-MB-231 as well as in R3-MB-231 cells. The lack of SSTR3 cytotoxic effect in these ER_{α}-negative cells emphasizes the importance of PI3K inhibition in apoptosis. The tyrosine phosphatase PTP-1C is highly expressed in breast tumors (Amin et al., 2011). In MCF-7 and HEK-293 cells, PTP-1C translocation from cytosol to the cell surface is an essential requirement for SSTR mediated apoptosis (Srikant and Shen, 1996; War et al., 2011). Moreover, OCT inhibits cell proliferation via PTP-1C mediated dephosphorylation and subsequent inactivation of PI3K (Cuevas et al., 2001; Theodoropoulou et al., 2006). In the present study, significantly high expression of membrane-bound PTP-1C was identified in R3-MCF-7 when compared to MCF-7 cells. These data are in line with a growing body of evidence suggesting that increased PTP-1C levels lead to apoptosis via PI3K inhibition (Cuevas et al., 2001; Srikant and Shen, 1996; Theodoropoulou et al., 2006). On the other hand, no discernable changes in expression levels of PTP-1C were observed between MDA-MB-231 and R3-MB-231 cells. The presence or absence of ER_{α} in these cells might be one of the possible explanations for the contrasting observations from MCF-7 and R3-MCF-7 cells.

ERK cascade is implicated in growth factor-mediated tumor progression (McCubrey et al., 2007). Previous studies have identified an intimate association

between SSTR mediated antiproliferation and dual stimulatory/inhibitory effect on ERK1/2 phosphorylation (Grant et al., 2008; Somvanshi et al., 2009; War and Kumar, 2012; War et al., 2011; Watt et al., 2009). Many cytotoxic drugs also promote apoptosis via ERK activation (Chambard et al., 2007; Hsu et al., 2005). In the present study, ERK2 but not ERK1 was constitutively phosphorylated in R3-MCF-7 when compared to MCF-7 cells. It is thus conceivable that SSTR3 mediated pro-apoptotic effects in R3-MCF-7 cells require ERK1/2 activation. Conversely, the basal ERK1/2 phosphorylation was significantly downregulated in R3-MB-231 in comparison with non-transfected cells. Whether this confers a predominantly cytostatic role to these cells remains to be investigated. Altogether, these observations provide a dual but distinct role for SSTR3 overexpression on ERK1/2 signaling in breast cancer cells.

Cell proliferation and apoptosis are regulated by p38 MAPK in a cell and receptor-specific manner (Alderton et al., 2001; Chuang et al., 2000; Wagner and Nebreda, 2009). In MCF-7 breast cancer cells, the significant increase in p38 phosphorylation in response to SST but not L-796778 might be attributed to presence of other SSTRs such as SSTR2. EST has been previously described to mediate anti-apoptotic function in MCF-7 cells (Zheng et al., 2007). In this context, EST mediated downregulation of p38 in MCF-7 cells might suggest an anti-apoptotic role. This study also provides evidence that R3-MCF-7 cells exhibit significantly high basal phosphorylated p38 levels in comparison to MCF-7 cells. Conversely, both MDA-MB-231 and R3-MB-231 cells failed to exhibit p38 signaling. It seems plausible that p38 MAPK is associated with SSTR3 pro-apoptotic effect in R3-MCF-7 cells.

Given these diverse signaling outcomes, it is clear that SSTR3-mediated regulation of downstream signaling molecules is cell-specific.

There is emerging evidence attesting the concept of ligand-independent constitutive activity for many GPCRs (Bond and Ijzerman, 2006; Costa and Cotecchia, 2005; Smit et al., 2007). Recent studies using receptor specific knockdown have revealed constitutive SSTR signaling in the pituitary (Ben-Shlomo and Melmed, 2010; Ben-Shlomo et al., 2007; Ben-Shlomo et al., 2010; Ben-Shlomo et al., 2009). Consistent with these observations, the present study showed that SSTR3 overexpression in R3-MCF-7 and R3-MB-231 cells promotes significant basal cytotoxic and cytostatic effects, respectively. The functionality of GPCRs is generally attributed to the cell surface expression (Conn et al., 2006). In contrast, the ligand independent regulation of ACTH in mouse anterior pituitary-derived cell line AtT-20 by SSTR3 has been attributed to the cytoplasmic receptor pool (Ben-Shlomo et al., 2007; Cervia et al., 2003; Strowski et al., 2002). These lines of evidence support the antiproliferative role for the predominantly intracellular SSTR3 in R3-MB-231 cells. In conclusion, the present study highlights the importance of SSTR3 overexpression in breast cancer cells. Whether the constitutive antiproliferative effects of SSTR3 are mediated in association with any other interacting partners such as SSTR2 or ER_{α} is not known. Future studies in this direction will lead to a novel therapeutic approach to explore the role of SSTR subtypes in breast cancer.

Chapter 5: Overall discussion and conclusions

5.1 Overall discussion

GPCRs constitute the largest family of cell surface proteins and represent approximately 1% of the human genome (Flower, 1999; Fredriksson et al., 2003; Pierce et al., 2002). Most importantly, the fact that majority (>50%) of the current therapeutic drugs target GPCRs either directly or indirectly, further strengthen the clinical importance of GPCRs (Flower, 1999; Fredriksson et al., 2003; Gudermann et al., 1995). GPCRs mediated downstream signaling cascades are initiated from a monomeric entity or an oligomeric complex, depending upon the nature of receptor, and the type of cells and/or tissues expressing that particular receptor (Jones et al., 1998; Kuner et al., 1999; Patel et al., 2002a; Rocheville et al., 2000b). Many GPCR oligomers exhibit exceptionally unique pharmacological and physiological characteristics when compared to the protomers (Rocheville et al., 2000a). Such an association has significantly improved the spectrum of action of numerous drugs targeting GPCRs. The concept of protein-protein interactions among GPCRs and/or non-GPCR proteins has indeed led to the identification of novel therapeutic targets for many diseases, notably cancer. Since its identification as a GH inhibitory peptide four decades ago, SST has attracted considerable interest owing to its diverse patho-/physiological roles (Kumar, 2011; Patel, 1999). One of the most important roles for SST is the ability to exert cytostatic and cytotoxic effects via different SSTRs (Acunzo et al., 2008; Ferrante et al., 2006; He et al., 2009; Pages et al., 1999; Pagliacci et al., 1991; Patel, 1999; Sharma et al., 1996, 1999; Sharma and Srikant, 1998b; Srikant, 1995; Srikant and Shen, 1996; Thangaraju et al., 1999a;

