Exploring Store Operated Calcium Entry Channels in Human Oral Squamous Cell Carcinomas (OSCC)

A thesis submitted for the degree of

Doctor of Philosophy

To

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By

Anuj Kumar Singh

Department of Biosciences and Bioengineering

Indian Institute of Technology Guwahati

Guwahati 781039, Assam, India

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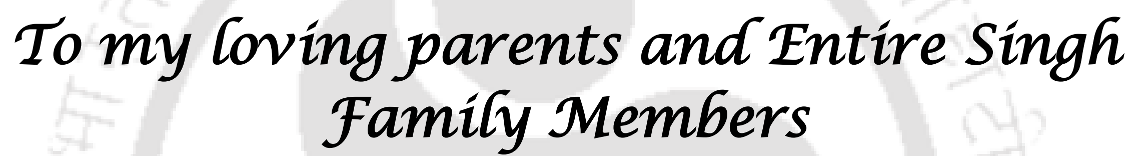

For always being supportive to me

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DECLARATION

I hereby declare that the contents of the research work described in this thesis titled ***"*Exploring Store Operated Calcium Entry Channels in Human Oral Squamous Cell Carcinomas (OSCC)*"***, is a presentation of my original research work carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology, Guwahati, India under the supervision of Dr. Ajaikumar B Kunnumakkara.

An honest effort has been made to duly acknowledge the contributions from others for their ideas, technical help, references or any other help which may be involved in the completion of this thesis work.

November, 2018

Anuj Kumar Singh
Roll no. 126106038
Department of Biosciences and Bioengineering
IIT Guwahati

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING
GUWAHATI-781039

CERTIFICATE

This is to certify that the work described in the thesis titled **"Exploring Store Operated Calcium Entry Channels in Human Oral Squamous Cell Carcinomas (OSCC)"**, submitted by Anuj Kumar Singh (Roll no: 126106038) to Indian Institute of Technology Guwahati, India, for the award of the Degree of Doctor of Philosophy is an authentic record of the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology, Guwahati, India.

This thesis or any part thereof has not been submitted elsewhere for award of any other degree or diploma.

November, 2018

Dr. Ajaikumar B Kunnumakkara
Associate Professor
Cancer Biology Laboratory
DBT-AIST International Laboratory for Advanced Biomedicine
Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati
Guwahati, India

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CHAPTER-1

Introduction and Review of Literature

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1. Introduction

Oral cancer is the sixth most common cancer in the world with an annual incidence of 3,00,373 and a mortality of approximately 1, 45, 328 (Gupta et al., 2016). This cancer is mainly confined to the regions of Melanesia, South Asia, Central Asia and Eastern Europe (Jemal et al., 2011). In India, it is the most prevalent cancer and constitutes 30% of all cancer cases in our country (Coelho and Coelho, 2012). North East region of India is regarded as the hub of oral cancer in our country due to the high incidence of this cancer in this region (Yamsani et al., 2014). Tobacco, alcohol, human papilloma viruses (HPV) etc. are the main risk factors of this disease (Iype et al., 2001; Sharma et al., 2018). Surgery, radiation therapy and chemotherapy are the major modalities of the treatment of this cancer; however, chemotherapy is effective in increasing patient survival by only 8-22% due to the emergence of chemoresistance and tumor recurrence (Furness et al., 2011; Tsai et al., 2012). Additionally, the adjuvant chemoradiotherapy can prolong the survival of oral cancer patients to a maximum of only 16% compared to chemotherapy alone (Furness et al., 2011). In addition, in the last couple of decades, our country has witnessed alarming incidence of this cancer in younger population due to high tobacco and alcohol consumption (Iype et al., 2001; Sharma et al., 2018). Therefore, the development of biomarkers for early diagnosis and prognosis, and novel targets for drug development are imperative for the management of this disease.

Recently, store operated calcium channels (SOC/SOCC) have gained much attention in the development of different cancers such as breast cancer, cervical cancer, hepatocellular carcinoma, lung cancer etc. The SOCs are highly upregulated in these cancers and help in the proliferation, survival, invasion, migration and metastases (Bose et al., 2015). Interestingly, the suppression of SOCs has been shown to inhibit the development of many different cancers in preclinical models. Therefore, targeting SOCs may have high potential in the

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prevention and treatment of cancer. However, the role of SOC and its related genes/proteins are not well understood in oral cancer. Therefore, the present study was aimed at examining the role of SOC and its related genes/proteins in oral cancer.

1.1. Oral cancer- overview

Oral cancer is defined as a neoplasm of oral cavity which includes gingiva, alveolar ridge, buccal mucosa, anterior two third of tongue, mandible and maxilla. In India, the most favorable sub-site for cancer of the oral cavity is the gingival–buccal complex which includes alveolar ridge, gingival–buccal sulcus and buccal mucosa whereas tongue is the most common sub-site in the western world (Curado MP et al. 2009). According to the report of GLOBOCAN 2012 for the cancer of lip and oral cavity, 198,975 new cases of male and 101,398 female have been diagnosed worldwide in 2012. The mortality from oral cancer was about 97,919 in male and 47,409 in female worldwide in the year 2012 ("The Global Cancer Atlas - World," n.d.). There is a wide variation in the geographical distribution of the disease. Approximately two-thirds of oral cancer patients have been reported in Southeast Asia, Eastern Europe and Latin America (Curado MP et al., 2009). According to recent reports it is also considered as 3rd most prominent cancer in between developing countries. However, in India oral cancer ranks 1st for men and 3rd for women compared to other cancers (Curado MP et al., 2009). In India, the rate of occurrence of oral cancer is 20 per 100,000 of population has been reported recently and it represents 30% of cancer patients in the country (Coelho and Coelho, 2012). Despite of all advancement in diagnosis and treatment options the five-year survival rate was found 62% for oral cancer (Weingart et al., 2008). The development of oral cancer is the consequences of long exposure of tobacco or related carcinogenic products which help in acquiring the genetic alterations in order to bring malignant transformation (Pérez-Sayáns et al., 2009).

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1.2 Multistage processes involved in oral cancer

Oral cancer is the result of multi-step processes in which normal cellular processes altered to become carcinogenic. In neoplastic cells alteration of regular signaling machineries and signal transduction pathways help the cells to divide uncontrollably by deregulating apoptosis and finally metastasizing to a distant location (Krishna et al., 2015). All these changes occurred in the development of oral cancer are divided into precancerous lesions, precancerous condition and oral neoplasms based on histology of oral tissues (Watanabe et al., 2015). According to WHO classification based on clinical criteria precancerous lesion was defined as “a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart” which develops precancerous conditions- “a generalized state associated with a significantly increased risk of cancer”. Later it may transform into malignant tissue (Watanabe et al., 2015).

1.2.1. Precancerous lesion

***Leukoplakia*:** Schwimmer used this term first time in 1877 for a white lesion of tongue which means syphilitic glossitis (Neville and Day, 2002). The WHO defined *leukoplakia* as “a white patch or plaque that cannot be characterized clinically or pathologically as any other disease”(Kramer et al., 1978). It is the most frequent in man of middle age (< 30yr) less than 1%, older age man 8% and less than 2% in older age female (*i.e.* > 70yr) (Neville and Day, 2002). ***Erythroplakia*:** This term was first used by Queyrat in order to describe a red, precancerous lesion found in penis (Neville and Day, 2002) but WHO definition is given as– “A fiery red patch that cannot be characterized clinically or pathologically as any other definable disease’’(Warnakulasuriya et al., 2007). Erythroplakia have more chance to become malignant than leukoplakia. More than 90% cases of erythroplakia were reported as

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carcinoma in situ or carcinoma or sever dysplasia (Watanabe et al., 2015). ***Epithelial dysplasia or Dysplasia*: ** According to medical dictionaries the term “dysplasia” means abnormal anatomical structure due to abnormal growth and development of cells (“Definition of DYSPLASIA,” n.d.). Dysplasia is the pre-malignant condition where lesion is about to becomes squamous cell carcinoma through carcinoma *in situ* (Watanabe et al., 2015). There are three grades of dysplasia- mild, moderate and severe. In a histopathologic condition when one fourth of the thickness of the tissue shows dysplasia, it is considered as mild dysplasia. If 50% of the thickness shows dysplasia then the case can be reported as moderate dysplasia appropriately and for three-fourth of the tissue with dysplasia is termed as sever dysplasia (Watanabe et al., 2015).

1.2.2. Oral neoplasm

***Squamous cell papilloma*:** This is the most common focal papillary lesion of oral mucosa and considered as a benign neoplasm of oral epithelial tissues (Firth, 2000). The lesion may be with stalk of tissues (pedunculated) or without stalk of tissues (sessile). In this condition tissue lesion may be colored or white (Watanabe et al., 2015). Histologically, the lesions in squamous cell papilloma are finger-like projections of growing stratified squamous epithelial cells which get support from the vascular connective tissue core and the covering of the epithelial neoplastic tissues may show hyperkeratosis. This condition is not considered as a premalignant lesion. ***Carcinoma in situ (CIS)*:** The CIS represents intraepithelial malignancy in which invasion is the forthcoming process towards the development of oral cancer. The epithelial dysplasia like features is common in CIS. The histopathological changes observed in CIS condition of oral neoplastic lesions were- cancer cells with abnormal and increased number of mitosis, nuclear atypia (*i.e.* abnormal nucleus) and loss of polarity. ***Squamous cell carcinoma (SCC)*:** In histology of oral SCC shows invasion and destruction of surrounding tissues. In the neoplastic SCC condition prickle cells are present within the peripheral basal

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cells layer. Additionally, the intracellular bridges are effortlessly visible. In SCC the keratin layer is present with the neoplastic invading cells (Watanabe et al., 2015). All the developmental processes are shown below in figure 1.2 (Krishna et al., 2015).

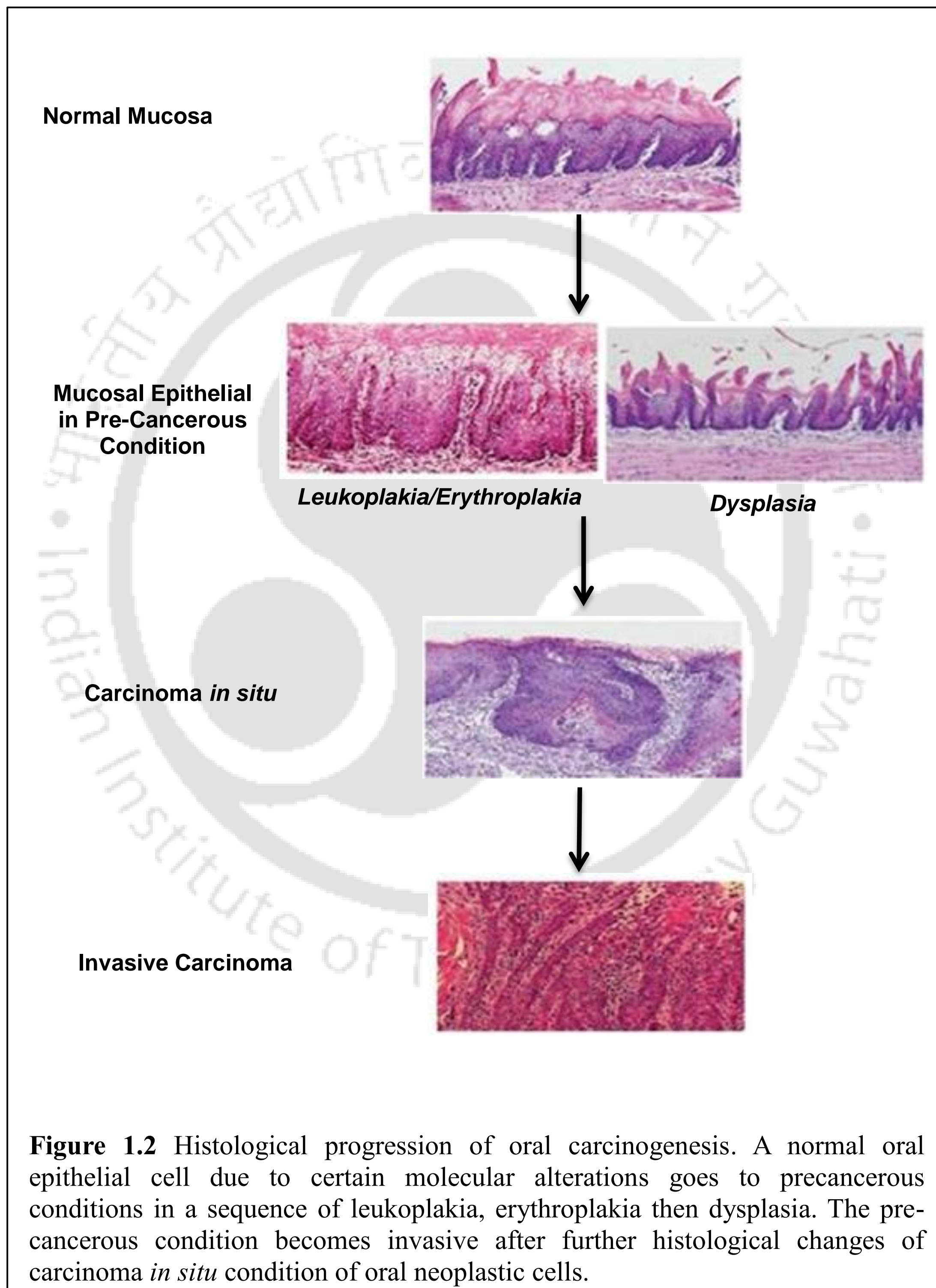

Figure 1.2 Histological progression of oral carcinogenesis. A normal oral epithelial cell due to certain molecular alterations goes to precancerous conditions in a sequence of leukoplakia, erythroplakia then dysplasia. The pre-cancerous condition becomes invasive after further histological changes of carcinoma *in situ* condition of oral neoplastic cells.

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1.3. Molecular alterations in oral cancer

The development of oral cancer is a multi-step process which is the outcome of several genetic alterations caused by tobacco, alcohol, environmental factors, chronic infections or viruses (Pérez-Sayáns et al., 2009). In consequences of these accumulated alterations normal cell becomes malignant and shows the characteristic features of cancer hallmarks such as- uncontrolled proliferation, altered cell migration, reprograming, aborted apoptosis and enhanced cell survival. The alterations were mainly observed in two types of genes *i.e.* tumor suppressor genes and oncogenes. The amendment of these genes increased the normal expression of growth factors (such as- TGF-α, TGF-β, platelet-derived growth factor etc.) or cell surface receptors (like- GPCR, EGFR etc.) or high intracellular signaling and dysregulated transcription factors (such as-*ras* gene family, *c-myc* gene) (Krishna et al., 2015). The alteration of tumor suppressor proteins (p53 and pRb) in oral cancer was dysregulated through HPV viral products (E6 and E7), reported recently (Krishna et al., 2015).

1.4. Risk factor for oral cancer

The above mentioned molecular alterations are the outcome of prolong exposure of smoking, smokeless tobacco (*i.e.* chewing tobacco which includes mainly betel quid, nut and tobacco leaves) and alcohol (Rodu and Jansson, 2004). In some cases human papilloma virus (HPV), herpes simplex virus (HSV), *Syphlis* and *Candida* were also reported for oral cancer (Sand and Jalouli, 2014). Several oncogenes are found to be associated with oral carcinoma which includes- *ras* family, *c-myc*, *int*-2, *hst*-1, PRAD-l and bel (Field 1992; Sidransky 1995; Riviére et al. 1990). Despite of these risk factors, poor dental hygiene and some nutritional habits could also be the reason for oral cancer (Neville and Day, 2002).

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1.4.1 Tobacco

Tobacco is the leading cause of oral cancer from many decades and the consumption varies in different group of population worldwide. Based on second edition of tobacco atlas the prediction was made that the population of smokers will reach up to 2 billion by 2030 and we know that cancer related to tobacco are more compared to other carcinogens (Asthana et al., 2016). Oral cancer is one of the most prevalent cancer which may develop among tobacco users especially smokers (Johnson, 2001; Rodu and Jansson, 2004; Sadri and Mahjub, 2007). Tobacco is consumed in two forms- smokeless and smoke, depending on the socioeconomic behavior of the society. The use of tobacco is 2 to 3 times higher in male compared to female which was reflected in the number of cases in both the sexes (Johnson, 2001). The WHO has estimated 250 harmful chemicals of tobacco smoke in which more than 60 can cause cancer ("Harms of Cigarette Smoking and Health Benefits of Quitting," 2017). In our work we used four tobacco components- Banzo[a]pyrene (BaP), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N′-nitrosonornicotine (NNN) and Nicotine (Hecht and Hoffmann, 1988; Talhout et al., 2011).

1.4.1.1 Banzo[a]pyrene (B[a]P)

B[a]P is the most explored polycyclic aromatic hydrocarbon (PAH) of tobacco smoke reported for causing cancer (Nebert et al., 2013; Shi et al., 2010; Walle et al., 2006). It is also present in the environment as an industrial pollutant, generated from burning of woods, cigarette smoke and some of the burned foods-such as coffee, grilled chickens etc. (Chen et al., 2012). It generates cancer promoting reactive species with the help of two enzymes Cytochrome P450 1A1 and 1B1 (*i.e.* CYC1A1 and CYC1B1) which reacts with DNA directly and form DNA adducts (Shi et al., 2010).

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1.4.1.2. Nicotine, NNN and NNK

Nicotine is known for the addiction with tobacco products. It can be absorbed directly across the lung epithelium, oral mucosa, through the skin and the nose (Sanner and Grimsrud, 2015). The metabolism of nicotine takes place in liver with the help of enzymes- CYP2A6, UDP glucuronosyl transferase and flavin-containing monooxygenase (Benowitz et al., 2009). Nicotine helps in cancer progression through activation of nAChR (Nicotinic acetylcholine receptors), as a known agonist for the receptor (Mishra et al., 2015). The exposure of nicotine in esophageal and oral keratinocytes promote epithelial-mesenchymal transition (EMT) through alterations of genes responsible for cell cycle and differentiation which includes Ki-67, cyclin D1, p21, PCNA, and p53 (Arredondo et al., 2001). Nicotine was considered as an addictive not as a carcinogen (Hukkanen et al., 2005) but recently it has been reported that reoccurrence and aggravation of cancer is caused by nicotine (Sanner and Grimsrud, 2015). However, N-Nitrosonornicotine (NNN) and 4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) are the nitrosamine derivatives of nicotine (Hecht and Hoffmann, 1988). The mechanisms behind the induced carcinogenesis by these two derivatives was forming DNA adducts and develop mutations which promotes receptor mediated tumor growth (Xue et al., 2014).

