A Dissertation

entitled

The role of store operated calcium entry in human carcinoid cell lines.

by

Sasi Arunachalam

Submitted to the Graduate Faculty as partial fulfillment of the
requirements for the Doctor of Philosophy Degree in Biomedical Sciences

________________________
Dr. David R. Giovannucci, Committee Chair

________________________
Dr. Joseph F. Margiotta, Committee Member

________________________
Dr. Surya Nauli, Committee Member

________________________
Dr. Guillermo Vazquez, Committee Member

________________________
Dr. Randall G. Worth, Committee Member

________________________
Dr. Kam C. Yeung, Committee Member

________________________
Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

University of Toledo

August 2010
An Abstract of

The role of store operated calcium channels in human carcinoid cell lines.

by

Sasi Arunachalam

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

Carcinoid tumors are a heterogeneous set of uncommon slow-growing
gastroenteropancreatic neuroendocrine cancers originating primarily from amine or
peptide secreting enteroendocrine cells of gastrointestinal tract. Here, we show that
carcinoid cell lines express an array of store operated calcium entry (SOCE) channels.
Ca^{2+} entry following ER Ca^{2+} depletion and store-operated Ca^{2+} channel activation can
regulate proliferation, migration and apoptosis in some cancer cells including those of
neuroendocrine phenotype. Consistent with these observations, SOCE was activated in
carcinoid cell lines following depletion of ER Ca^{2+} stores by artificial or physiological
agonist. Moreover, treatment with pharmacological inhibitors of SOCE generally reduced
Ca^{2+} entry. SOCE in carcinoid cell lines evoked by GPCR activation suggested that Ca^{2+}
entry may be important for mediating neural, paracrine or autocrine signals in the gut.
Promising molecular candidates artificially or physiologically activated for Ca^{2+} entry
pathways are the recently discovered plasma membrane channel protein Orai and ER
calcium sensor protein STIM. Molecular profiling by RT-PCR indicated that STIM 1 and
Orai1-Orai3 were robustly expressed in GEPNET cell lines derived from foregut and
midgut tumors. Thus to gain insight into the roles of STIM1 and Orai1 in foregut and
midgut cancers we used targeted gene silencing and over expression techniques in combination with live cell imaging of Ca^{2+} entry. These studies demonstrated significant reductions in Ca^{2+} entry following store depletion in cells where STIM1 was reduced by targeted knocked down compared to controls. Conversely, overexpression of STIM1 and Orai1 or Orai3 dramatically enhanced Ca^{2+} entry that was largely abolished by coexpression with its corresponding dominant negative/ mutant Orai proteins. This study points to a dominant role for STIM1 and Orai1 in mediating SOCE in foregut and midgut carcinoid cells. Furthermore, STIM1 and Orai1 have been proven critical for breast cancer migration and invasion. Hence to study the role of these proteins in carcinoid tumor genesis we developed a novel ex vivo xenograft organotypic slice culture technique. This provided the model system that resembled the three-dimensional multi cellular tumor microenvironment. Using organotypic slice culture, we observed that stable BON cells over expressing shRNA for Orai1 showed altered cell movement characteristics and their ability to form tumorlets were greatly reduced in contrast to control cells. Broadly, this work contributes to the search for strategies to inhibit metastasis of carcinoid tumors.
For my Mom and Dad.
Acknowledgements

It's my pleasure to acknowledge all those who made this dissertation possible.

First and foremost, I would like to thank and express my deepest gratitude to Dr. Giovannucci. He is an epitome of infinite patience and knowledge who had taught me the basics of doing research through his excellent guidance, mentoring, motivation and providing me with an excellent atmosphere. He taught me to think independently and always encouraged new ideas. Besides science, I have also learnt a lot from him. Very special thanks to him for instilling quality of integrity in me, not through didactic lectures but through living them.

I would like to thank my committee comprised of Dr. Margiotta, Dr. Yeung, Dr. Vazquez, Dr. Nauli and Dr. Worth for their great insight and constructive criticism towards the success of this project. Dr. Yeung and Dr. Margiotta have taught me how to be your own critic and think critically. Dr. Vasquez was always supportive and he taught me how to answer questions when your working in unexplored field and be a better presenter. Dr. Worth has taught me to think laterally and approach a problem in creative way. Dr. Nauli was very encouraging and motivative.

I would also like to thank all present and past members of lab especially Christian Peters, Tatiana Zhelay and Jennifer Warner. I cannot imagine myself in lab at early stage without Chris help. Chris deserves a special note for his humbleness. Tanya is a nice
colleague to have and I would like to thank her for her help in making few dissertation figures. Special thanks for Jenny for all her pronunciation lessons.

It's said that great friendship happens by chance and here I would like to thank my friend Dr. Riaz Nasim, who was a visiting professor in our lab and helped me in developing organotypic slice culture method.

I am also thankful and grateful to all my friends in the US, who made me feel at home in this country. Finally, and most importantly, I would like to extend my heartfelt thanks to my parents and sister for their love, unconditional support and immense confidence in me.
# Table of Contents

Abstract iii

Contents viii

List of Tables ix

List of Figures xi

List of Abbreviations xxxi

Chapter 1: Introduction 1

Chapter 2: Results 35

Chapter 3: Discussion 69

Chapter 4: Conclusion 76

Chapter 5: Materials & Methods 78

Chapter 6: References 86
Table 2-1: List of PCR primers used to screen possible candidate SOCE channels.

<table>
<thead>
<tr>
<th>No</th>
<th>Gene</th>
<th>Accession no</th>
<th>Primer sequence</th>
<th>Product size in bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>TRPC1</td>
<td>NM_003304</td>
<td>F:GACTCTGGTATGAGGGGTGGA R:CATACTGGTGTTGTGATGTCGATCCAATTG</td>
<td>357</td>
</tr>
<tr>
<td>2.</td>
<td>TRPC3</td>
<td>U47050</td>
<td>F:CTTCTCTAGGTCCATGGAGGGA R:TCAGAGTGAACGTGCTGTCG</td>
<td>419</td>
</tr>
<tr>
<td>3.</td>
<td>TRPC4</td>
<td>AF063822</td>
<td>F:CTCTGGTTTCTCTAATCATG R:CCTGTTGACGGCAAATCTCTCT</td>
<td>804</td>
</tr>
<tr>
<td>4.</td>
<td>TRPC5</td>
<td>NM_012471</td>
<td>F:GTCGTGGAATGGAATGATATG R:GTAGGCTCATCAGATGCTTG</td>
<td>450</td>
</tr>
<tr>
<td>5.</td>
<td>TRPC6</td>
<td>AJ006276</td>
<td>F:GACTTACATGCAAATGAGGAC T:CATATGATCCATATACTCC</td>
<td>626</td>
</tr>
<tr>
<td>6.</td>
<td>TRPC7</td>
<td>NM_020389</td>
<td>F:ACTCTATAGCTACGAGGAC R:CCTGTTGACGGCAAATCTCTCT</td>
<td>309</td>
</tr>
<tr>
<td>7.</td>
<td>TRPV5</td>
<td>NM_019841</td>
<td>F:GGCCTATGAGACACTGAGAATGATATG R:ATAGAATTGCCCAGACTGCTTG</td>
<td>448</td>
</tr>
<tr>
<td>8.</td>
<td>TRPV6</td>
<td>NM_018646</td>
<td>F:AGCCTACATGAGCCACCCCTAGGAGGAC R:GTAGAATTGCCCAGACTGCTTG</td>
<td>448</td>
</tr>
<tr>
<td>9.</td>
<td>TRPM5</td>
<td>NM_014555</td>
<td>F:GTCCTTCACAGACCGCTGAGGAC R:AGTCCTCAGACAGCACTTG</td>
<td>471</td>
</tr>
<tr>
<td>10.</td>
<td>TRPM6</td>
<td>NM_024080</td>
<td>F:GACCTGTGGAATGTGATGGA R:CAGCAGGGATGTGCTGGGT</td>
<td>528</td>
</tr>
<tr>
<td>11.</td>
<td>ORAI1</td>
<td>NM_032790</td>
<td>F:AGTTACTCCGAGGATGAGGCC R:GACCGAGTGATATGTCGAC</td>
<td>306</td>
</tr>
<tr>
<td>12.</td>
<td>ORAI2</td>
<td>NM_032831</td>
<td>F:ATGAGTGGCTAGCTGAGGAC R:AGCTGCTGAGGAGGTCCAG</td>
<td>410</td>
</tr>
<tr>
<td>13.</td>
<td>ORAI3</td>
<td>BC006126</td>
<td>F:CCACATTGAGCTGAGGAGCC R:AGCTGCTGAGGAGGTCCAG</td>
<td>502</td>
</tr>
<tr>
<td>14.</td>
<td>STIM1</td>
<td>NM_003156</td>
<td>F:AGCTCAGGCTGAGGAGC R:CCCTGAGGTTATATGCGGAC</td>
<td>300</td>
</tr>
<tr>
<td>15.</td>
<td>CASR</td>
<td>NM_000388</td>
<td>F:GGAGTCTGGAATGTATCAC GAG R:CCAGGGAATACTCGATGA</td>
<td>453</td>
</tr>
<tr>
<td>Cell line</td>
<td>Origin</td>
<td>Metastatic</td>
<td>Features</td>
<td>References</td>
</tr>
<tr>
<td>-----------</td>
<td>--------</td>
<td>------------</td>
<td>----------</td>
<td>------------</td>
</tr>
<tr>
<td>CNDT 2.5</td>
<td>Midgut</td>
<td>Yes</td>
<td>Secretes serotonin(5-HT); Expresses IGF receptor, platelet-derived growth factor receptor, EGFR, and cMET, VGFR and SSTR1 to SSTR5</td>
<td>Clin Cancer Res. 2007 Aug 15;13(16):4704-12</td>
</tr>
</tbody>
</table>
List of Figures

**Figure 1-21.2**: Predicted structures of the STIM1 and Orai protein families. A) Structures of STIM1 and STIM2. They both have N-terminal Ca\(^{2+}\) sensing EF hand and SAM domain, and coiled-coil motifs and ERM domain in C-terminals. B) Structures of Orai1, Orai2 and Orai3. Orai proteins have four trans- membrane regions and coiled-coil C-terminals. Only Orai1 has two proline and arginine rich region in N-terminal and Orai3 has longer second extracellular loop than Orai1 and Orai2.

Figure modified from Irene et al, channels 2:4(2008).
Figure 2-1: Semi-quantitative endpoint RT-PCR identifying transcripts for the pore forming channel proteins Orai 1-3 and the Ca\(^{2+}\) sensor STIM1 in a set of carcinoid cell lines. Multimeric assemblies of Orai and STIM proteins are believed to form a store-operated Ca\(^{2+}\) permeable channel. Gel band signal intensities were determined by densitometry and normalized to \(\beta\)-actin expression levels. Mean ratio values represent result of at least independent 3 experiments. Inset: an example of agarose gel electrophoresis showing the presence of Orai1 (O1), Orai2 (O2) and Orai3 (O3) transcripts in BON cells. The expected base pair size of Orai1, Orai1, Orai3 and \(\beta\)-actin are 306, 410, 502 and 200 respectively.
**Figure 2-2:** Semi-quantitative RT-PCR identifying some TRPC and TRPV transcripts in carcinoid cell lines. These channels are non-selective or highly selective for $\text{Ca}^{2+}$ respectively, and are generally thought to play sensory roles in a variety of cell types. In addition some TRP channels have been proposed to SOCE in some cell types. Gel band signal intensities were determined as in Fig 1.
Figure 2-3: Functional assessment of SOCE in carcinoid cell lines. A. Representative fura-2 trace in a BON cell showing SOCE response (arrow 2) induced by restoration of extracellular Ca\(^{2+}\) to bath solution following store-depletion (arrow 1) with CPA in nominal Ca\(^{2+}\) containing bath solution. B. Averaged peak amplitudes of SOCE responses. C. Induced influx was augmented by increasing [Ca\(^{2+}\)]\(_o\) to 20 mM, reduced by membrane depolarization and was not significantly altered by treatment with 10 μM nifedipine compared to control (influx in presence of 2.2mM of [Ca\(^{2+}\)]\(_o\)). D. Averaged amplitudes of SOCE activated following treatments indicated in C. Legend: r.u stands for ratio units.
**Figure 2-4:** Representative fura-2 traces in BON cells (A) and average peak amplitudes in cell lines tested (B) showing effects of various channel inhibitors on SOCE following restoration of extracellular Ca2+(shaded box). For comparison, traces in A were normalized to Ca2+ release peak amplitude. B. Reduction in peak amplitude of SOCE following treatment with inhibitors was compared to control. For BON, H727, CNDT2.5, HC45 AND HC49, the percent reductions in SOCE are listed respectively. **Gd**$^{2+}$: 15±3, 49±25, 6±4, 178±32, 0.75±10; **2APB**: 107±9.3, 10.4±4, 2.2±6.3, 170±65, 38±30; **CAI**: 89±6, 81±12, 34±5, 15±5, 0.5±5; **BTP2**: 12±1.5, 7.4±1.5, 5.5±1, 129±44, 7±3.2; 6≤ n ≤104; for significance, p value was ≤0.05. Legend: r.u stands for ratio units.
**Figure 2-5:** Transiently transfected BON cells. A) BON cells were transiently transfected with 0.5 µg of GFP plasmid. The cells were imaged exciting at 488 nm and monitoring fluorescence at 510 nm. B) Over expression of Orai and STIM1 proteins. BON cells were transiently transfected with 0.5 µg of wild type human Orai1, Orai3, STIM1 and GFP plasmids. Functional assessment of SOCE in these transiently transfected BON cells were induced by restoration of extracellular Ca2+ to bath solution following store-depletion with CPA in nominal Ca2+ containing bath solution. The enhanced averaged peak amplitudes of SOCE responses in BON cells over expressing Orai1+ STIM1 and Orai3+ STIM1 is shown below. Traces besides the graph show the enhancement of SOCE in contrast to control. For significance, \( p \) value was \( \leq 0.001 \).
**Figure 2-6:** Over expression of mutant and shRNA proteins. A) BON cells were transiently transfected with 1-2 µg of OraiE106A, Orai3E81A, shRNA STIM1 and GFP plasmids. Functional assessment of SOCE in these transiently transfected BON cells were induced by restoration of extracellular Ca$^{2+}$ to bath solution following store-depletion with CPA in nominal Ca$^{2+}$ containing bath solution. A) Bar graph showing reduction in calcium influx in BON cells transfected with dominant negative Orai1E106A plasmid (O1DN), Orai3E81A plasmid (O3MUT) and shRNA STIM1 plasmid. Corresponding traces show the reduction in SOCE in contrast control. Bar graph showing the slope of fluorescence quenching of fura-2 by Mn$^{2+}$ in BON cells transfected with dominant negative Orai1 and mutant Orai3. For significance, p value of * was ≤0.05 and ** was ≤0.001 respectively. B) Manganese quenching experiment. In this set of experiments, intracellular stores were depleted by CPA in absence of calcium and manganese was added to the bath solution of BON cells over expressing mutant plasmids. Manganese influx into the cytoplasm quenches the fluorescence of calcium indicator dye fura-2 F$_{360}$ signal which corresponds to plasma membrane channel activity. Fluorescence quenching of the signal was measured by line fit to the initial phase of quenching which indicated the influx rate of calcium and shown as a negative slope. For significance, p value of * was ≤0.05.

![Graph A](image1.png)

![Graph B](image2.png)
**Figure 2-7:** Extracellular Ca\(^{2+}\) is required for maintaining sustained calcium oscillations. A) BON cells stimulated by 10 μM CCh in presence and absence of extracellular calcium. B) The total number of spikes observed over a period of time in CCh stimulated BON cells in presence and absence of calcium were 20±2 n=17 and 5±0.5 n=31. For significance, p value was ≤ 0.001. C) The frequency of the oscillations in CCh stimulated BON cells in presence and absence of calcium was represented as graph plot of Ca\(^{2+}\) spikes vs time. Legend: r.u stands for ratio units.
**Figure 2-8:** Oscillatory changes in cytosolic Ca$^{2+}$ in response to application of carbachol (CCh). A. Representative traces of Ca$^{2+}$ dynamics induced by CCh in the absence of extracellular Ca$^{2+}$ 30 μM CPA was applied following CCh evoked response to assess ER Ca$^{2+}$ content. CPA-evoked Ca$^{2+}$ release following treatment with CCh shows a concentration dependent decrease in ER store content. B. A representative trace showing determination of rate of influx by exponential fit (red colored trace) to the slope of Mn$^{2+}$ induced fura-2 fluorescence quench. C. Representative traces of unstimulated (-CCh) influx and following application of 30 μM CPA or 100 μM CCh in the presence or absence of 1 μM Gd$^{3+}$ or BTP2 to measure influx determined by Mn$^{2+}$ quench method in fura-2 loaded BON cells. D. Averaged rates (τ) of influx under different conditions of activation and block of SOCE. Legend: r.u stands for ratio units.
Figure 2-9: Correlation of store depletion and SOC influx in BON cells. Monitoring SOC influx by addition of barium to BON cells stimulated with CCh in absence of extracellular Ca$^{2+}$ showed direct relationship of store depletion to SOC influx. Enhanced SOCE was observed in response to more store depletion (red traces). In contrast, black trace shows lesser store depletion reduced the magnitude of SOCE. Integrated area under the depletion curve Vs SOCE amplitude graph shows the total amount of Ca$^{2+}$ influx actually reflects the amount of store depletion. Legend: r.u stands for ratio units.
**Figure 2-10:** Role of Orai in CCh-evoked entry and its effect on calcium oscillations. BON cells transiently transfected with 0.5 µg of GFP plasmid (mock transfected/control) were stimulated in the presence of extracellular calcium with 10 µM CCh in presence and absence of 1 µM BTP2 tested for oscillations. Similarly, BON cells were transiently transfected with 0.5 µg of wild type STIM1, Orai1 and GFP plasmids in one set of experiments, and 1-2 µg of O1DN and GFP plasmids in another set of experiments in BON cells were then stimulated in the presence of extracellular calcium with 10 µM CCh and tested for oscillations. B) The total number of spikes observed over a period of time (500 secs) in CCh stimulated BON cells over expressing GFP plasmid in presence and absence of 1 µM BTP2 treatment, over expressing O1DN plasmid, and over expressing wild type STIM1, Orai1 plasmids were listed respectively. The values are 13±2, 9±1, 7±0.5 and 15±2; 10≤n≤18; for significance p value was ≤ 0.05. Legend: r.u stands for ratio units.
Figure 2-11: Role of Orai1 in cellular processes. A and B). Cell cycle phase analysis. Untransfected (BON) and transiently transfected BON cells with GFP plasmid (GFP), O1DN+GFP plasmids (O1DN) and Orai1+STIM1+GFP plasmids in absence (A) and presence (B) of CPA were subjected to cycle analysis by flow cytometry. Bar graph showing the different phases of cell cycle in BON cells over expressing O1DN (Orai1E106A), O1+S1 (Stim1+Orai1), transfection control (GFP), untransfected cells (BON) with/without CPA treatment. * denotes CPA treatment. B) Cell proliferation analysis. Transiently transfected BON cells with GFP plasmid (CON) and O1DN+GFP plasmids (O1DN) were subjected to Ki-67 staining. Transfected cells appear green and the cells stained with Ki-67 appear red.
Figure 2-12: A) Diagram of mouse liver structure. Human BON cells in intact LLL of mouse liver vasculature at lower (B) and higher magnification (C).
Figure 2-13: A) Periodic monitoring of selected region from an organotypic slice. B) The selected region was monitored using fluorescence macroscopy typically 24 hrs intervals to see any morphological changes and tumor formation of the seeded cells over a period of time.
Figure 2-14: Confocal images of portal vascular structure (red), seeded BON cells (green) and tumorlet arrangements in slices at 5 and 288 hrs post injection respectively. Inset: Tumorlet structure at higher magnification.
**Figure 2-15:** Ameboid and mesenchymal movements. A) Image montage of cells (20X) in a cluster showing mesenchymal migration characterized by cell membrane extensions namely filopodia and lamellipodia. B) Image montage of tumor cells (20X) showing blebbing process indicated by *, a characteristic feature of amoeboid migration movements.
Figure 2-16: A) Image montage of two tumor cell groups showing amoeboid migrations (20X). Cells at the left group migrate into surrounding parenchyma tissue and exhibit amoeboid movement and join the cells at right group. B) Image montage at 0 hrs (top) and 14 hrs (bottom). The red trace shows the boundary/area of cells at 0 hrs and black trace shows the boundary/area of cells after 14 hours of amoeboid migration.
Figure 2-17: Functional assessment of Orai1 stable knock down BON cells. Stable knockdown of Orai1 protein in BON cells was achieved using shRNA Orai1 plasmid vector from OriGene. A) Reduction of SOCE in O1 knockdown BON cells. B) Reduction in mRNA message level in O1 knockdown BON cells. For significance, $p$ value of * was $\geq 0.05$. 
Figure 2-18: Tumorlet formation. Stably transfected BON cells expressing GFP and scrambled shRNA (control) proliferates in mouse liver as “tumorlets”. In contrast ORAI1 knockdown BON cells do not.
Figure 2-19: Analysis of selected tumor area of Orai1 knockdown and normal BON cells over a period of time in slices was done using ImageJ and fold area increase of the tumor was plotted against time. Tumor area fold increase in control (black) and Orai1 knockdown (red) BON cells.
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>2APB</td>
<td>2-amino-ethyl diphenyl borate</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ARC</td>
<td>Arachidonic acid-Regulated Ca(^{2+}) channels</td>
</tr>
<tr>
<td>BTP2</td>
<td>N-(4-[3, 5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide</td>
</tr>
<tr>
<td>cADPR</td>
<td>Cyclic ADP ribose messenger</td>
</tr>
<tr>
<td>CAI</td>
<td>Carboxyamido-triazole</td>
</tr>
<tr>
<td>CAM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CASR</td>
<td>Calcium sensing receptor</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium-induced calcium release</td>
</tr>
<tr>
<td>CIF</td>
<td>Calcium influx factor</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium release activated calcium</td>
</tr>
<tr>
<td>CRACM</td>
<td>Calcium release activated calcium channel modulator</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-responsive element binding protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enterochromaffin-like</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin-radixin-moesin</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GEPNETs</td>
<td>Gastroenteropancreatic neuroendocrine tumors</td>
</tr>
<tr>
<td>GPCR</td>
<td>Guanine nucleotide-binding protein coupled receptor</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>I(_{\text{CRAC}})</td>
<td>Calcium release activated calcium current</td>
</tr>
<tr>
<td>IP</td>
<td>Inositol 1, 4, 5-trisphosphate</td>
</tr>
<tr>
<td>IP(_3)</td>
<td>Inositol 1, 4, 5-trisphosphate receptor</td>
</tr>
<tr>
<td>HVA</td>
<td>High voltage activated channels</td>
</tr>
<tr>
<td>LVA</td>
<td>Low voltage activated channels</td>
</tr>
<tr>
<td>MEN</td>
<td>Multiple endocrine neoplasia</td>
</tr>
<tr>
<td>MMP</td>
<td>Metalloproteinase</td>
</tr>
<tr>
<td>ML-9</td>
<td>1-(5-chloronaphthalene-1-sulfonyl) homopiperazine</td>
</tr>
<tr>
<td>MOCC</td>
<td>Mechanically activated calcium channels</td>
</tr>
<tr>
<td>NAADPR</td>
<td>Nicotinic acid adenine di-nucleotide phosphate receptor</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
</tbody>
</table>
PC12……...Pheochromocytoma 12 cells
PET…………Positron emission tomography
PLC…………Phospholipase C
PIP₂………Phosphatidylinositol 4, 5-biphosphate
PM…………Plasma membrane
PMCA……..Plasma Membrane Calcium ATPase
ROCC……..Receptor operated calcium channels
RYRs………Ryanodine receptors
SCaMPER ….Sphingolipid calcium release mediating protein of endoplasmic reticulum.
SCID……..Severe combined immunodeficiency syndrome
SERCA……Sarco/Endoplasmic Reticulum Calcium ATPase
SKF-96365…1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy] ethyl-1H-imidazole hydrochloride
SOCC……..Store operated calcium channels
SOCE……..Store operated calcium entry
SR/ER……..Sarco/endoplasmic reticulum
STIM……..Stromal Interacting Molecule
TRP………Transient receptor potential
TRPC……..Transient receptor potential canonical channel
TRPV……..Transient receptor potential vanilloid channel
TRPM…….Transient receptor potential melastatin channel
VOCC……..Voltage operated calcium channels
Chapter 1: Introduction