Theodoropoulou et al., 2006; Vernejoul et al., 2002; Weckbecker et al., 1994). The receptor subtypes associated with apoptosis include SSTR2 and SSTR3 (Acunzo et al., 2008; Ferrante et al., 2006; Guillermet et al., 2003; He et al., 2009; Sharma et al., 1996; Vernejoul et al., 2002). SSTRs demonstrate homo- and/or heterodimerization within the family as well as with other GPCRs and RTKs, with altered signaling properties (Baragli et al., 2007; Grant et al., 2008; Grant et al., 2004a; Grant et al., 2004b; Kharmate et al., 2011a, b; Pfeiffer et al., 2001; Pfeiffer et al., 2002; Rajput et al., 2012; Rocheville et al., 2000a; Rocheville et al., 2000b; Somvanshi et al., 2009; Somvanshi et al., 2011a; Somvanshi et al., 2011b; Watt et al., 2009). Of note, human SSTR5/dopamine receptor-2 heterodimer exhibited enhanced pharmacological and signaling properties than the protomers (Rocheville et al., 2000a). Conversely, SSTR2 functions were intact in rodent SSTR2/SSTR3 heterodimer, whereas, SSTR3 characteristics such as ligand binding, receptor internalization and cAMP inhibition were abolished (Pfeiffer et al., 2001). It was speculated that a similar interaction might exist in humans expressing SSTR2/SSTR3 subtypes, hence potentially contributing to the reported ineffectiveness of SST-based therapeutics. Whether the loss of SSTR3 functionality in SSTR2/SSTR3 heteromeric complex represented a regulatory mechanism to protect normal cells from apoptosis is not clear. In view of that, it is necessary to delineate whether cross-talk between human SSTR2 and SSTR3 would correspond to their rat counterpart, considering that SSTR3 of human and rodent origins share least aa homology in comparison to other SSTR subtypes (Patel, 1999). It should also be noted that SSTRs C-tail plays a prominent role in modulating dimerization,

trafficking and antiproliferative signaling (Grant et al., 2004b; Hukovic et al., 1998; Hukovic et al., 1999; Roth et al., 1997a; Sharma et al., 1999; Somvanshi et al., 2009). In spite of the robust negative regulation of cell proliferation *in vitro*, SST analogs failed to exhibit potent tumor suppressive role in clinical studies, raising many questions concerning the paucity of data on the precise cellular mechanisms of SST/SSTRs (Bajetta et al., 2002; Ingle et al., 1999a; Ingle et al., 1999b; Orlando et al., 2004). Indeed, low receptor expression at the tumor cell surface has been attributed as one of the key reasons for the limited success of SST and its analogs in inhibiting cell proliferation in the clinical trials. Whether the diminished SSTR3 expression is a contributing factor in breast tumorigenesis is not well understood. Most importantly, the role of SSTR3 overexpression on breast tumor progression has not been previously elucidated in detail. This thesis focused on the comprehensive characterization of human SSTR3. The core issues that were investigated included the molecular determinants of SSTR3 signaling and apoptosis, its functional cross-talk with human SSTR2, and finally the characterization of antiproliferative signaling in breast cancer cells in response to SSTR3 overexpression.

Accumulating evidence suggests that C-tail of many GPCRs including SSTRs not only regulates plasma membrane targeting, oligomerization and trafficking, but also governs an ability to modulate second messengers and intracellular signaling molecules (Cvejic and Devi, 1997; Hukovic et al., 1998; Jones et al., 1998; Liu et al., 2008; Roth et al., 1997a; Sharma et al., 1999; Trapaidze et al., 1996). Studies on human SSTR5 showed that C-tail deletion resulted in impaired receptor

internalization and AC coupling, as well as, loss of antiproliferative signaling which was proportional to the number of aa deleted from the C-tail (Hukovic et al., 1999; Sharma et al., 1999). SSTR3 is unique among other SSTR subtypes in possessing the longest C-tail containing 100 aa (Patel, 1999). Whether the difference in the aa composition of C-tail might account for its specific ability to activate apoptotic cascades is not well understood. Using C-tail truncated rat SSTR3, Pfeiffer et al demonstrated that the mutant receptor existed as preformed homodimer, an indication that dimerization domain of rat SSTR3 is not present in the C-tail (Pfeiffer et al., 2001). Human SSTR3 undergoes agonist-dependent internalization, but unlike rat SSTR3, it does not recycle back to the cell surface, rather targeted to lysosomal degradation (Hukovic et al., 1996; Roth et al., 1997b). This is not an isolated finding, since rat SSTRs have been reported to respond differently to agonist activation in comparison to human SSTRs (Cescato et al., 2006; Hukovic et al., 1996).