1.4.2 Alcohol

Alcohol together with tobacco is considered as a risk factor for approximately 75% cancer for upper aero-digestive part of the body (Ogden, 2005). However, on the basis of oral cancer case study published from 1988 to 2009 the relative risks for more than 60g/day or 4 drinks/day was found 3.2–9.2 (Goldstein et al., 2010). The mechanism for carcinogenesis due to alcohol has not been understood fully but the some of the convincing studies support following events are associated such as- acetaldehyde regulated gene toxicity, generation of

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reactive oxygen species through cytochrome P450 2E1(CYP2E1), increased action of estrogen, genetic polymorphisms and dysregulated folate and retinoids metabolism (Ratna and Mandrekar, 2017)

1.4.3 Viruses

HPV and herpes viruses are reported to cause oral carcinomas (Shillitoe, 2009). In a recent study, it was found that HPV positive tonsillar cancer cases increased from 23% to 93% in the course of time *i.e.* 1970 to 2007 (Näsman et al., 2009). Gupta K et al reviewed the HPV reported cases of OSCC in different geographic location of the world and concluded that significant number oral cancer cases were infected with HPV (Gupta and Metgud, 2013). In the study an Indian oral cancer report suggested that out of 60 OSCC samples 48% cases were HPV 16 positive (Elango et al., 2011; Gupta and Metgud, 2013). Till date, more than 200 genotypes of HPV were identified but the HPV1, 6, 11, 16 and 18 were found to be associated with oral cancer cases (Ragin et al., 2007). HPV express two types of proteins- early proteins (E1 to E7) and late proteins (L1-L2). Among these, E6 and E7 were found to be tumorigenic transformation of cells. These proteins are involved in immortalization of cells, degradation of tumor suppressor proteins (p53 and pRb), telomerase activity and anti-apoptotic effects, transactivation of E2F dependent promoters and induction of genomic instability (Gupta and Metgud, 2013). However, other oncogenic viruses such as Herpes Simplex Virus Type 1 and Epsteine Barr Virus were reported to be involved in oral cancer (Sand and Jalouli, 2014).

1.4.4 Other factors

Additionally, there are many other factors which might be associated to oral cancer development and progression. The poor hygiene, age, use of therapeutic drugs and injury was also found to be associated with oral cancer at some extent (Oji and Chukwuneke, 2012).

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1.5. Current Approach for Oral Cancer Screening, Diagnosis and Treatments

1.5.1 Screening

The visual examination of oral cavity and sensing by touching the lesion is accepted a gold standard internationally for screening of any mouth cancer. It includes oral examination and mucosal examination which has been shown an efficient discriminatory ability till today (Moles et al., 2002). While observation if the examiner found change in color, texture, presence of ulcer or swelling then it must go for further diagnosis of oral cancer (Silverman, 1987). Other than visual oral examination from outer side and inside of mouth there are some additional screening tools are developed for better screening such as Oral CDx , Vizilite Plus with TBlue630, VELscope, Identafi, Orascoptic DK and Microlux Diagnostic Light (McCullough et al., 2010; "Mouth Cancer Screening Protocol | Mouth Cancer Foundation," n.d.).

1.5.2 Treatment

There are three common modalities of treatments for oral cancer similar to other cancers like surgery, chemotherapy and radiation therapy. Surgery and radiation therapy are given in early stages (*i.e.* stage I and stage II) of oral cancer either one by one or in combination. Since the advance stage (*i.e.* stage III and stage IV) tumors are aggressive therefore a combined treatment approach of surgery, radiation and chemotherapy are usually applied with proper planning and team work (Sankaranarayanan et al., 2015).

1.5.2.1 Surgery

Surgery is used for the patients with early stage and the factors to be considered mainly is the size of the tumor, site of the tumor, depth of penetration and the proximity of the tumor towards mandible or maxilla. The most easy operational site of primary tumor are removed

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surgically by peroral approach, for inner site which is generally difficult resect directly mandibulotomy approach, visor flap approach or upper cheek flap approach and sometime lower cheek flap approach are taken. The reconstructive surgery is considered after the removal of the tumor if there is functional or aesthetic loss (Shah and Gil, 2009).

1.5.2.2. Chemotherapy

The anti-cancer drugs are used to treat the oral cancer patients is known as chemotherapy or chemo. The most commonly used chemotherapeutic drugs are- Cisplatin, Carboplatin, 5-fluorouracil (5-FU), Paclitaxel, Docetaxel and Hydroxyurea (Furness et al., 2011; Hartner, 2018). Chemotherapy is either given after surgery or in combination with radiation therapy or surgery. The neoadjuvant and adjuvant chemotherapy showed 10% - 20% beneficial to increase survival in oral cancer cases (Furness et al., 2011). The most common drug used in oral cancer therapy is cisplatin (100mg/m^2) and 5-florouracil (15mg/Kg) (Schuler et al., 2010). Recently, cisplatin showed a positive response in the adjuvant chemoradiation therapy in 25% to 30% oral cancer patients with positive resection (Hartner, 2018). However, cisplatin and carboplatin showed cross reactivity in a patient originated cell line based study (Schuler et al., 2010). Additionally, cisplatin was also found to promote chemoresistance, enhancing cancer stem cell property, and decrease in DNA damage response in oral cancer (Li et al., 2018; Tsai et al., 2012; Wang et al., 2012).

1.5.2.3. Radiation therapy

Radiotherapy is often used in combination with either surgery or chemotherapy or both but rarely as single therapy (Zakrzewska, 1999). The high energy particle or X-ray is used for radiation therapy to destroy cancer cells or retard the cell growth. Currently most common radiation therapy is *external beam radiation therapy* or *EBRT*. The treatment is usually given

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for 6 to 7 week (5day per week) ("Radiation Therapy for Oral Cavity and Oropharyngeal Cancer," n.d.).

1.5.2.4 Other therapies

Apart from these a new treatment strategy has recently been adapted for the management of oral complications is Hematopoietic Cell Transplantation (HTC) (Epstein et al., 2012). There are many targeted therapies are also available basically targeting epithelial or vascular growth factor receptors (EGFR or VGFR), mec bhanistic target of rapamycin (mTOR) or some tyrosine kinases inhibitors (*i.e.* KIs) ("Cancers of the Oral Mucosa Treatment & Management," 2016) but there are always some side effects associated with all the therapies mentioned here such as development of chemoresistance and tumor recurrences. Therefore, it necessitates the need of new targets or new approach to overcome from the existing problems associated with oral cancer treatment.

Moreover, cancer is still not been cataloged under channelopathy but the growing evidences are suggesting the role of ion channels and pumps in cancer progression (Litan and Langhans, 2015). In recent research related to calcium (Ca^{2+}) regulation and associated channels has shown a good hope as a new target for cancer therapy (Bose et al., 2015). There is lack of information about oral cancer and Ca^{2+}-channels which showed the need of investigation in this area.

1.6 Ca^{2+} Regulation and Channels

1.6.1 Ca^{2+} Regulation

A hypothetic concept came in last few decades for cell signaling suggested that at the time of evolution when cell was developing in a continuously changing environment there was a need of cell signaling and signaling messengers which can pass the information to the

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required destination inside the cell. It can be possible through changing the concentration of the messenger time-to-time. To full fill this need calcium ion (Ca^{2+}) and phosphate ion {$(PO_4)^{2-}$} evolved to control most of the cell signaling (Clapham, 2007). Both the ions have the ability to change the local electrostatic environment and confirmation of the targeted proteins which is the key of signal transduction (Clapham, 2007). There is one more divalent ion, Mg^{2+}, but its tightly binding capacity with water molecule disqualify it to become an efficient signaling molecule compared to Ca^{2+} (Clapham, 2007). Ca^{2+} signalling is involved in almost all functions of cells including- cell division , gene transcription, secretion, motility, muscle contraction, exocytosis etc. (Izant, 1983; Santella, 1998, Berridge et al., 2000). It is reported that in resting condition cell shows less calcium ions inside the cell (*i.e.* approximately 100nM) however it was observed almost 10000 time higher in the outer environment (*i.e.* 1mM) (Yamakage and Namiki, 2002). It means in resting stage there are more positive calcium ions on the extracellular side of the plasma membrane. Therefore cells always try to maintain its low cytosolic Ca^{2+} concentration by any of the three ways which was represented in figure 1.1 (adopted from Borle, 1981).

1. Actively pump calcium out of the cell.
2. Chelate calcium using Ca^{2+} binding proteins.
3. Compartmentalize Ca^{2+} into intracellular stores.

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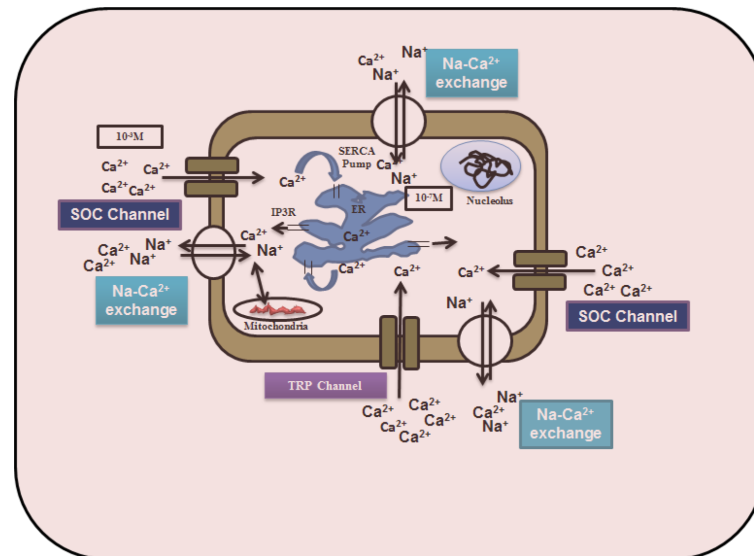

Figure 1.6.1 Regulation and distribution of intracellular concentration of free Ca^{2+}. The intracellular [Ca^{2+}] concentration is ~ 10^{-7} M and extracellular [Ca^{2+}] is ~ 10^{-3} M. These concentration gradients are maintain through SOC channel, TRP channels or Na-Ca^{2+}.

Therefore cell developed many tools to handle the Ca^{2+} concentration which is shown in figure 1.6.2 in a summarized way (Berridge, 2012).

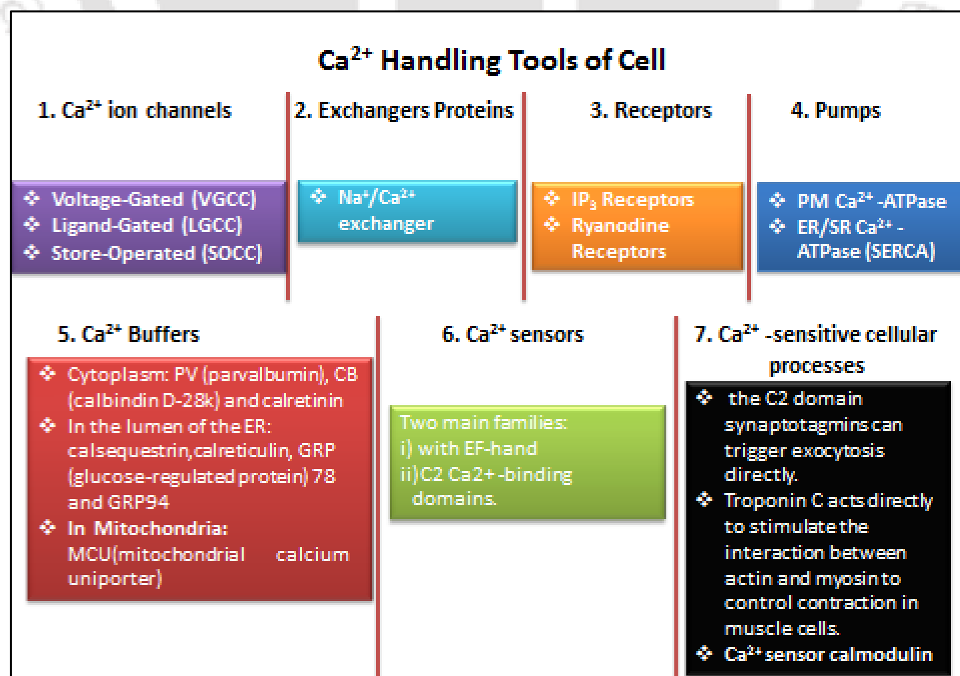

Figure 1.6.2 Ca^{2+} handling tools of cell

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1.6.2 Calcium Ion Channel

These channels are complex proteins found either on the cell membrane or into the cytoplasm on the outer layer of cell organelles (Berridge, 2012). On the basis of their primary activation mechanisms Ca^{2+} channels can be broadly divided into three different categories. Such as-

1) Voltage-gated Ca^{2+} channels (VGCC)
2) Ligand-gated Ca^{2+} channels (LGCC)
3) Store-operated Ca^{2+} channels (SOCC)

1.6.2.1 Voltage-Gated Ca^{2+} Channels (VGCC) or (Ca_V)

As the name suggest, opening and closing of the channel is controlled by a voltage around the channel. These types of channels are mostly present on the membrane of excitable cells (*e.g.*, muscle, glial cells, neurons, etc.) with permeability to the calcium ion Ca^{2+}. VGCC is also categorized into two main categories namely:

A) High voltage activated channels and
B) Low voltage activated channels

A) High voltage activated (HVA) channels

These Ca^{2+} channels are getting activated at high membrane potential range (e.g. - 40 mV) and are further classified into L-type (long lasting) $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, $Ca_V1.4$), P/Q-type (Ca_V 2.1), N- type (Ca_V 2.2), and R-type (Ca_V 2.3) based upon distinct pharmacological sensitivities (Catterall et al., 1993). Here N-type is more negative type current and R-type current is resistant to Ca^{2+} channel blockers.

B) Low voltage activated (LVA) channels

These channels are otherwise known as ‘‘T-type’’ because it open transiently. It needs negative membrane potential to activate the channel (e.g. - 60 mV). It is also classified into three subtypes- Ca_V 3.1, Ca_V 3.2, and Ca_V 3.3 (Cain and Snutch, 2011).

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1.6.2.2 Ligand-gated Ca^{2+} channels (LGCC)

These Ca^{2+} channels are getting activated when specific substances (i.e. ligand) bind to its special receptor domain (Burnashev, 1998)**.** The LGCC superfamily, which is also known as ionotropic receptors, is broadly represented by three different classes based on its topology such as- trimeric P2X purinoceptors, tetrameric glutamate receptors and pentameric receptors which is known to form acetylcholine and serotonin receptor in vertebrata (Pankratov and Lalo, 2014). On the basis of way of operation the LGCC is divided into two categories:

A) Directly operated LGCC- Here the agonist bind directly on the receptor and then gate open for Ca2+ entry. It includes N-methyl D-aspartate (NMDA) receptors which get activated by glutamate, P2X receptors activated by ATP and the Nicotinic acetylcholine (nACh) receptor which opened by acetylcholine.

B) Indirectly Operated LGCC- The best example for this type of Ca^{2+} channel is G-protein coupled receptors (GPCR). This membrane receptor is capable to sense various physiological stimuli like light, odor, hormones, metal ions and neurotransmitters. GPCR is composed of G_{α}-GDP and $G_{\beta\Upsilon}$ subunits, an effector and a regulator respectively. When any kind of external stimuli (e.g. hormone) binds to GPCR complex extracellular receptor then these two subunits get dissociated upon GDP exchanged with cellular GTP and become an active effector subunit of the heterotrimeric G protein as G_{α}-GTP. Later it stimulate an enzyme phospholipase C_{β} (PLC_{β}) to hydrolyze a membrane lipid called phosphatydyl-inositol-4, 5-bisphosphate (PIP_2) to generate inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). This generated IP_3 then act as a ligand for IP_3 receptor of endoplasmic reticulum (ER) membrane to release Ca^{2+} from the store. The concept was well explained in figure 1.4 (Kiselyov et al., 2003).

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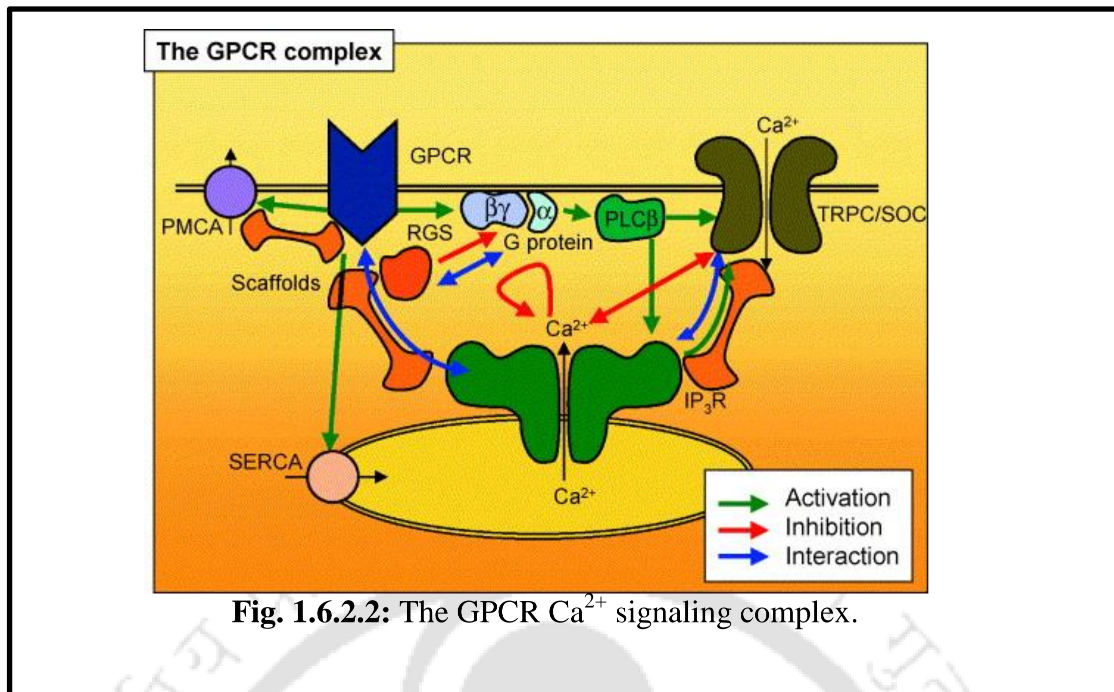

Fig. 1.6.2.2: The GPCR Ca^{2+} signaling complex.