1.1 Carcinoid: A brief history

Neuroendocrine tumors can arise from cells derived from the embryonic neural crest, neuroectoderm or endoderm (Van Buren, et al., 2007). Neuroendocrine tumors which originate from dispersed enteroendocrine cells such as enterochromaffin cells are termed as Gastroenteropancreatic neuroendocrine tumors (GEPNETs) which are commonly known as carcinoids (Modlin, Kidd, Latich, Zikusoka, & Shapiro, 2005). Carcinoids are rare, slow growing cancer and they are often described as cancer in slow motion. Carcinoid was first characterized by Lubarsh in 1888 (Schnirer, Yao, & Ajani, 2003) and Ranson in 1890 (Schnirer, et al., 2003) first described carcinoid of the ileum. The term ‘Karzinoide’ was coined by Oberndorfer (Schnirer, et al., 2003) in 1907 to distinguish these slow growing tumors from typical adenocarcinoma. Masson stated that carcinoids should be considered as endocrine tumors (Masson, 1928). The carcinoid tumor was not widely recognized as neuroendocrine tumor until the work of Erspamer and Lemberk (Pasieka, 2009). The demonstration of serotonin in enterochromaffin cells and extraction of serotonin from carcinoid tumor were two major findings that related carcinoid tumor to endocrine origin and henceforth, it was widely accepted as neuroendocrine tumor (Pasieka, 2009).
1.2 Significance:

According to American Cancer Society, 11,000 to 12,000 people are diagnosed with a carcinoid tumor each year and the number has been increasing around 6% per year. These tumors are more common in females and are often diagnosed in a person’s fifth or sixth decade of life with a higher incidence in African American population (Modlin & Sandor, 1997; Schnirer, et al., 2003). These tumors occur most commonly in gastrointestinal tract and they are not diagnosed until they metastasize. The average survival rate of carcinoid patient is 5 years. Almost 50% of identified carcinoid tumors occur in digestive system with 30% in the lungs and 20% in other organs (Schnirer, et al., 2003).

1.3 General features:

Carcinoid tumors commonly consist of epithelial cells grouped in columns of various sizes, with a centrally placed nucleus and fibrous stroma. The shape of the tumor depends on its location and supporting stroma (Fasolino, Consoli, Barroetavena, & Parodi, 1963). The cells of these tumors are characterized histologically by positive reactions to silver stains and to neuroendocrine markers such as neuron specific enolase, synaptophysin, and chromogranin. Their cytoplasm contains numerous membrane-bound neurosecretory granules, which contain a variety of hormones and biogenic amines as described in the following sections.

1.4 Classification:

There is no consensus on a single practical classification of carcinoid tumors. Broadly these tumors can be classified into gastrointestinal and non- gastrointestinal carcinoids (Schnirer, et al., 2003). Alternatively carcinoid tumors can be classified by
clinical methods, embryonic divisions of gut, silver impregnation method or serotonin secretion (Nilsson, 1996). The clinical method of classification is based on the aggressiveness and secretory properties of the tumor. Silver impregnation method is based on the ability to reduce the silver ions consistent with A.P.U.D phenotype. The most accepted form of classification is based on the site of origin or embryonic division of gut, which is described in detail in following sections.

1.5 Gastrointestinal carcinoids:

Tumors of gastroenteropancreatic axis can be further classified by their site of origin or by the product they release. For example, they can be classified into foregut, midgut or hindgut tumors (Kirshbom, Kherani, Onaitis, Feldman, & Tyler, 1998; Thompson, et al., 1985). The tumors arising from different segments of embryologic gut differ widely in their histological, immunohistochemical and bioactive amine production. The classification and the properties of this tumor are described in detail in following sections.

1.5. A. Foregut carcinoids:

Foregut carcinoid includes carcinoid of the bronchus, lung, thymus, stomach, proximal portion of the duodenum and pancreas. Gastric carcinoids originate from enterochromaffin-like (ECL) cells which secrete histamine regulating gastric acid secretion (Jensen, 1996). Gastric carcinoids have been classified into chronic atropic gastritis type A, Zollinger-Ellison syndrome and sporadic gastric carcinoid by clinical and histological method (Solcia, et al., 1988). 75% percent of gastric carcinoid patients also have chronic gastritis type A, where tumor sizes are usually less than 1 cm in diameter and metastasize in 10% of cases (Moertel & Hanley, 1979). 5 to 10% of gastric
carcinoids are associated with Zollinger-Ellison syndrome where tumor sizes are less than 1.5 cm and metastasize in 25% of the cases (Rindi, Luinetti, Cornaggia, Capella, & Solcia, 1993). Sporadic carcinoid constitutes 15 to 25% of gastric carcinoids. In these cases the tumor sizes are larger than 1 cm with a high metastatic potential (Moertel & Hanley, 1979).

Foregut carcinoids are typically argentaffin negative because they fail to reduce the silver ions; contain low serotonin content and present with a higher incidence of (Multiple endocrine neoplasia) MEN-1 gene mutations and deletion in chromosome 11 (Dong, et al., 1997). Foregut carcinoids produce peptide hormones such as gastrin, glucagon or insulin. Foregut carcinoids have various molecular and genetic markers such as p53, bcl2 and MEN1 (Leotlela, Jauch, Holtgreve-Grez, & Thakker, 2003). P53 mutation in carcinoid tumor is limited to atypical carcinoid of the lung and 44% of sporadic lung carcinoma has inactivated copies of MEN1 gene. Also lung carcinoma has higher expression of bax and lower bcl-2 expression (Brambilla, et al., 1996). Liver and bone is the most common site of metastasis for foregut tumors.

1.5. B. Midgut carcinoids:

Midgut carcinoids are the most common type of carcinoid typically responsible for distinct syndrome such as flushing and diarrhea (Kazi & Grover, 1969). Carcinoid syndrome is explained in detail in following section. The midgut carcinoid includes distal portion of the duodenum, jejunum, ileum, appendix and ascending colon. They are argentaffin positive as they reduce the silver ion and become stained brown or black, often have a deletion mutation in chromosome 18, and secrete high serotonin (5HT), prostaglandins and bradykinin (Caplin, et al., 1998; Memon & Nelson, 1997). So far
there are no specific molecular and genetic markers for midgut tumors. The primary metastatic site is liver through predicted hepatic portal circulation.

1.5. C. Hindgut carcinoids:

The hindgut carcinoid includes transverse colon, descending colon and rectum and they are argentaffin negative (Schnirer, et al., 2003). Hindgut tumors have no specific molecular or genetic markers and they secrete somatostatin, low level of serotonin, 5HTP, peptide YY (Kema, de Vries, Slooff, Biesma, & Muskiet, 1994). These tumors express transforming growth factors-α and epidermal growth factor receptor. Hindgut tumor metastasizes mostly into bone but rarely metastasize into liver (Schnirer, et al., 2003).

1.6 Non- gastrointestinal carcinoids:

Carcinoid tumor mostly occurs in gastrointestinal tract. However, these tumors sometimes occur in other organs such as lung and ovary. Lung carcinoid tumor was first described in 1972 and it was shown that it accounts for 2% of all lung cancer (Dusmet & McKneally, 1996). Symptoms are highly variable and classical carcinoid syndromes occur in fewer than 5% of the cases. Carcinoid tumor also occurs in ovary and ovarian carcinoid tumor secrets high level of peptide YY (Shigeta, et al., 1999).

1.7 Carcinoid syndrome:

Carcinoid tumors secrete numerous hormones and biogenic amines. Besides the above mentioned secretions like serotonin, gastrin, insulin, prostaglandins and bradykinin, the carcinoid tumor also produce and secrete chromogranins-A and C, growth hormone, neurotensin, pancreatic polypeptide, calcitonin, tachykinins, growth hormone-releasing hormone, bombesin, adrenocorticotropic hormone (ACTH), kallikrein,
glucagon, substance P, substance K, neuropeptide (K), enkephalin, glicentin, VIP, pancreastatin, and various growth factor (TGF-), platelet-derived growth factor (PDGF), and β-fibroblast growth factor (Lembeck, 1954; Schnirer, et al., 2003). They also secrete biogenic amines such as 5-hydroxytryptophan (5-HTP), histamine, and catecholamine (Lembeck, 1954; Neary, Redmond, Houghton, Watson, & Bouchier-Hayes, 1997; Page, Corcoran, Udenfriend, Szoedsma, & Weissbach, 1955; Schnirer, et al., 2003). These secreted amines and peptides gives rise to carcinoid syndrome such as flushing, diarrhea, abdominal pain, bronchospasm, pellagra, teleangiectasia and dyspnea (Grahame-Smith, 1968; Memon & Nelson, 1997; Strodel, Talpos, Eckhauser, & Thompson, 1983). Foregut and hindgut carcinoids secrete low amounts or no serotonin hence they rarely cause carcinoid syndrome. However production of ACTH by foregut tumor can cause Cushing syndrome (Feldman, 1989). Cushing syndrome is the hormone disorder caused by high level of cortisol in the blood. The midgut carcinoid secretes high serotonin and hence they cause carcinoid syndrome. Serotonin plays a major role in diarrhea and also causes bronchospasm. High concentration of circulating amines causes fibrosis, which in turn causes carcinoid heart disease leading to failure of right side of the heart (Feldman, 1989; Vinik, et al., 1989). Massive release of catecholamines from the tumor was also thought to be reason for carcinoid crisis.

1.8 Diagnosis:

The diagnosis of carcinoid tumor is often done after the symptoms of flushing and diarrhea are exhibited. Although Chromogranin A serves as a useful neuroendocrine marker and also serum marker for carcinoid, it is also elevated in pancreatic neuroendocrine tumors and other types of neuroendocrine tumor (Stridsberg, Oberg, Li,
Engstrom, & Lundqvist, 1995). Carcinoid tumor can also be diagnosed by patient’s urine sample. Increased urinary 5-HIAA (5-hydroxyindoleacetic acid) was observed in patients with carcinoid syndrome (Feldman & Lee, 1985). Hence, diagnosis of carcinoid tumors by measuring the level of plasma chromogranin-A, and also by measuring the levels of 5-HIAA is only possible after the tumors are metastasized.

Staging of carcinoid tumor can be determined by nuclear and radiographic imaging. There are three types of staging namely localized, regional spread and distant spread. To aid in staging octreoscan is commonly used (Westlin, et al., 1993). Octreoscan is a radioactive imaging agent, which is helpful in finding primary and metastatic neuroendocrine tumors. In Octreoscan imaging, a small amount of indium$^{111}$-labeled DTPA-octreotide is injected into vein. Octreotide being a somatostatin analogue easily binds to Somatostatin receptor 2, which is over expressed and regulates the secretion of hormones responsible for carcinoid syndrome and hence the receptor serves a marker for carcinoid tumor (Krenning, et al., 1994). Following octreotide injection into vein, its attachment to carcinoid cells is scanned to reveal the spread of gastrointestinal carcinoid tumors to other areas of the body. To test the spread and whereabouts of carcinoid tumor, a special kind of non-invasive radiological technique called positron emission tomography (PET) is used. In PET scanning, a radioactive form of 5-hydroxytryptophan is used which is readily taken up and used by carcinoid cells (Eriksson, et al., 1993). Computed tomography (CT) and magnetic resonance imaging is also used for screening and staging of the carcinoid tumors (Krenning, et al., 1994). Once the carcinoid tumor is diagnosed and staged, there are few treatment options available. Unfortunately, all available treatment options have limited effectiveness.
**1.9 Treatment:**

The principle factors in selecting the treatment option of carcinoid tumor depend on its size, location and migration of the tumor. Of all the treatment option surgery seems to be the only curative therapy for carcinoid (Caplin, et al., 1998). For the tumors with diameters less than 2 cm, local excision is done. For the tumor size more than 2 cm in diameter, more extensive excision such as segmental colon resection or hemicolectomy or low anterior resection is done. In some cases abdominoperineal resection and liver resection are done to remove the metastases or part of the organ to cure or reduce the symptoms of carcinoid tumor (Kulke & Mayer, 1999). For the treatment of advanced disease, chemotherapeutic or biotherapeutic methods like somatostatin receptor-directed hormonal therapy or interferon treatment are employed. Chemotherapy is used only on carcinoid tumors that have metastasized and are unresponsive to other treatments. Chemotherapy drugs such as 5-fluorouracil (5-FU), doxorubicin, etoposide, dacarbazine, streptozotocin, cisplatin, and cyclophosphamide are used as single drugs or with combinations of two or three drugs (Moertel, 1983). Somatostatin regulates secretion and inhibits the release of hormones and bioamines that are responsible for carcinoid syndrome (Reichlin, 1983). So the somatostatin analogue, octreotide is used to control carcinoid syndrome. Further alpha-interferon which activates the body immune system is used to shrink some metastatic carcinoid tumor and also to alleviate the symptoms of carcinoid syndrome (Oberg, Eriksson, & Janson, 1994). In spite of all the treatment options available, the disease remains incurable and treatments of metastatic tumors have low biological activity and are not promising enough to tackle down this tumor. These factors clearly underline the need of early diagnosis and effective treatment options.
against this cancer, which can be helped by exploring the basic biology of these tumor cells.

1.10 Calcium and cancer:

Cancers differ widely in their biology and causes. Tumor promoting agents like carcinogens, radiation and viral infections can alter the genetic makeup of a cell to promote malignant growth. For the past four decades, war on cancer armed with intensive scientific research employing molecular and cellular biology approaches has helped us to understand the disease processes, oncogenes, tumor suppressing genes but limited success in therapies and treatment of cancers.

In recent years, numerous studies have been focusing on prevention since more than 30% of the cancer can be prevented by avoiding the risk factors. For example, one of the leading ideologies is to prevent and treat colon cancer by calcium (Ca\(^{2+}\)) and vitamin D (Holick, 2005). According to American Cancer Society, Ca\(^{2+}\) may lower the risk of colon cancer and there is an inverse relationship of Ca\(^{2+}\) uptake and colorectal cancer. Impairment of vitamin D signaling and Ca\(^{2+}\) insufficiency causes cellular dysfunction which increases the risk of diseases such as osteoporosis, colorectal and breast cancer, inflammatory bowel disease, insulin-dependent diabetes mellitus type I, metabolic syndrome, diabetes mellitus type II, hypertension and cardiovascular diseases (Holick, 2005; Peterlik, Grant, & Cross, 2009). Vitamin D and Ca\(^{2+}\) has been portrayed as wonder drug in prevention of some cancers.

Extracellular Ca\(^{2+}\) has been shown to be important for maintenance and growth of various cell types including carcinoid cancer cell lines. It has been known that Ca\(^{2+}\) signaling plays a vital role in neuroendocrine secretion and hence it is worth to look into
Ca\(^{2+}\) homeostasis in neuroendocrine cancers such as carcinoid cancers. Carcinoid cancer cell line expresses store operated and voltage operated Ca\(^{2+}\) channels but little is known about the role of luminal Ca\(^{2+}\) and channels that mediate the Ca\(^{2+}\) entry in small intestine and bowel carcinoid tumors. Hence, studying the role of Ca\(^{2+}\) entry through these channels might shed more light in development, progression and possible treatment of these carcinoid tumors. Ca\(^{2+}\) signaling controls majority of cellular reaction and inadequate Ca\(^{2+}\) in cell is one of the important risk factor of cancer (Peterlik, et al., 2009). In the light of these recent data we decided to study the role of Ca\(^{2+}\) in human carcinoid cell lines.