The main objective in chapter 2 was to elucidate the role of C-tail in governing SSTR3 properties. The study was designed in HEK-293 cells primarily because these cells are well defined model systems to characterize GPCRs, and importantly, they are also devoid of endogenous SSTRs expression (Thomas and Smart, 2005). To accomplish this aim, HEK-293 expressing four different C-tail deleted mutants; 3 aa deleted (R3 Δ415), 30 aa deleted (R3 Δ388), 76 aa deleted (R3 Δ342) and 100 aa deleted (complete C-tail deletion or R3 ΔCT), were investigated for cell surface expression, dimerization, ability to inhibit AC, signal transduction molecules and cytotoxic functions, and compared with *wt*-SSTR3. In this study, *wt*-SSTR3 and C-tail truncated mutants display comparable level of cell surface expression,

suggesting that the plasma membrane targeting sequence in human SSTR3 is not present in the C-tail. Previous work by Ammon C et al., demonstrated that the residues present in N-terminal region of rat SSTR3 played an important role in regulating receptor routing to the cell surface (Ammon et al., 2002). In agreement, it is possible that plasma membrane routing sequence of human SSTR3 is also located in N-terminal region. Hukovic et al., demonstrating that the ligand binding property was unaffected in C-tail deleted human SSTR5 mutant (Hukovic et al., 1999). Consistently, the data from the present study show no significant changes in the ligand binding profile of C-tail mutants vs. *wt*-SSTR3. Also, previous reports have suggested that the ligand binding pocket of GPCRs including SSTRs is confined in ECLs or TMDs (Gether et al., 1993; Greenwood et al., 1997; Schwartz and Rosenkilde, 1996; Strader et al., 1995). Internalization plays a crucial role in regulating GPCRs transcription as well as their functions (Boudin et al., 2000; Csaba and Dournaud, 2001). Human SSTRs with the exception of SSTR1 display agonist-dependent internalization (Hukovic et al., 1996). Importantly, Roth et al revealed impaired rat SSTR3 internalization upon mutations in Ser and Thr residues present in the C-tail (Roth et al., 1997a; Roth et al., 1997b). Conversely, in the current study, the comparable cell surface and intracellular distribution of *wt*-SSTR3 *vs.* C-tail mutants in response to agonist indicates that some other regions of human SSTR3 such as ECLs, ICLs or TMDs might contain the internalization signal, and warrants further investigation. Several previous studies have examined the role of C-tail in homo- and/or heterodimerization of SSTRs (Grant et al., 2004b; Pfeiffer et al., 2001; Rajput et al., 2012; Rocheville et al., 2000b; Somvanshi et al., 2009). Using CO-IP,

Pfeiffer et al described rat SSTR3 homodimerization in HEK-293 cells even upon C-tail deletion (Pfeiffer et al., 2001). The present study provides biophysical and biochemical evidence for human SSTR3 dimerization, which was decreased upon agonist exposure. A possible explanation for decreased SSTR3 dimerization could be receptor internalization as a homodimer, which was also previously reported for rat SSTR3 (Pfeiffer et al., 2001). Another justification could be the effect of agonist on critical regulators of dimerization, such as receptor conformation and orientation at the cell surface. The data from the present study demonstrate no discernable changes in the status of homodimerization between *wt*-SSTR3 and C-tail mutants, suggesting that C-tail is not an essential requirement for human SSTR3 dimerization. Metabotropic glutamate receptor-5 and Ca^{2+} sensing receptor dimerize via N-terminal interactions (Bai et al., 1998; Romano et al., 1996). Thus, the role of N-terminal or TMDs in regulating human SSTR3 dimerization cannot be excluded from this discussion. It is well recognized that the physiological functions of SSTRs are governed by key phosphorylation sites present in the C-tail (Hukovic et al., 1998; Hukovic et al., 1999; Liu et al., 2009b; Liu et al., 2008; Sharma et al., 1999). Therefore, the next step in this study was to ascertain the role of C-tail on SSTR3 functionality. There was a gradual loss of receptor's ability to inhibit cAMP proportional to the number of aa deleted from the C-tail. These data highlight the determinant role of SSTR3 C-tail residues in regulation of second messengers. It is well recognized that SST/SSTRs mediated inhibition of cAMP governs functional consequences. The next step towards understanding the impact of C-tail deletions in SSTR3 mutants was to elucidate the antiproliferative function including apoptosis,

which is a characteristic feature of SSTR3. The inhibition of cell proliferation as well as increased apoptosis in PCNA-positive HEK-293 cells expressing *wt*-SSTR3 indicates that actively dividing cells are susceptible to agonist-mediated apoptosis. SSTR3-mediated antiproliferative effects were associated with increased expression of PARP-1 and PTP-1C, and ERK1/2 inhibition. In an attempt to dissect the precise role of C-tail on SSTR3 antiproliferative effects, the data from this study show the failure of C-tail deleted mutants to induce apoptosis upon agonist activation. Mutations in basic aa Arg and Lys of MCHR-1 C-tail significantly altered the status of receptor expression and functions (Saito et al., 2005). C-tail mutants of human SSTR3 contain an increased proportion of acidic aa residues with progressive C-tail deletions. Hence, it is conceivable that the lack of basic aa in SSTR3 mutants might be responsible for the loss of agonist-induced apoptosis. An earlier study described C-tail deleted FSHR to function more efficiently than *wt* (Hipkin et al., 1995). In the present study, R3 Δ388 and R3 Δ342 exhibited distinct response to agonist-mediated ERK1/2 and PARP-1 than other C-tail mutants. This unexpected response might be due to the exposure of previously non-accessible phosphorylation sites upon C-tail deletion. Not all GPCRs agonists are associated with consistently identical effects on signaling molecules governed by the respective receptor (Cescato et al., 2010; Kao et al., 2011; Schonbrunn, 2008). The phenomenon termed as agonist bias, which critically depends upon receptor state, results in distinct functional response through the activation of same receptor by different ligands (Kenakin and Christopoulos, 2013a, b). This notion holds tremendous therapeutic promise for drugs targeting GPCRs. In the current study, the differential

actions on signaling molecules between SST and receptor-specific agonists might be attributed to the ligand activation of different receptor conformational states. Taken together, C-tail is not absolutely essential for plasma membrane targeting, internalization and homodimerization of human SSTR3, whereas the receptor functionality and apoptosis was lost upon C-tail deletion.