1.6.2.3 Store-operated Ca^{2+} channels (SOCC)

1.6.2.3.1 Introduction

The cytoplasmic Ca^{2+} get stored in the some cell organelles like ER/SR, mitochondria, Golgi apparatus, lysosomes, nuclear envelope and secretory granules in which ER/SR is considered as main storage organelle. Study about the distribution of calcium inside the cell is based on two Ca^{2+} reporter proteins like aequorin (jelly fish) and the cameleons together (Parekh and Putney, 2005). The stored Ca^{2+} is being utilized by various cellular activities which lead to empty the cytoplasmic store. Here the role of Store-operated Ca^{2+} channels comes into picture when Putney JW Jr. in the year 1986, first time introduced the concept of SOCE based on his findings (Putney, 1986). He defined store operated Ca^{2+} entry (SOCE) as- "store-operated calcium entry is a process by which the depletion of calcium from the endoplasmic reticulum activates calcium influx across the plasma membrane" (Roos et al., 2005). Based on last few years of findings now we know the players of SOCE, they are mainly ER/SR-Stromal interaction molecules (STIMs) (Liou et al., 2005; Roos et al., 2005; Zhang et al., 2005) and Orai (Monika Vig et al., 2006; M. Vig et al., 2006; Yeromin et al., 2006; Zhang et al., 2006) together which is known as SOC Channel. Here STIMs are sensor

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protein found on ER membrane however Orai1, Orai2 and Orai3 is present on plasma membrane through Ca^{2+} entry takes place. Now it is known that resting cells have very low Ca^{2+} permeability but at very minute increase in the permeability the huge amount of Ca^{2+} influx happens which is induced by Ca^{2+} release. Therefore the channel formed is known as Ca^{2+} release activated Ca^{2+} channels (i.e. CRAC) and the current generated by this electrochemical gradient is known as I_{CRAC} (Putney, 2010).

1.6.2.3.2 Mechanism for Activation of SOCC or SOCE by ER Store of Ca^{2+}

In LGCC we show that plasma membrane GPCR bind to a ligand and activate PLC breaks PIP_2 into DAG & IP_3. This secondary ligand i.e. IP_3 binds to IP_3 receptor which exists on ER membrane. It allows the Ca^{2+} to release from ER lumen then STIM1 senses the depletion of ER lumen Ca^{2+} stock and redistributes into discrete puncta near the plasma membrane Orai1 of PM form a pore with the help of STIM1 to influx of Ca^{2+} which is shown in figure 1.5 (A) (Putney, 2010) and (B) (Lewis, 2007). This SOCE induced Ca^{2+} is equally important for various cellular processes as well as the repletion of the ER lumen Ca^{2+} store by a pumping mechanism of sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA) which is located on the ER membrane (Stathopulos and Ikura, 2013).

1.6.2.3.3 Orai and STIM- main players of SOCE

A) Orai

The name was given on Greek goddess for heaven’s gate who has three sisters -Orai is approximately 33 kDa plasma membrane protein divided into 4 transmembrane domains (M1,M2,M3 and M4) with α helices found no sequence homology with other type of channels (Hou et al., 2012; Lewis, 2007). In human there are three forms of Orai has been found- Orai1, Orai2 and Orai3 (Hou et al., 2012). Orai1 has shown a significant role in SOCE

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but other two Orai's functions are not so clear although their overexpression with STIM1 in HEK cells showed some role in SOC activity (Mercer et al., 2006).

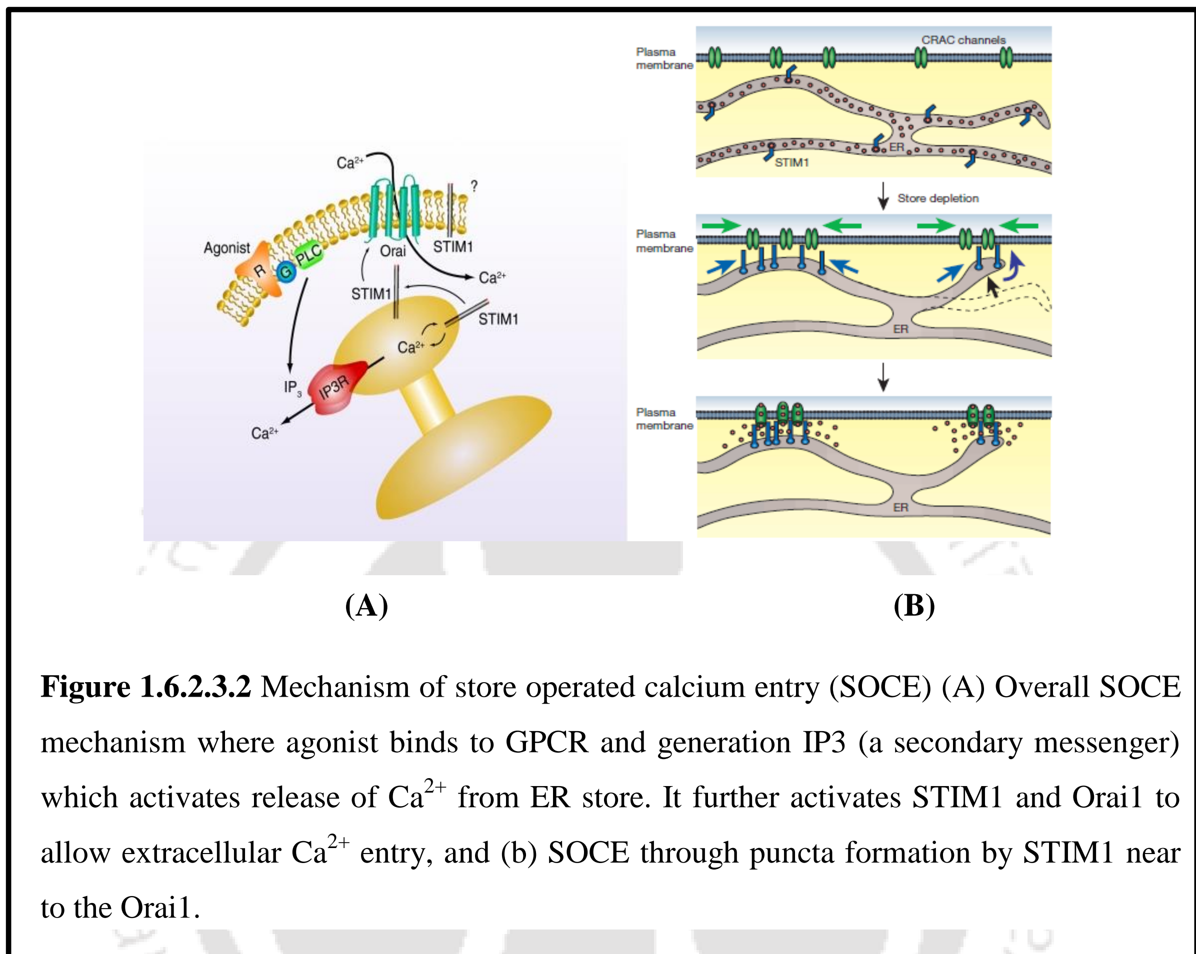

Figure 1.6.2.3.2 Mechanism of store operated calcium entry (SOCE) (A) Overall SOCE mechanism where agonist binds to GPCR and generation IP3 (a secondary messenger) which activates release of Ca^{2+} from ER store. It further activates STIM1 and Orai1 to allow extracellular Ca^{2+} entry, and (b) SOCE through puncta formation by STIM1 near to the Orai1.

B) STIM

STIM proteins are reported as a single type I transmembrane (TM) proteins. It contain two Ca^{2+} binding domains canonical and non-canonical (cEF & nEF or simply EF) inside the ER lumen along with a sterile α motif (SAM). On the other side (*i.e.* in cytosol) there is an overlapping coiled-coil segment (CC); an ERM (*i.e.* ezrin-radixinmoesin) domain then sequentially arranged serine-proline rich domain (S/P) and lysine-rich (K) region (Lewis, 2007). In vertebrates two isoforms of STIM has been observed STIM1 and STIM2. The protein database suggest that STIM1 is a small protein with 685 aa, (75kDa) and STIM2 is

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with 746 aa (82kDa) (Stathopulos and Ikura, 2013). The structure of Orai and STIM is shown in figure 1.6 (A) & (B) adopted from Lewis, 2007 & Stathopulos and Ikura, 2013.

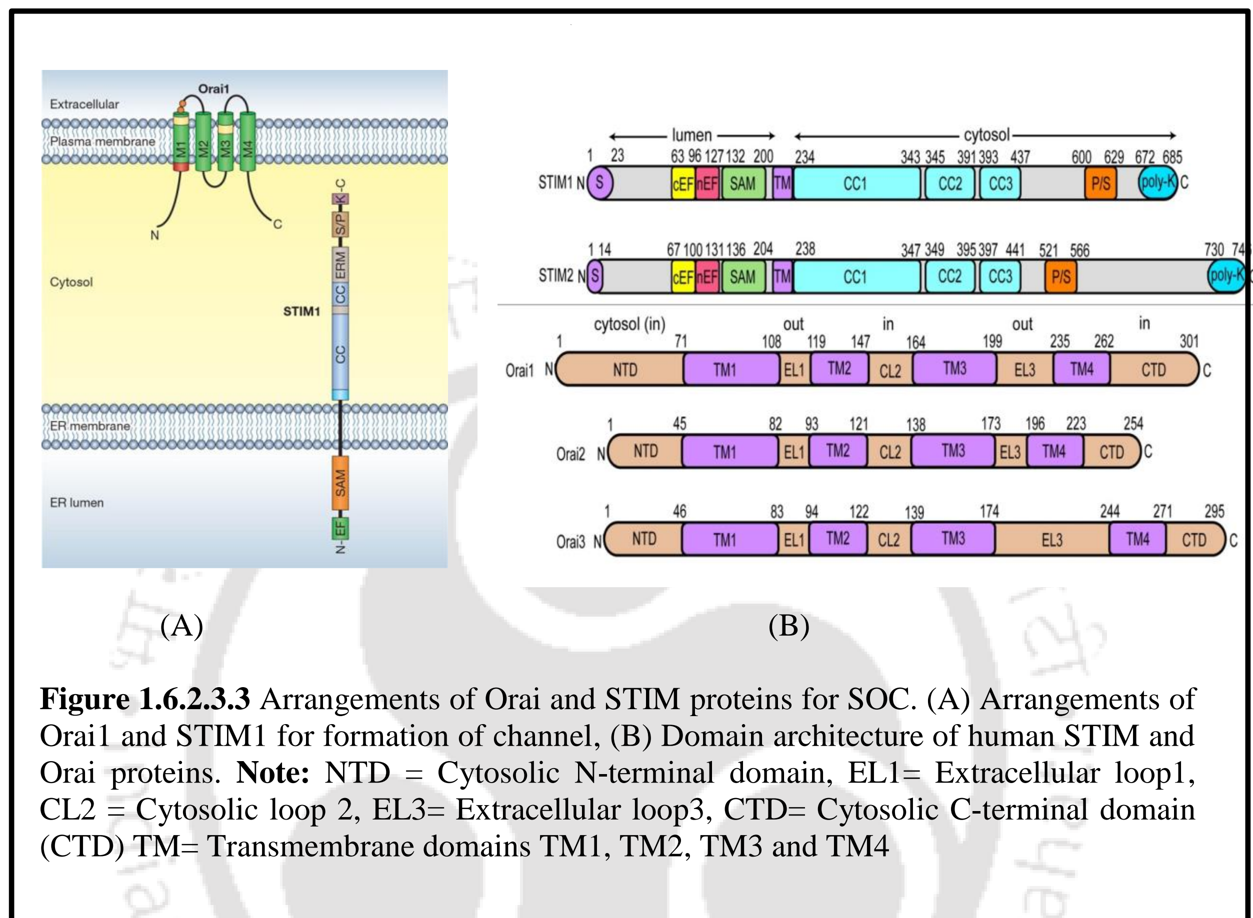

Figure 1.6.2.3.3 Arrangements of Orai and STIM proteins for SOC. (A) Arrangements of Orai1 and STIM1 for formation of channel, (B) Domain architecture of human STIM and Orai proteins. **Note:** NTD = Cytosolic N-terminal domain, EL1= Extracellular loop1, CL2 = Cytosolic loop 2, EL3= Extracellular loop3, CTD= Cytosolic C-terminal domain (CTD) TM= Transmembrane domains TM1, TM2, TM3 and TM4

1.6.2.3.4 Transient receptor potential (TRP) channels

TRP channels are the member of a family of relatively non-selective divalent cation channels. It has been proposed that their molecular entity is associated with SOCE activity because the TRP activity required PIP_2 and depletion of the phospholipid. Moreover, with the help of gene silencing and overexpression experiments it is now established that TRPs promote SOCE (Parekh and Putney, 2005).

A large super-family of cation channels that are expressed in many cell types from yeast to mammals were first identified in *Drosophila melanogaster* photo-cells (i.e. cone cell and rod

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cells).The mammalian TRP channel family is divided into 6 subfamilies based upon sequence similarity:

i. TRPC (Canonical)
ii. TRPV (Vanilloid)
iii. TRPM (Melastatin)
iv. TRPP (Polycystin)
v. TRPML (Mucolipin) and
vi. TRPA (Ankyrin)

Another Seventh subfamily TRPN (NompC) was identified which is reported only in worms and flies and considered as mechanosensitive (Montell et al., 2002). Based on the two above mentioned experiments it was observed that most of the TRPCs as well as TRPV6 are store-operated.

1.6.2.3.5 TRPCs and SOCE

In 1995, two independent research groups identified TRPC1 with full length of cDNA was the first mammalian TRP homologous (Wes et al., 1995, p. 1; Zhu et al., 1995). TRPCs are further sub-divided into seven canonical forms as TRPC (1 to 7). In case of human TRPC2 is reported as pseudo gene (Parekh and Putney, 2005). There are evidences which supported that TRPC1 play important role in SOCE (Salido et al., 2009). TRPC1 interaction with ER's STIM1 and PM's Orai1 has been reported recently (Cheng et al., 2008, p. 1; Huang et al., 2006; Ong et al., 2007; Philipp et al., 1996).

1.6.2.3.6 SOC Channels and Cancer

Ion channels play a crucial role in tumor development starting from a malignant transformation of cells for abnormal differentiation which leads to high proliferation rate,

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altered apoptosis, cell migration and invasion (Bose et al., 2015). It is shown in tumor cells that SOC mediated cytosolic Ca^{2+} increase leads to trigger apoptosis (Flourakis et al., 2010). There are many reports which support the role of STIM1 and Orai1 for migration and metastasis in various cancers like cervical cancer, breast cancer and hepatocarcinoma (Chen et al., 2011; Huang and Jan, 2014; Yang et al., 2013, 2009). CRAC channels are also reported as an angiogenesis promoting factor via VEGF-activated Ca^{2+} influx (Yang et al., 2009). Similarly many TRPs are also reported to show their activity in many cancer types. The consolidated findings and co-relation with TRPs and SOCs are shown below in table 1 (adopted from Bose et al., 2015).

Table 1: The role of TRP and SOC channels in cancer development and progression

Cancer Hallmarks	**Ion Channels (TRPs & SOCs)**	**Expression Profile (for Gene and protein)**	**Cancer Type**
Proliferation	TRPs(TRPC6, TRPV6, TRPM7, TRPM8)	Up-regulation	Breast cancer, prostate cancer, head and neck cancer, human glioblastoma cell line
	SOCs (STIM1/Orai1)	Down-regulation	Lung cancer cells, cervical cancer
	SOCs (STIM1/Orai1)	Up-regulation	Cervical cancer, glioblastoma cells
Cell Migration & Metastasis	TRP (TRPM7, TRPM8, TRPV1, TRPV6)	Up-regulation	Lung cancer cells, primary breast cancer, prostate cancer cells, squamous carcinoma, hepatoblastoma
	SOCs (STIM1/Orai1)	Up-regulation	Breast cancer, cervical cancer, hepatocarcinoma, glioblastoma

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Tumor Angiogenesis	TRP (TRPC6)	Up-regulation	Human glioblastoma cell line
	SOCs (STIM1/Orai1)	siRNA- or dominant negative mutant-mediated knockdown	VEGF-induced angiogenesis observed in Tumors
Apoptosis Resistance	TRP (TRPA1)	Up-regulation	human glioblastoma cell line
	SOC (Orai1)	siRNA mediated knockdown	Prostate cancer cell line

1.7 Ca^{2+} Channels/Pumps associated with oral cancer

The influence of Ca^{2+} homeostasis on oral cancer was first shown by Liu *et al*. 2001where the study suggested that any alteration in Ca^{2+} signaling gene, such as ATP2A2 gene (for encoding sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase isoform 2 or SERCA2), may lead to oral squamous cell carcinomas (OSCC). Further, Endo *et al.* in 2004 investigated and suggested that the epigenetic mechanism may be responsible for the changes in ATP2A2 at early stage of oral squamous cell carcinomas. In another study a crucial gene for intracellular calcium regulation, *PMCA1*, was investigated in OSCC by Saito *et al*. in 2006. He suggested that *PMCA1* gene inactivation through epigenetic mechanism is one of the early events in OC. Recently TRP channels and Orai1 has been reported for OC where TRPs were associated with invasion-migration and tumor pain but Orai1 controls the stemness (Gonzales et al., 2014, p. 1; Lee et al., 2016, p. 1; Okamoto et al., 2012, p. 8; Ruparel et al., 2015). A consolidated table 2 has been given below for Ca^{2+} Channels/Pumps reported in oral cancer.

In summary SOC channels are shown to play important role in cancer cell proliferation, migration and metastasis like various cancer hallmarks in different cancers. Many studies suggested that SOC channel protein mainly Orai1 and STIM1 could be a potential therapeutic

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target. But there are very few studies have been done on oral cancer to find the role of SOC channels. Therefore my study will be- "Exploring Store Operated Calcium Entry Channels in Human Oral Carcinomas".