Intracellular Ca\(^{2+}\) is often considered a “life giving signal” because it regulates various cellular processes including those relevant to tumor genesis, such as proliferation, apoptosis, gene transcription and angiogenesis by activating or inhibiting cellular signaling pathways and Ca\(^{2+}\)-regulated proteins (Berridge, Bootman, & Roderick, 2003; Monteith, McAndrew, Faddy, & Roberts-Thomson, 2007; Rizzuto & Pozzan, 2006). Tight regulation of intracellular Ca\(^{2+}\) concentration plays a crucial role in gene transcription and proliferation. For example, Ca\(^{2+}\) is shown to regulate transcription without the intermediation of kinases and phosphatases and the transcription factor CREB is activated by Ca\(^{2+}\) carrying out gene expression (Cartin, Lounsbury, & Nelson, 2000) and Ca\(^{2+}\) regulates cell cycle, and hence proliferation, through various pathways including regulation of ras activity (Cullen & Lockyer, 2002). Further, it has been reported that elevated intracellular Ca\(^{2+}\) increases the proliferation of human astrocytoma and mouse neuroblastoma cell lines (Panner, et al., 2005).
Intracellular Ca\textsuperscript{2+} abnormality leads to various dysfunctions in cell that leads to apoptosis, necrosis and tumor genesis. For example, elevation of intracellular Ca\textsuperscript{2+} levels in the presence of normal androgen levels has been implicated in apoptotic prostate cell death (Gong, Blok, Perry, Lindzey, & Tindall, 1995). Further, in CHO cells, store-dependent entry of Ca\textsuperscript{2+} can accelerate apoptosis by accelerating the expression of \textit{GADD153} and by inducing a prolonged efflux of K\textsuperscript{+} out of the cell (Pigozzi, Tombal, Ducret, Vacher, & Gailly, 2004). It has been also shown that apoptosis and necrosis are often linked to accumulation of excessive Ca\textsuperscript{2+} and SERCA2 haploinsufficiency predisposes mice to tumor development (Prasad, et al., 2005; Rizzuto, et al., 2003).

In neuroendocrine tumors Ca\textsuperscript{2+} plays an important role in secretion of amines and peptides, which promotes carcinoid syndrome symptoms (Mergler, et al., 2007). However, the role of Ca\textsuperscript{2+} dynamics in neuroendocrine cancers is not well studied. So targeting Ca\textsuperscript{2+} channels and pumps may pave a way for cancer prognosis and treatment. In the following section the importance of Ca\textsuperscript{2+} and function is briefly addressed.

\textbf{1.11 Calcium as a ubiquitous signal:}

Calcium was long known as a structural element for bone stability until the serendipitous finding of Ringer in 1883 linking Ca\textsuperscript{2+} to initiation of heart cell contraction. In 1940, Heilbrunn showed Ca\textsuperscript{2+} causes contraction in frog muscle fiber and Bailey (Bailey, 1942) in 1942 showed that ATPase activity of myosin was strongly activated by Ca\textsuperscript{2+}. Further the research work of Weber (Weber, 1959), Ebashi (Ebashi & Kodama, 1965), Lipmann (Ebashi & Lipmann, 1962) and Kodama (Ebashi & Kodama, 1965) confirmed that Ca\textsuperscript{2+} had a function in cell biochemistry. Following those early discoveries, Ca\textsuperscript{2+} was recognized as a carrier of signals and their signaling role of Ca\textsuperscript{2+}
has been extensively studied. Ca$^{2+}$ is now known as universal intracellular messenger, regulating all important cellular processes such as triggering developmental program in embryo, gene transcription, muscle contraction, cell proliferation, hormonal regulation and cellular apoptosis.

1.12 Versatility and uniqueness of Ca$^{2+}$ signaling:

Calcium has number of properties that makes it an ideal signaling agent. Ca$^{2+}$ signaling is so versatile that it acts as first, second or third messenger (Carafoli, 2004a). Ca$^{2+}$ can act on the outside of the plasma membrane like hormones or growth factors by binding to G-protein coupled receptor (GPCR) or Ca$^{2+}$ sensing receptor (CASR) and functioning as a bona fide first messenger. Ca$^{2+}$ liberated from intercellular stores acts as a second messenger, and the Ca$^{2+}$ from calcium induced calcium release (CICR) acts as a third messenger. Ca$^{2+}$ can also auto regulate its messenger function by controlling the production and movements of Ca$^{2+}$ inside through membrane transporters (Carafoli, 2004a). Ca$^{2+}$ signals are essential for proper cellular functions but if the concentration and movements of Ca$^{2+}$ inside the cell becomes abnormal it causes apoptosis/necrosis. The Ca$^{2+}$ signaling mediating death of the cells exposed to toxic environment is often seen as negative connotation where as terminating the cell life by apoptosis is seen as positive connotation (Carafoli, 2004b).

1.13 Cytosolic Ca$^{2+}$ entry and release:

1.13. A. Extracellular sources:

Changes in cytosolic Ca$^{2+}$ concentration can be provided by two major sources; extracellular environment and intracellular stores. Extracellular Ca$^{2+}$ can get into cells through different plasma membrane channels based on their activation mechanism
(Berridge, Lipp, & Bootman, 2000). Some of the major Ca$^{2+}$ channels are voltage
operated Ca$^{2+}$ channels (VOCC), receptor operated Ca$^{2+}$ channels (ROCC), mechanically
activated Ca$^{2+}$ channels (MOCC) and store operated Ca$^{2+}$ channels (SOCC).

Excitable cell types like muscle and neuronal cells employ VOCC. These
channels are activated by depolarization of plasma membrane (PM). Mammalian VOCCs
are formed as a complex of five protein subunits ($\alpha_1$, $\alpha_2\delta$, $\beta_1-4$, and $\gamma$). The $\alpha_1$ subunit acts
as the Ca$^{2+}$ conducting portion of the channel while the associated subunit regulates
channel gating (Bootman, et al., 2001). VOCCs are classified into two classes: high
voltage activated channels (HVA) and low voltage activated channels (LVA). HVA are
further divided into different types; L-type channel (long lasting/DHP receptor), P-type
(purkinje)/Q-type channel and N-type channel (neural). Although usually grouped as
HVA channel, R-type Ca$^{2+}$ (residual) channels falls under intermediate voltage activated
channels (Dolphin, 2006). The only known LVA channel is T-type (transient) channel.

ROCCs are structurally and functionally diverse. Secretory cells and nerve
terminal endings have large amount of receptor-operated Ca$^{2+}$ channels. Agonist binding
to the extracellular domain of the channel typically activates ROCCs. Nicotinic
acetylcholine receptor and the N-methyl- D-aspartate receptor are examples of ROCCs.
Although nicotinic acetylcholine receptor is mainly permeable to Na$^+$ and K$^+$, $\alpha$ nAChR
is highly permeable to Ca$^{2+}$ (Bertrand, Galzi, Devillers-Thiery, Bertrand, & Changeux,
1993). These types of channels are activated by wide variety of agonist such as ATP,
serotonin, glutamate and acetylcholine (Bootman, et al., 2001).

Mechanically operated Ca$^{2+}$ channels are present in many cell types and these
channels are activated by mechanical deformation of cell membrane in response to
stress/shape changes of a cell. Stretching heart cells can lead to Ca\(^{2+}\) signaling and subsequent release of paracrine factors leading to cardiac hypertrophy (Dostal & Baker, 1998). Another example of MOCC is epithelial cells of trachea where deformation of a single cell radiates Ca\(^{2+}\) wave that synchronizes the Ca\(^{2+}\) sensitive beating of neighboring cilia cells (Boitano, Dirksen, & Sanderson, 1992).

First indentified in non-excitable cells, store operated Ca\(^{2+}\) channels (SOCCs) plays a major role in mode of Ca\(^{2+}\) influx (Berridge, 1995) termed as store operated Ca\(^{2+}\) entry (SOCE). SOCCs are activated in response to intracellular Ca\(^{2+}\) store depletion. Store operated Ca\(^{2+}\) entry is explained in detail in the following section. In addition, there is another set of Ca\(^{2+}\) channels that are store independent. Although these channels were once implicated as SOCCs, the activation of these types of channels does not require intracellular store depletion. Intracellular lipid messengers such as diacylglycerol and arachidonic acid can activate these channels. Activation of these channel leads to substantial Ca\(^{2+}\) influx without requiring intracellular Ca\(^{2+}\) store depletion (Mignen & Shuttleworth, 2000). Thus, this mode of activation is independent of store depletion and requires a chemical activator or physical activator (i.e temperature).

1.13. B. Intracellular sources/stores:

There are two main types of Ca\(^{2+}\) channels that are present in sarco/endoplasmic reticulum (SR/ER) that empties the store content. They are inositol 1, 4, 5- trisphosphate receptor (IP\(_3\)R) and Ryanodine receptors (RYRs) (Berridge, 1993a). The IP\(_3\) (inositol 1, 4, 5- trisphosphate) acts as a second messenger of Ca\(^{2+}\) controlling many cellular processes by generating internal Ca\(^{2+}\) signals. Two major receptor-mediated pathways stimulate the formation of IP\(_3\). Ligands binding to GPCRs on the PM leads to the
activation of phospholipase Cβ1 (PLCβ1) and ligands binding to tyrosine receptor kinases activate phospholipase Cγ1 (PLCγ1). The activated PLCβ1 and PLCγ1 catalyze the hydrolysis of phosphatidylinositol 4, 5-biphosphate (PIP2) to produce IP3 and diacylglycerol (DAG) (Berridge, 1993b). DAG stays in the membrane whereas IP3 is highly soluble and easily diffuses into the cytoplasm and binds to and activates the IP3 receptor on the ER/SR. The binding of IP3 to IP3 receptors causes conformational changes of the receptor, which opens a Ca2+ channels, resulting in the release of Ca2+. IP3 receptors are tetramers which mediate Ca2+ release from internal stores by acting as ligand-gated channels (Foskett, White, Cheung, & Mak, 2007). The IP3 receptor has 3 domains: an inositol triphosphate binding domain near the N terminus where IP3 binds to the receptor, a transmembrane spanning domain near the C terminus which anchors the protein in the ER membrane and a coupling domain in the middle of the molecule (Foskett, et al., 2007). The IP3 receptor family has three members termed as Type1, 2 and 3, and broadly distributed in various tissues.

Ryanodine receptors (RYRs) are a class of intracellular Ca2+ channels found in muscles and neurons. Although IP3R engage in Ca2+ induced Ca2+ release (CICR), RYR mediates most of CICR in animal cells (Endo, 1977). There are multiple isoforms of ryanodine receptors such as RYR1, RYR2 and RYR3 and fourth isoforms found only in fish (Bennett, et al., 1996). RYR1 found in skeletal muscle and certain neurons, RYR2 found in cardiac muscle and brain, RYR3 found in smooth muscle and brain. Ryanodine receptors are controlled by Ca2+ and by cyclic ADP ribose (cADPR) messenger (Berridge, 1993a). cADPR normally binds to RYR2 and also to RYR3 and it has no effect on RYR1. Ryanodine receptor share many similarities with IP3 receptor and causes Ca2+...
induced Ca\(^{2+}\) release by recognizing cytosolic Ca\(^{2+}\) near the receptor thus providing a positive feedback mechanism (Berridge, 1993a).

The IP\(_3\)R and RYR are highly Ca\(^{2+}\) sensitive which makes them important for generation of complex patterns of Ca\(^{2+}\) signaling. Ca\(^{2+}\) has a biphasic effect on these channels. These channels are opened when concentration of Ca\(^{2+}\) increases as a positive feedback but once concentration of Ca\(^{2+}\) reaches certain level it exerts the negative feedback and inhibits the channel. In general, the opening of IP\(_3\)R and RYRs are modulated by many factors such as phosphorylation, adenine nucleotides, thiol reactive compounds, pH and Ca\(^{2+}\) content of ER/SR (Berridge, et al., 2000).

Besides these two major receptors in ER, sphingolipid Ca\(^{2+}\) release mediating protein of endoplasmic reticulum (SCaMPER) is expressed in cardiac muscle, pancreas and liver and nicotinic acid adenine dinucleotide phosphate receptor (NAADPR) are less characterized but also known to cause Ca\(^{2+}\) release into cytoplasm from intracellular stores (Betto, et al., 1997).

**1.13. C. Ca\(^{2+}\) homeostasis:**

Cytosolic Ca\(^{2+}\) homeostasis is achieved by balancing the intracellular Ca\(^{2+}\) increases by removal of Ca\(^{2+}\) using pumps that are present in ER or PM. Plasma Membrane Ca\(^{2+}\) ATPase (PMCA) in PM and Sarco/Endoplasmic Reticulum Ca\(^{2+}\) ATPase (SERCA) in ER are the two major Ca\(^{2+}\) pumps that pumps Ca\(^{2+}\) outside cytoplasm and ensure that cytosolic Ca\(^{2+}\) concentration remains low and stores are loaded with right amount of Ca\(^{2+}\) (Lipskaia & Lompre, 2004). In addition mitochondria play an important role in regulating cytosolic Ca\(^{2+}\) concentration primarily through the production of ATP, but also via uniporter, Ca\(^{2+}\) transporter and Ca\(^{2+}\) exchangers resident in the inner
mitochondria membrane. Ca$^{2+}$ uptake into mitochondria is performed by mitochondrial Ca$^{2+}$ uniporter driven by mitochondrial membrane potential. Under specific conditions, Ca$^{2+}$ can be released into cytoplasm by H$^+$/Ca$^{2+}$ and Na$^{2+}$/Ca$^{2+}$ exchangers, and the permeability transition pore (PTP) (Rizzuto, Bernardi, & Pozzan, 2000). All these channels, stores and pumps act in co-ordination in shaping cytosolic Ca$^{2+}$ signals necessary for effective Ca$^{2+}$ signaling.

1. 14 Ca$^{2+}$ signals:

Ca$^{2+}$ concentration increases in the cytoplasm when plasma membrane Ca$^{2+}$ channels and intracellular store receptors are activated. However, the channels open only for short time allowing Ca$^{2+}$ in pulses. Since Ca$^{2+}$ increases are seen in vast majority of cells, identifying and characterizing the Ca$^{2+}$ signals might give better understanding of Ca$^{2+}$ signaling processes.

1.14.1. Elementary and global signaling:

Ca$^{2+}$ can mobilize itself to various signaling forms. The two main types of Ca$^{2+}$ signaling events are; elementary events (puffs, sparks and oscillations) and global events (regenerative waves) (Bootman & Berridge, 1995; Lipp, Thomas, Berridge, & Bootman, 1997). Wave form of Ca$^{2+}$ signaling was first observed in fertilized oocytes in 1977 (Ridgway, Gilkey, & Jaffe, 1977). Such wave forms were later termed as global Ca$^{2+}$ signaling (Gilkey, Jaffe, Ridgway, & Reynolds, 1978). Global Ca$^{2+}$ signals pass between same cell types. Such intercellular Ca$^{2+}$ signals happen through gap junction of coupled cells. In cilia on tracheal epithelial cells intercellular Ca$^{2+}$ waves are required for spreading activation. Ca$^{2+}$ signals can also travel between different cell types such as smooth muscle/endothelial cells and neurons/glial cells (Bootman, et al., 2001).
Elementary Ca\(^{2+}\) signals are responsible for generation of global signals because sustained Ca\(^{2+}\) release in cytoplasm is toxic, cells prefer Ca\(^{2+}\) in pulses or spikes or oscillations (Bootman & Berridge, 1995). This type of repetitive Ca\(^{2+}\) signal was first observed in hepatocytes in response to hormonal stimulation (Hajnoczky, Robb-Gaspers, Seitz, & Thomas, 1995). Periodic opening of plasma membrane or periodic emptying of stores makes repetitive spikes of Ca\(^{2+}\) possible. The frequency of Ca\(^{2+}\) spikes depends on the concentration of hormones or ligands and also on the duration of agonist action on receptors. Pulsatile Ca\(^{2+}\) signals are less prone to noisy fluctuations and hence they are much preferred and have higher fidelity of information transfer (Bootman, et al., 2001). However, the intimate mechanism for the initiation of the spiking is still unclear.

Specific gene expression depends on specific type of Ca\(^{2+}\) signaling. For example, transient Ca\(^{2+}\) spike through VOCC activated CREB (cAMP-responsive element binding protein) where as sustained increase in cytosolic Ca\(^{2+}\) is necessary for activation of the transcription factor, NFAT (nuclear factor of activated T-cells) (Cartin, et al., 2000; Dolmetsch, Lewis, Goodnow, & Healy, 1997). Thus, alteration of Ca\(^{2+}\) signaling can result to re program genetic expression.

1.15 **SOCE:**

Ca\(^{2+}\) can get into cytoplasm through many channels in plasma membrane (PM) as described above and one of the least characterized molecularly and a channel of my research interest is the SOCC. The story of SOCE started when Michell in 1975 (Michell, 1975) proposed that cleavage of membrane inositol causes generation of Ca\(^{2+}\) signals. Kinetic study of intracellular Ca\(^{2+}\) refilling following depletion suggested that release and entry of Ca\(^{2+}\) across PM might be linked by common mechanism and this was speculated.
even before the discovery of IP3 (Parod & Putney, 1978; J. W. Putney, Jr., 1977). In 1983 Berridge (Berridge, 1983) reported that IP3, the breakdown product of membrane inositol causes release of Ca^{2+} from intracellular stores. It was Putney (J. W. Putney, Jr., 1986) who formulated a hypothesis that filling state of intracellular Ca^{2+} store signals the activation of plasma membrane channels and named them capacitative Ca^{2+} entry. The idea of capacitative Ca^{2+} entry was postulated as Ca^{2+} entry was shown to persist even after the removal of phospholipase C- activating agonist (Takemura & Putney, 1989) and gained acceptance after the discovery of thapsigargin (Thastrup, Linnebjerg, Bjerrum, Knudsen, & Christensen, 1987). Thapsigargin inhibits SERCA pump, releasing intracellular Ca^{2+} which doesn’t require the activation of phospholipase C or the formation of IP3 and this was noted first in parotid acinar cells (Jackson, Patterson, Thastrup, & Hanley, 1988). Similarly, cyclopiazonic acid and 2, 5,-di-(tert-butyl)-1, 4-benzohydroquinone depletion of endoplasmic reticulum activates the Ca^{2+} entry (Deng & Kwan, 1991; Oldershaw & Taylor, 1990). The term capacitative Ca^{2+} entry becomes a misnomer as it was initially thought that the store and the channels behaves like capacitor and resistor in series circuit i.e., charge must load a capacitor before current can flow through it and the Ca^{2+} store provided a reservoir of Ca^{2+}. Store operated Ca^{2+} entry (SOCE) is now more accepted and accurate description of the mechanism.

1.16 Mechanism of SOCE:

Following the work of Berridge (Berridge, 1983), Thastrup (Thastrup, et al., 1987) and Putney(J. W. Putney, Jr., 1986), the process of SOCE was well known. This process was characterized by activation of PM receptor coupled with G protein that leads to production of IP3, which activates the IP3 receptor in ER (as described intracellular
store section) in causing store depletion. The depletion of intracellular stores activates SOC channels and causes Ca\(^{2+}\) influx from extracellular environment and hence the name SOCE. SOCE also refills the intracellular stores.

### 1.17 SOCE in various cell types:

SOCE has been found in many cell types especially in non-excitable cell types. SOCE is best characterized in lymphocytes. SOCE is the major Ca\(^{2+}\) influx pathway in lymphocytes where it controls various cellular events like cell proliferation, cytokine production, regulation of transcription factors, and organization of the cytoskeleton and cell motility (Lewis, 2001). Glioma C6 also exhibits SOCE and is one of the best models to study SOCE since they do not have voltage-operated Ca\(^{2+}\) channels and lack active purinergic receptors (Baranska, Przybylek, & Sabala, 1999).

SOCE was thought to be an integral part of non-excitable cells. However, it has been recently shown that numerous types of excitable cells also exhibit SOCE (Obukhov & Nowycky, 2002). SOCE activated by thapsigargin depletion of intracellular Ca\(^{2+}\) stores in dorsal root ganglion neurons is known to inhibit neurite initiation and elongation (Mattson, et al., 2000). SOCE has also been implicated in taste receptor function, long term potentiation and also it has been found in different smooth and skeletal muscle lines (Hopf, Reddy, Hong, & Steinhardt, 1996; Perez, Margolskee, Kinnamon, & Ogura, 2003).

### 1.18 Role of SOCE in cell growth, differentiation and apoptosis:

Cellular Ca\(^{2+}\) homeostasis plays an important role in a cell’s life and function. Waldron (Waldron, Short, & Gill, 1997) reported that ER Ca\(^{2+}\) homeostasis is crucial for cell growth and the Ca\(^{2+}\) signal are necessary for induction of expression of many
immediate–early response genes. SOCE mediate cellular processes by influencing ER Ca\(^{2+}\) homeostasis. Various laboratories showed evidence for SOCE importance in cell division, proliferation, apoptosis and differentiation. For example, Golovina found that SOCE regulates cell division (Golovina, et al., 2001) and inhibition of store operated Ca\(^{2+}\) entry inhibits cell growth and cell proliferation (Gill, et al., 1996). Ca\(^{2+}\) oscillations that are important for gene expression could be changed by disturbing SOCE (Enfissi, Prigent, Colosetti, & Capiod, 2004). Lewis showed that SOCE is very important for lymphocyte proliferation (Lewis, 2001). Enfissi reported that SOCE is very well correlated with cell proliferation in hepatoma cells and proliferation of hepatoma cells were inhibited by SOC blockers (Enfissi, et al., 2004).