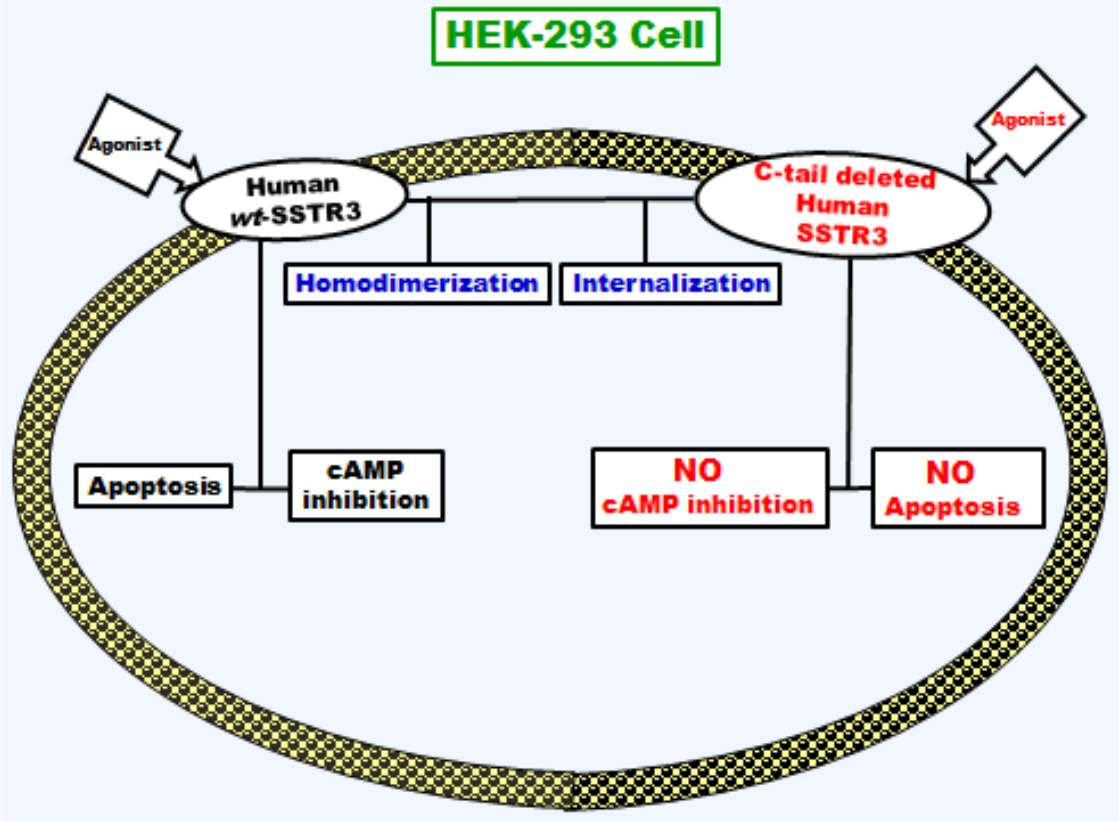

Figure 5.1 A schematic illustration for the crucial role of C-tail in human SSTR3 mediated signaling and apoptosis. Human *wt*-SSTR3 exists as a homodimer with or without agonist. The receptor activation leads to internalization, cAMP inhibition and apoptosis. C-tail deleted SSTR3 mutants display homodimerization and internalize in response to agonist. The ability to inhibit cAMP and induce apoptosis was lost in C-tail mutants.

In the present study so far, characterization of human SSTR3 and C-tail mutants provided useful insights into receptor functions. Human SSTR3 response to agonist treatment was strikingly different when compared to SSTR3 of rat origin as described in previous studies (Roth et al., 1997a; Roth et al., 1997b). Such divergent receptor properties might be attributed to the presence or absence of distinct phosphorylation sites in rat *vs.* human SSTR3. Furthermore, rat SSTR3 C-tail contains 75 aa, whereas C-tail of human SSTR3 consists of 100 aa with 11 Ser and 6 Thr residues (Patel, 1999; Pfeiffer et al., 2001). Whether the different aa composition between rodent and human SSTR3 plays a decisive role in functional diversity remains an open question. From the preceding discussion, it is plausible that the observations from rodent SSTRs might not essentially hold true for human SSTRs. Several lines of evidence have described SSTRs heterodimerization with exceptional signaling properties, and direct pathophysiological relevance in neuroendocrine and cardiovascular disorders (Baragli et al., 2007; Grant et al., 2008; Rocheville et al., 2000a; Rocheville et al., 2000b; Somvanshi et al., 2011a; Somvanshi et al., 2012; Somvanshi et al., 2011b). Conversely, the receptor functionality such as ligand binding and effector coupling, of SSTR3 but not SSTR2 was lost in HEK-293 cells coexpressing rat SSTR2/SSTR3 (Pfeiffer et al., 2001). The data from chapter 2 of this thesis provide a rationale as to why human SSTR2/SSTR3 might interact differently from their rat counterpart.