Table 2: Ca^{2+} Channels/Pumps reported in OC so far

Ca^{2+} Channels or Pumps in OC	**Findings**	**References**
SERCA2	The SERCA2 gene (*i.e.* Atp2a2) was found to be responsible for tumors of squamous cells such as oral mucosa, skin, fore stomach and esophagus.	Liu *et al*. (2001).
SERCA2	The down regulation of human SERCA2 gene (ATP2A2) is an early event probably by DNA methylation induced gene inactivation in case of OSCC.	Endo, Y. *et al*. (2004).
PMCA1 pump	Due to DNA methylation in OSCC the early event occurred is inactivation of PMCA 1 (down regulated) **.**	Saito, K. *et al*. (2006).
TRPM8	Its role in migration and invasion of oral cancer cell.	Okamoto Y*et al*. (2012).
TRPV1 and TRPA1	Both proteins are associated with oral cancer pain and the apoptosis mechanism, induced by vanilloid, is found to be independent of TRPV1 activation**.**	Ruparel *et al*. (2015) and Cara B. Gonzales et al. (2014).
Orai1	Orai1 role in OSCC through promoting cancer stemness.	Lee SH *et al*. (2016).

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1.8 Aims of the study

Based on the literature reviewed following objectives were formulated-

1. Status of SOCE /SOCC in normal cells, oral cancer cell lines and patients' samples
2. Effect of tobacco and its components on oral cancer cell proliferation, SOCC genes and SOCE
3. Effect of SOCE modulation in oral cancer cells using SOCE inhibitors and siRNAs

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CHAPTER-2

SOCE In Oral Cancer Cells and Patients Samples

Indian Institute of Technology Guwahati

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2.1. Introduction

As mentioned in the previous chapter the rate of incidence and deaths due to oral cancer in India especially the northeastern states have increased drastically over the years due to excessive consumption of tobacco in this region. In addition, the current available treatment modalities are not sufficient to cure the patients and control the death rate. Additionally, we also found that SOCs are highly upregulated in different cancers and help in oncogenesis (Bose et al., 2015). However, the role of SOCs in oral cancer (OC) is poorly understood. Over the last two decades, few store independent calcium channels and pumps associated proteins has been reported for OC such as PMCA1, SERCA2, and TRPM8 (Endo et al., 2004; Liu et al., 2001; Okamoto et al., 2012). These studies indicated that Ca^{2+}- homeostasis is a key factor for cancer initiation and progression. Until the year 2015, there was no report for SOCE and oral cancer, however a recent study demonstrated that Orai1 was responsible for oral cancer stem cell like properties (Lee et al., 2016). This study shows that Orai1 induced tumorigenicity and stemness through a downstream effector molecule, NFATc3. Moreover, the study also suggested that overexpression of Orai1 also induced cytokines with high cancer stem cell (CSC) properties. There are other channel proteins such as Orai2, Orai3, STIM1 and STIM2. However, the role of these proteins in oral cancer is not known. Therefore, in this chapter we have examined the status of SOCs and the expression of SOCs associated proteins in oral cancer. In this chapter, first we examined the status of SOCs in oral cancer cells versus normal cells; second the expression of SOCs associated gene expression in oral cancer cells compared to normal cells and finally the expression of Orai1 & 2 in human oral cancer tissues compared to normal tissues.

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Material and Methods

2.2.1. Cell Culture

Two cell lines were used in this study- SAS (oral cancer) and HaCaT (non-cancerous skin epithelial). The SAS cells were obtained from RGCB (Rajiv Gandhi Centre for Biotechnology), Trivandrum and HaCaT cells were obtained from NCCS, Pune. The DMEM (Dulbecco's Modified Eagle Medium) high glucose (Invitrogen) media with 10% heat inactivated fetal bovine serum (FBS) (Invitrogen-Gibco) and 1% Penicillin-Streptomycin mixtures (Invitrogen) was used to maintain the cells at 37°C in 5% CO_2 and 95% R.H. incubator (Chiou et al., 2008).

2.2.2. Store Operated Ca^{2+}-influx measurement in cell lines

To study the SOCs in oral cancer cells vs normal cells 30,000 cells were seeded per well in triplicate for each type of cells and grown in DMEM media supplemented with 10% FBS and 1% PS until it forms a mono layer. After forming the monolayer, wells were washed with SBS with Ca^{2+} (NaCl 135 mM, KCl 5 mM, MgCl2 1.2 mM, Glucose 8 mM, HEPES 10 mM and CaCl2 1.5 mM; pH adjusted to 7.4) two times. Now 2µM Fura 2-AM with 0.02% of Pluronic F-127 was prepared in SBS with Ca^{2+} and 100µL/well loaded in the cell plate. The plate was covered with aluminium foil after loading the probe and incubated for 1h at room temperature. After 1h cells were washed with SBS (with Ca^{2+}) twice and re-incubated with SBS with Ca^{2+} for 15 minutes to cleave the acetoxymethyl (AM) esters group and allow Fura-2 to enter inside the cells. After loading the dye, cell monolayers were washed with zero Ca^{2+} SBS (NaCl 135 mM, KCl 5 mM, $MgCl_2$ 1.2 mM, Glucose 8 mM, HEPES 10 mM and pH adjusted to 7.4) and pre-incubation with 2µM thapsigargin at 37°C for 30 min. The thapsigargin added mixture was replaced with 200 µl zero Ca^{2+} SBS. In a U-bottom 96 well plate 15mM $CaCl_2$ was prepared which will be

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diluted to 3mM after injection (50µl) in the cell plate by using the pipetting system of the fluorometer (BMG NOVOstar). The data was recorded for both 340nm and 380nm in excel sheet which was used to analyse the results and plotted. The statistics was checked using one way t-test. For statistical significance (*) was considered at $p < 0.05$.

2.2.3. Expression of SOCC Genes at mRNA level using $\Delta\Delta C_T$ method

To determine the expression of different SOCE genes expression at mRNA level in SAS and HaCaT cells total RNA was extracted using TRIzol methods (Sigma) from both the cell lines and the cDNA was prepared from 1µg of RNA using cDNA synthesis kit method (Applied Biosystems). The quantitative polymerase chain reaction (qPCR) was performed for the SOC genes- Orai1, Orai2, Orai3, STIM1 and STIM2. The primer list is given in the table 2.1. The reaction mixture was prepared using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) and the reaction condition was taken initial denaturation 95° C for 10 min, 40 cycles of each set of denaturation (95° C/15s) then combined annealing and extension (60° C/1min). After the 40 cycles a fixed melt curve was used in real time PCR 7500 (Applied Biosystems). The obtained data was compared using $\Delta\Delta C_T$ method or comparative C_T method and calculated the fold change for each gene between SAS and HaCaT using MS Excel (* is for $p < 0.05$ *i.e.* significance). The overall data was plotted using MS Excel.

Table-2.1: Primer list real-time PCR

Gene Name		Sequence 5' to 3'	Tm	Amplicon Size	References
Orai 1 (NM_032790.2)	F R	ACCTCGGCTCTGCTCTCC GATCATGAGCGCAAACAGG	60.76 57.09	147bp	Chin-Smith *et.al* 2014
Orai 2 (NM_032831.1)	F R	TACCTGAGCAGGGCCAAG GGTACTGGTACTGCGTCTCAA	58.60 59.46	109bp	Chin-Smith *et.al* 2014
Orai 3 (NM_152288.2)	F R	ACGTCTGCCTTGCTCTCG GAGTGCAAAGAGGTGCACAG	59.74 59.41	141bp	Chin-Smith *et. al* 2014
STIM1 (NM_1277961.1)	F R	AAGGCTCTGGATACAGTGCTCTTT AGCATGAAGTCCTTGAGGTGAT	61.85 60.10	71bp	*Respiratory Research* 2006, **7**:119

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		TAT			
STIM-2 (NM_001169118.1)	F R	ACGACACTTCCCAGGATAGCA GACTCCGGTCACTGATTTTCAA C	60.90 59.81	73bp	*Respiratory Research* 2006, **7**:119
β – Actin (NM_001101.3)	F R	CCCTGGCACCCAGCAC GCCGATCCACACGGAGTAC	59.97 60.23	71bp	Sherin Bakhashab et al (2014)

2.2.4. Immunohistochemistry for Checking Expression of Orai1 and Orai2 in Patient's Tissue Samples

Two tissue micro array (TMA) slides with OC patient's tissue samples (Cat no. - OR802) were purchased from U. S. Biomax, (USA) to determine the expression of Orai1 and Orai2 proteins. Overall detail information about the slides are given below-

Oral cavity disease spectrum (oral cavity cancer progression) TMA with 79 cases/80 cores

Name: OR802

Panel: Oral cavity disease spectrum (oral cavity carcinoma progression) tissue microarray

Cases: 79

Cores: 80

Diameter: 1.5mm

Rows: 8

Columns: 10

Table 2: Oral cavity tissue array details (OR802)

Position	Age	Sex	Organ	Pathology diagnosis	TNM	Grade	Stage
A1[a]	40	M	Gingiva	Squamous cell carcinoma	T4N0M0	1	IV
A2[a]	47	F	Tongue	Squamous cell carcinoma	T1N0M0	1	I
A3[a]	81	M	Lip	Squamous cell carcinoma	T2N0M0	1	II
A4[a]	57	M	Tongue	Squamous cell carcinoma	T1N0M0	1	I
A5[a]	52	F	Lip	Squamous cell carcinoma	T1N0M0	2	I
A6[a]	53	M	Cheek	Squamous cell carcinoma	T2N0M0	1	II
A7[a]	62	F	Cheek	Squamous cell carcinoma	T1N0M0	1	I
A8[a]	48	M	Base of tongue	Squamous cell carcinoma	T2N0M0	-	II
A9[a]	68	M	Right palate	Squamous cell carcinoma	T2N0M0	1	II
A10[a]	56	F	Cheek	Squamous cell carcinoma	T2N0M0	1	II
B1[a]	79	M	Cheek	Squamous cell carcinoma	T2N0M0	1	II
B2[a]	60	M	Gingiva	Squamous cell carcinoma	T1N0M0	1	I
B3[a]	55	M	Cheek	Squamous cell carcinoma	T1N0M0	1	I
B4[a]	66	M	Tongue	Squamous cell carcinoma	T1N0M0	1	I
B5[a]	46	F	Tongue	Squamous cell carcinoma	T1N0M1	1	IV

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B6[a]	39	F	Tongue	Squamous cell carcinoma	T1N0M0	1	I
B7[a]	78	M	Tongue	Squamous cell carcinoma	T2N0M0	1	II
B8[a]	78	F	Lip	Squamous cell carcinoma	T1N0M1	1	IV
B9[a]	54	F	Lip	Squamous cell carcinoma	T1N0M1	1	IV
B10[a]	75	F	Lip	Squamous cell carcinoma	T1N0M1	1	IV
C1[a]	60	M	Tongue	Squamous cell carcinoma	T1N0M0	1	I
C2[a]	73	M	Lip	Squamous cell carcinoma	T1N0M0	1	I
C3[a]	60	M	Gingiva	Squamous cell carcinoma	T1N0M0	2	I
C4[a]	78	M	Lip	Squamous cell carcinoma	T1N0M0	2	I
C5[a]	55	M	Gingiva	Squamous cell carcinoma	T1N0M0	2--3	I
C6[a]	47	M	Lower mandible	Squamous cell carcinoma	T2N0M0	3	II
C7[a]	41	M	Dental alveoli	Squamous cell carcinoma	T1N0M0	3	I
C8[a]	60	M	Tongue	Squamous cell carcinoma	T2N0M0	3	II
C9[a]	40	F	Palate	Adenoid cystic carcinoma	T1N0M0	-	I
C10[a]	45	M	Left lower mandible	Adenoid cystic carcinoma (sparse)	T1N0M0	-	I
D1[a]	64	M	Palate	Adenoid cystic carcinoma	T2N0M0	-	II
D2[a]	66	M	Parotid gland	Acinic cell carcinoma	T2N0M0	-	II
D3[a]	71	M	Mouth floor	Mucoepidermoid carcinoma	T1N0M0	1	I
D4[a]	57	M	Palate	Mucoepidermoid carcinoma	T2N0M0	1	II
D5[a]	50	F	Cheek	Mucoepidermoid carcinoma	T2N0M0	1	II
D6[a]	57	M	Upper lip	Mucoepidermoid carcinoma (skeletal muscle and blood vessel)	T1N0M0	-	I
D7[a]	48	F	Right lower mandible	Mucoepidermoid carcinoma	T1N0M0	2	I
D8[a]	55	M	Gingiva	Mucoepidermoid carcinoma	T1N0M0	3	I
D9[a]	60	M	Right lower mandible	Mucoepidermoid carcinoma	T3N0M0	3	III
D10[a]	50	M	Root of tongue	Mucoepidermoid carcinoma (sparse)	T1N0M0	-	I
E1[a]	79	F	Lip	Basal cell carcinoma (sparse)	T2N0M0	-	II
E2[a]	48	F	Lip	Basal cell carcinoma	T2N0M0	-	II
E3[b]	70	F	Lymph node	Metastatic squamous cell carcinoma of neck from cheek	-	2	-
E4[b]	79	M	Lymph node	Metastatic squamous cell carcinoma of neck from tongue	-	1	-
E5[b]	59	F	Lymph node	Metastatic squamous cell carcinoma of neck from mandible	-	2	-
E6[b]	40	F	Lymph node	Metastatic mucoepidermoid carcinoma of neck from mandible	-	3	-
E7[e]	11	M	Mandible	Adamantinoma	-	-	-
E8[e]	28	M	Left mandible	Adamantinoma	-	-	-
E9[e]	51	M	Right mandible	Adamantinoma	-	-	-
E10[e]	64	M	Mandible	Adamantinoma (fibrous tissue and blood vessel)	-	-	-
F1[e]	37	F	Mandible	Adamantinoma	-	-	-
F2[e]	40	M	Lower mandible	Adamantinoma	-	-	-

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F3[e]	47	F	Mandible	Adamantinoma	-	-	-
F4[e]	70	F	Right jaw bones	Adamantinoma	-	-	-
F5[f]	67	M	Lip	Hyperplasia of squamous epithelium	-	-	-
F6[f]	40	M	Lip	Mild atypical hyperplasia of squamous epithelium	-	-	-
F7[f]	82	M	Lip	Hyperplasia of squamous epithelium	-	-	-
F8[f]	46	M	Tongue	Hyperplasia of squamous epithelium (skeletal muscle and blood vessel)	-	-	-
F9[f]	60	F	Tongue	Hyperplasia of squamous epithelium	-	-	-
F10[f]	3	F	Tongue	Hyperplasia of squamous epithelium	-	-	-
G1[g]	68	M	Parotid gland	Cancer adjacent tissue (with squamous cell carcioma sparse)	-	-	-
G2[g]	53	F	Tongue	Cancer adjacent tissue	-	-	-
G3[g]	53	M	Tongue	Cancer adjacent tissue (hyperplasia of squamous epithelium)	-	-	-
G4[g]	70	M	Parotid gland	Cancer adjacent tissue (with mucoepidermoid carcinoma)	-	2	-
G5[g]	63	M	Lip	Cancer adjacent tissue (chronic inflammation of fibrous tissue and blood vessel)	-	-	-
G6[h]	43	M	Sub maxillary gland	Chronic submaxillaritis	-	-	-
G7[h]	23	F	Parotid gland	Chronic parotitis	-	-	-
G8[h]	66	F	Parotid gland	Chronic parotitis	-	-	-
G9[h]	40	F	Right cheek	Chronic inflammation of mucosa	-	-	-
G10[h]	75	F	Lower lip	Chronic inflammation of mucosa of No. 20	-	-	-
H1[d]	48	F	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-
H2[d]	48	F	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-
H3[d]	37	M	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-
H4[d]	63	M	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-
H5[d]	56	M	Salivary gland	Cancer adjacent normal salivary gland tissue	-	-	-
H6[c]	42	F	Tongue	Normal tongue tissue	-	-	-
H7[c]	38	F	Salivary gland	Normal salivary gland tissue	-	-	-
H8[c]	48	M	Tongue	Normal tongue tissue	-	-	-
H9[c]	50	M	Salivary gland	Normal salivary gland tissue	-	-	-
H10[c]	22	M	Salivary gland	Normal salivary gland tissue	-	-	-

a: malignant tissues, b: metastatic tissues, c: Normal tissues, d: Normal adjacent tissue, e: benign tissues, f: hyperplastic tissues, g: cancer adjacent tissue, h: inflammation

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The expression of Orai1 and Orai2 protein was determined by immunohistochemistry (IHC) using Histostain plus kit (Life technologies; Cat no: 859043) and specific antibodies [Abcam- Orai1 (Cat no: ab86748) & Orai2 (Cat no: ab155216)], according to manufacturer's protocol. The TMA were deparaffinised and rehydrated using xylene and decreasing concentrations of ethanol (*i.e.* from 100% to 50%) respectively. To overcome with endogenous peroxidase activity the slides were blocked in 3% H_2O_2 in methanol for 30 minutes and epitope exposure was done by breaking the protein crosslink while heating the slides at 60°C in sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20; pH 6.0) for 30min. Then the slides were washed with PBS and then incubated for 30 min in the blocking solution of the kit in a humidified chamber. The antibodies were prepared in the blocking buffer as per recommended by the antibodies manufacturer- Orai1 (1:25) and Orai2 (1:50) and incubated in the moist chamber at 4 °C for overnight. On day2 the slides with tissue sections were incubated with the secondary antibody provided with the kit containing reagent for 1h at room temperature before washing the tissue sections with PBS. Then after PBS wash it was stained with Metal Enhanced DAB (3'-Diaminobenzidine) Substrate Kit (Cat no- 34065) and counter stained with haematoxylin to stain the nucleus. After developing the DAB colour and counter stain, the tissue sections were dehydrated using increasing concentrations of ethanol (from 50% to 100%). Finally the sections were mounted using D.P.X. (distyrene, a plasticizer, and xylene) and put the cover slip. The slides were the allowed to dry until it became hard and ready for scoring.

Scoring:

After performing the IHC experiment, scores were given to each stained tissue sections based on overall DAB intensity (I) and percentage of cells (P) with the stain. The final score (Q) for each tissue section was calculated by the multiplication of I and P (Q = I x P).

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The score for I was visually determined as low (1+), moderate (2+) and high (3+). Later, the score was provided to each section for P as- 0 for ≤10% stained cells, 1 for ≤10-25% stained cells, 2 for ≤25-50% stained cells, 3 for ≤50-75% stained cells and 4 for ≤75-100% stained cells (Kraus et al., 2012; McDonald and Pilgram, 1999). The statistical significance (*) was compared with normal tissues using T-test for unequal variances (where * represents $p< 0.05$) in Microsoft Excel.