SOCE has been also shown to play a role in cell differentiation. Wu (X. Wu, Zagranichnaya, Gurda, Eves, & Villereal, 2004) showed that SOCE plays an important role in differentiation of H19-7 neuronal cells and knocking down a particular set of proteins result in reduced SOCE and causes apoptosis instead of proliferation. Also, expression of SOC channels and SOCE has been shown to induce differentiation in various cells such as muscle cells, monocytic line U937 and HL60 cells (Floto, Mahaut-Smith, Allen, & Somasundaram, 1996; Gardner, Balasubramanyam, & Studzinski, 1997). Furthermore, in dendritic cells, SOCE is the main Ca\(^{2+}\) influx pathway that promotes cell maturation and same pattern is observed in monocytes and bone marrow (Hsu, et al., 2001; Koski, et al., 1999).

SOCE has been linked to cell apoptosis. Vandenabeele (Vanden Abeele, Roudbaraki, Shuba, Skryma, & Prevaskaya, 2003) showed that inhibition of SOCE augments cell death in prostate cancer cells and results in great enhancement of ER
depletion-induced apoptosis in osteoclasts (Mentaverri, Kamel, & Brazier, 2003). Also, SOCE that activates the mature T-cells causes apoptosis in immature T-cells (Blackshaw, et al., 2000). Consistent with a role for SOCE in apoptosis, high concentration of IP3 receptors which can activate SOCE is linked to apoptosis in lymphocytes and prostate cancer cells (Blackshaw, et al., 2000).

1.19 Role of SOCE in other cell functions:

SOCE has been found to play a role in cell shape, motility, secretion and neurotransmission signaling. This section will briefly describe some of the reports that link SOCE to various cell functions. SOCE regulates exocytosis and release of various hormones and neurotransmitters. Emptage (Emptage, Reid, & Fine, 2001) showed that SOCE regulates spontaneous release of neurotransmitters in neurons and it’s also one of the regulators of synaptic plasticity. It has been also shown that in hippocampal neurons SOCE is involved in neurotransmitter release (Bouron, 2000). In non-excitable cells, the role of SOCE in exocytosis (Parekh & Penner, 1997) has been well documented. SOCE influx occurs in many endothelial cells such as vascular and pulmonary endothelium and endothelial SOCE was responsible for nitric oxide production and prostaglandin synthesis (Freichel, et al., 2004).

1.20 SOCE and diseases:

SOCE maintains Ca2+ homeostasis in cell and dysregulation of SOCE leads to various diseases. For example, in acute pancreatitis, premature activation of trypsin precursor by prolonged cytosolic Ca2+ elevation by SOCE leads to autodigestion of the pancreas (Parekh, 2000). SOCE has also been implicated in immune system defect. Lymphocytes from primary immunodeficiency patients cannot cause T cell activation as
they have dysfunctional SOC channels (Partiseti, et al., 1994). In another disease called Duchenne’s dystrophy, the patients show enhanced SOCE, which activates Ca$^{2+}$-dependent proteases beyond normal levels (Vandebrouck, Martin, Colson-Van Schoor, Debaix, & Gailly, 2002). Another group has shown, significant deficits of SOCE in neurofibromatosis NF1 (Korkiamaki, Yla-Outinen, Koivunen, Karvonen, & Peltonen, 2002).

The role of SOCE in cancer cell is controversial and also not thoroughly investigated. However, there are some cases where a particular SOCE channels have been implicated to play a role in cancer. SOCE is also linked to cell tumor genesis. Kohn (Kohn, et al., 1996) showed that lack of SOCE can inhibit production of metalloproteinase MMP-2 and attenuate collagenolysis and inhibition of SOCE by CAI(Carboxyamido-triazole) stops angiogenesis, cancer cell proliferation and invasion. In addition, Yang (S. Yang, Zhang, & Huang, 2009) showed that SOCE channel proteins are critical for breast tumor migration and metastasis.

In addition to classical transient receptor potential (TRP) channels, TRPV6 expression and its involvement in SOCE in proliferation and apoptosis of cancer cells make SOC channels modulator as a potent target for anticancer therapy (Vanden Abeele, et al., 2003). Also certain SOCE channel blockers act as cancer therapy agent. Kohn reported that CAI, a Ca$^{2+}$ influx inhibitor is one of the best candidates for cancer therapy drugs as they stop proliferation, invasion and tumor angiogenesis of cancer cells (Kohn, et al., 1996). Since SOCE channels and it blockers have been linked to cancer, studying SOC channels and identifying the molecular players of SOCE regulation might pave a way to find novel drugs or therapy for certain cancers.
1.21 Molecular candidates for SOCE:

Much effort has been invested in identifying the genes that encode for SOCE and understanding how store depletion activates the Ca^{2+} entry. The genes that encode the SOCCs remain controversial in spite of intense investigation and recent discoveries.

Initial focus was placed on the transient receptor potential (TRP) mutant in *Drosophila* was found to have defective Ca^{2+} influx. Moreover, the TRP channel was found to be selective for Ca^{2+} ions suggesting that TRP might be the long sought store operated channel (Hardie & Minke, 1993). Hardie and Minke showed that the *Drosophila* TRP was activated downstream of phospholipase C. Hence, the best candidate for studying SOCE was thought to be a mammalian homologous of the *Drosophila* trp genes (Harteneck, Plant, & Schultz, 2000). Many of the early results from various labs encouraged the idea of TRP channels as potential SOCC genes. Mammalian TRP super families have been divided into seven subfamilies. Ca^{2+} signaling have been reported in the three major subfamilies namely TRPC, TRPV and TRPM, all of which have six trans-membrane domains (Minke & Cook, 2002). TRPC stands for classical or canonical channel due to the highest homology with the molecular identity of TRP channels in *Drosophila*. There are at least seven members (TRPC 1–7) of this subfamily. TRPC2 is a pseudogene in man. TRPV, where V stands for vanilloid, channels (TRPV 1–6) are closely related to the vanilloid receptors. In TRPM, M stands for melastatin (a tumor suppressor) (Nilius, 2003).

1.21.1 TRPC1:

TRPC were the first mammalian TRPs to be cloned. Based on structural and functional similarity, TRP channels fall in two groups: TRPC1, 4 and 5 and TRPC3, 6 and 7.
Among all the TRPC channels, the role of TRPC1 in store operated influx is extensively studied and argued. The functional expression of human TRPC1 in COS cells by Zhu (Zhu, et al., 1996) showed modest increase in Thapsigargin-evoked Ca$^{2+}$ influx. Furthermore, whole cells recording on CHO cells expressing TRPC1 by Zitt (Zitt, et al., 1996) showed IP$_3$ or Thapsigargin activates non selective currents suggesting that TRPC1 encoded a nonselective channel gated by store depletion. Ambudkar (Liu, et al., 2000) showed that TRPC1 is intimately involved in store operated entry in salivary gland cells. Over expression of mutated TRPC1 in HSG cells significantly reduced store operated Ca$^{2+}$ channel activity by 70%. The role of TRPC1 in store operated Ca$^{2+}$ entry is further validated by knockdown and inhibition strategies. Antisense oligonucleotides directed against TRPC1 in A549 endothelial cells reduced thapsigargin-induced Ca$^{2+}$ signals by 25% and the endogenous store operated current called $I_{soc}$ by 50% (S. Wu, et al., 2001). Mori (Mori, et al., 2002) showed TRPC1 was expressed in mouse spleen and in a mutant DT40 B cell lines, and knock down of TRPC1 in DT40 cells demonstrated that store operated current i.e, Ca$^{2+}$ release activated Ca$^{2+}$ current ($I_{CRAC}$) was lost in majority of cells. All this evidence supports the idea that TRPC1 might play a role in store operated Ca$^{2+}$ influx. Although Zitt lab (Zitt, et al., 1996), Ambudkar lab (Liu, et al., 2000) and Zhu lab (Zhu, et al., 1996) showed convincing evidence that TRPC1 was store operated channel, other labs have failed to observe store operated activity of exogenously expressed TRPC1 (Lintschinger, et al., 2000; Sinkins, Estacion, & Schilling, 1998; Strubing, Krapivinsky, Krapivinsky, & Clapham, 2001).
1.21.2 TRPC3:

Over expression of TRPC3 on HEK293 cells showed enhanced store operated Ca\(^{2+}\) (Hurst, Zhu, Boulay, Birnbaumer, & Stefani, 1998). The studies conducted on TRPC3 channels by Muallem’s (Kiselyov, Mignery, Zhu, & Muallem, 1999) and Birnbaumers’s lab (Kiselyov, et al., 1998; Z. Zhang, et al., 2001) showed that TRPC3 are store operated Ca\(^{2+}\) channels and these channels were activated by interaction with IP\(_3\) receptors in the ER membrane. Based on the level of expression of the TRPC3 protein, Vazquez (Vazquez, Lievremont, St, & Putney, 2001) showed that these channels display difference in pharmacology and modes of regulation. The idea of TRPC3 as store operated Ca\(^{2+}\) channel was seriously challenged by Zitt (Zitt, et al., 1997) and Zhu lab (Zhu, Jiang, & Birnbaumer, 1998) and Hoffmann (Hofmann, et al., 1999) showed that TRPC3 and TRPC6 channels expressed in CHO-k1 cells were not activated by store depletion. Recently, Smedlund and Vazquez have shown TRPC3 can function as a constitutively active channel (Smedlund, Tano, & Vazquez).

1.21.3 TRPC4 and TRPC5:

TRPC4 and TRPC5 have a close structural relationship by phylogenetic analysis (Philipp, et al., 1998). Philip lab (Philipp, et al., 1996) was first to clone TRPC4 and reported that they act like store operated Ca\(^{2+}\) channels when expressed in HEK293 cells. Stable expression of TRPC4 in CHO cells also resulted in store operated Ca\(^{2+}\) entry (Warnat, Philipp, Zimmer, Flockerzi, & Cavalie, 1999). Adrenal cortex has abundant TRPC4 and TRPC4 expressed in bovine adrenal cortex cell line SBAC exhibits small store operated CRAC like current. Antisense oligonucleotide against TRPC4 resulted in 50% reduction in store operated current (Philipp, et al., 2000). Two-fold potentiation of
external Ca\(^{2+}\) dependent chloride currents were seen in Xenopus oocytes that were over expressed with rat TRPC4 (Tomita, et al., 1998). Furthermore, knock down of TRPC4 impaired the ability of acetylcholine to relax precontracted aortic smooth muscle strips (Freichel, et al., 2001). However, Schaefer (Schaefer, et al., 2000) found that in HEK293 cells over expressing TRPC4 and TRPC5 store depletion failed to induce any whole currents suggesting that murine TRPC4 and TRPC5 are not store operated. Using an antisense oligonucleotide against TRPC4 in HEK293 cells, Wu (X. Wu, Babnigg, Zagranichnaya, & Villereal, 2002) found that TRPC4 was not involved in store-operated entry but they exhibit arachidonic acid-dependent Ca\(^{2+}\) influx.

1.21.4 TRPC6 and TRPC7:

The TRPC6 appears to be Diacylglycerol (DAG)-activated (Hofmann, et al., 1999) and there were no published reports suggesting its role in store operated Ca\(^{2+}\) entry. TRPC7 is the least studied of the TRP channels and Mori (Okada, et al., 1998) demonstrated that these channels are not store operated Ca\(^{2+}\) channel and in fact they are DAG-operated.

1.21.5 New molecular players:

For a decade TRP channels were thought to be store operated Ca\(^{2+}\) channels and initial experimental results from various lab were encouraging. Although under certain circumstances TRPC channels can exhibit store operated Ca\(^{2+}\) activity when ectopically expressed and SOCE is diminished whenever TRPC expression is suppressed (Vazquez, et al., 2001). However, subsequent work on *Drosophila* TRP channels demonstrated that these channels are not clearly store-operated Ca\(^{2+}\) influx channels (Schaefer, et al., 2000; Zitt, et al., 1997). The major problem in accepting these channels as store operated Ca\(^{2+}\)
entry is underscored by the observation that they don’t recapitulate the properties of $I_{CRAC}$ when they were experimentally introduced in the cells. Recent work in endothelial cells provided solid evidence that TRPC channels are not store operated; Abdullev showed STIM1 (Stromal Interacting Molecule1) and Orai1 mediate CRAC current and store operated $Ca^{2+}$ entry (Abdullaev, et al., 2008). These two recently discovered molecular players play an important role in store operated $Ca^{2+}$ entry as described below. RNAi screening experiments have identified two major proteins in store operated $Ca^{2+}$ entry; STIM and Orai.

1.21.2. A. STIM:

STIM is an acronym for Stromal Interacting Molecule and this protein had been known earlier for its role as tumor suppressing gene product (Sabbioni, Barbanti-Brodano, Croce, & Negrini, 1997). RNAi screening experiments in *Drosophila* S2 cells (Roos, et al., 2005) and human-derived HeLa cells (Liou, et al., 2005) showed STIM1 plays a major role in store operated entry. STIM1 is a single transmembrane domain containing protein located in ER with N-terminal stretching into ER lumen and C-terminal in cytoplasm (Liou, et al., 2005) shown in Figure 1-21.2A. The N-terminal has a single EF-hand $Ca^{2+}$ binding motif that acts as luminal $Ca^{2+}$ sensor and also sterile-alpha motif (SAM) (Liou, et al., 2005; Smyth, et al., 2006). The C-terminal has glutamate, serine/proline, serine/threonine and lysine rich region and it also has ezrin-radixin-moesin (ERM) like domain and two coiled regions overlapping ERM domains (Huang, et al., 2006) shown in Figure 1-21.2A. Mammals have additional protein in STIM family i.e, STIM2, Figure 1-21.2A, which is structurally related to STIM1 (Zheng, Stathopulos, Li, & Ikura, 2008). Soboloff (Soboloff, Spassova, Hewavitharana, et al., 2006) showed that
STIM1 is found in both PM and ER whereas STIM2 is located in ER not in PM. Its unclear about the variants of STIM1 that resides both in PM and ER. STIM1 activates the store operated entry by sensing the decrease in luminal Ca$^{2+}$ concentration and forming a punctae that couples with Orai1 channels in plasma membrane (Lewis, 2007). These diverse actions are made possible due to its distinct structure. Based on mutation and deletion studies it has been shown that each of its domains plays a crucial role in the process. Deletion of SAM domain and lysine-rich domain prevents punctae formation and hence CRAC activation and disrupting the C-terminus after ERM domain leads to defective STIM1 redistribution and fails to activate Orai1 channels (Baba, et al., 2006; Liou, Fivaz, Inoue, & Meyer, 2007). In general STIM1 mutant lacking the whole C-terminal fails to co-localize with ORAI1 upon Store depletion (Li, et al., 2007). All these mutant and deletion studies points out that each of the domain plays its role in store operate Ca$^{2+}$ entry and disruptions of any one of its structure collapses the whole process. Mammalian STIM1 forms heteromers with STIM2 and Soboloff (Soboloff, Spassova, Hewavitharana, et al., 2006) showed that STIM2 is a potent SOC inhibitor when expressed in HEK293, PC12, A7r5 and Jurkat T cells. It has been also shown that the Ca$^{2+}$ sensing protein, STIM also plays a role in ER integrity.

1.21.2. B. Orai:

RNAi screening experiments in Drosophila S2 cells and elucidation of STIM1 as Ca$^{2+}$ sensor protein allowed for the identification of Orai (Feske, et al., 2006; Vig, Beck, et al., 2006; S. L. Zhang, et al., 2006). This protein was discovered simultaneously by three independent laboratories: Feske (Feske, et al., 2006), Vig (Vig, Peinelt, et al., 2006) and Zhang (S. L. Zhang, et al., 2006). This protein has been known by different names in
literature as CRACM (CRAC modulator), olf186-F and Orai. However, Orai is widely accepted to denote this protein. There are three human homologs that have been found so far, termed as Orai1, Orai2 and Orai3 as shown in Figure 1-21.2B. Linkage analysis showed that single point mutation at position 91 of human Orai1 results in defective T-cell signaling that leads to severe combined immunodeficiency (SCID) syndrome (Feske, et al., 2006). The structure of Orai does not bear homology to any known channel and all three Orai proteins have four transmembrane domains (Cahalan, et al., 2007). Both the N-terminal and C-terminal lies in cytoplasm. N-terminal has proline/arginine –rich region and C-terminal region has coiled-coil domain, a common protein interaction motif (Cahalan, et al., 2007) as shown in Figure 1-21.2B. Co-expression of STIM1 and Orai1 fully reconstitute CRAC currents (Peinelt, et al., 2006). Orai is a pore forming subunit of CRAC channels, which is evident from the single amino acid mutations. Mutation of glutamate to alanine at position 106 in human Orai1 of first trans-membrane region results in complete loss of function and it serves as a dominant negative (Feske, et al., 2006; Prakriya, et al., 2006). Also, mutation of second glutamate at position 190 in human Orai1 leads to reduction in channel selectivity (Vig, Beck, et al., 2006). These two glutamate residues are well conserved in all three Orais, Figure 1-21.2B and also in various species.

1.22 STIM/ Orai coupling and SOCE:

To activate the store operated Ca\(^{2+}\) entry following store depletion, the STIM1 molecules must convey the message to Orai channels. There are two proposed mechanisms. The first one is the straight forward model that involves direct protein-protein interaction of STIM1 and Orai 1 proteins (Lewis, 2007). As per the model,
STIM1 and Orai 1 are dispersed throughout the ER and PM respectively in resting cells. Following store depletion STIM1 accumulates at junctional ER locations where ER is juxtaposed to the PM. Then the STIM1 couples with Orai1 protein in PM that resides directly opposite to the STIM1 clusters (Luik, Wu, Buchanan, & Lewis, 2006). There has been considerable evidence to prove this model. Yeromin (Yeromin, et al., 2006) showed that STIM1-Orai interact each other following store depletion by co-immunoprecipitation. Barr (Barr, et al., 2008), Prakriya (Navarro-Borelly, et al., 2008), Calloway (Calloway, Vig, Kinet, Holowka, & Baird, 2009) and Muik (Muik, et al., 2008) demonstrated the interaction of STIM1 and Orai1 using fused fluorophores and fluorescence resonance energy transfer (FRET) method. They reported that STIM1 and Orai1 come sufficiently closer to produce FRET signals. Muik (Muik, et al., 2009), Yuan (Yuan, et al., 2009) and Park (Park, et al., 2009) reported that peptide fragments of STIM1 C-terminals activate the Orai channels. The second method for the mechanism of activation postulates a soluble Ca\textsuperscript{2+} influx factor (CIF) (Randriamampita & Tsien, 1993). Following store depletion STIM1 triggers the production of CIF, which transmits the signal from STIM1 to ORAI1. The CIF stimulates lysophospholipid generation by displacing calmodulin (CAM) from phospholipase A2 and thereby activating the CRAC proteins (Csutora, et al., 2008). However, lack of solid evidence for second theory and unknown structure of the CIF makes it more complicated. So, the direct protein-protein interaction theory is widely accepted now.

1.23 Orai /STIM Stoichiometry:

STIM and Orai protein interaction is required for the activation of SOCE. These proteins form a functional ion pore which requires assembly of various subunits into
multimers (Mignen, Thompson, & Shuttleworth, 2008). Since SOCE depends on interaction of STIM and Orai proteins, studying the stoichiometry of STIM1 and Orai helps in better understanding of the process. Orai1 which is primarily responsible for SOCE in HEK 293 cells, fibroblast and human T-cells exists as a dimer in PM under resting condition (Penna, et al., 2008). However, when it forms active CRAC channels/SOCE channels it undergoes Stim-induced dimerization to form tetramer which indicates four Orai1 monomers assembles to form active SOCE/CRAC channels (Penna, et al., 2008). Employing single-molecule imaging technique, Wei (Ji, et al., 2008) demonstrated that two STIM1 molecules combine with one active CRAC/SOCE channel.