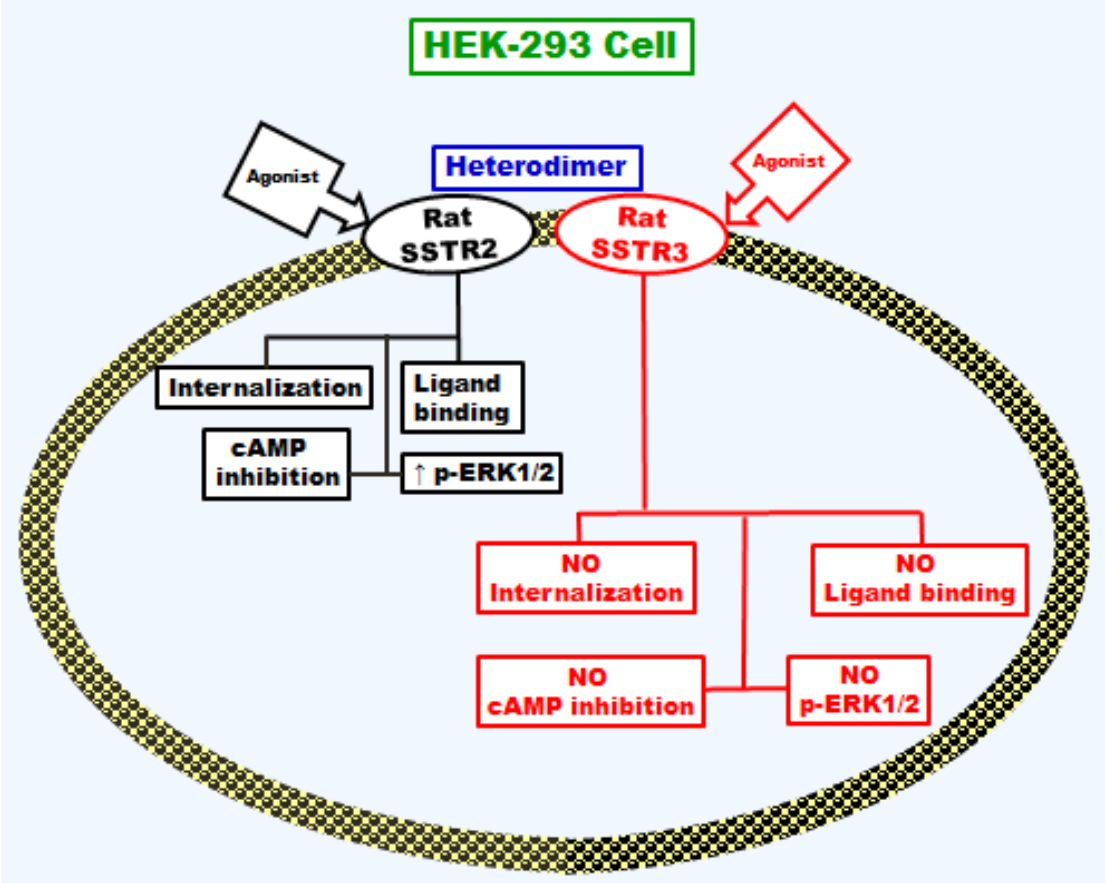

Figure 5.2 Inactivation of rat SSTR3 functions by heterodimerization with rat SSTR2 in HEK-293 cells, as reported by Pfeiffer et al. Rat SSTR2/SSTR3 heterodimer functions like SSTR2, whereas SSTR3-like properties such as ligand binding, internalization, cAMP inhibition and ERK1/2 phosphorylation were abolished (Pfeiffer et al., 2001).

The objective in chapter 3 of this thesis was to investigate functional cross-talk between SSTR2 and SSTR3 of human origin. HEK-293 cells were transfected with human SSTR2 and SSTR3, and studied for heterodimerization, trafficking, cAMP and cell proliferation including apoptosis. The biochemical (CO-IP) and biophysical (Pb-FRET analysis) data suggest that human SSTR2/SSTR3 exist as preformed heterodimers at the plasma membrane. The heteromeric complex was stable in

response to agonist, albeit with a reduced FRET efficiency when compared to control. The next step was to examine the role of heterodimerization on agonist-mediated receptor internalization, which is a typical hallmark of human SSTR2 and SSTR3. The results from immunocytochemistry experiments revealed human SSTR2/SSTR3 colocalization at the cell surface. Notably, selective activation of either SSTR2 or SSTR3 resulted in loss of cell surface distribution for both receptors, and enhanced intracellular colocalization possibly suggesting the internalization of SSTR2/SSTR3 as heterodimer. These findings are contrary to a previous report in cells coexpressing rat SSTR2/SSTR3, showing the failure of SSTR3 endocytosis upon agonist treatment (Pfeiffer et al., 2001). It is well recognized that cAMP inhibition is a characteristic feature of functionally active SSTRs (Kumar, 2011; Patel, 1999). As a next step, functional analysis was performed in cotransfected cells, and the data revealed significant inhibition of cAMP in response to SSTR2 and SSTR3 activation, albeit at a lesser degree when compared to monotransfected cells. This finding confirmed the functionality of both SSTR2 and SSTR3 in the heteromeric complex. The slight attenuation of cAMP inhibition in cotransfected *vs*. monotransfected cells might be attributed to the selective contribution of monomeric, homodimeric or heteromeric sub-populations. Next, antiproliferative signaling was examined to elucidate the functional significance of human SSTR2/SSTR3 heterodimerization. Cells coexpressing SSTR2/SSTR3 displayed significant antiproliferation upon agonist activation when compared to monotransfectants in a G_i-dependent manner. These results are in agreement with previous studies demonstrating pronounced effect of SSTR2/SSTR5 or

SSTR4/SSTR5 oligomerization on agonist mediated inhibition of cell proliferation (Grant et al., 2008; Somvanshi et al., 2009). In an effort to further dissect the nature of antiproliferative signal, the markers for cell cycle arrest (p21 and $p27^{Kip1}$) and apoptosis (PARP-1 and TUNEL) were examined. The results demonstrate prominent cytostatic and cytotoxic roles for human SSTR2 and SSTR3 in cotransfected cells. The data indicating intact antiproliferative functions for SSTR2/SSTR3 in cotransfected cells reinforce my hypothesis that human SSTR3 could be exploited as a potential therapeutic target in tumors.