2.2.5. Statistical analysis

The statistical analysis was done in MS Excel for calculation and simple t-test or ANOVA was performed to determine the confidence interval at p<0.05.

2.3. Results and Discussion

In the present chapter we have studied the level of SOCE in oral cancer cells compared to normal cells. We have also studied the expression of SOCE associated genes such as Orai1, Orao2, Orai3, STIM1 and STIM2 in oral cancer cells compared to normal cells. In our study, we have also examined the Orai1 and Orai2 in human oral cancer tissues compared to normal tissues. This is the first report that shows the level of SOCE and SOCs related gene expression in oral cancer cells compared to normal cells. The preliminary data suggested that high SOCE in oral cancer cells were associated with high expression of Orai1 and Orai2 genes which we confirmed with patients samples.

2.3.1. High SOCE in oral cancer cells compared to normal cells

In the first step we studied the level of SOCE in oral cancer cells compared to normal cells to understand the Ca^{2+}-uptake behaviour in oral cancer cells. We performed Ca^{2+}-influx measurement after depletion of cellular calcium store (ER) using thapsigargin and Fura-

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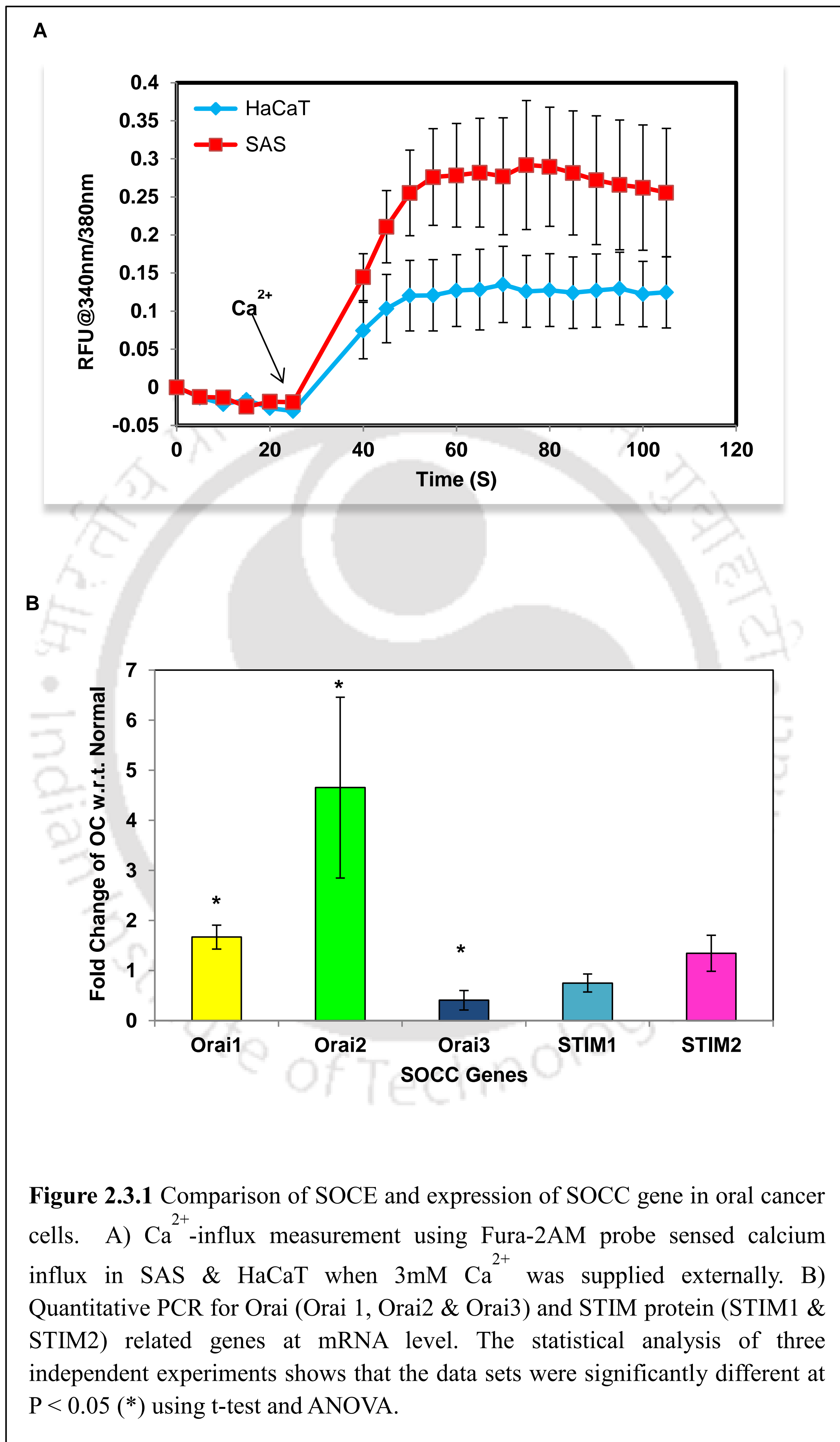

Figure 2.3.1 Comparison of SOCE and expression of SOCC gene in oral cancer cells. A) Ca^{2+}-influx measurement using Fura-2AM probe sensed calcium influx in SAS & HaCaT when 3mM Ca^{2+} was supplied externally. B) Quantitative PCR for Orai (Orai 1, Orai2 & Orai3) and STIM protein (STIM1 & STIM2) related genes at mRNA level. The statistical analysis of three independent experiments shows that the data sets were significantly different at $P < 0.05$ (*) using t-test and ANOVA.

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2AM, a calcium sensing probe. Our results show that there was a significant upregulation of SOCE in oral cancer cells compared to normal cells (Figure 2.3.1A). High Ca^{2+}-influx (*i.e.* SOCE) is probably due to the aggressiveness of the cancer cells compared to normal cells. Since the cancer cells are known for high proliferation, invasion and migration, it requires high amount of Ca^{2+} ions to perform cell division and metastasis (Parkash and Asotra, 2010). This must be due to some alteration in cellular calcium handling tools such as calcium channels, pumps, exchangers and binding proteins etc. (Stewart et al., 2015). Similar to our study, upregulation of SOCE and associated channels has been reported in breast cancer, liver cancer, prostate cancer and cervical cancer (Bose et al., 2015).

2.3.2. Overexpression of Orai1 and Orai2 expression in oral cancer cells

In the previous experiment we found that the SOCE is highly upregulated in oral cancer cells. To find out why SOCE is upregulated we examined the SOC genes in oral cancer cells compared to normal cells by real-time PCR. The relative SOC genes expression were done for SAS cells using comparative C_T method where HaCaT cells were considered as control for fold change comparison of each gene (Figure 2.3.1B). In our study Orai1 and Orai2 showed significant upregulation by 1.6 folds and 4.6 folds respectively in oral carcinoma cells compared to normal cells. The Orai1 upregulation was reported in many cancer cases including oral cancer (Guéguinou et al., 2016; Lee et al., 2016; McAndrew et al., 2011; Pla et al., 2016) but this is the first report of upregulation of Orai2 in oral cancer. Notably, very few studies have been performed on Orai2 and Orai3 due to which these two are considered as ignored CRAC proteins (Hoth and Niemeyer, 2013). However, the Ca^{2+}-influx rate varies for Orai1, Orai2 and Orai3 among them Orai3 does not show any detectable calcium current in the study (DeHaven et al., 2007). In line with our study, Orai1 overexpression in oral cancer cells was found to regulate stem cell property through

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nuclear factor of activated T cells (NFAT) signaling pathway (Lee et al., 2016). Orai1 in association with STIM1 was found to be regulating cell migration in breast cancer through regulation of focal adhesion kinases (FAK) and calcium-dependent protease calpain which further regulates the focal adhesion turnover in breast cancer cells (Yang et al., 2009). Recently, Orai1 and Orai2 were reported to form CRAC channel by direct coupling in human chondrosarcoma where it activated by STIM1 (Inayama et al., 2015). In our study, we concluded that Orai1 and Orai2 genes were upregulated in oral cancer compared to normal cells.

2.3.3. Orai1 and Orai2 protein overexpressed in oral cancer patients' samples

To evaluate the status of Orai1 and Orai2 in patient samples, we performed IHC on a tissue microarray slide with oral cancer patients' tissue samples using Orai1 and Orai2 antibodies. We found that Orai1 and Orai2 were upregulated in oral cancer and its distribution in different sexes, stages, grades and location in mouth compared to normal tissues were analysed. In the current study, we observed a significant upregulation of Orai1 and Orai2 in overall oral cancer tissue samples compared to normal tissues (Figure 2.3.2C). Furthermore, we also observed that Orai1 and Orai2 showed significant upregulation in male tissues but in case of female only Orai1 showed upregulation (Figure 2.3.3). This data directly correlates with the amount of tobacco and alcohol consumption which is more in males than females (Asthana et al., 2016; Yue et al., 2015). We also found that expression of Orai1 and Orai2 was significantly upregulated in metastatic patient's tissue samples (Figure 2.3.4). Moreover, we observed that the tissues with different stages and grades showed significant upregulation of Orai1 but not Orai2 (Figure 2.3.5). Overall our IHC based analysis indicated that Orai1 and Orai2 was overexpressed in oral cancer tissues compared to normal tissues and the data also supported their significant role in advance

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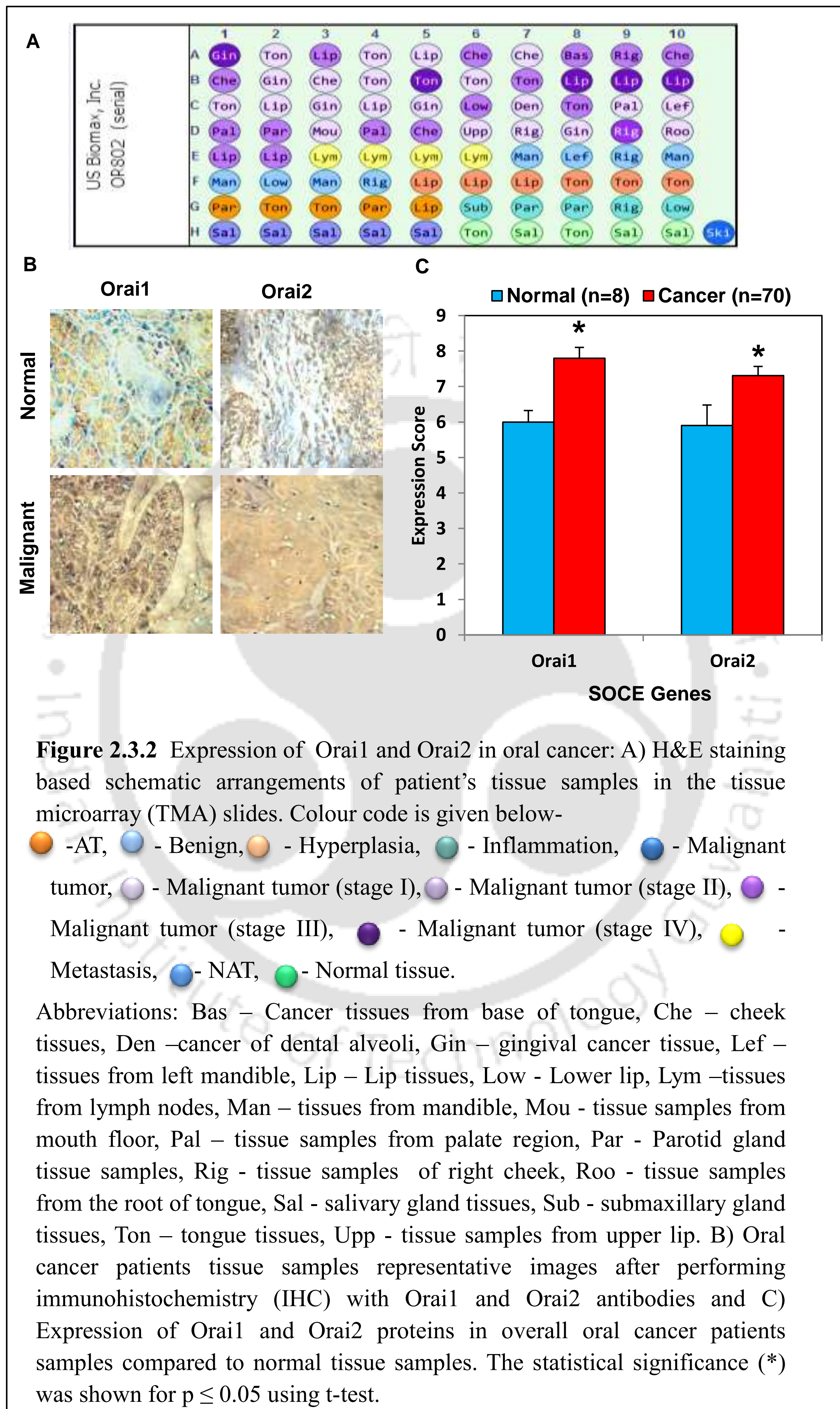

Figure 2.3.2 Expression of Orai1 and Orai2 in oral cancer: A) H&E staining based schematic arrangements of patient's tissue samples in the tissue microarray (TMA) slides. Colour code is given below- -AT, - Benign, - Hyperplasia, - Inflammation, - Malignant tumor, - Malignant tumor (stage I), - Malignant tumor (stage II), - Malignant tumor (stage III), - Malignant tumor (stage IV), - Metastasis, - NAT, - Normal tissue.

Abbreviations: Bas – Cancer tissues from base of tongue, Che – cheek tissues, Den –cancer of dental alveoli, Gin – gingival cancer tissue, Lef – tissues from left mandible, Lip – Lip tissues, Low - Lower lip, Lym –tissues from lymph nodes, Man – tissues from mandible, Mou - tissue samples from mouth floor, Pal – tissue samples from palate region, Par - Parotid gland tissue samples, Rig - tissue samples of right cheek, Roo - tissue samples from the root of tongue, Sal - salivary gland tissues, Sub - submaxillary gland tissues, Ton – tongue tissues, Upp - tissue samples from upper lip. B) Oral cancer patients tissue samples representative images after performing immunohistochemistry (IHC) with Orai1 and Orai2 antibodies and C) Expression of Orai1 and Orai2 proteins in overall oral cancer patients samples compared to normal tissue samples. The statistical significance (*) was shown for $p \leq 0.05$ using t-test.

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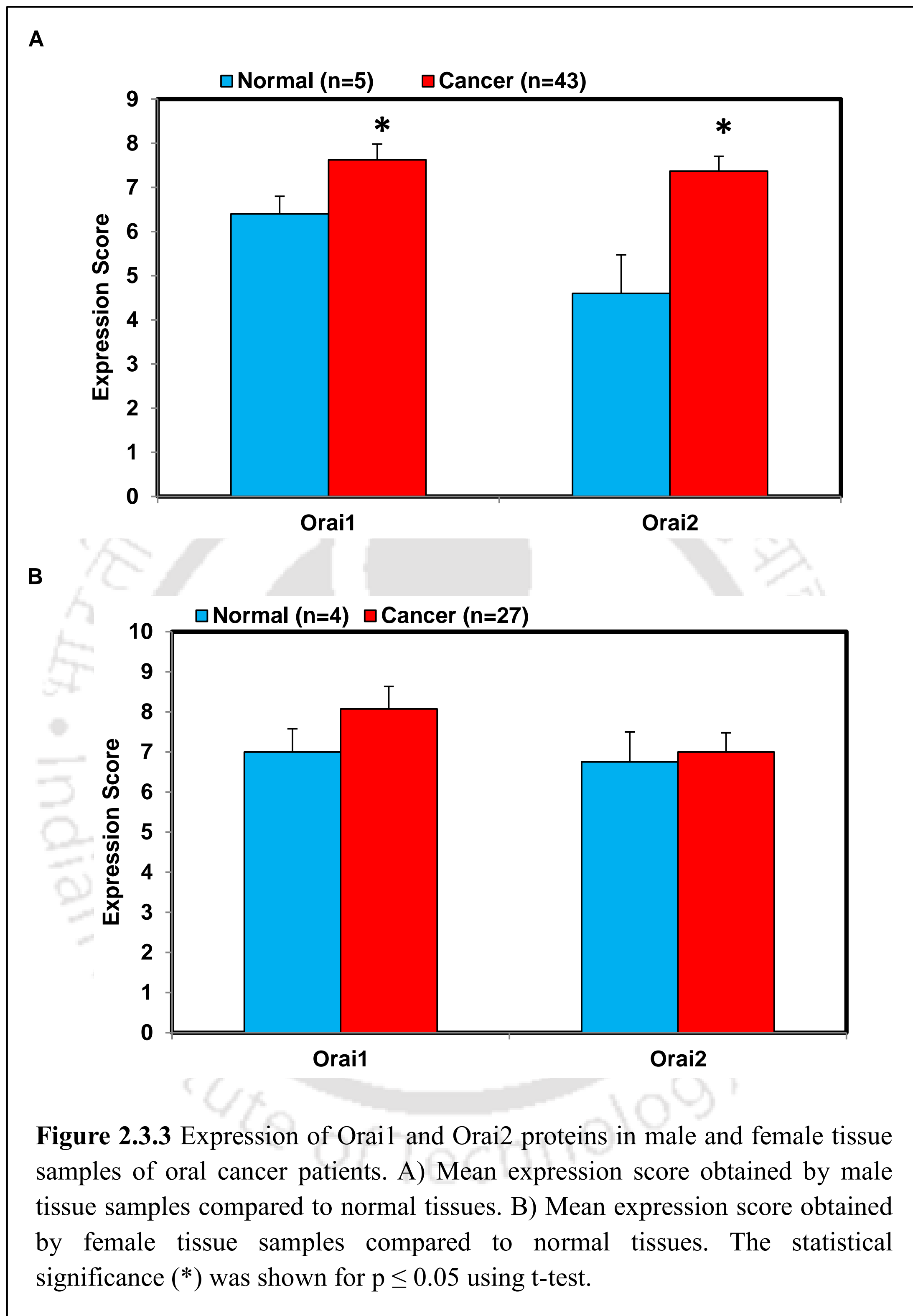

Figure 2.3.3 Expression of Orai1 and Orai2 proteins in male and female tissue samples of oral cancer patients. A) Mean expression score obtained by male tissue samples compared to normal tissues. B) Mean expression score obtained by female tissue samples compared to normal tissues. The statistical significance (*) was shown for $p \leq 0.05$ using t-test.