1.24 Arachidonic acid-Regulated Ca^{2+} (ARC) channels:

STIM1 and Orai1 protein which plays crucial role in SOCE also forms an integral component of ARC channels (Shuttleworth, 2009). Arachidonic acid-Regulated Ca^{2+} channels are activated by low concentration of arachidonic acid and this mechanism of activation is independent of intracellular store depletion and seen in most non-excitatory cells (Shuttleworth, 2009). STIM1 tightly regulates both CRAC/SOCE channel and ARC channels. Unlike in CRAC channels where its activation depends on STIM1 in ER, the activation of ARC channel depends on plasma membrane STIM1 (Shuttleworth, 2009; Shuttleworth, Thompson, & Mignen, 2007).

1.25 Mechanism of activation and stoichiometry of ARC channels:

ARC channels activation requires involvement of Orai3, Orai1 and STIM1 proteins residing in PM (Shuttleworth, 2009). STIM1 present in PM behaves differently than STIM1 in ER (Shuttleworth, et al., 2007). The extracellular location of N-terminal EF-hand of STIM1 residing in PM always bound to Ca^{2+} and SAM domain does not
undergoes any conformational changes unlike in CRAC/SOCE channels (Li, et al., 2007). The activation of ARC channels involves binding of arachidonic acid to PM STIM1 and/or Orai3 subunit of the active ARC channel (Shuttleworth, 2009). It has been also predicted that arachidonic acid induces an interaction between these proteins enabling the channel activity. The functional stoichiometry of Orai1, Orai3 and STIM1, which forms an ARC channel, is well elucidated by Shuttleworth. Shuttleworth demonstrated that functional ARC channel is a heteropentameric assembly of three Orai1 subunits and two Orai3 subunits and it require a pool of STIM1 residing in PM.

1.26 Orai/STIM pharmacology:

The store operated Ca\textsuperscript{2+} entry mechanism was difficult to study as there are no available specific inhibitors prior to the discovery of STIM and Orai (J. W. Putney, Jr., Broad, Braun, Lievremont, & Bird, 2001). For long time, divalent cations and trivalent lanthanides have been known to inhibit Ca\textsuperscript{2+} entry pathways. La\textsuperscript{3+} (IC\textsubscript{50} =10-100µM) and Gd\textsuperscript{3+} (IC\textsubscript{50} <1.0µM) have been widely used to inhibit Ca\textsuperscript{2+} entry (J. W. Putney, 2009). However, these ions inhibit wide range of Ca\textsuperscript{2+} entry channels and they are not specific for SOCE channels. The drugs that inhibits movements of STIM1 following store depletion such as ML-9[1-(5-chloronaph-thalene-1-sulfonyl)homopiperazine] and 2APB (2-amino-ethylidiphenyl borate) also blocks SOCE. ML-9 is a member of myosin light chain kinase inhibitors, which prevents the movement of STIM1 to the PM following store depletion, and prevents the formation of punctae (Q. K. Tran, et al., 2001). 2APB, which was originally identified as membrane permeant inhibitor of IP3 receptors, have been shown my many laboratories as potent inhibitor of store operated channels (Maruyama, Kanaji, Nakade, Kanno, & Mikoshiba, 1997). Also, in some cases 2APB
causes transient activation of $I_{\text{CRAC}}$ currents at lower concentrations due to the presence and activation of Orai3 proteins by 2APB (DeHaven, Smyth, Boyles, Bird, & Putney, 2008; Prakriya & Lewis, 2001; S. L. Zhang, et al., 2008). At 20-30 µM range, an imidazole compound SKF-96365 (1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy] ethyl-1H-imidazole hydrochloride) was shown to inhibit SOCE and $I_{\text{CRAC}}$ in various cell lines (Prakriya & Lewis, 2002). This compound also inhibits TRP channels, voltage-gated channels and potassium channels (Merritt, et al., 1990). Very specific and potent SOCE inhibitor i.e, BTP2 was first reported by Ishikawa (Ishikawa, et al., 2003). The study showed that BTP2 (Bistrifluoromethyl-pyrazole derivate) inhibits SOCE in lymphocytes (IC$_{50}$ =10nM) (Ohga, Takezawa, Arakida, Shimizu, & Ishikawa, 2008; Zitt, et al., 2004). Ishikawa (Ishikawa, et al., 2003) also showed that it inhibits interleukin -2 productions and NFAT-dependent transcription in lymphocytes In addition to these blockers; SOCE and $I_{\text{CRAC}}$ have been potently inhibited by inhibitors of PLC and polyphosphoinositide synthesis (Broad, et al., 2001).
Chapter 2: Results

2.1. Molecular identification and functional assessment of candidate Store operated calcium entry (SOCE) channels:

2.1. A. Molecular identification:

In contrast to the voltage dependent channel in excitable cells, the channels involved in Ca\(^{2+}\) entry in non-excitable cells are not well defined and very little is known about SOCE in carcinoid cell lines. However, many candidates SOCE proteins have been identified over last two decades and extensive research have been done to elucidate the mechanism of SOCE, and importantly recent breakthrough in SOCE have identified the key molecular players, namely Stromal Interaction Molecule, STIM and the ion channel protein Orai. SOCE is the major Ca\(^{2+}\) influx pathway in many cells and in lymphocytes they control proliferation (Lewis, 2001). SOCE has also been shown critical for breast cancer cell migration (S. Yang, et al., 2009). Although voltage operated calcium channels were extensively studied in neuroendocrine tumors, SOCE is least studied. So, to investigate the role of SOCE in carcinoid tumors, we therefore undertook experiments to screen/identify candidate SOCE channels in human carcinoid cell lines. Semi quantitative conventional endpoint RT-PCR was done to screen carcinoid cell lines for an array of possible SOCE channel transcripts. Carcinoid cells were trypsinized, total RNA was extracted using Qiagen RNeasy Mini Kit, the extracted RNA was reverse transcribed to
cDNA using superscript II RT and used as template for identifying candidate genes. Specific PCR primers, which identify the transcripts of candidate proteins, were designed by primer3 software. The primer sets used are given in Table2.1. PCR reactions as described in method sections were carried out using the specific primers. Products were identified based on predicted base pair size and band signal intensity was normalized to β-actin gene expressions levels. (Figure 2-1 and Figure 2-2) shows the RT-PCR results for the store operated Ca$^{2+}$ channels (SOCC) transcripts normalized to the house keeping gene, β-actin. RT-PCR profiling of carcinoid cell lines identified various channel transcripts that have been implicated as candidate proteins for SOCE. The transcripts of STIM1 and Orai1, which are now believed to be the key molecular players in SOCE, were identified for the first time to our knowledge in carcinoid cell lines. Not only have STIM1 and Orai1 proteins implicated as central players in regulating SOCE in T lymphocytes (Feske, 2007) but these proteins have also been recently tied to breast cancer cell migration and invasion (S. Yang, et al., 2009). In addition to Orai1, the other homologs such as Orai2 and Orai3 were also detected in carcinoid cell lines. Carcinoid cell lines expressed robust levels of STIM1 and Orai1 messages as seen in HEK293 cells and normal intestinal cell lines, FhsInt74.

We also identified other potential SOCCs transcripts in carcinoid cell lines. For example, we identified canonical TRP channels, (TRPC) and also the Ca$^{2+}$-selective TRPV5 and TRPV6 channels that are expressed in the intestinal mucosa. Although there is evidence that some TRP channels can acts as a SOCE channel in some contexts, the general consensus is that TRP channel activation is independent of store depletion. Nevertheless, we noticed strong expression of TRPC1 in all carcinoid cell lines tested.
Transcripts for TRPC3-7 were also identified in various carcinoid cell lines (Figure 2-2). However, the expression of certain transcripts seems to be cell line specific that depends on the sites of origin in the gut. For example, TRPC6 was exclusively identified in H727 (Figure 2-2). Some cell lines seem to lack certain transcripts. TRPC7 and TRPC5 were expressed in all carcinoid lines except BON and HC49 respectively (Figure 2-2).

Furthermore, we also identified the other TRP family channels involved in Ca^{2+} transport, temperature sensitivity or taste detection in some carcinoid lines. TRPV6, implicated in vitamin D-dependent Ca^{2+} absorption in the human small intestine were detected in low levels in all carcinoid cell lines. We also identified TRPM8 and TRPM5 transcripts in BON cells, which were consistent with the work of Mergler (Mergler, et al., 2007).

TRPV2 was identified in BON and CNDT2.5 cells and TRPV1 was detected in H727. Having identified an array of SOCE candidate genes in carcinoid cell lines, we decided to functionally assess the SOCE. Next section describes the functional assessment of SOCE in various carcinoid cells lines.

2.1. B. Functional assessment of SOCC in carcinoid cell lines:

To assess SOCE in human carcinoid cell lines, we used artificial depletion of intracellular stores with or without the addition of pharmacological blockers. In addition to pharmacology blockers, we used over expressing methods to over express candidate proteins to reconstitute SOCE and blocking the SOCE by employing dominant negative mutant. We also used gene-silencing methods to knock down specific selected protein using shRNA.
2.1. B1. Artificial activation of SOCE:

Carcinoid cell lines have variety of SOCC proteins and to assess the SOCE we used digital fluorescence imaging to monitor the Ca\(^{2+}\) dynamics in cytoplasm of the cell. To test SOCE in carcinoid cell lines, the cells were loaded with fura-2 dye in Ca\(^{2+}\) containing physiological saline (2.2mM Ca\(^{2+}\)). We then applied 30µM of cyclopiazonic acid (CPA), a reversible SERCA pump inhibitor to fura-2 loaded cells in absence of extracellular calium to deplete intracellular Ca\(^{2+}\) stores and activate SOCE pathways. CPA caused a slow transient rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) that likely reflected the leak of ER Ca\(^{2+}\) and subsequent extrusion from the cytoplasm. Re-introduction of Ca\(^{2+}\) containing solution to extracellular medium induced Ca\(^{2+}\) influx and caused rise in cytosolic Ca\(^{2+}\) concentration, which were detected by rapid rise in fluorescence signal. The general protocol for testing store operated entry is shown schematically in Figure 2-3A. All carcinoid cell lines tested by this paradigm exhibited SOCE and the averaged peak influx induced by store depletion for BON, H727, HC45, CNDT2.5 and HC49 is shown in bar graph in Figure 2-3B. To validate the rise in influx is primarily through store operated Ca\(^{2+}\) channels we enhanced the driving force of Ca\(^{2+}\) by increasing the concentration of Ca\(^{2+}\) in the extracellular medium. We observed enhanced Ca\(^{2+}\) influx following store depletion by increasing the extracellular Ca\(^{2+}\) concentration to 20 mM (Figure 2-3C&D). Furthermore, we reduced the driving force of Ca\(^{2+}\) by membrane depolarization. BON cells were depolarized by treatment with 50 mM K\(^+\) before and during the restoration of extracellular Ca\(^{2+}\) diminished the Ca\(^{2+}\) influx. To access whether this depolarization and/or activation of VOCC was caused by SOCE, we treated BON cells nifedipine to block HVA VOCCs. BON cells treated with nifedipine
produced only minor diminishment in SOC influx (Figure 2-3C&D) suggesting that the majority of Ca\(^{2+}\) entry induced by intracellular store depletion was through SOCCs and not through VOCCs.

2.1. B2. Pharmacological characterization of SOCE channels:

The SOCE channels were further characterized by using pharmacological blockers. To assess whether Ca\(^{2+}\) influx occurs through SOCCs we used number of inorganic and organic inhibitors of SOCCs that have been reported to block the Ca\(^{2+}\) influx. These blockers vary in potency and selectivity. For example, lanthanoids, such as La\(^{3+}\) and Gd\(^{3+}\) or divalent metals such as Ni\(^{2+}\) or Cd\(^{2+}\) are commonly used as inorganic blockers of SOCE. Of all the inorganic blockers, Gd\(^{3+}\) was reported to be most effective, specific and best characterized inorganic blockers. Aminoethoxydiphenyl borate (2-APB), carboxy-amidotriazole (L651582 or CAI) and N-(4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP2) are examples of organic blockers of SOCE (Bootman, et al., 2002; Munaron, Antoniotti, Fiorio Pla, & Lovisolo, 2004; J. L. Yang, Qu, Yu, Kohn, & Friedlander, 2008), out of which BTP2 is a very effective blocker of SOCE in lymphocytes.

In the following studies we chose to compare the actions of Gd\(^{3+}\), 2-APB, CAI and BTP2 on SOCE between various carcinoid cell lines. Figure 2-4A shows the representative traces showing the treatment of various pharmacological blockers of SOCE channels in BON cells. 10 µM La\(^{3+}\) blocked SOCE and the blocking was further enhanced at the 100 µM concentrations. The use of La\(^{3+}\) was highly non-specific, and toxic to cells as determined by growth assay (Data not shown). So we switched to better specific blocker, Gd\(^{3+}\).
2.1. B2.1. Gd^{3+} Treatment:

As shown in the representative traces, treatment with 1 µM of Gd^{3+} had no effect on Ca^{2+} release but significantly blocked SOCE in BON cells following re-introduction of Ca^{2+} in the extracellular medium. We also treated other carcinoid cell lines such as H727, CNDT2.5, HC45 and HC49 with 1 µM of Gd^{3+} to observe the reduction in SOC influx. We found that Gd^{3+} significantly reduced SOCE in H727, CNDT2.5 and HC49 cell lines but it has no effect in HC45 cell lines. The bar graph of average percent reduction of Ca^{2+} entry is shown in Figure 2-4B.

2.1. B2.2. 2-APB Treatment:

Treatment with 100 µM 2-APB significantly reduced the Ca^{2+} entry in H727, CNDT2.5 and HC49 cell lines. 2-APB treatment produced a complex reaction in BON cells. Treatment with 2-APB was generally ineffective at diminishing entry. However in a subset of cells, 2-APB caused reduction in SOCE in 50% of BON cells and augmented SOCE in remaining cells. This complex action of 2-APB has been previously reported for other cell lines and one possible reason for this complex action is the presence of other Orai paralogues, especially Orai3 which are known to be activated by 2-APB (DeHaven, et al., 2008).

Like Gd^{3+}, 2-APB had no effect on SOCE in HC45 cell lines. (Figure 2-4B).

2.1. B2.3. CAI treatment:

Additionally, we treated carcinoid cell lines with an anti proliferative compound called CAI, which stops angiogenesis, cancer cell proliferation and invasion by inhibiting SOCE. CAI has been shown to inhibit SOCE in breast cancer and HEK cells (Mignen, et al., 2005; J. L. Yang, et al., 2008). Treatment with 10 µM CAI reduced
the influx in midgut and hindgut carcinoid cell lines (Figure 2-4B). However, CAI had little/no effect in foregut carcinoid cell lines.

2.1. B2.4. BTP2 treatment:

We then used more potent and specific SOCE inhibitor, BTP2 to block the SOCE in carcinoid cell lines. BTP2 completely blocks SOCE in lymphocytes and inhibits interleukin-2 productions and NFAT-dependent transcription. Treatment with 1 µM BTP2 significantly blocked SOCE in BON, H727, CNDT2.5 and HC49 cells. However, these treatments had no effect on HC45 cells. The average percent reductions of SOCE following the pharmacological blockers treatments are summarized in Figure 2-4B. The observed difference in the action of SOCE blockers in different carcinoid cell lines might point to the fact that carcinoid cell lines arising from various embryonic gut regions behave differently as they expresses distinctive SOCCs. Although pharmacological inhibitors were helpful to validate SOCE in carcinoid cells, the complexity of channel expression and broad specificity limited their effectiveness at identifying SOCC. Hence we decided to do molecular studies, which are described in next section.

2.1. B3. Over expression of candidate SOCE proteins:

The screening of carcinoid cell lines revealed the expression of various SOCE transcripts. The identification of Orai homologs and STIM transcripts in carcinoid cell lines placed these candidates as a central focus in our experiments since these two proteins are implicated in SOCE in lymphocytes (Feske, 2007) and other cell types. Over expression of these proteins in HEK293 cells increased the SOCE and electrophysiological analysis showed that these two proteins largely reconstitute SOCE in HEK293 cells (Soboloff, Spassova, Tang, et al., 2006). Also, endogenous SOCCs form
an active SOCE channels in HEK293 cells (Zagranichnaya, Wu, & Villereal, 2005). Henceforth, we decided to over express these genes in carcinoid cell lines and characterize the SOCE in those cell lines. To do this we obtained wild type human Orai1, Orai3 and STIM1 plasmids from Origene. These plasmids were produced in large quantity by using Qiagen Maxi kit from the overnight culture of transformed DH5α Escherichia coli. The plasmids were transiently transfected by electroporation method using Amaxa Nucleofection system. We chose BON cells as a model in our further studies since it is an established carcinoid cell lines that exhibits some of the secretory properties of midgut carcinoid cancers and also they are no other good model system available.

In the over expression studies, the wild type plasmids were co-transfected with GFP plasmid. GFP acts as a fluorescent marker of the transfected cells. Identification of transiently transfected cells by exciting at 488 nm and monitoring fluorescence at 510 nm is shown in the Figure 2-5A. BON cells transiently transfected with Orai1 and STIM1 wild type plasmids were grown on glass coverslips for 48 hours for assessing SOCE. Although expressions of GFP proteins were seen in 4 hours after transfection, we chose to assess SOCE after 48 hours because we noted significant changes in SOCE and the cells attained their typical morphology after 48 hours. Again in these studies, the intracellular stores were depleted using CPA to assess the SOCE.

We noted that BON cells over expressed with 0.5-2 µg of STIM1 and 0.5-2 µg Orai1 wild type resulted in four to five fold increase in SOCE when compared to control, mock transfected cells (BON cells transfected with GFP plasmid alone) as shown in Figure 2-5C. The ΔCa^{2+} peak amplitude was expressed as percent of control for BON
cells over expressing STIM1 and Orai1 plasmids together (n=3, p value < 0.0001) in
Figure 2-5C. Similarly, over expression of Orai3 along with STIM1 in BON cells
significantly increased the SOCE. The ΔCa^{2+} peak amplitude was expressed as percent of
control for BON cells over expressing STIM1 and Orai3 plasmids together (n=3, p value
< 0.0009) in Figure 2-5C. Representative traces showing enhanced Ca^{2+} influxes are
shown in inset in Figure 2-5C. All the statistics were done by pairing the control and test
experiments independently done on same day. The enhanced Ca^{2+} influx observed in
BON cells overexpressing Orai1 and STIM1 together, and Orai3 and STIM1 together
suggested that these proteins might play a significant role in mediating SOCE. Next
section will address the role of these proteins in SOCE using mutant and knock down
strategies.

2.1. B4. Functional knockdown of SOCE proteins:

Having observed that Orai1, Orai3 and STIM1 over expression enhanced SOCE in BON cells we decided to silence these genes to validate if the enhanced influx is primarily through these channels. We performed site directed mutagenesis of Orai1 and Orai3 wild type plasmids as described in Stratgene mutagenesis kit. We mutated glutamate to alanine at position 106 in wild type human Orai1 plasmid and this mutation had a dominant negative (DN) function (Feske, et al., 2006; Prakriya, et al., 2006). In addition, glutamate was mutated to alanine at position 81 in wild type human Orai3 splasmid. Although this mutation does not serve as a dominant negative, it significantly reduces the Ca^{2+} influx in BON cells. For easy representation in further section we termed Orai1 DN mutation as Orai1E106A or O1DN and Orai3 mutation as Orai3E81A or ΔO3.
To validate the DN construct, we co-transfected the mutant plasmid with its corresponding wild type plasmids to suppress the enhanced Ca$^{2+}$ influx effect caused by over expression of wild type plasmids in BON cells. As predicted, we observed that the enhanced Ca$^{2+}$ influx caused by over expression of wild type Orai1 and STIM1 were completely abolished by co-expressing with its mutant protein, O1DN (Data not shown). Similarly in BON cells co-expressing wild type Orai3 and its mutant ΔO3 plasmids significantly reduced SOCE (Data not shown). The above experiments showed that mutant plasmid was functionally effective in reducing the enhanced Ca$^{2+}$ influx caused by exogenous over expression of wild type proteins. However, this did not demonstrate any role of endogenous Orai and STIM1 protein in mediating SOCE.