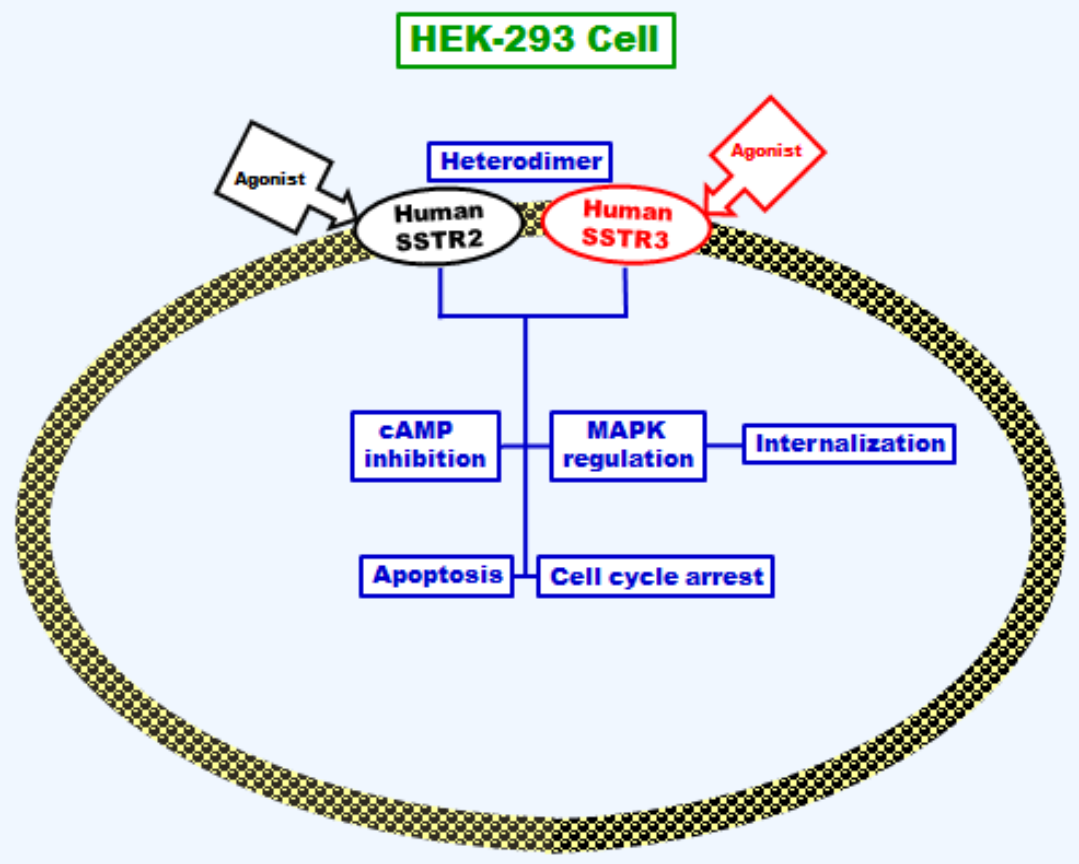

Figure 5.3 Schematic representation of human SSTR2/SSTR3 heterodimerization in HEK-293 cells. Human SSTR2/SSTR3 exist in a heteromeric complex. The heterodimer is functional as

evident by cAMP inhibition, receptors internalization, apoptosis and cell cycle arrest in response to SSTR2 and SSTR3-specific agonists.

The role of SSTRs in pathophysiological conditions, specifically in the clinical management of many tumors is well recognized (Bousquet et al., 1998; Bousquet et al., 2006; Bousquet et al., 2004; Cameron Smith et al., 2003; Weckbecker et al., 2003). SSTR subtypes are not only expressed in normal cells, but also in tissues adjacent to tumors (Qin et al., 2004). SSTRs are perceived to act as endogenous tumor suppressors (Bousquet et al., 2006). Despite remarkable antiproliferative properties of SST analogs *in vitro*, clinical trials have yielded poor results (Burch et al., 2000; Buscail et al., 1996; Delesque et al., 1997; Fisher et al., 1998; Qin et al., 2004; Reubi et al., 1988b; Sulkowski et al., 1999). Although, the reasons for the apparent failure of SST analogs in clinical setting are not clear, it has been argued that the diminished SSTRs expression at the tumor site might play a crucial role. It also remained elusive, whether the loss of SSTR3 at the tumor site was intimately associated with the aberrant cell proliferation and tumorigenicity. Whether tumor cell proliferation would be negatively impacted by reinstating SSTR3 expression has not been reported so far. So far, the interpretations as described in chapters 2 and 3 were derived from an experimental model using transfected HEK-293 cells which lack endogenous SSTR3 expression. Many tumors including breast cancer exhibit low SSTR3 expression (Watt and Kumar, 2006). Several previous studies have established that tumor progression and treatment success depend on the presence or absence of ER_{α} (Burns and Korach, 2012). The precise role of SSTR subtypes in tumor with ER_{α} or devoid of ER_{α} is not well defined.

Accordingly in chapter 4, the pathophysiological relevance of SSTR3 in breast cancer in ER_{α} dependent or independent manner was put to the test. In an effort to delineate the therapeutic potential of human SSTR3 as a tumor target in breast cancer, MCF-7 and MDA-MB-231 cells were stably transfected to overexpress SSTR3. Western blot analysis data revealed significantly high SSTR3 expression at the cell surface in R3-MCF-7 (SSTR3-overexpressing) cells when compared to MCF-7 cells. In contrast, SSTR3 localization was predominantly intracellular in R3-MB-231 (SSTR3-overexpressing) cells. Although, there is no direct evidence, this might possibly suggest a defect in receptor targeting to the plasma membrane. It is also possible that intracellular receptor pool acts as a reservoir for cell surface distribution upon agonist treatment. This was also previously reported in COS-7 cells where agonist activation recruited rat SSTR5 from intracellular stores to the plasma membrane (Stroh et al., 2000). In the next step, MTT assay was performed to examine the role of SSTR3 overexpression on cell proliferation in breast cancer cells. SSTR3-overexpressing cells exhibited significantly low basal proliferation when compared to non-transfected cells. The results also indicate diminished EGF-induced cell proliferation in R3-MCF-7 and R3-MB-231 cells, when compared to non-transfected cells. Cells overexpressing SSTR3 displayed enhanced antiproliferative response to agonist treatment. Importantly, SSTR3-induced apoptosis in monotransfected cells, whereas in cotransfected cells, the receptor exhibited dual cytostatic/cytotoxic role (Sharma et al., 1996; War and Kumar, 2012; War et al., 2011). The next objective was to ascertain whether SSTR3-mediated antiproliferation in breast cancer cells was cytostatic or cytotoxic, or both. The data