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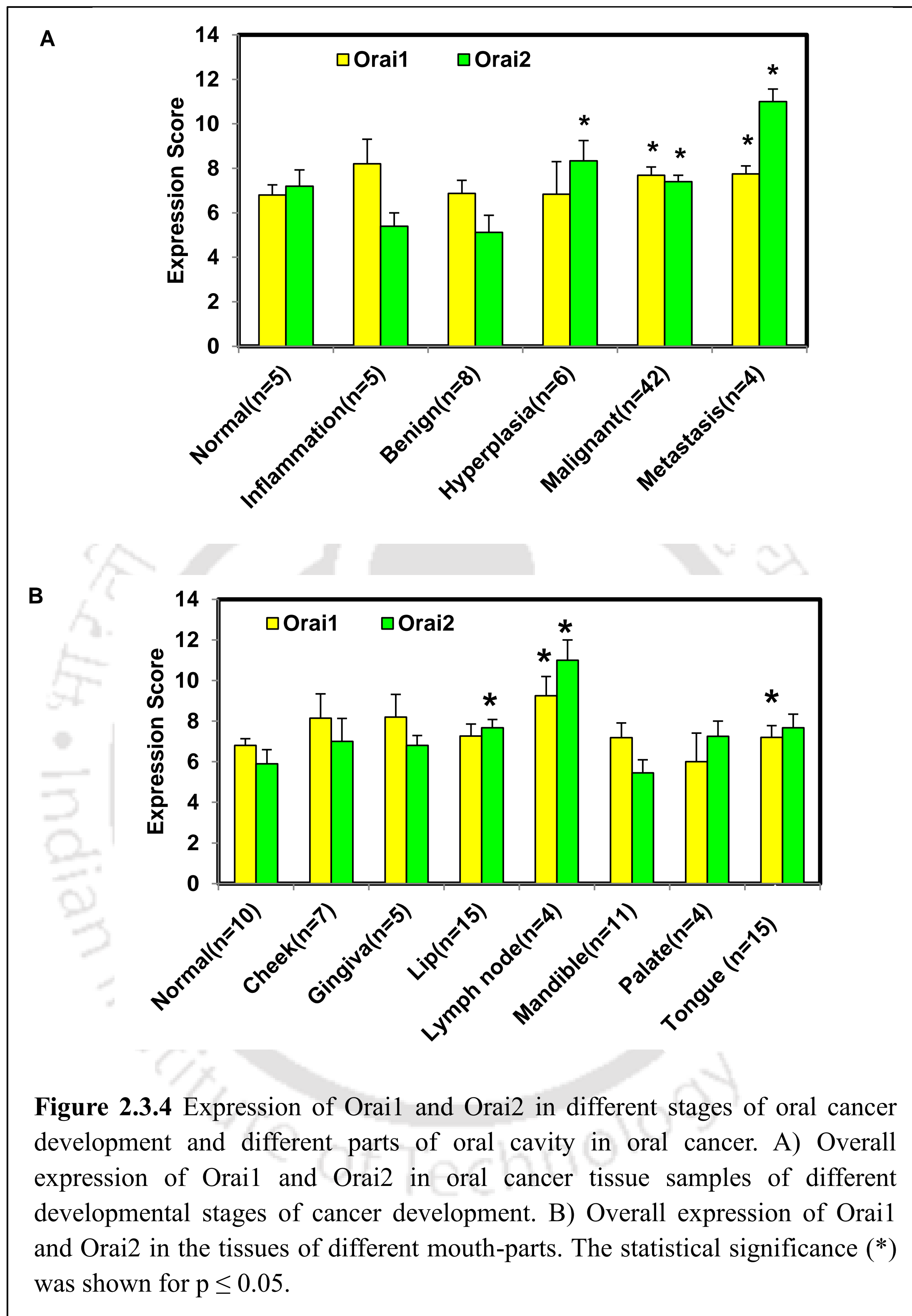

Figure 2.3.4 Expression of Orai1 and Orai2 in different stages of oral cancer development and different parts of oral cavity in oral cancer. A) Overall expression of Orai1 and Orai2 in oral cancer tissue samples of different developmental stages of cancer development. B) Overall expression of Orai1 and Orai2 in the tissues of different mouth-parts. The statistical significance (*) was shown for $p \leq 0.05$.

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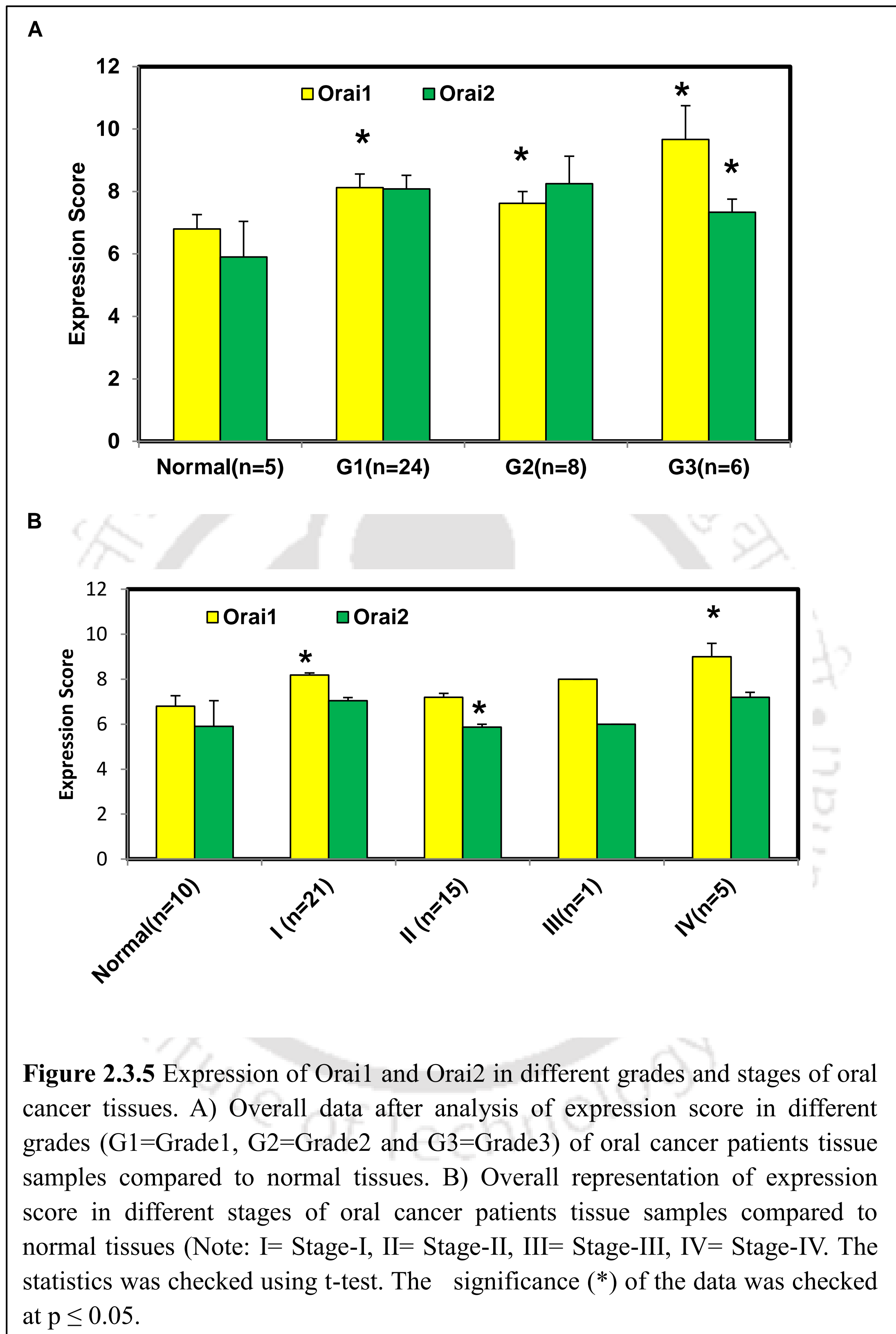

Figure 2.3.5 Expression of Orai1 and Orai2 in different grades and stages of oral cancer tissues. A) Overall data after analysis of expression score in different grades (G1=Grade1, G2=Grade2 and G3=Grade3) of oral cancer patients tissue samples compared to normal tissues. B) Overall representation of expression score in different stages of oral cancer patients tissue samples compared to normal tissues (Note: I= Stage-I, II= Stage-II, III= Stage-III, IV= Stage-IV. The statistics was checked using t-test. The significance (*) of the data was checked at $p \leq 0.05$.

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metastatic stage patients. Recently, few studies were reported that Orai1 and STIM1 regulates metastasis in breast cancer and liver cancer through focal adhesion turnover (Yang et al., 2013, 2009). Here, we have reported first time that Orai2 was significantly upregulated in oral cancer tissue samples. The IHC based analysis for OSCC was shown in a recent study where normal human oral epithelial tissues showed weak staining, dysplasia tissue showed moderate staining and most of the OSSC tissues where with strong staining. Finally, they found that Orai1/NFAT axis was regulating the CSC in oral/oropharyngeal carcinoma (Lee et al., 2016). So far there were no report on Orai2 role in oral cancer but its role was explored in human leukemia cell line (HL60) where phosphorylation of focal adhesion kinase was found to be synchronized by Orai1 and Orai2 which further regulated the cell migration (Diez-Bello et al., 2017). Therefore, we further investigated the role of Orai1 and Orai2 in oral cancer to elucidate the exact mechanism of action which will be discussed in the chapter 4.

2.4. Conclusion

In this chapter our aim was to check the status of SOCE in OC cells compared to non-cancerous (*i.e.* normal) cells. We also wanted to know the key proteins which may be responsible for high SOCE (*i.e.* Ca^{2+}-influx) and verify that with OC patient's samples. Overall by using three different techniques (Ca^{2+}-influx measurement, qPCR and IHC), we understand that SOCE was high in OC cell compared to normal cells which may be due to overexpressed Orai1 and Orai2 as suggested by qPCR data. Later, the IHC data also supported this hypothesis that high Orai1 and Orai2 may have significant role in OC progression which may have important role in metastasis. We further investigated that how tobacco associated carcinogens can affect SOCE/SOCC in OC cells and if we disrupt the Ca^{2+}-influx then what could be the changes in OC cells?

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CHAPTER-3

Effect of Tobacco on SOCE of Oral Cancer Cells

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3.1 Introduction

From our previous chapters, we understand that Orai1 and Orai2 were upregulated in oral cancer cells and tissues which might be associated with the influence of risk factors. The several decades of research revealed that tobacco is a major risk factor for oral cancer and incidence of this cancer is more common among tobacco smokers and chewers (Johnson, 2001; Rodu and Jansson, 2004; Sadri and Mahjub, 2007). Recently, it has been found that the incidence of oral cancer is higher in males than females which could be attributed to the increased tobacco consumption among males (Asthana et al., 2016). More than 200 potential carcinogens have been identified to be present in tobacco and benzo[a]pyrene (B[a]P), nicotine, *N-nitrosonornicotine* (NNN) and nicotine-derived nitrosamine ketone (NNK) are the major carcinogens among them (Nebert et al., 2013; Talhout et al., 2011; Xue et al., 2014). Likewise, 4NQO is a synthetic carcinogen which has been extensively used to mimic the developmental stages and associated molecular alteration of oral cancer in animal models (Hawkins et al., 1994; Rivera, 2016; Arima et al., 2006). In the multi-step developmental processes of carcinogenesis through poly aromatic hydrocarbons such as B[a]P, nitrosamines (*i.e.* NNN and NNK) or 4NQO the formation of DNA adduct and ROS is common (Nebert et al., 2013; Rivera, 2016; Xue et al., 2014). Therefore, we determined the effect of B[a]P, NNN, NNK and 4NQO on SOCE/SOCC of oral cancer cells in our study. Moreover, Ca^{2+} oscillation was found to be affected by B[a]P in liver cancer (Barhoumi et al., 2002) which suggested that other tobacco carcinogens might also have some role in Ca^{2+}-regulation which may leads to the development of oral cancer. Therefore, we hypothesized that these tobacco associated carcinogens and 4NQO might be involved in the dysregulation of Ca^{2+}-influx thus resulting in oral cancer. In order to identify whether these carcinogens play any role in the regulation of Ca^{2+}- homeostasis, effect of tobacco and its associated carcinogens on SOCE/SOCC were further studied in this chapter. To understand that we first analysed the

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Ca^{2+}-influx after treatment with these carcinogens and analysed the expression of SOC genes such as Orai1, Orai2, STIM1, STIM2 and TRPC1 in oral cancer cells at the mRNA level.

3.2 Materials and Methods

3.2.1 Cell Culture

SAS cells were received from RGCB (Rajiv Gandhi Centre for Biotechnology), Trivandrum. DMEM (Dulbecco's Modified Eagle Medium) high glucose (Invitrogen) media along with 10% FBS (Invitrogen-Gibco) and 1% of Penicillin-Streptomycin (Invitrogen) was used to grow the cells at 37°C in a 5% CO_2 and 95% R.H. in incubator (Chiou et al., 2008).

3.2.2 Chemical Carcinogens

In our experiments we used crude tobacco extract (TE) prepared from tobacco which was purchased from local tobacco shop where people directly buy and consume tobacco. The purchased tobacco was finely chopped and grinded using motor-pestle then the powdered form of the tobacco was dissolved in water and filtered with filter paper. Later, the filtrate was lyophilized and the powder was re-dissolved in water for experimental uses. Other tobacco components such as B[a]P (Cat. no.- B1760), NNN (Cat. no.-78013) and NNK (Cat. no.-78013) purchased from Sigma-Aldrich. Apart from these tobacco components a synthetic carcinogen, 4NQO (Cat. no.- N8141) from Sigma-Aldrich, was also used in our experiments.

3.2.3 Cell Proliferation Assay

2000 cells per well were seeded in two 96 well plates, one for 0h and one for 24h treatment with the carcinogens. Next day, the cells were treated with the chemical carcinogens and MTT assay was performed for one plate at 0h and another plate at 24h treatment. MTT (*i.e.* (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that is

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used to quantify cell proliferation. After the incubation period over, 10 µl of MTT was added per well and the plates were incubated at 37°C for 2h in the CO_2 regulated incubator. After 2h incubation, the MTT added media was replaced with DMSO (100 µl/well) and the absorbance was recorded at 570nm using Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland) microplate reader. The percentage of cell proliferation was calculated using the formula:

$$\% \text{ Proliferation} = \frac{\text{Absorbance (Control−Treatment)}}{\text{Absorbance (Control)}} \times 100$$

3.2.4 Store Operated Ca^{2+}-influx measurement after treating cells with the carcinogens

To study the role of tobacco and associated carcinogens in Ca^{2+}- homeostasis through SOCs in oral cancer 30,000 cells were seeded per well in triplicate and grown in DMEM media with 10% FBS and 1% PS. On day2 treatments were given with different concentrations of tobacco and associated carcinogens prepared in the DMEM complete media. Same steps were performed for 4NQO. The treated cells were incubated for 24h or till it formed monolayer. After formation of monolayer, all wells were washed twice with Ca^{2+} -SBS (NaCl 135 mM, KCl 5 mM, MgCl2 1.2 mM, Glucose 8 mM, HEPES 10 mM and $CaCl_2$ 1.5 mM; with pH 7.4). Then 2µM Fura 2-AM mixture was prepared with 0.02% of Pluronic F-127 in Ca^{2+} -SBS and 100µL/well was added in the cell plate. The plate was incubated in dark for 1h at room temperature. After 1h cell monolayers were washed with Ca^{2+}-SBS two times and incubated again with the Ca^{2+}-SBS for another 15 minutes to break the bond of acetoxymethyl (AM) esters group which allowed Fura-2 to enter in the cell cytoplasm. After loading Fura-2, cell monolayers were rinsed with zero Ca^{2+} -SBS (NaCl 135 mM, KCl 5 mM, $MgCl_2$ 1.2 mM, Glucose 8 mM, HEPES 10 mM and pH 7.4) and pre-treated with 2µM thapsigargin for 30 min at 37°C. Finally, the pre-treatment solution was replaced with 200 µl zero Ca^{2+} SBS. In a 96 well U-bottom plate 15mM $CaCl_2$ was simultaneously prepared from which 50µl will be used to injection in the cell plate by using the pipetting system of the

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fluorometer (BMG NOVOstar) and become 3mM in cell plate. After setting the wells and pipetting system the program was ran and florescence data was recorded for the dual excitations 340nm and 380nm with single emission wavelength at 510nm. The obtained data was analysed with MS excel. The statistics were analysed through t-test. Statistical significance (*) was considered at $p < 0.05$.

3.2.5 Expression of SOCC Genes at mRNA level using $\Delta\Delta C_T$ method

To study the effect of tobacco and associated carcinogens total RNA from the treated SAS cells (*i.e.* with crude tobacco extract, NNN, NNK and 4NQO) and untreated control cells were extracted using TRIzol from Sigma-Aldrich. Then cDNAs were prepared from 1µg of each RNA types using cDNA synthesis kit (Cat. no. – 4368814; Invitrogen). The quantitative polymerase chain reaction (qPCR) was performed for the SOC genes- Orai1, Orai2, TRPC1, STIM1 and STIM2. The primer list is given in table 2.1. The reaction mixture was prepared for 10µl each in triplicates using PowerUp™ SYBR® Green Master Mix (Cat. no.- A25742, Invitrogen) and the reaction condition was set as- initial denaturation (95° C for 10 min), 40 cycles of each set of denaturation (95° C/15s) followed by combined annealing-extension (60° C for 1min). After the 40 cycles a default program for melt curve was used in real time PCR7500 (Cat. no.- 4351105, Applied Biosystems). The C_T values obtained from the experiments were analysed using comparative C_T method or $\Delta\Delta C_T$ method and the fold change for the genes were calculated individually with respect to controls. The three independent experiments were compared for statistical significance (*) using Student t-test for p-value < 0.05.