To address the role of endogenous Orai and STIM1 in SOCE, BON cells were transiently transfected with mutant plasmid alone and SOCE was studied. In the mutant studies, 1-2 µg of mutant plasmid was transiently transfected along with 0.5-2 µg of GFP plasmid into BON cells. Intracellular stores were depleted using CPA and SOCE was assessed after 48 hours after transfection. We observed that in BON cells transiently transfected with O1DN plasmid SOCE was completely abolished (Figure 2-6A). The ΔCa$^{2+}$ peak amplitude was expressed as percent of control for BON cells transiently transfected with O1DN plasmid (n=3, p value < 0.0001). However, complete reduction of SOCE was not observed in BON cells over expressing ΔO3 plasmid but we noted significant reduction in Ca$^{2+}$ influx as shown in Figure 2-6A. The ΔCa$^{2+}$ peak amplitude was expressed as percent of control for BON cells transiently transfected with ΔO3 plasmid (n=3, p value <0.0001). Using transient transfection approaches, these mutant studies indicate that Orai channel proteins are the vital regulators of SOCE in BON cells.
Following the mutant studies of Orai proteins, the role of Ca\(^{2+}\) sensing protein STIM1 in SOCE was studied. STIM1 that resides in ER senses the Ca\(^{2+}\) depletion from the stores and interacts with Orai proteins in PM to activate the Ca\(^{2+}\) influx. To do the targeted knock down of STIM1 we obtained shRNA STIM1 plasmid from Origene. These vectors also express GFP protein, which serves as a fluorescent marker of the transfected cells. The shRNA plasmids were transiently transfected in BON cells and SOCE was assessed after 72 hours. We observed that BON cells transiently transfected with shRNA plasmid caused significant reduction in SOCE as shown in Figure 2-6A. The \(\Delta \text{Ca}^{2+}\) peak amplitude was expressed as percent of control for BON cells transiently transfected with shRNA STIM1 plasmid (n=3, p value < 0.0001). To summarize the above findings, functional knockdown of Orai1 channel using its DN appeared to completely block the SOCE in BON cells and silencing STIM1 using shRNA significantly reduced the SOCE in BON cells. Thus, the mutant and knock down studies done above strongly suggest that STIM and Orai form a functional SOCE channels.

Having found that these channels are functionally involved in SOCE, we decided to test the protein channel activity in BON cells. Therefore, to further investigate the role of Orai1 and 3 in SOCE, we performed manganese-quenching experiment to directly track the influx. Like Ca\(^{2+}\), Mn\(^{2+}\) is a divalent cation and in many cell types Mn\(^{2+}\) seems to enter the cytosol through the same pathways as Ca\(^{2+}\). Therefore, Mn\(^{2+}\) is often used as a surrogate of Ca\(^{2+}\) to trace receptor-stimulated Ca\(^{2+}\) influx mechanisms (Fasolato, Hoth, & Penner, 1993). Manganese entry into the cells often corresponds to filling state of intracellular Ca\(^{2+}\) stores. Manganese influx into the cytoplasm quenches the fluorescence of Ca\(^{2+}\) indicator dye fura-2 F\(_{360}\) signal that corresponds to plasma membrane channel.
activity. In this set of experiments, intracellular stores were depleted by CPA in absence of Ca$^{2+}$ and manganese was subsequently added to the bath solution of BON cells over expressing mutant plasmids. Fluorescence quenching of the signal was measured by line fit to the initial phase of quenching and depicted as negative slope in a bar graph in (Figure 2-6B).

We observed greater negative slope by line fit graph in BON cells over expressing O1DN than BON cells transfected with GFP alone suggesting that O1DN reduced SOCE. However in BON cells over expressing ΔO3 plasmids, the negative slope were alike the control BON cells indicating that SOCE was not reduced. The reduction of SOCE in BON cells transfected with O1DN plasmids once again validated the previous findings that SOCE in BON cells appear to occur predominantly through Orai1. The failure to alter the SOCE in BON cells transfected with ΔO3 plasmid suggests that Orai3 may play only a contributory role in SOCE in BON cells.

Taken together, artificial depletion of intracellular stores activates SOCE in all carcinoid cell lines and pharmacological blockers were successful in blocking SOCE with varying specificity and effectiveness on different carcinoid cell lines. DN and knock down strategies reduced both endogenous SOCE and SOCE enhanced by overexpression. In the following section, physiological activation of SOCE and the findings were discussed in detail.
2.2 Physiological activator of SOCE and role of SOCE in foregut carcinoid cells

2.2. A. Physiological activator of SOCE:

In the previous chapter, depleting intracellular stores using non-physiological molecule, CPA, activated SOCE in carcinoid cell lines. The use of SERCA pump inhibitor, CPA was very helpful to functionally assess and characterize the SOCE in carcinoid cell lines. However, under physiologically relevant conditions, Ca\(^{2+}\) influx is normally generated by reduction of intracellular store content following activation of G-protein coupled receptor or growth factor receptor. Ligands binding to G protein–coupled receptors on the PM leads to the activation of phospholipase C\(\beta\)1 (PLC\(\beta\)1) and ligands binding to Tyrosine receptor kinases activates phospholipase C\(\gamma\)1 (PLC\(\gamma\)1). The activated PLC\(\beta\)1 and PLC\(\gamma\)1 catalyze the hydrolysis of phosphatidylinositol 4, 5-biphosphate (PIP\(_2\)) to produce inositol 1, 4, 5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG) (Berridge, 1993b). DAG stays in the membrane whereas IP\(_3\) is highly soluble and easily diffuses into the cytoplasm and binds to and activates the IP\(_3\) receptor on the ER/SR. The binding of IP\(_3\) to IP\(_3\) receptors causes conformational changes of the receptor, which opens a Ca\(^{2+}\) channels, resulting in the release and diminishment of store Ca\(^{2+}\) content. Muscarinic receptors (mACHRs) or G protein-coupled acetylcholine receptors are a class of G-protein couple receptors that are broadly expressed in gastrointestinal tract and have been found to regulate release of neurotransmitters, hormones, and other substances including gastrointestinal peptides (Anini & Brubaker, 2003; Anini, Hansotia, & Brubaker, 2002). The gastrointestinal tissue is rich in nerve receptors that communicates chemically or electrically to carry out its basic cellular
functions. The gastrointestinal system is well embedded and completely controlled by enteric nervous system (ENS). ENS is a subdivision of the peripheral nervous system (PNS) that are derived from neural crest capable of operating autonomously (Lundgren, Svanvik, & Jivegard, 1989). ENS includes efferent neurons, afferent neurons, and interneurons that can function independently of central nervous system (CNS) and communicate with the gut via neurotransmitter release (Lundgren, et al., 1989). One such messenger/ neurotransmitter that plays an important role in gastrointestinal signaling is acetylcholine. The submucosal neurons influence the intestinal epithelium by release of acetylcholine (Bornstein, Costa, & Furness, 1986). Acetylcholine has been shown to stimulate 5-HT secretion in BON cells primarily through muscarinic receptor activation (V. S. Tran, et al., 2004). Because muscarinic receptor was coupled to Ca\(^{2+}\) release from internal store, we decided to test whether activation of muscarinic receptor evoke SOCE in our cell model cell line, BON. To activate the muscarinic receptors we used acetylcholine receptor agonist carbachol (CCh). Although CCh will activate both mAChRs and nAChRs, only muscarinic receptor activation is expected to induce significant store depletion. Nevertheless, a contribution from nicotinic receptors cannot be excluded. In this chapter we carried out experiments to functionally assess SOCE evoked by CCh, a physiological agonist and the effects of SOCE on CCh evoked Ca\(^{2+}\) oscillation.

First to demonstrate that extracellular Ca\(^{2+}\) or SOCE was required for maintaining sustained Ca\(^{2+}\) oscillations, we stimulated BON cells with 10 μM CCh in presence and absence of extracellular Ca\(^{2+}\) (Figure 2-7A). We found that in absence of extracellular Ca\(^{2+}\), Ca\(^{2+}\) oscillations induced by CCh were not maintained. The frequency
of the oscillations was quantified by counting number of spikes/100 secs. We observed 7±1 spikes in first 100 seconds in BON cells stimulated with CCh in presence of Ca$^{2+}$, whereas in BON cells stimulated with CCh in absence of extracellular Ca$^{2+}$ we observed 3.5±1 spikes in first 100 seconds (Figure 2-7C). The frequencies of oscillation were still maintained at lower rate in presence of Ca$^{2+}$ but oscillations were abolished after 300 seconds in absence of Ca$^{2+}$. The total number of spikes observed in first 500 secs of CCh stimulated BON cells in presence and absence of Ca$^{2+}$ were 20±2, n=17 and 5±0.5, n=31 respectively (p value <0.001) (Figure 2-7B). These finding indicated that the maintenance and/or frequency of oscillations in BON cells were dependent on the presence of extracellular Ca$^{2+}$.

Next, we wanted to test whether activation of muscarinic receptor induces intracellular store depletion, which leads to SOCE. Accordingly, to correlate the relationship between muscarinic receptor activation and intracellular store depletion, we treated BON cells with CCh in absence of extracellular Ca$^{2+}$ and monitored the cytosolic Ca$^{2+}$ dynamics, and subsequently applied CPA to assay the store content. We found that 100 µM of carbachol evoked diminishment of intracellular stores (Figure 2-8A) whereas 0.1 µM CCh failed to deplete the store content. The above experiment demonstrated concentration-dependent reduction of the intracellular store content in absence of extracellular Ca$^{2+}$.

Since CCh evoked diminishment of intracellular stores, we next wanted to test whether CCh can activate Ca$^{2+}$ entry. To test the hypothesis, we used manganese quenching experiment to track the Ca$^{2+}$ influx. In this method, BON cells were treated with 10 µM and 100 µM of CCh in absence of extracellular Ca$^{2+}$ solution supplemented
with 250-500 µM EGTA, followed by application of 1 µM of manganese solution (Figure 2-8B). Application of manganese quenched the fura-2AM F_{360} signal that was represented as inverse rate constant (τ) in Figure 2-8C indicated the Ca^{2+} influx through plasma membrane channels. Application of CCh and 30 µM CPA resulted in reduced τ indicating increased Ca^{2+} channel activity than unstimulated BON cells. This indicated that CCh treatment activated Ca^{2+} influx. However, we also noticed basal channel activity attributed by entry of Ca^{2+} in low levels in unstimulated BON cells as shown in Figure 2-8C. To test whether the influx was through SOCCs we employed known pharmacological blockers of SOCE in BON cells. BON cells were then stimulated with 100 µM CCh in presence BTP2 or Gd^{3+} (SOCC blockers). Application of Mn^{2+} to the bath solution produced Mn^{2+} dependent quenching of fura2 signal (Figure 2-8C). The Ca^{2+} influx stimulated by CCh treatment was blocked by BTP2 and Gd^{3+} indicating that majority of Ca^{2+} influx is through SOCE channels. The rates of Ca^{2+} entry were expressed as time contants (τ), which were determined from the fits of single exponential lines to the quench of the F_{360} signals (Figure 2-8D). The τ for unstimulated cells, 30 µM CPA-treated cell, 100 µM CCh-treated cells, 100 µM CCh-treated cells in the presence of 1 µM Gd^{3+} and 100 µM CCh- treated cells in the presence of 1 µM BTP2 were 473±79 s, 106± 9.4 s, 85±11 s, 221±30 s, 223±26; respectively, 8≤n≤57; for significance p value was ≤ 0.05. CCh-treated BON cells in presence of SOCCs blockers showed increased inverse rate constant whereas CCh or CPA-treated cells in absence of SOCCs showed decreased inverse rate constant. This indicated that Ca^{2+} influxes activated by CCh treatment were largely blocked by known SOCE inhibitors.
In addition, we also studied the correlation of store depletion and SOC influx. Monitoring SOC influx by addition of barium to BON cells stimulated with CCh in absence of extracellular Ca\(^{2+}\) showed direct relationship of store depletion to SOC influx (Figure 2-9). Barium is a Ca\(^{2+}\) entry substitute and the calcium channels are more permeable to Ba\(^{2+}\) than Ca\(^{2+}\) ions. By comparing the integrated area under the depletion curve and SOCE amplitude, we observed that the total amount of Ba\(^{2+}\) influx actually reflects the amount of store depletion. This suggested that mode of activation of SOCE in BON cells was graded. Taken together, the above set of experiments showed CCh can physiologically activate SOCE in BON cells.

To assess the role of Orai in CCh-evoked entry and its effect on Ca\(^{2+}\) oscillations we used transient over expression methods and pharmacological blockers. In the transient over expression studies, BON cells were transiently transfected with 0.5-2 µg of GFP plasmid as a control. These GFP transfected control BON cells were stimulated in the presence of extracellular Ca\(^{2+}\) with 10 µM CCh in presence and absence of 1 µM BTP2 (Figure 2-10A). CCh treated BON cells in absence of BTP2 produced more oscillations than in presence of BTP2. The oscillations were quantified by counting the total number of spikes in both the experiments. The total number of spikes observed in first 500 secs of CCh stimulated BON cells in presence and absence of BTP2 were 13±2 and 9±1 respectively; 10≤n≤18 (p value < 0.05) (Figure 2-10B). The amplitude and the frequency of oscillation were more in BON cells in absence of BTP2 than in presence of BTP2, and Ca\(^{2+}\) oscillation in BON cells in absence of BTP2 attained higher steady state than the resting Ca\(^{2+}\) levels. In contrast, Ca\(^{2+}\) oscillation in BON cells in presence of BTP2 fell back to the resting Ca\(^{2+}\) level. These findings indicated that Ca\(^{2+}\) oscillation induced by
SOCE was reduced by BTP2 suggesting that the maintenance of oscillations in BON cells were dependent on SOCCs. In addition, we also over expressed Orai1 and STIM1 together, and O1DN in BON cells to test whether the maintenance of oscillations was Orai1 dependent. The transfected BON cells were then stimulated in presence of extracellular Ca$^{2+}$ with 10 µM CCh (Figure 2-10A). We observed that CCh treatment in BON cells co-transfected with Orai1 and STIM1 showed more sustained Ca$^{2+}$ oscillation than in BON cells transfected with O1DN. The oscillations were quantified by counting the total number of spikes in both the experiments. The total number of spikes observed in first 500 secs of CCh stimulated BON cells co-transfected with STIM1 and Orai1 together, and O1DN were 15±2 and 7±0.5 respectively; 10≤n≤18 (p value < 0.05) (Figure 2-10B). The amplitude and the frequency of oscillation were more in BON cells transfected with STIM1 and Orai1 together than in BON cells transfected with O1DN. Further, the Ca$^{2+}$ oscillation in STIM1 and Orai1 transfected BON cells have a steady state much higher than the resting Ca$^{2+}$ levels. In contrast, Ca$^{2+}$ oscillation in O1DN transfected BON cells fell back to the resting Ca$^{2+}$ level. The inhibition of Ca$^{2+}$ oscillation in BON cells over expressing mutant Orai1 protein suggested that the maintenance and/or frequency of oscillations in BON cells were dependent on Orai1 channel.

To summarize the chapter, we found that in the presence of extracellular Ca$^{2+}$, the CCh treatment in BON cells evoked sustained Ca$^{2+}$ oscillation, which was reduced by application of BTP2. BON cells over expressing Orai1 and STIM1 protein showed increased frequency and more sustained oscillation than control BON cells when treated
with CCh. The sustained oscillation and the frequency of the oscillation were greatly reduced in BON cells transfected with Orai1 mutant plasmid.

The above findings indicated that Orai1 is a critical component of SOCE and for the maintenance and/or control of oscillations in BON cells. In next section, we investigated the role of Orai1 in basic cellular functions such as cell proliferation, apoptosis and cycle phases.

2.2. B. Role of Orai1 in cellular processes:

The experimental results from the previous sections showed that Orai1 played a major role in SOCE in BON cells. However, both transient transfection of mutant Orai1 plasmid and stable knockdown of Orai1 protein in BON cells did not affect the cell viability. This was surprising since others have shown Orai1 play a role in the control of cell proliferation (El Boustany, et al.). Hence, we decided to check more closely if Orai1 influence any of the cellular processes such as cell cycle, growth, proliferation and apoptosis in BON cells.

To investigate the role of Orai1 in cell cycle phases, BON cells transiently transfected with Orai1E106A plasmid were subjected to cell cycle analysis by propidium iodide (PI) staining. Propidium iodide staining is widely used in the quantification of apoptosis and cell cycle distribution by Flow cytometry analysis (Krishan, 1975). The relative content of DNA at different phases like G0/G1 (diploid; 2n), G2/M (4n), S phases (2n>S<4n) and apoptotic cells (<2n; sub-diploid) are quantified using this method.

In a first set of experiments, cell cycle phase analysis was performed on BON cells over expressing both wild type STIM1 and Orai1 plasmids or O1DN plasmids. The transfected cells were cultured for 48 hours, trypzinied and stained with propidium iodide
as described in method section. The stained cells were sorted and analyzed using flow
cytometry to observe any changes in cell cycle phases. We observed no difference in cell
cycle phase in BON cells expressing either mutant or wild type Orai1 plasmids and no
significant difference was observed in BON cells stimulated with CPA expressing either
mutant or wild type Orai1 plasmids as shown in Figure 2-11A&B. Further, apoptosis
was not noted in either cases which suggest that Orai1 might not play a role in cell cycle
and apoptosis. Caspase-3 luciferase apoptosis assay done on BON cells over expressing
wild type Stim1 and Orai1 plasmids together, and O1 DN plasmids showed no sign of
apoptosis when compared to control (Data not shown). To validate our findings we used
Ki-67 staining. Ki-67 staining in BON cells transfected with O1DN plasmids also
showed no difference from the control cells as shown in Figure 2-11C. Taken together, it
appeared that Orai1 played no role in cell cycle, apoptosis and proliferation because the
dominant negative of Orai1 failed to alter any of the above-mentioned cellular processes
in BON cells under these conditions.

A recent paper by Yang and group (S. Yang, et al., 2009) demonstrated the role
of Orai1 and STIM1 in breast cancer migration and invasion, and henceforth we wanted
to test the role of Orai1 and STIM1 in migration and invasion in BON cells. In initial
experiments, BON cells were subjected to wound healing assay. The wound-healing
assay is simple and inexpensive in vitro methods to study unidirectional cell migration. In
this experiment, a wound was made on confluent flask of BON cells and cultured in
presence and absence of SOCC blockers and monitored until 96 hours. We found that
BON cells were poor in healing the wound when compared to aggressive (MDAMB231)
and non-aggressive (MCF-7) breast cancer. MDAMB231 healed the wound in 24 hours
whereas MCF-7 healed the wound in 48 hours (Data not shown) but BON cells took more than 96 hours to heal the wound that showed that they are poorly migrating cells. Moreover, in this assay the presence of SOCC blockers did not significantly alter the wound healing rate of BON cells (Data not shown).

However, for this latter set of experiments a couple of important caveats should be noted. One should keep in mind that all the over expression and mutant studies were done in transiently transfected BON cells. The results may vary if one employs stably transfected BON cells. Furthermore, in vitro migration assay did not answer any questions about the ability of BON cell to invade or migrate. The cells growing in two-dimensional (2D) model system has a limited space for migration and invasion process cannot be addressed. One possible explanation is the absence of specific tumor microenvironment in Petri dish. In contrast, cells growing in three-dimensional (3D) model system which replicates it’s naturally environment effectively undergoes the process of invasion and migration if any. Henceforth, we developed a novel system, which addressed the tumor microenvironment to study the invasion and migration of human carcinoid cell line.
2.3. Organotypic mouse liver slices mimic natural microenvironment for human carcinoid cell lines: a novel study to address migration and invasion of carcinoid tumors.

2.3. A. Development of organotypic slice culture method:

Organotypic slice cultures have been used to study complex tissue dynamics including carcinogenesis in breast cancer tissue, gene expression profiles and the interaction of human epithelial cells with their microenvironment (Lee, Kenny, Lee, & Bissell, 2007). For migration and invasion of tumor, the microenvironment plays an important role because cancer progresses through the complex interaction of many components. In addition to cancer cells, this complex system can include endothelial cells, pericytes, smooth muscle cells, fibroblasts, lymphocytes, macrophages, dendritic cells, neutrophils, granulocytes and the extracellular matrix (ECM) that participate in tumor progression. This complex system is termed as the tumor microenvironment. The components of tumor microenvironment are grouped into four categories including cancer cells, non-cancer cells, secreted soluble factors and non-cellular solid material, including the ECM. The communication between the tumor cells and its environment helps drive the process of tumor progression (Mbeunkui & Johann, 2009). Carcinogenesis and tumor angiogenesis, which are the two hallmarks of cancer development, depends on its microenvironment. Also, the microenvironment of the cancer cells can provide signals that turn on/off specific transcription factors (Mbeunkui & Johann, 2009). Hence, monitoring the changes in tumor microenvironment is very important in identifying the molecular targets for cancer treatment and prevention.
To study invasion and migration, matrigel assays and fluoroblock assays are typically done. These so-called 2D assays do not adequately address the microenvironment model. Hence, a better system is needed that address these key issues. One widely used model system is transgenic or nude mouse model but cost of maintaining immunocompromised mice and screening drugs in them is very high (Duff, Noble, Gaynor, & Matsuoka, 2002). Alternatively, tissue slice cultures have been used as model systems to study physiology, pharmacology, morphology, endocrinology in a broad array of studies investigating but not limited to chronic application of drugs in liver, synaptic transmission in various brain areas, and alteration in gene expression by viral vectors (Bergold, Casaccia-Bonnefil, Zeng, & Federoff, 1993; Muller, Gahwiler, Rietschin, & Thompson, 1993; Plenz & Aertsen, 1996).