show SST and SSTR3-specific agonist induced apoptosis in MCF-7 but not MDA-MB-231 cells. The intriguing observation that R3-MCF-7 but not R3-MB-231 cells exhibited apoptosis even in the absence of agonist suggests constitutive cytotoxic role for SSTR3 in a cell-specific manner. Although, the exact mechanistic explanation for such a finding was beyond the scope of this thesis, the potential role of interacting partners such as SSTR2 cannot be ruled out. It is also likely that SSTR3 transfection in R3-MCF-7 cells might result in local production of SST, to constitutively activate pro-apoptotic signal. An observation of similar nature has been previously reported in pancreatic cancer cell line transfected with SSTR2 (Benali et al., 2000; Delesque et al., 1997; Rauly et al., 1996). Further studies are warranted to explore such possibility. Upregulation of PI3K pathway plays a critical role in tumor progression, and its inhibition triggers cell death (Kauffmann-Zeh et al., 1997; Kulik et al., 1997). Importantly, the inhibition of PI3K by PTP-1C has been previously demonstrated (Cuevas et al., 2001). In addition, OCT was shown to block PI3K pathway in pituitary tumor cells via PTP-1C (Theodoropoulou et al., 2006). Accordingly, in R3-MCF-7 cells, the downregulation of PI3K phosphorylation and increased membrane expression of PTP-1C are associated with SSTR3-mediated antiproliferation. Moreover, p38 MAPK phosphorylation in R3-MCF-7 but not in R3-MB-231 cells might indicate its role as a pro-apoptotic MAPK. The constitutive cytostatic but not cytotoxic effects observed upon SSTR3 overexpression in R3-MB-231 cells which lack $ER\alpha$ suggests that the presence or absence of $ER\alpha$ might play a role in SSTR3-mediated antiproliferative effects, which cannot be ignored from this discussion. Whether $ER\alpha$ is a preferred partner for SSTR3 cytotoxic effects in R3-

MCF-7 cells cannot be concluded at this point, and needs further investigation. This speculation gains support from a previous study in MDA-MB-231 cells, where ERα transfection + EST treatment resulted in inhibition of EGF-mediated mitogenic signaling (Boerner et al., 2005). Therefore, the loss of ERα expression might provide a possible explanation for the aberrant proliferation of ERα-negative breast tumors.

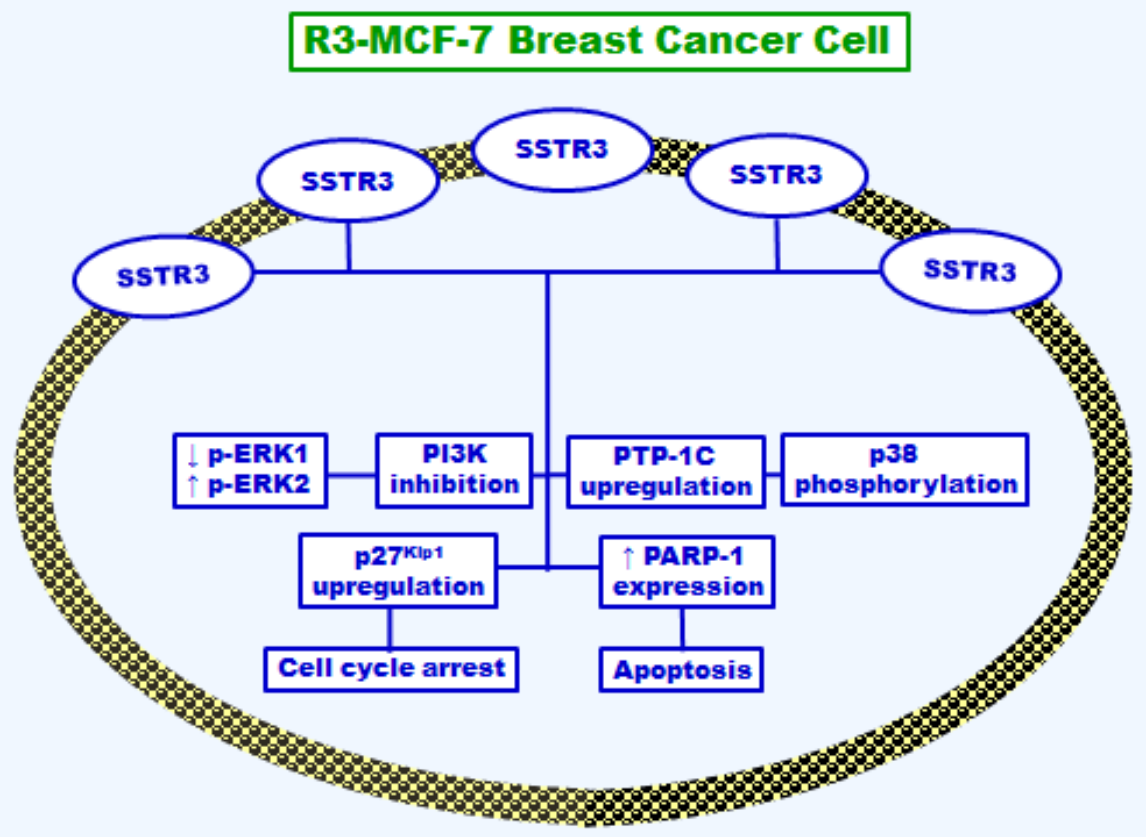

Figure 5.4 Summary of intracellular events following SSTR3 overexpression in R3-MCF-7 breast cancer cells. SSTR3 was largely distributed at the cell surface in R3-MCF-7 cells. Receptor overexpression was associated with isoform-specific modulation of ERK1/2 phosphorylation and inhibition of basal/EGF-mediated PI3K phosphorylation. In comparison to non-transfected cells, R3-MCF-7 cells exhibited increased expression of basal PARP-1, $p27^{Kip1}$ and PTP-1C, and p38 phosphorylation suggesting cell cycle arrest and apoptosis.