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Table 2.1: Primer list for real-time PCR

Gene Name		Sequence 5' to 3'	Tm	Amplicon Size	References
Orai 1 (NM_032790.2)	F R	ACCTCGGCTCTGCTCTCC GATCATGAGCGCAAACAGG	60.76 57.09	147bp	Chin-Smith *et.al* 2014
Orai 2 (NM_032831.1)	F R	TACCTGAGCAGGGCCAAG GGTACTGGTACTGCGTCTCAA	58.60 59.46	109bp	Chin-Smith *et.al* 2014
STIM1 (NM_1277961.1)	F R	AAGGCTCTGGATACAGTGCTCTTT AGCATGAAGTCCTTGAGGTGATTAT	61.85 60.10	71bp	*Respiratory Research* 2006, **7**:119
STIM-2 (NM_001169118.1)	F R	ACGACACTTCCCAGGATAGCA GACTCCGGTCACTGATTTTCAAC	60.90 59.81	73bp	*Respiratory Research* 2006, **7**:119
hTRPC1 (U31110)	F R	GAGGTGATGGCGCTGAAGG GCACGCCAGCAAGAAAAGC	60.80 61.02	78bp	A. Riccio et al. 2002, 95–104
β – Actin (NM_001101.3)	F R	CCCTGGCACCCAGCAC GCCGATCCACACGGAGTAC	59.97 60.23	71bp	Sherin Bakhashab et al (2014)

3.3 Result and Discussion

In this chapter we have studied the effect of crude tobacco, B[a]P, NNN, NNK and 4NQO on the Ca^{2+} -influx of oral cancer cells through SOCCs. To know the status of responsible channel proteins we examined the expression of Orai1, Orai2, STIM1, STIM2 and TRPC1 genes at mRNA level. We found that these carcinogens are able to induce SOCE with very low dose and overexpression of SOCC genes at mRNA level using real-time PCR.

3.3.1. Crude Tobacco Extract (TE) and its role in SOC of oral cancer

Aforementioned, tobacco has been reported as a main causative agent for oral cancer (Johnson, 2001; Rodu and Jansson, 2004; Sadri and Mahjub, 2007). In our study we first determined the non-toxic concentration of TE by cell proliferation assay. Then we determined the TE concentrations which enhanced the SOCE. These concentrations were used to treat the cells for 24hr to find the changes occurred in SOCC genes expression in oral epithelial cells. In our study we checked the expression of Orai1, Orai2, STIM1, STIM2 and TRPC1 using real-time PCR. Interestingly, we observed a high SOCE upon treatment with

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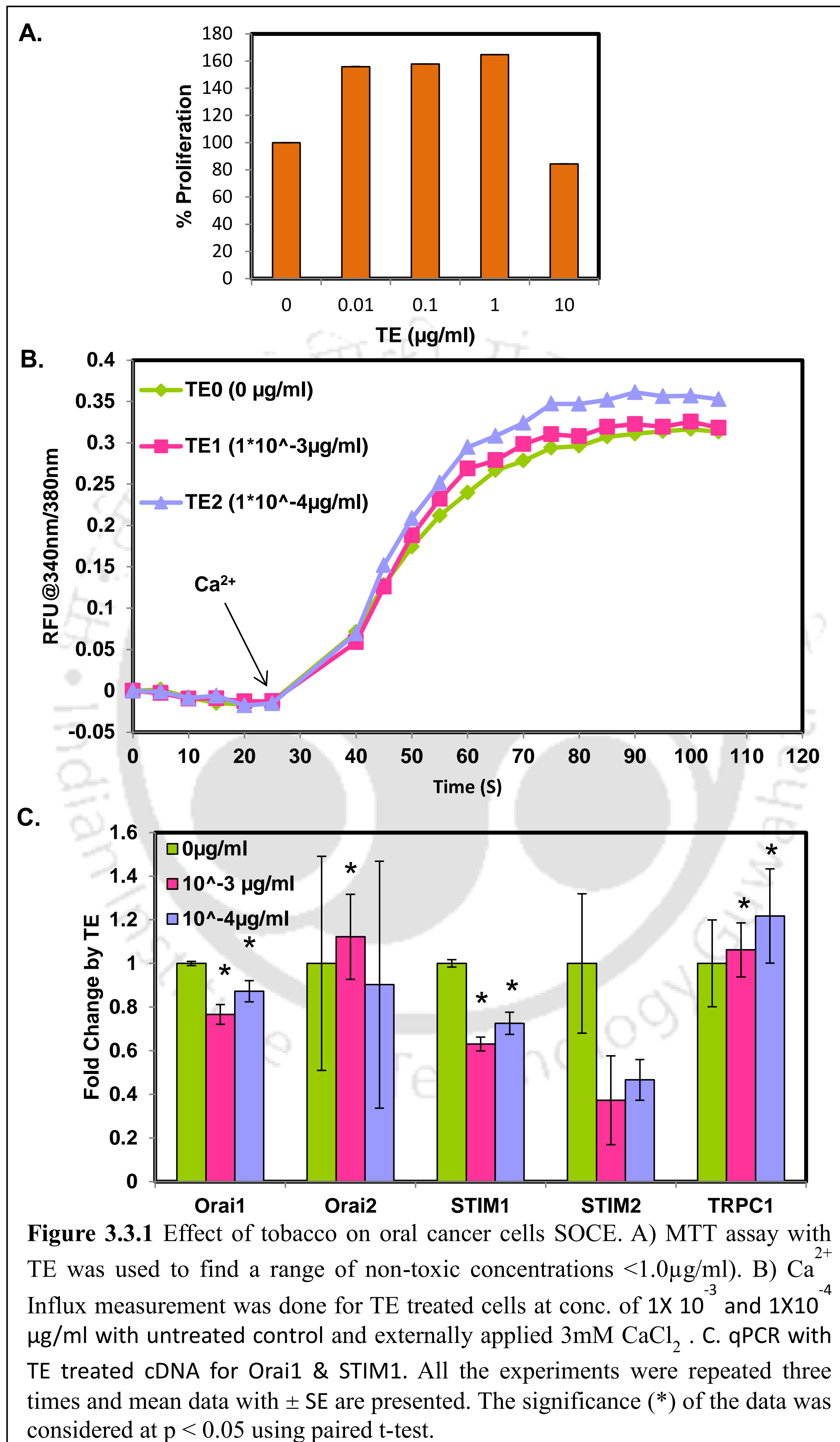

Figure 3.3.1 Effect of tobacco on oral cancer cells SOCE. A) MTT assay with TE was used to find a range of non-toxic concentrations <1.0µg/ml). B) Ca^{2+} Influx measurement was done for TE treated cells at conc. of 1X 10^{-3} and 1X10^{-4} µg/ml with untreated control and externally applied 3mM $CaCl_2$. C. qPCR with TE treated cDNA for Orai1 & STIM1. All the experiments were repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $p < 0.05$ using paired t-test.

TE (*i.e.* 10^{-4}µg/ml and 10^{-3}µg/ml) compared to the untreated control. In line with this, when OC cells were treated with these two concentrations of TE for 24hr, we found that Orai2 and TRPC1 genes were upregulated (~ 1.5 fold) compare to untreated control (figure 3.3.1). However, other genes– Orai1, STIM1 and STIM2 did not show any upregulation. These results are in accordance with a recent article which showed that tobacco smoke increases the cytoplasmic calcium release (Sassano et al., 2017). We are reporting for the first time here that treatment of oral cancer cells with TE increased the Ca^{2+}-influx at the concentrations as low as 10^{-4}µg/ml and 10^{-3}µg/ml. This crude tobacco extract induced elevated Ca^{2+}-influx and SOCC gene expressions cued us to further analyse the effect of individual tobacco carcinogens (B[a]P, NNN and NNK).

3.3.2. Role of B[a]P in the regulation of SOC of oral cancer

Benzo[a]pyrene, B[a]P, is a well-known carcinogen used for the development of various tumor models for research purpose and was also reported earlier for regulating Ca^{2+}-oscillation in clone 9 cells, rat hepatoma cells ((Labib et al., 2012; Shi et al., 2010; Barhoumi et al., 2002). However, so far there is no report available on the B[a]P mediated regulation of SOCE in cancer. In the current study, for the first time, we attempted to analyse the effect of B[a]P on SOCE and SOCC gene expressions in oral cancer. Similar to TE, B[a]P also induced SOCE however at the concentrations lower than that of TE (*i.e.* 10^{-7}µg/ml and 10^{-8}µg/ml). These two concentrations were used for further analysing the SOCC gene expression levels. Treatment of SAS cells with B[a]P resulted in 2-fold and 8-fold increase in the levels of TRPC1 and Orai2 respectively. However, other SOCC genes were unaffected by B[a]P. This is the first report were effect of B[a]P on SOCE/SOCC has been shown (figure 3.3.2). Altogether, it was found that very little amount (10^{-8}µg/ml) of B[a]P would be able to induce Ca^{2+}-entry and up-regulate the associated channel proteins which would finally help in cancer progression.

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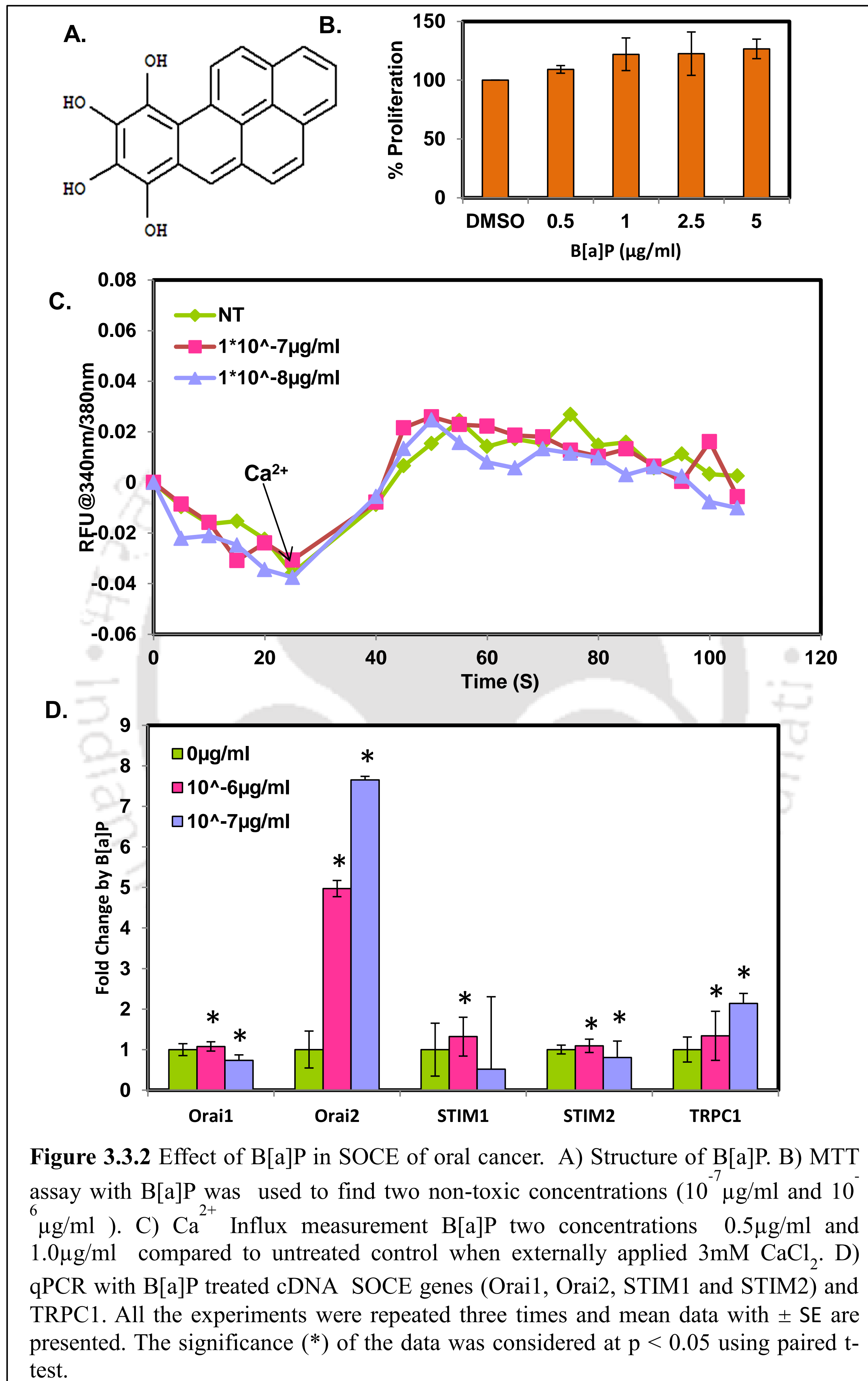

Figure 3.3.2 Effect of B[a]P in SOCE of oral cancer. A) Structure of B[a]P. B) MTT assay with B[a]P was used to find two non-toxic concentrations (10^{-7} µg/ml and 10^{-6} µg/ml). C) Ca^{2+} Influx measurement B[a]P two concentrations 0.5µg/ml and 1.0µg/ml compared to untreated control when externally applied 3mM $CaCl_2$. D) qPCR with B[a]P treated cDNA SOCE genes (Orai1, Orai2, STIM1 and STIM2) and TRPC1. All the experiments were repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $p < 0.05$ using paired t-test.

3.3.3. Role of NNK and NNN in the regulation of SOC of oral cancer

NNK and NNN are the nitrosamines that are formed from nitrosation of nicotine while curing process (Carmella et al., 1997; Hecht and Hoffmann, 1988). These nitrosamines have been reported for causing lung cancer through nAChr but their effect on SOC is yet to be deciphered (Boo et al., 2016; Song et al., 2008). In our study we found that treatment of oral cancer cells with NNN resulted in the up-regulation of SOCE at 1nM and 10nM concentrations (figure 3.3.3.1). Similarly, NNK treatment also upregulated SOCE in oral cancer cells at 0.25µM and 1.0µM concentrations. The same concentrations were used to treat the cells for analysing the effect of these nitrosamines on the expression of SOCC genes at mRNA level by qPCR. We found very high expression of Orai2 *i.e.* 10 fold in NNN and 3.4 fold in NNK treated oral cancer cells. Treatment with NNN did not affect other SOCC genes (Orai1, STIM1 and STIM2) but TRPC1 expression was found to be 3.4 fold upregulated (fig. 3.3.3.2). However, NNK showed up-regulation of Orai1 (2.4 fold), STIM1 (3.4 fold) and TRPC1 (1.4 fold). Overall, nitrosamines activated more Ca^{2+}-uptake machineries in oral cancer cells which full fill the need for tumor progression.

3.3.3. Role of 4NQO in the regulation of SOC of oral cancer

4NQO is a well-known chemical carcinogen used for mimicking the development of oral cancer in animal models for research (Hawkins et al., 1994; Rivera, 2016). So far 4NQO has been extensively used to understand the changes occurred in oral cancer progression at molecular level such as decreased Rarb2, upregulated p-ERK1/2, and upregulated COX-2 (YANG et al., 2013). In a recent study, Peng et al. (2015) identified that 4NQO induced oral carcinogenesis in rat is associated with the alteration of almost 3500 genes using microarray and PCR techniques and the author states that 1735 genes were upregulated and 1803 genes were downregulated in the malignant tissues compare to normal tissues (Peng et al., 2015).

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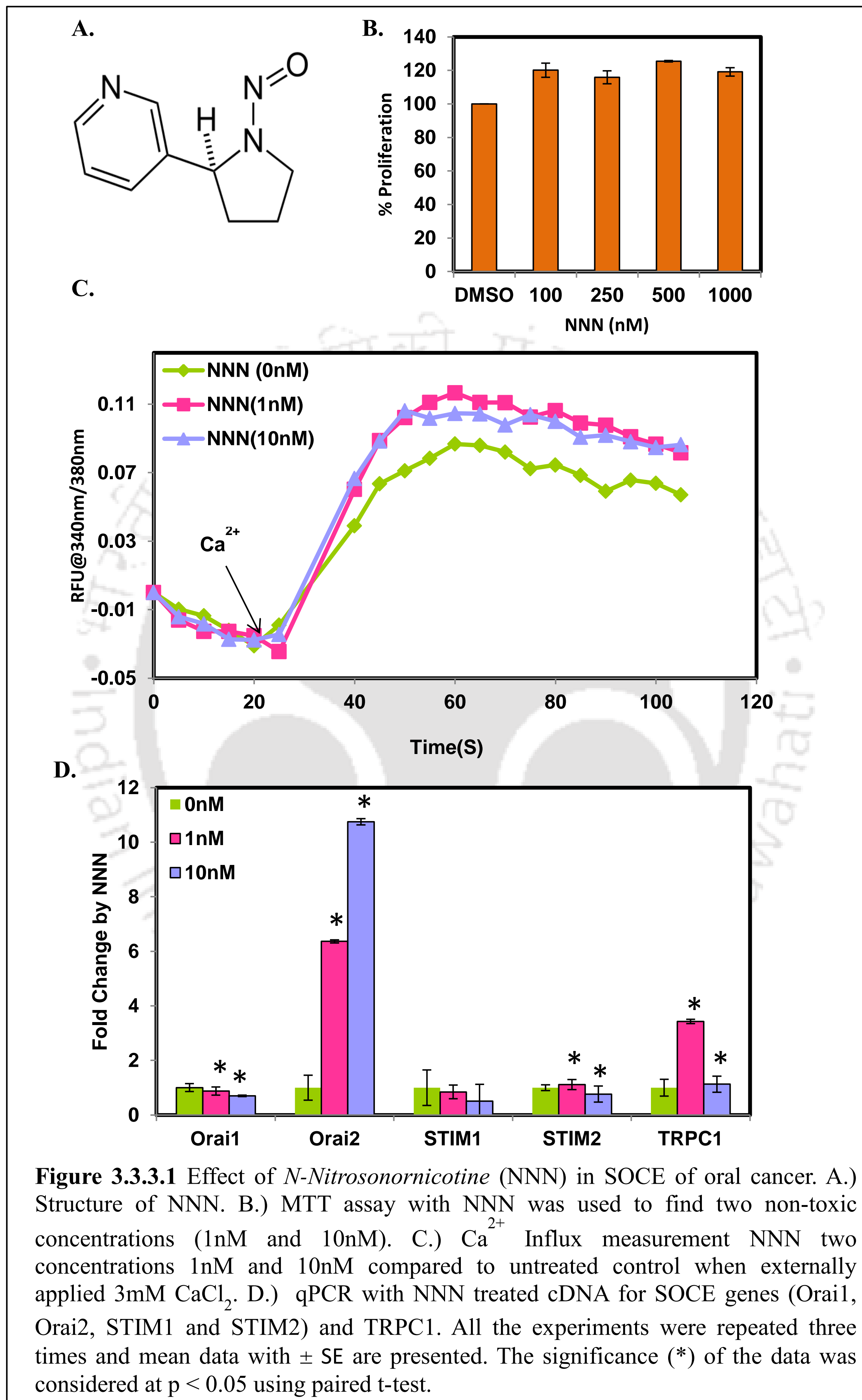

Figure 3.3.3.1 Effect of *N-Nitrosonornicotine* (NNN) in SOCE of oral cancer. A.) Structure of NNN. B.) MTT assay with NNN was used to find two non-toxic concentrations (1nM and 10nM). C.) Ca^{2+} Influx measurement NNN two concentrations 1nM and 10nM compared to untreated control when externally applied 3mM $CaCl_2$. D.) qPCR with NNN treated cDNA for SOCE genes (Orai1, Orai2, STIM1 and STIM2) and TRPC1. All the experiments were repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $p < 0.05$ using paired t-test.