Therefore to overcome the limitation of the 2D model systems, we developed a tractable, cost-effective 3D model system to better investigate cancer cell dynamics in a more physiological setting. This ex vivo model system has the potential to study migration, invasion, proliferation and extravasation of human foregut carcinoid cell line. The ex vivo system we developed used an organotypic mouse liver slice culture. Organotypic culture is defined as in vitro maintenance and growth of tissue explants and multi-cellular cultures that mimic cell interactions within tissues. In general, the success of the organotypic culture experiment depends on slice preparation and culture method. Typically, the tissues are removed and precision cut into sections of 100-400 µm in thickness using a tissue chopper or vibratome. The tissues are cut into cubes and embedded into low temperature melting point (LTMP) agarose gel before sectioning. The cut slices are then washed in balanced salt solution and cultured over prolonged time.
There are three methods of culturing sectioned tissue namely roller-tube cultures, membrane cultures and dish cultures. In roller tube cultures, the sectioned tissue is embedded in plasma clot and undergoes continuous slow rotation facilitating oxygenation of cells. In membrane cultures, the slices are placed at air-medium interface on a semi-porous membrane in a transwell plate. The slices obtain oxygen from medium below and above air surface. In dish culture, the slices are directly placed dish coated with collagen that contains gas-permeable bottom. However, oxygenation of tissue is very poor in this method (Gahwiler, Capogna, Debanne, McKinney, & Thompson, 1997).

We chose to use liver slices because carcinoid cancers typically metastasize to liver and in human carcinoid cancers, the hepatic portal vein circulation is a predicted route for carcinoid tumor metastasis into liver. Thus, a brief description of mouse liver anatomy is presented here. The mouse liver is divided into four lobes namely left lateral lobe, median lobe, right lobe and caudate lobe (Figure 2-12 A). The hepatic portal vein drains venous blood from spleen, stomach, duodenum and intestine. Thus in our method, the human carcinoid cell lines were seeded into mouse liver by injection into hepatic vasculature to study the tumorogenesis process. The hepatic artery supplies arterial blood to liver. The blood from hepatic portal vein and hepatic artery mixes as it reaches the terminal branches and flow into sinusoids and empties into the central vein of each lobule. Central veins coalesce into hepatic veins, which leave the liver and empty into the vena cava.

Our xenograft model used a human carcinoid cell line. We chose foregut carcinoid cell line, BON. BON cells are one of the few available models to study neuroendocrine tumor and it also secretes serotonin, which is thought to be a major
causative factor in classical carcinoid syndrome. Also, they have been shown to ne
tumorogenic when intra-spleenically injected in nude mice. Control BON cells used were
stably transfected with a plasmid vector from OriGene expressing GFP and a scrambled
shRNA. Stably transfected cells were grown under selection pressure (puromycin) for
two weeks and then sorted by flow cytometry. GFP was used as the fluorescent marker to
identify the stably transfected cells. The reason for creating these stably transfected BON
cells were to ultimately use as a control against Orai1 knockdown BON cells.

Stably transfected green BON cells were introduced by injection into hepatic
portal vein following surgical exposure of the portal vein. The liver was first flushed with
physiologically balanced saline solutions. Subsequently, the hepatic vasculature was
stained by perfusion through the hepatic vein using an irreversible vital fluorescent
marker called CellTracker CM-DiI. DiI is a lipophilic membrane stain that diffuses
laterally to stain the entire cell and its derivative CellTracker CM-DiI is more water-
soluble than DiI which is used in staining of cell suspensions and fixed cells. The
advantage of CellTracker CM-DiI is that its labeling is well retained in some cells
throughout fixation and permeabilization steps. For perfusion of liver, 20 ml of 4 µM
CM-DiI concentration in PBS was injected into hepatic vein using 30-gauge hypodermic
needle. The needle was held in place using metal clamp. The solution was left in
vasculature for 10 min to allow uptake of concentration of dye in endothelial cells of
hepatic vasculature as shown in **Figure 2-12B**. Following the vasculature staining,
approximately six million green BON cells were injected at the rate of 1cc/min through
hepatic vein in mouse. Then, the entire liver was removed and distribution of cells was
assessed using macroscope. The macroscopic merged image of BON cells seeded into
mouse liver vasculature at lower and higher magnification is shown in the Figure 2-12B. In these images, the BON cells appear green due to the distribution of green fluorescent protein expressing cancer cells in the vasculature and the vasculature were completely stained red by the cell tracker CM-DiI. The red and green signal largely co-localized indicated that BON cells injected through hepatic portal vein were distributed throughout the hepatic vasculature.

For organ slice preparation, we selected the well-flushed and stained lobes where distributions of cells were even. In most cases we chose left lateral and median lobes. Then, the selected lobes were cut into 5mm cubes and embedded in LTMP gel agarose. The embedded liver tissue was then sliced using vibratome into 200 µm thickness. The slices were maintained at 37°C at 5% CO2/95% O2 and cultured in DMEM medium for up to 2 weeks in a 6 well plates on a semi porous membrane supports. Periodically, organotypic slices obtained from the liver were monitored by fluorescence macroscopy/confocal microscopy typically 24 hrs intervals to see any morphological changes in the seeded cells. The slices were also tested for viability by trypan blue assay. We observed that the periphery of the slice picked up more dye than the interior suggesting that the edges were damaged during the precision cut using vibratome. In general, the liver slices maintained its shape and texture for a week and then started to shrink. Then, the liver slice structure began to visibly degrade after 2 weeks so in most cases we terminated the experiment.

Following injection, initially BON cells were rounded but within 1- 2 hrs we observed that the seeded BON cells typically adopted elongated morphology. We observed in larger vessels where the diameter is typically more than 20 µm, BON cells
were rounded up. The average diameters of the BON cells are 10-20 µm and the adopted elongated morphology may be induced by residence in microvasculature of lesser diameter than BON cells. Interestingly we observed no BON cells in the sinusoids. Sinusoids are small blood vessels with a fenestrated endothelium i.e, vessels with small pores allowing rapid exchange of molecules between blood vessels and surrounding tissue. Liver sinusoids can take up and destroy foreign materials such as bacteria. One explanation of absence of cells in sinusoids is of filtration effect i.e, the diameter of the sinusoids was about 1 µm, atleast an order of magnitude smaller than the size of BON cells. This idea is consistent with our observation that the central vein which receives its blood circulation from sinusoids was also devoid of cells as the cells never appeared to get pass the sinusoids. Further, the CM-DiI staining appeared to be much diffused and did not clearly mark the sinusoids. Another explanatory is that BON cells were cleared by immune cells resident in the sinusoids.

Throughout the experiment the vasculature staining was well marked with a slight decreased intensity and fragmentation of the fluorescence after a week. The seeded cells decreased in number in first 48 hours and the surviving cells appear to proliferate after 72 hours and either expand or coalesce as a small group, we termed as “tumorlet”. There was detectable increase in GFP signal from 72 hours and cells appeared to drastically increase in area every 24 hours. After 140 hours the proliferating cells formed a spheroid-like structure that appeared to extend beyond the vasculature (Figure 2-13). To picture the three-dimensional topology of the tumorlet, we obtained high-resolution images using confocal microscopy. Confocal microscopy rejects out of focus fluorescent light allowing in focus images to be collected at each optical section. Confocal images of
the tumorlet showed that the tumor mass (green) clearly expand beyond vasculature (red) and migrated into liver surrounding liver parenchyma tissue. Figure 2-14 shows confocal images of seeded BON cells (green) at 5 hours and tumorlet formation at 288 hrs post injection. Typically, once the tumorlet invaded into the surrounding tissue it continued to grow until it reached the size of 300 microns in diameter after which they typically disintegrated and collapsed. One possible reason for the tumorlet disintegration was the cancer cells at center of the tumor mass get deoxygenated and deprived of nutrients, and they start to die from inside. Figure 2-13 shows tumorlet formation of seeded BON cells in cultured mouse liver slice over a period of time.

Monitoring the seeded cells in liver slice using fluorescence macroscopy suggested that BON cells were capable of proliferating in liver after metastasis. However, to study the other process of tumor genesis such as migration and invasion we did live-cell imaging. We used multi-photon microscopy (MP) to observe BON cell dynamics in mouse liver slice. The use of multi-photon microscopy has many advantages since multi-photon microscopy uses longer wavelength light that causes less damage than short-wavelength light and also it gives deeper penetration of tissue. Moreover, the signal to noise ratio is very low because MP is inherently confocal. Hence, this method is well suited for live cell imaging for longer periods with fewer toxic effects.

To image the cells using multi-photon microscopy, the liver slice was stabilized on a glass dish using a 100-micron stainless steel mesh. Experiments were performed using 20X air objective lens (NA=1.0). The entire microscope was in temperature-controlled enclosure, and well-aerated environmental chamber emulating the culture conditions (37°C, 5% CO₂/95% O₂). Using 4D-imaging methods (see description below),
BON cells in liver slice were imaged overnight typically 12-16 hours. We did a sequential imaging which captured both the vasculature, which were stained red and the seeded cells, which were green. The red CM-Dil (vasculature) was excited using 755 nm light and the green fluorescence (BON cells) was imaged using excitation at 820 nm. Even though we used long and low energy wavelength lights in multi-photon imaging, we minimized the intensity of exposure by adjusting the gain and exposure time. The optical slice thickness of exposure was about 0.5 µm. The slices were imaged in the X, Y and Z-axis at 10 µm interval for a total stack size of about 100-160 µm. Although the slices were precision cut into 200 µm thickness, after four days they shrink to 140-160 µm. The areas of the slice where cells appeared to be well distributed throughout the Z-axis (generally 96 to 140 µm thickness) were chosen for imaging typically with a time interval of 10-15 minutes. The stacked images obtained were collapsed into a projected image and these images over time course of the experiment were made into movie to observe the cancer cell movements.

In order for the tumor cells to invade and migrate into surrounding and distant tissues, it has to overcome both endothelial cells and ECM barriers (Kopfstein & Christofori, 2006). There are two modes of invasion by which tumor cells migrate namely mesenchymal and ameboid cell migrations (Pankova, Rosel, Novotny, & Brabek). In mesenchymal mode of migration/invasion the cells exhibit elongated fibroblast-like morphology. This cell attaches firmly to ECM via integrin clusters, degrades the ECM by matrix metalloproteinases (MMPs) and migrates into surrounding tissues. This type of migration is slow and characterized by cell membrane extensions namely filopodia and lamellipodia by organization of actin cytoskeleton. The initial
propulsions and elongation of leading pseudopods are driven by actin polymerization and assembly of filaments (Cramer, 1999).

In contrast, amoeboid mode of migration the cells are typically rounded in morphology and show weak adhesion into ECM. Generally they squeeze and push through the ECM. The rate of amoeboid migration is much faster than mesenchymal migration. The amoeboid migrations are characterized by formation of intensive blebbing by contractile actin cortex movements (Lammermann & Sixt, 2009; Pankova, et al.).

In the current experiments we note both the types of migration. We observed that the seeded BON cells after 80 hours exhibited what appeared to be mesenchymal migration behavior. This behavior was typified by extension and retraction of cytoplasmic protrusions as shown as image montage in Figure 2-15A. BON cells also exhibited blebbing process, a characteristic feature of amoeboid movement as shown in Figure 2-15B. A third type of characteristic behavior was also identified, most often seen after 96 hrs in culture. This was typified by elongated morphology but very rapid cell migration. Speed of the movement through tissue was estimated approximately to be 5 µm /min, which is quite rapid and similar to the rate reported for lymphocyte movement (Friedl, Noble, Shields, & Zanker, 1994). The migration usually started with a single cell from one group of cancer cells approaching the neighboring cell group. It appeared as if the migrating cell either laid a track or secreted chemoattractant, which initiated the rapid migration of rest of the cells in the group. BON cells migrated a total distance of 100 µm to coalesce into a larger tumor cell mass. A montage of images taken at 15 mins intervals demonstrates migration is shown in Figure 2-16A&B.
2.3. B. Role of Orai1 in invasion and migration:

Having developed and established the organotypic culture method to study carcinoid cancer cell dynamics and tumorlet formation, we next decided to study the role of Orai1 in BON cell proliferation, invasion and migration. The rationale for these sets of experiments was the growing evidence that SOCE plays a role in tumorogenesis in variety of cancers. For example, SOCE can inhibit production of metalloproteinase MMP-2 and attenuate collagenolysis and inhibition of SOCE by CAI stops angiogenesis, cancer cell proliferation and invasion (Kohn, et al., 1996). Also, Yang showed that SOCE and Orai1 proteins are critical for breast tumor migration and metastasis (S. Yang, et al., 2009). Given the importance of Orai1 in the metastatic behavior of other cancers, we decided to use conditional knockdown of the SOCE protein Orai1 to determine if it plays a role in the metastatic process in carcinoid cancers.

In this set of experiments we used shRNA gene silencing approach to achieve stable knockdown of Orai1 protein in BON cells using shRNA Orai1 plasmid vector from OriGene. Also, BON cells stably transfected with scrambled shRNA discussed in the previous section were used as control. The Orai1 knockdown was verified by real time PCR which shows 40-60% reduction in message levels as shown in Figure 2-17B and also by live cell Ca^{2+} imaging (Figure 2-17A) using fura-2AM that showed significant reduction (40%) in SOCE amplitude in stably knockdown BON cells. The relative Orai1 messages for control and O1 knockdown BON cells were 103.3 ± 3.333 n=3 and 56.67 ± 7.265, n=3 respectively (p value < 0.0043).

Knock down BON cells and control BON cells expressing scrambled shRNA were seeded into mouse hepatic vasculature as previously described, sliced, cultured and
monitored for 2 weeks using fluorescence macroscopy. We observed gradual decrease in cell numbers for first 48 hours post injection and the surviving seeded cells appeared to proliferate at slower rate. Unlike cells expressing the scrambled shRNA, Orai1 knockdown cells did not appear to proliferate and the ability to form tumorlets was greatly reduced as shown in Figure 2-18. However, in two cases, a tumorlet formed from seeded knock down cells but they never reach a spheroid structure nor grew beyond 100 microns in the time frame we assayed (2 weeks). In contrast, in control cells we noticed multiple tumorlet formations in ninety percent of the slices. We analyzed the tumor area of knockdown and normal BON cells over a period of time in each slice using ImageJ and fold area increase of the tumor was plotted against time as shown in Figure 2-19. We noted that normal BON cells seeded into the vasculature exhibits increase in fold area. Typically the tumorlet exhibited 300-400 percent increase in fold area but some of the tumors show 600 percent increase in fold area after seeding into the liver. The knockdown BON cells generally did not exhibit fold area increase except in 2 cases out of 16. Total number of tumorlet formation varied in slice harboring knockdown cells and control cells. We noticed just 2 to 4 tumorlet formation in liver slice containing knockdown BON cells where as in the slice harboring control BON cells we noticed 10-20 tumorlet formations. The seeded Bon cells initially probes inner wall of vasculature and appeared to proliferate in the vasculature. Later they either coalesce or invade the surrounding parenchyma tissue to form tumorlets. Although the BON cells appear to extravasate the vasculature in live cell imaging, it has yet to be confirmed by markers of migration.
BON cells lacking Orai1 protein failed to form tumorlets and also the fold area increase of tumor mass is greatly reduced which suggest that Orai1 might be required in early events of tumor genesis. These initial findings suggests that Orai1 might play a significant role in proliferation of tumor since the proliferation of Orai1 knockdown BON cells are much slower than control cells and also their failure to form tumorlets.

Knocking down of Orai1 appeared to affect the migration movements of BON cells. Orai1 knockdown BON cells undergo massive blebbing and yet they failed to show amoeboid movement. Cell membrane extension namely filopodia and lamellipodia movements were not observed extensively in Orai knockdown BON cells. This suggested that Orai1 might play a role in metastatic movements in carcinoid cell lines.

Using an *ex vivo* organotypic slice culture method that we developed in our lab, we showed there is a correlation between expression Orai1 protein and early events of metastasis of foregut carcinoid tumor genesis in a model system. The above findings also correlate with Yang observation (S. Yang, et al., 2009). Further investigation is needed and it is under progress to validate the role of Orai1 in migration and invasion of foregut carcinoid tumor.

To summarize the chapter, we found that BON cells appeared to stay in the vasculature initially and later they proliferate in the vasculature. Then, they either coalesce or invade the surrounding parenchyma tissue. BON cells in tissue show different characteristic (i.e., movements) than in 2D substrate (glass bottom dish). BON cells show multiple modes of characteristic movement namely slow elongated mesenchymal movement, fast rounded amoeboid movement, or elongated but rapid movement. The modes of movements are dynamic. They switch/interconvert to other modes of movement simultaneously. Orai1
may play a role in metastatic movement in BON cells perhaps in altering some types of movement. This study may have implication for strategies to inhibit metastasis.
Chapter 3: Discussion

Calcium entry through VOCCs and SOCCs are necessary for various cellular functions such as gene transcription, cell growth and cell death (Parekh & Putney, 2005). In this study we focused on the role of SOCCs in human carcinoid cell lines. Here, we demonstrate that artificial and physiological activators of SOCCs in human carcinoid cell lines could induce SOCE. We report for the first time to our knowledge that SOCE in BON cells could be evoked by muscarinic receptor activation. The activation of SOCE pathway in BON cells was consistent with our identification of STIM1 (originally identified as tumor suppressor gene) and the pore-forming channel subunits Orai1, 2 and 3. Although various TRP channel family proteins such as TRPC1-7, and TRPV6, TRPM5 and TRPM8 that have been speculated to play a role in SOCE were indentified, we concentrated on STIM and Orai proteins since there are growing and substantial evidence that STIM and Orai proteins reconstitute SOCE in many cell types.

The identification of STIM1 and Orai paralogues is of our great interest because not only have these proteins been identified as the structural determinants of Ca\(^{2+}\) release activated current (i.e, SOCE in lymphocytes) but also critical for breast tumor cell migration and metastasis (S. Yang, et al., 2009). The pharmacological profiling was done using SOCE blockers such as BTP2, 2-APB, CAI and Gd\(^{3+}\). Pharmacological profiling of SOCE in carcinoid cell lines largely correlated with our identification of STIM and Orai with the exception of the midgut cell line HC45. 2-APB, Gd\(^{3+}\) and BTP2 substantially
reduced SOCE in foregut and hindgut but these blockers have no effect on HC45. One possible reason for the refractory behavior of HC45 cells are those cell lines has lowest level of STIM1 transcript expression when compared to other carcinoid cell lines. In addition to the unexpected insensitivity of HC45 to SOCE blockers, there were other more subtle differences between blockers among the cell line. For example, 2-APB treatment induced complex response in BON cells. In some cases the SOCE was enhanced by 2-APB. One possible explanation for the observation was that BON cells express more Orai3 than Orai1 that can be activated by 2-APB. Moreover, the use of the anti-tumor compound CAI inhibited SOCE in midgut and hindgut carcinoid lines but did not consistently block Ca\(^{2+}\) entry in the foregut cell lines. This shows the possibility of CAI as a potential compound of interest to target Ca\(^{2+}\) entry in ileal carcinoids. The observed variability of these treatments reflect many factors such as limited selectivity of the blockers used, the complexity of channel subunit expressions and interaction, and use of non-clonal cell lines. Thus, these findings while informative regarding the identification of functional SOCE in carcinoid cell lines was relatively inconclusive concerning molecular details of SOCE. To overcome this limitation, we undertook a molecular approach to functionally assess STIM1 and Orai proteins by site-directed mutagenesis and gene silencing methods.