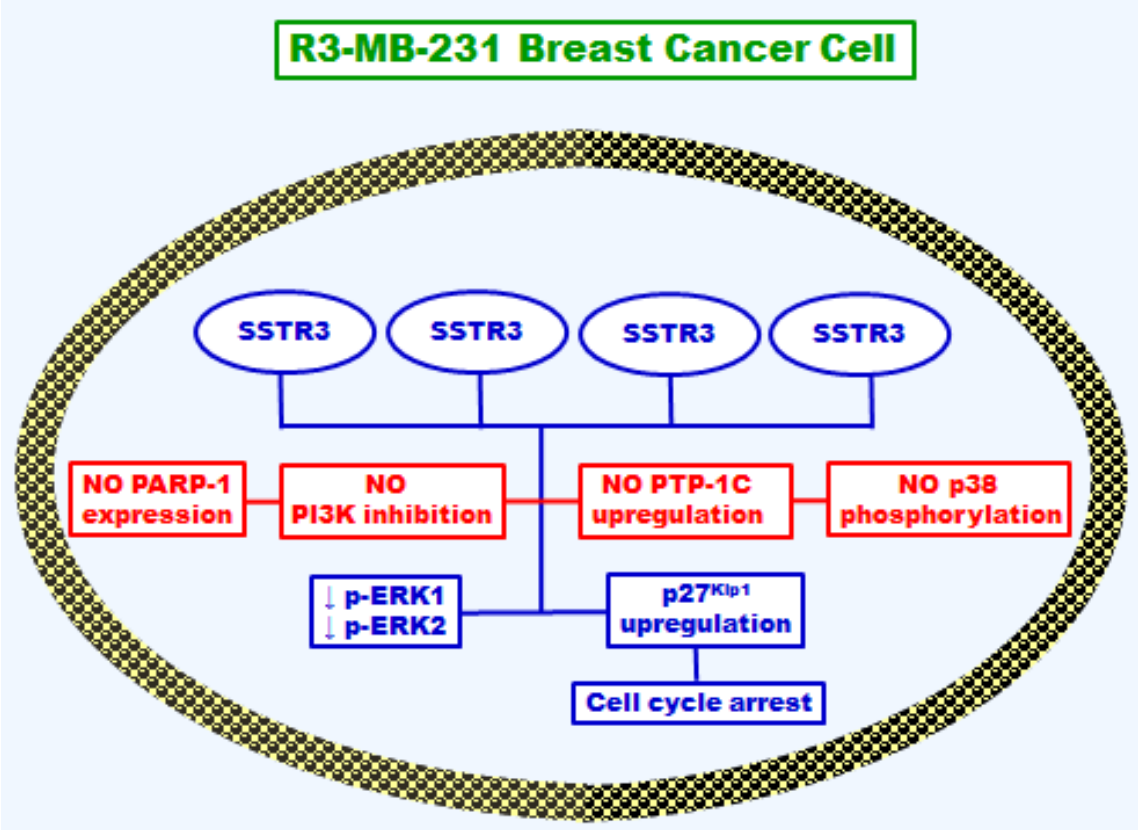

Figure 5.5 Functional consequences upon SSTR3 overexpression in R3-MB-231 cells. SSTR3 was predominantly localized in the intracellular compartment upon overexpression in R3-MB-231 cells. The receptor overexpression resulted in downregulation of basal pERK1/2 when compared to MDA-MB-231 cells. The inhibition of cell proliferation correlated with constitutive upregulation of $p27^{Kip1}$ indicating cytostatic effect.

5.2 Overall conclusions

1. In HEK-293 cells, human SSTR3 exists as a preformed homodimer at the cell surface, displayed agonist-induced internalization, cAMP inhibition, apoptosis and modulation of downstream signaling molecules. C-tail mutants exhibited homodimerization and agonist-mediated internalization. The ability to inhibit

cAMP, induce apoptosis and regulate intracellular signaling cascades was lost upon C-tail deletions.

2. Using HEK-293 cells coexpressing human SSTR2/SSTR3 as a model, the data from this study have challenged the earlier notion based on SSTRs of rodent origin that SSTR3 functions are abolished in the heteromeric complex with SSTR2. Human SSTR3 exerted a dual cytostatic/cytotoxic role in presence of SSTR2.
3. Overexpression of human SSTR3 specifically induced apoptosis in ER_{α}-positive R3-MCF-7 and cell cycle arrest in ER_{α}-negative R3-MB-231 cells. This study improved the scope for human SSTR3 as a potential therapeutic target in breast cancer.
4. Previously, the role of SSTR3 in breast cancer was not well defined, due to the concept of SSTR3 inactivation in presence of SSTR2 (most predominant SSTR subtype in normal and tumoral tissues). The data from this study provide compelling evidence for potential antiproliferative role of SSTR3 in breast cancer cells. There are many questions that need to be addressed in future studies to elucidate the role of SSTRs in several pathophysiological states.

5.3 Future directions

1. The status of homodimers was not altered in C-tail deleted human SSTR3 mutants. To identify the putative dimerization domain, future studies could be targeted in HEK-293 cells expressing human SSTR3 devoid of ECLs, ICLs or

TMDs. A similar strategy could also be used to identify the specific motif in human SSTR3 responsible for plasma membrane targeting and internalization.

2. Overexpression of human SSTR3 in breast cancer cells selectively triggered cytotoxic (R3-MCF-7) and cytostatic (R3-MB-231) effects. To identify whether SSTR2 plays a determinant role, knock-down experiments using siRNA could be conducted in future to delineate the underlying mechanisms for such diverse functional consequences.

3. Whether the presence or absence of ER_{α} plays a decisive role in SSTR3 mediated apoptosis and/or cell cycle arrest upon overexpression warrants further investigation. Future studies using MDA-MB-231 cells transfected with ER_{α} preceding SSTR3 overexpression, as well as, ER_{α} knock-down in MCF-7 cells followed by SSTR3 overexpression, could provide key mechanistic insights.

4. The fact that no *in vivo* experiments were conducted in the present study raises the question whether a similar outcome could be translated in human subjects. In this direction, future studies should focus on animal models of breast cancer.

5.4 Study limitations

1. One of the potential limitations of the current study is that the cell lines used were derived from a single biological source. Therefore, the results presented might not accurately predict the outcomes in a large population sample. However, replicates of three identical experiments are scientifically acceptable in case of *in vitro* cell culture experiments.
2. The frequent culture of cell lines especially MCF-7 breast cancer cells makes them susceptible to genotypic and phenotypic drift (Burdall et al., 2003; Osborne et al., 1987). Although, similar in morphological features, a different pattern of growth rates and status of hormone receptors have been observed for the same cell lines used in different laboratories.

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