Over expression of wild type Orai1 and STIM1 in BON cells has been shown by others to reconstitute the SOCE in HEK cells (Soboloff, Spassova, Tang, et al., 2006). Consistent with this idea, we showed that BON cells over expressing STIM1 and Orai1 or STIM1 and Orai3 had a 5 fold and 2 fold increases in SOCE respectively. The enhanced SOCE was subsequently abolished by use dominant negative Orai1 where as
the mutant form of Orai3 significantly reduced the SOCE. However, these experiments did not answer the ability of endogenous Orai that participates in SOCE. To address the role of endogenous activity of Orai1, we over expressed dominant negative of Orai1 with a speculation that it might interfere with the regular function of endogenous Orai1. As predicted, we noticed complete inhibition of SOCE in BON cells. Further, shRNA against STIM1 significantly reduced SOCE in BON cells which is consistent with the other published data and also shows it has been involved as a component in SOCE pathway in BON cells. Thus, these experiments strongly suggested Orai1 and STIM1 play a major role in SOCE in foregut carcinoid cells, BON and perhaps in midgut carcinoid cell line CNDT2.5 as we have noticed similar effect (Data not shown).

Our demonstration of Orai1 mediate SOCE in BON cells did not clarify what possible physiological activators could evoke influx. In physiologically relevant conditions, G-protein coupled receptors or various growth factor receptors activation cause Ca\(^{2+}\) influx. Ligands binding to these signaling pathways activate PLC\(\beta\)1 and PLC\(\gamma\)1 which in turn start a cascade of reaction that ends up in activating SOCE channels (Berridge, 1993a). In the current study we chose muscarinic receptors (mAChRs). mAChRs are a class of G-protein coupled receptors that are broadly expressed in gastrointestinal tract. Acetylcholine is the dominant excitatory neurotransmitter in the ENS that communicates with gut (Anini, et al., 2002). We found that frequency and maintenance of Ca\(^{2+}\) evoked by muscarinic receptor activation in BON cells required extracellular Ca\(^{2+}\). These observations were consistent with our findings of STIM1 and Orai messages in BON cells and the requirement of SOCE for maintenance of Ca\(^{2+}\) oscillation in other cell types. The frequency and amplitude of the oscillation in CCh
treated BON cells were greatly reduced by BTP2 and dominant negative Orai, supporting a dominant role of Orai1 in SOCE in BON cells.

Interestingly, our data suggested that the amplitude of SOCE in BON cells might be related to the concentration of the agonist and the amount of store depletion. Such a graded rise in SOCE is in contrast to that of reported by others where SOCE was activated as an all-or-none signal in response to a certain threshold concentration of agonist.

Although, others have shown that Orai1 and STIM1 plays a role in cell cycle and proliferation (El Boustany, et al.), we observed that the transfected cells appear to grow normally. We failed to observe an effect of perturbing Orai1 function using cell cycle, proliferation, apoptosis and migration in BON cells. One possibility for the lack of an effect was that we used transient transfections of wild type and mutant plasmids in BON cells whereas others (El Boustany, et al.) used Orai-directed siRNA plasmids that may induce longer term perturbation of Orai signaling.

*Similarly, in vitro* migration assays did not indicate that Orai was involved in regulating cell mobility. The ability of BON cell to invade or migrate in 2D model system does not recapitulate a specific tumor microenvironment. To address this shortcoming, we developed a novel system that better represented the tumor microenvironment to study the extravasation, proliferation, invasion, and migration dynamics of human carcinoid cell line.

Although in this study we did not directly assess proliferation and extravasation using the *ex vivo* organotypic slice model system we found that Orai1 appeared to alter the migration of BON cell in the liver slice. Overnight multiphoton imaging
demonstrated that control BON cells (expressing Orai1) exhibited three modes of movements. 1) slow elongated (mesenchymal) movement 2) fast rounded (amoeboid) movement 3) elongated but rapid movement. The latter elongated and rapid movement was generally noticed only after 96 hours when two groups of cells coalesce. This may suggest that the cell movements are induced or guided by chemoattractants or paracrine secretions.

It also appeared that BON cells could rapidly interconvert between mesenchymal and amoeboid movement. In mesenchymal mode of migration, the lamellipodia protrusion of the leading edge is achieved by actin polymerization by actin nucleating complex ARP2/3 (Friedl & Wolf, 2003; Yamazaki, Kurisu, & Takenawa, 2009). Polymerized actin then bind to members of Wiscott-Aldrich syndrome protein (WASP) family especially WAVE2. The formation of lamellipodia that initiate migration is regulated by Rac1 through WAVE2/Arp2/3 mediated actin polymerization (Yamazaki, et al., 2009). Further, the formations of cell-substrate adhesion during elongated cell migration are regulated by Rac1 and Rac3 (Yamazaki, et al., 2009). In contrast, in amoeboid mode of migration, the blebbing protrusions that direct the movement is achieved by contractile forces of cortical actomyosin network contraction. The contraction of cortical actomyosin network is dependent of RHO effector, ROCK (Pinner & Sahai, 2008). Inhibition of Rac1 signaling causes mesenchymal to amoeboid transition (Pankova, et al.). In contrast, Orai1 knockdown BON cells appear to show confused movement characterized by intensive blebbing formation. It is generally believed that in contrast to mesenchymal movement blebbing is not dependent on filopodia/lamellopodia formation (Lammermann & Sixt, 2009). Thus knock down of Orai1 might have altered
or inhibited actin remodeling used in mesenchymal migration and facilitated the cells to adopt amoeboid movement. Based on the observed results and role of Rac1 and Rho in cellular migration, we hypothesize that Orai1 might alter the Rac1 signaling and drives the cell toward amoeboid mode of migration as shown below in schematic representation.

Carcinoid tumor metastasis is a complex process and for any tumor migration cell movements are critical factors. We hypothesize that Orai1 might play a crucial role in cell movements. Lack of Orai1 appeared to cause mesenchymal to amoeboid transition movements. We speculate that Orai1 might interfere with Rho/Rac signaling and thus favoring amoeboid movement. Cancer therapeutic drugs against MMPs are of limited success since the tumor cell can interchange between mesenchymal and amoeboid mode of migration. Thus addressing the molecular mechanism of transition between mesenchymal and amoeboid movements might pave a better way to treat migrating cancer cells.
It would be worth investigating the Orai1 knockdown effect in carcinoid tumors in patients. Targeted delivery of siRNA against Orai1 to carcinoid tumor can be easily achieved by binding the interference RNA with octreotide that binds to somatostatin receptor 2, which serves as carcinoid tumor marker.

To conclude, the molecular and pharmacologically profiling in current studies provided evidence for functional SOCE in human foregut, midgut and hindgut carcinoid cell lines. Moreover, we demonstrated that SOCE can be activated either by artificial depletion of ER Ca\(^{2+}\) stores or by muscarinic acetylcholine receptor activation in foregut carcinoid cell lines. Also we provide solid evidence that Orai1 is the major SOCCs in foregut carcinoid cell line, BON. Ca\(^{2+}\) entry through SOCCs controls important processes in tumorogenesis and we observed that entry of Ca\(^{2+}\) through Orai1 appear to play a significant role in metastatic movement in BON cells. This study may have implication for strategies to inhibit metastasis of carcinoid tumors and clarify the role of SOCE pathways in enteroendocrine cell biology.
Chapter 4: Conclusions

- Carcinoid cell lines express variety of candidate SOCCs including STIM and Orai channels.
- SOCE was evoked in carcinoid cell lines by treatment with artificial and physiological activators of Ca$^{2+}$ store depletion and subsequently SOCE was reduced or blocked by pharmacological blockers.
- Overexpression with wild type STIM1 and Orai1 plasmids or with wild type STIM1 and Orai3 plasmids enhanced SOCE in BON cells. Enhanced SOCE was abolished or reduced by co-transfecting with corresponding plasmids coding for non-functional proteins.
- Overexpression of dominant negative Orai1 in BON cells completely abolished the endogenous SOCE.
- Activation of muscarinic acetylcholine receptors in BON cells evoked sustained Ca$^{2+}$ oscillations that were reduced by application of the SOCE blocker, BTP2.
- BON cells over expressing Orai1 and STIM1 protein showed increased frequency and more sustained CCh- evoked oscillation than control BON cells when treated with CCh.
- The sustained oscillation and the frequency of the oscillation were greatly reduced in BON cells transfected with Orai1 mutant plasmid.
• Dominant negative Orai1 reduced or abolished SOCE in BON cells and also altered carbachol induced Ca$^{2+}$ signals.

• BON cells in tissue show different characteristic in an organ slice model (i.e, movements) than on 2D substrate (glass bottom dish).

• Three modes of characteristic movement were observed in BON cells introduced into liver organotypic slice: 1) Slow elongated (mesenchymal) movement. 2) Fast rounded (amoeboid) movement. 3) Elongated but rapid movement.

• The modes of movements are dynamic. They can rapidly switch/interconvert between mesenchymal and amoeboid movement.

• Orai1 may play a role in metastatic movement in BON cells perhaps in altering some types of movement.

• This study may have implication for strategies to inhibit metastasis.
Chapter 5: Materials and Methods

Cell culture:

A variety of human foregut, midgut and hindgut carcinoid cell lines and a non-cancer small intestine epithelial cell line were used for the current study (Table 1). The foregut carcinoid cell line, BON originally derived from a carcinoid tumor metastatic to the pancreas was grown in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 10% FBS. The bronchial carcinoid cell line H727 was grown in RPMI with L-glutamine and supplemented with 10% FBS, 1% sodium pyruvate (100mM) and 1% HEPES (1M). HC45 and HC49 cell lines originally derived from human ileal and rectal carcinoids, respectively were grown in RPMI with L-glutamine supplemented with 10% FBS, 5% horse serum and 1ug/mL of insulin. Another ileal carcinoid cell line, CNDT2.5 was maintained in DMEM supplemented with 10% FBS and 1% sodium pyruvate and 1% HEPES. The non-transformed, fetal small intestine epithelium cell line, FHs 74 Int, was grown in Hybri-Care medium supplemented with 10% FBS. Penicillin/streptomycin Gibco #15410 (1%) was added to all media. All the cell lines were maintained at 37 °C in a humidified incubator set at 5% CO2. The cell lines were harvested following brief treatment with Trypsin/EDTA (0.25%). All cell lines were passaged at the ratios recommended by provider. Dr. Kjell Oberg, Uppsala Sweden, provided BON cells and H727 cells were purchased from American type culture
collection (ATCC). Human ileal and rectal carcinoid cell lines derived from liver metastases, HC45 and HC49 cells, respectively were gift of Dr. Ricardo Lloyd, Mayo Clinic Rochester, MN. The ileal carcinoid line CNDT2.5 was provided by Dr. Lee Ellis, M.D. Anderson Cancer Center, Houston, TX.

**Live cell imaging:**

For live cell imaging experiments, cells were grown on clean glass coverslips that were used to form the bottom of a recording chamber. Changes in cytosolic Ca\(^{2+}\) levels were monitored using fura-2 fluorescence method. For loading with fura-2 AM, cells were incubated with physiological saline solution (containing 140 mM NaCl, 5 mM KCl, 2.2 mM CaCl\(_2\), and 1 mM MgCl\(_2\), and 10 mM HEPES, and 5 mM glucose, pH 7.35) containing 2 μM fura-2 AM for 30-40 min at 25°C. Following loading protocol, cells were mounted on the stage of an Olympus IX-71 microscope equipped with a 40X oil immersion objective (NA = 1.4). An air pressure driven device was used to exchange physiological saline or solutions containing agonists or antagonists by local application through a glass capillary placed at the edge of the field of view. Cells were alternately illuminated at 340 and 380 nm light focused using a fiber optic guide and epifluorescence condenser onto the image plane by dichroic mirror (Semrock) using a monochromator-based Polychrome IV imaging system (TILL Photonics) and emission was detected using 510 nm ±25 nm band pass filter (Semrock) and IMAGO QE camera. Changes in intracellular Ca\(^{2+}\) were represented by and expressed as the ratio of fura-2 fluorescence (F340/F380).
**Ca^{2+} entry measurements by Mn^{2+} quenching of fura-2 fluorescence:**

Cells were loaded with fura-2 AM as described in the preceding section. Following CCh or CPA treatment to induce a reduction in ER Ca^{2+} content, Mn^{2+} which is known to bind to fura-2 with very high affinity and to strongly quench its fluorescence was added at a concentration of 1 mM to the bath solution. The rate of Mn^{2+} quenching of the fura-2 fluorescence signal at the Ca^{2+} insensitive isosbestic excitation wavelength (360 nm) was determined and used as a semiquantitative indicator of plasma membrane mediated Ca^{2+} entry.

**RT-PCR profiling:**

Carcinoid cell lines were trypsinized at 75-90% confluency and mRNA was extracted using Qiagen RNeasy as described by the manufacturer. Extracted RNA was transcribed to cDNA using SuperScript II RT (Invitrogen) and used as template for identifying candidate genes. Specific primer sets were designed using Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm) unless otherwise noted and were synthesized by Integrated DNA Technologies. Primer selection was made with the following parameters: length between 18-27 bases, Tm between 53°-63°C, and primer GC% between 20-80%. The web-based Blast program (available at the NCBI website) was used to determine specificity of the sequences. The PCR reactions using specific primers were carried out using Platinum® Taq DNA polymerase (Invitrogen) under the following conditions: denaturing at 95°C for 30 s, annealing at 53°C for 30 s, and primer extending at 72°C for 30 s for 30 cycles. PCR products were separated by electrophoresis (120 V for 50-60 minutes) on a 1.5% agarose gel stained with ethidium bromide and sizes
determined using a 50 bp DNA ladder. For each cell line/experiment, actin signal was used to normalize to relative levels.

**Cell Cycle Analysis:**

Following synchronization by overnight treatment with medium containing reduced serum (0.1 %), cells were grown on plastic flasks in standard culture medium (or in medium containing reduced or elevated Ca$^{2+}$) to 50-75% confluence. Cells were harvested and fixed in ice cold 70% ethanol for 45 minutes. Next, cells were centrifuged at 1200 rpm for 10 minutes at 4 ºC and the supernatant removed. The pellet re-suspended at a final cell density of 0.5X10$^6$ cells/ml in Ca$^{2+}$ and Mg$^{2+}$ free PBS containing 40 μg/mL propidium iodide (PI), 100 μg/ml RNase (DNase-free) 70% ethanol. Prior to analysis by flow cytometry, samples were incubated at 37ºC for 30 minutes. Cell cycle analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and sample analysis quantified using CellQuest Pro software and ModFit 3.0.

**Transfection:**

The siRNA construct, Dominant negative and plasmid DNA were introduced into carcinoid cell lines using Amaxa biosystems as described by manufacturer. Program T-20 was used to transfect the neuroendocrine cells. Estimated efficiency of transfection is approximately 60 -75%.

**Mutation:**

Site-Directed mutagenesis of Orai1E106A and Orai3E81A was performed as mentioned in Quikchange II XL site –directed mutagenesis kit from stratagene.
Organotypic liver slice:

One to two months old mice were killed by CO$_2$ asphyxiation. Liver was exposed surgically and vena cava was nicked to allow perfusion of liver. A 30 gauge hypodermic needle was inserted in hepatic portal vein and liver was flushed with Slice Lockes (140mM NaCl, 5mM KCl, 1mM MgCl$_2$, 2.2mM CaCl$_2$, 10mM HEPES, 10mM glucose and 0.4mM ascorbic acid and then pH was adjusted to 7.2 with osmolarity around 300-305) containing 4µm of DiI-CM (invitrogen C7001) to stain the hepatic vasculatures. Six to eight million GFP stably transfected BON cells were injected through hepatic portal vein and liver was cut into 5mm$^3$ cubes. The liver cubes were embedded in agarose gel and sliced into 200µm thick slices in an ice-cold slice lockes bath using a vibratome (series 3000, zero-z technology, USA). Slices were placed in a semi porous membrane (100µm cell strainer, BD Falcon) and maintained in an incubator at 37°C in 5% CO$_2$. The culture medium consists of Dulbecco modified eagles medium (GIBCO # 10566) supplemented with 10% fetal bovine serum (GIBCO #16140), 5% penicillin/streptomycin (GIBCO #15140). The medium was changed every day.

Fixing and microtome slicing:

Tissues were fixed overnight at 4°C in 4% w/v Para formaldehyde (Aldrich #441244) then they were washed in ice cold PBS for 1-2 hr and stored in 30% s ucrose (Acros #57501) at 4°C for one to two months. Fixed tissue samples were placed inside a plastic custom mould on dry ice and partially filled with OCT. OCT (Optimal Cutting Temperature, Tissue-Tek, Terrance, CA) is a cryo-embedding compound. More OCT is poured inside the mould to completely immerse the tissue in OCT. Once casted; the mould assembly was set at -20 °C for brief time and placed inside the cryo-microtome
chamber for equalizing the specimen temperature to the cryo-microtome temperature. The mould was removed from the specimen after 30-45 mins and the frozen specimen was mounted on the microtome stage using more OCT. An initial “facing-off” cycle continuously slices the block at maximum thickness until liver tissue become visible. Then 10–20 µm thickness slices were made and stored at -20 °C. In case the cryo-imaging was about to be done later, the mould assembly is stored in airtight bags in a –20°C freezer.

**Multiphoton imaging:**

The liver slice with seeded BON cells was placed on glass bottom dish and stainless steel mesh was used to immobilize the region of interest. The glass bottom dish was placed in an environmental chamber emulating the culture conditions (37°C, 5% CO₂/95% O₂). Experiments were performed using 20X air objective lens (NA=1.0). Two-photon imaging was performed using an upright microscope (DMI 6000B) with an SP5 confocal head (Leica Microsystems). Excitation was provided by Chameleon Ultra Ti:Sapphire laser (Coherent). Emitted fluorescence was split using dichroic mirror and passed through LP560 filters (Chroma Technology) to nondescanned detectors (Leica Microsystems). Acquisition mode was set to xyzt for 4-D imaging and sequential imaging was done to minimize the cross talk between different fluorochromes using 820nm light to excite GFP and 755nm light to excite red CM-DiI. The frame size was usually set at 1024X1024 and scanning speed was set to 400 Hz. To improve the signal to noise ratio line average was set at 2. The optical slice thickness of exposure was about 0.5 µm. The slices were imaged for X, Y and Z-axis at 10 µm interval for a total stack size of about 100-160 µm. Typically, the slices were imaged at 15 minutes interval overnight
and the stacked images obtained were collapsed into a projected image and these images over time course of the experiment were made into movie and further processed using ImageJ software.

**Confocal imaging:**

Confocal images were obtained using an upright microscope (DMI 6000B) with an SP5 confocal head (Leica Microsystems). Green BON cells were excited at 488 nm excitation, with emission filters of 535. Argon laser was used as a source of light and experiments were performed using 20X air objective lens (NA=1.0). For three-dimensional image projection of a tumorlets, z-scans in 10-μm increments were taken and the stacked images obtained were collapsed into a final projected image. The frame size was usually set at 1024X1024 and scanning speed was set to 400 Hz. To improve the signal to noise ratio line average was set at 2 and frame average was set at 3.

**Ki-67 Staining:**

BON cells plated on glass coverslips were washed two times in PBS and fixed in methanol for 10 mins. Following fixation, cells were rinsed with PBS and incubated for 5 mins in cell permeabilization buffer (PBS, 0.3% Triton X-100, and 0.1% bovine serum albumin), and then incubated in blocking buffer (PBS, 0.2% Triton X-100, 5% goat serum) for 60 mins to block non-specific IgG binding sites. BON cells were incubated at 4 °C overnight with anti-Ki-67 polyclonal antibody (1:100) (from abcam, #ab833-500) in antibody dilution buffer (PBS, 5% goat serum, 0.1% Triton X-100). On the following day, BON cells were washed two times for 5-10 mins each in PBS and then incubated at room temperature with Alexa Fluor 488-conjugate and AlexaFluor 555-conjugated anti-rabbit IgG (H + L) antibodies (from invitogen) in antibody dilution
buffer for 1 hour. Cells were washed three times for 5 min each in PBS. The coverslips were then mounted using Vectashield mounting medium (Vector) and imaged using fluorescence microscopy.
Chapter 6: References


sustained Ca2+ influx and IL-2 production in T lymphocytes. *J Immunol*, 170(9), 4441-4449.


Nilius, B. (2003). From TRPs to SOCs, CCEs, and CRACs: consensus and controversies. *Cell Calcium*, 33(5-6), 293-298.