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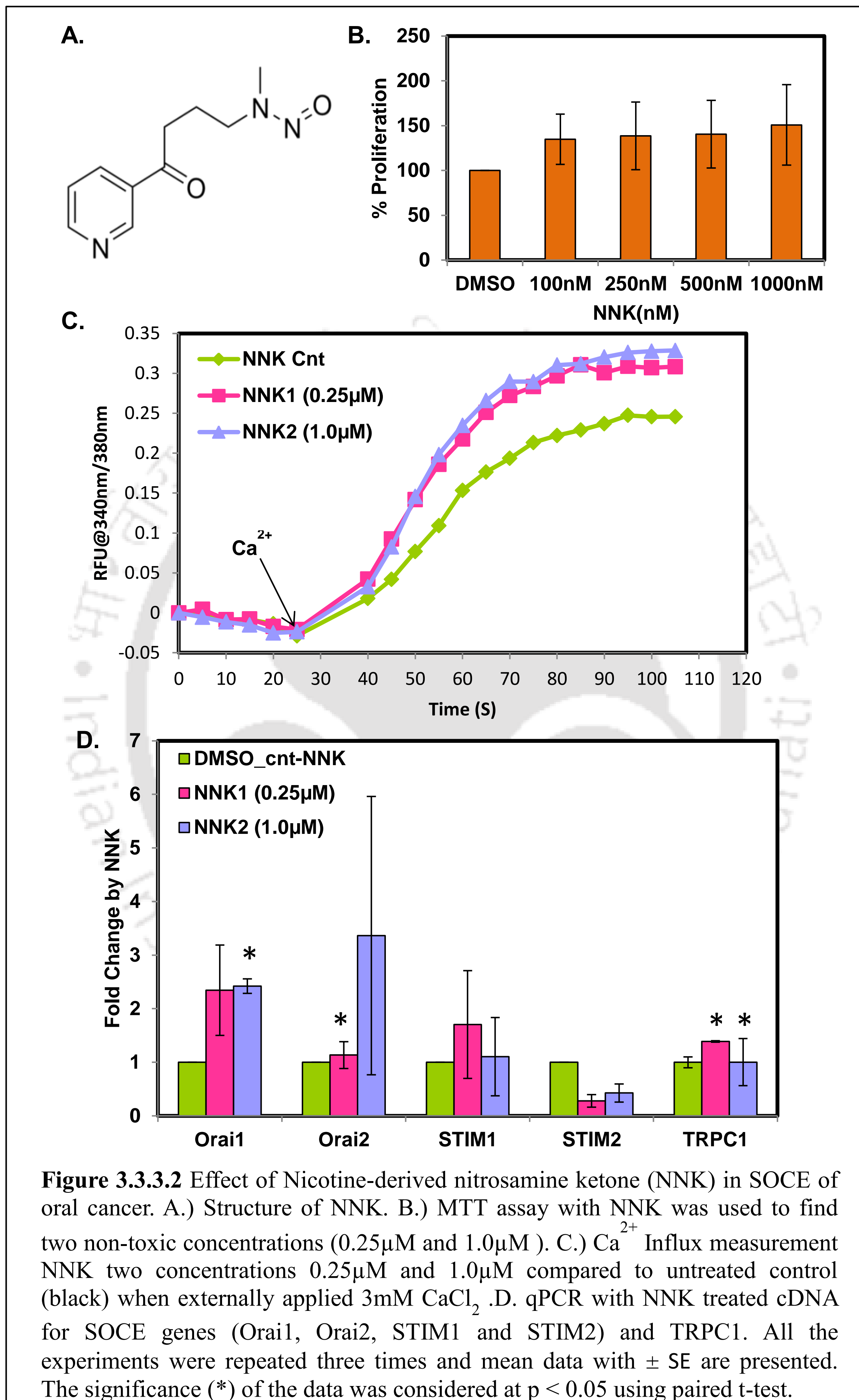

Figure 3.3.3.2 Effect of Nicotine-derived nitrosamine ketone (NNK) in SOCE of oral cancer. A.) Structure of NNK. B.) MTT assay with NNK was used to find two non-toxic concentrations (0.25µM and 1.0µM). C.) Ca^{2+} Influx measurement NNK two concentrations 0.25µM and 1.0µM compared to untreated control (black) when externally applied 3mM $CaCl_2$ .D. qPCR with NNK treated cDNA for SOCE genes (Orai1, Orai2, STIM1 and STIM2) and TRPC1. All the experiments were repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $p < 0.05$ using paired t-test.

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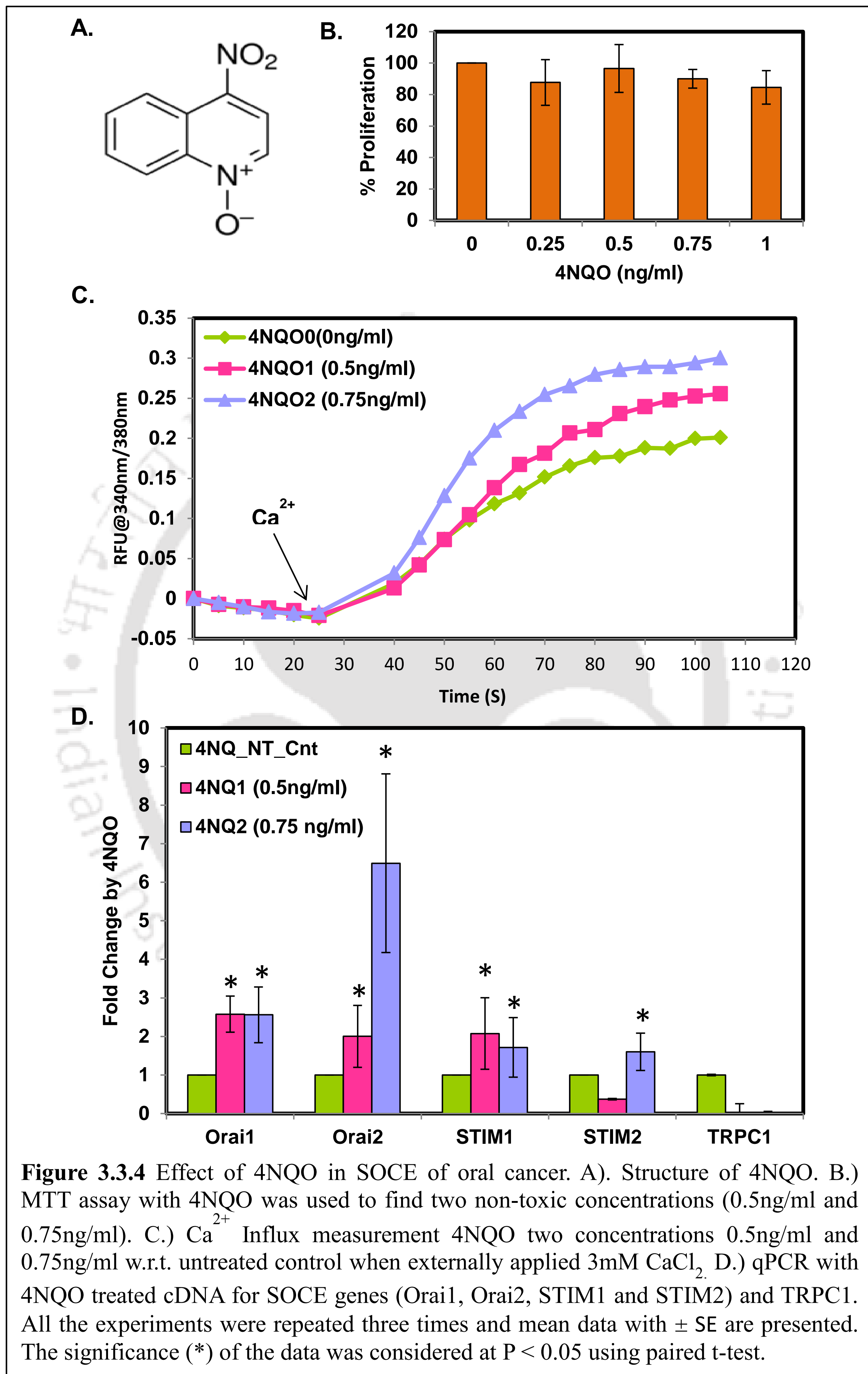

Figure 3.3.4 Effect of 4NQO in SOCE of oral cancer. A). Structure of 4NQO. B.) MTT assay with 4NQO was used to find two non-toxic concentrations (0.5ng/ml and 0.75ng/ml). C.) Ca^{2+} Influx measurement 4NQO two concentrations 0.5ng/ml and 0.75ng/ml w.r.t. untreated control when externally applied 3mM $CaCl_2$. D.) qPCR with 4NQO treated cDNA for SOCE genes (Orai1, Orai2, STIM1 and STIM2) and TRPC1. All the experiments were repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $P < 0.05$ using paired t-test.

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There was no information about the effect of 4NQO on SOCE/SOCC before this report. We found that 0.5ng/ml and 0.75ng/ml of 4NQO induced SOCE in OC cells. The same concentration was used for RT-PCR to find out the expression of SOCC genes and we observed that the expression of Orai1 (2.5 fold), Orai2 (6.5 fold), STIM1 (2 fold) and STIM2 (1.6 fold) genes were upregulated but TRPC1 gene was downregulated compared to DMSO treated oral cancer cells. Overall, we conclude that 4NQO based carcinogenicity may be associated with enhanced SOCE.

Conclusion

Tobacco is a major risk factor for oral cancer. In this chapter, we studied effect of tobacco and associated carcinogens on the SOCE and its regulatory genes. We found that B[a]P, nitrosamines (NNN & NNK) and 4NQO enhanced Ca^{2+}-influx in oral cancer cells after 24h treatment. We also observed that SOCC genes (Orai1, Orai2, STIM1 and STIM2) along with a TRP channel gene (*i.e.* TRPC1), act as SOCC, were upregulated upon treating with tobacco components. Mainly Orai2 and TRPC1 were found to be up-regulated by all the tobacco components and crude tobacco extract. In our study, the synthetic carcinogen, 4NQO, also showed the significant effect. Therefore, we concluded that use of tobacco is responsible for upregulation of SOCE and overexpression of SOC regulatory genes which further promote oral cancer development.

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CHAPTER-4

Modulation of SOCE

and

Changes in Oral Cancer

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4.1 Introduction

In our previous chapters, we found that SOCE was high in oral cancer cells compared to normal cells. In addition we found that Orai1 and Orai2 expressions were higher in oral cancer compared to normal cells and tissues, in qPCR and IHC analysis. We also found that tobacco and associated carcinogens are able to upregulate SOCE and SOCCs through Orai1, Orai2 and TRPC1 which were discussed in chapter 3. Recently, a study showed that Orai1 helps in progression of oral cancer by promoting its stem cell properties (Lee et al., 2016). The role of Orai1 has been explored in various cancer types such as breast cancer, colon cancer, lung cancer and prostate cancer (Ashmole et al., 2013; Guéguinou et al., 2016; Yang et al., 2009). The study revealed that Orai1 controls SOCE through STIM1. Recently, it was also noticed that TRPC1, TRPC6 and SK3 also play some important role in cancer progression which are associated with Orai1 dependent SOCE (Guéguinou et al., 2016; Rigalli et al., 2015). But, the role of Orai2 has not been explored much in cancer cases but Orai1 and Orai2 actions were found to be interrelated (Diez-Bello et al., 2017; Inayama et al., 2015; Vaeth et al., 2017). The CRAC channel can be made through STIM1 and/or TRPC1 in combination with either Orai1 (homomer) or with Orai1-Orai2 (heteromer) in plasma membrane (Cheng et al., 2008a; Inayama et al., 2015). Our studies suggested that Orai1 and Orai2 are associated with high SOCE which was found to be overexpressed in advanced stage tissues of oral cancer. These results show that SOCE may be highly important in the different processes of oral cancer such as proliferation, survival, invasion and metastases. Therefore, in this chapter we determined the effect of SOCE and SOCE related gene on the above mentioned processes. In the current chapter, to determine the effect of SOCE and SOCE related genes on oral cancer we used chemical SOCE inhibitors (such as- 2-APB, $LaCl_3$ & SKF96365) and gene knockdown methods.

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Materials and Methods

4.2.1 Cell Culture

SAS cells were obtained from RGCB (Rajiv Gandhi Centre for Biotechnology), Trivandrum. The DMEM (Dulbecco's Modified Eagle Medium) high glucose (Invitrogen) media with 10% FBS (Invitrogen-Gibco) and 1% Penicillin-Streptomycin (PS) mixtures (Invitrogen) was used to maintain the cells at 37°C in a 5% CO_2 and 95% R.H. in incubator (Chiou et al., 2008).

4.2.2 Preparation of SOCE inhibitors stock

To analyse the effect of Ca^{2+} ions in oral cancer cells proliferation and migration it has to be blocked by known SOCE inhibitors such as 2-APB, La^{3+} and SKF96365. The stock solution was prepared and used for the various experiments.

4.2.2.1 Preparation of 100mM 2-APB stock

100mM stock solution was prepared by adding 2-APB (Sigma-Aldrich) 22.5 mg powder in 1ml of cell culture grade DMSO (Sigma). The 1ml stock was aliquoted 50μL each in 10 vials for protection from freezing thawing effect on efficiency. To protect it from light aluminium foil was used to wrap and stored at -20°C.

4.2.2.2 Preparation of 100mM $LaCl_3.7H2O$ stock

100mM stock of $LaCl_3.7H_2O$ (SRL) was prepared by dissolving 37.1 mg of the powder in 1ml of autoclaved deionized water and sterilized by using 0.22μm syringe filter freshly for each experiment.

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4.2.2.3 Preparation of 10mM SKF96365 stock

10mM sock of SKF96365 (Sigma-Aldrich) was prepared by dissolving 5 mg of SKF96365 in 1.241 ml of autoclaved deionized water and sterilized by using 0.22µm syringe filter. The stock was aliquoted 20µl each in 5 vials to protect it from freezing and thawing effect on efficiency and stored at -20°C.

4.2.3 Cell proliferation assay with SOCE inhibitors

To study the effect of SOCE inhibitors on oral cancer cells 2000 cells per well were seeded in three 96 well plates in triplicate for each concentration, for 0h, 24h and 48h incubation with the SOCE inhibitors. The MTT or (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium salt is used to quantify the viable cells (Riss et al., 2004). After the incubation period over media with the drug was replaced with MTT (5mg/ml) added media (i.e. 10 µl MTT per 100 of media). The plates were incubated at 37°C for 3h- 4h in the CO_2 incubator. After 3h- 4h incubation the MTT added media was replaced with DMSO (100 µl/well) and the absorbance was recorded at 570nm in a multimode reader (Tecan Group Ltd., Männedorf, Switzerland). The percentage of cell proliferation was calculated using the formula:

$$\% \text{ Proliferation} = \frac{\text{Absorbance (Control−Treatment)}}{\text{Absorbance (Control)}} \times 100$$

4.2.4 Store operated Ca^{2+}-influx measurement protocol for SOCE inhibitors

To study the Ca^{2+}-influx inhibition efficiency of 2-APB, La^{3+} and SKF96365 in oral cancer 30,000 cells per well were seeded in triplicate for each types of inhibitors and cells were grown in DMEM media with 10% FBS and 1% PenStrep until it forms a monolayer. After forming monolayer, all the wells were washed twice with Ca^{2+}-SBS (NaCl 135 mM, KCl 5 mM, MgCl2 1.2 mM, Glucose 8 mM, HEPES 10 mM and $CaCl_2$ 1.5 mM, and pH 7.4). After washing 2µM Fura-2AM was prepared in Ca^{2+}-SBS with 0.02% of Pluronic F-

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127 and loaded 100µL/well in the cell plate. After loading plate was covered with aluminium foil and incubated for 1h at room temperature. After 1h, cells were washed with Ca^{2+}-SBS twice and re-incubated with Ca^{2+}-SBS for another 15 minutes to make Fura-2 free from the Acetoxymethyl (AM) esters group and allow it to enter inside the cells. After loading the probe, cell monolayers were rinsed with zero Ca^{2+} SBS (NaCl 135 mM, KCl 5 mM, $MgCl_2$ 1.2 mM, Glucose 8 mM, HEPES 10 mM and pH 7.4) twice and 2µM thapsigargin (100µL/well) was used for pre-incubation at 37°C for 30 min. After 30 min thapsigargin solution was replaced with inhibitor added zero Ca^{2+} SBS. The SOCE inhibitors were prepared with desired concentration in zero Ca^{2+} SBS which was loaded (200µL/well) in the cell plate just before the measuring fluorescence. Simultaneously, a reagent plate was prepared with 15mM $CaCl_2$ which was used for injecting (50µL/well) by the fluorimeter pipetting system. After injection the $CaCl_2$ becomes 3mM $CaCl_2$ in the cell plate and gave fluorescence signal when Fura-2 interacts with Ca^{2+}. The data was recorded for both 340nm and 380nm in excel sheet which was used to analyse the results. The statistics was analysed using one way ANOVA. For statistical significance (*) was considered at $p < 0.05$.

4.2.5 siRNA knockdown of Orai1 and Orai2 genes

To understand the role of Orai1 and Orai2 in oral cancer progression, the genes were silenced in SAS cells using siRNA for Orai1 and Orai2. The siRNA based gene knockdown was performed using lipofectamine (siRNAMAX, Invitrogen) and the custom siRNAs with the given sequences in the table 4.1 were synthesized from Eurogentec, Belgium. Protocol suggested by the lipofectamine manufacturer was used for siRNA transfection. To silence Orai1 and Orai2, 2 x 10^6 SAS cells per well were seeded in a 6 well plate and grown in the DMEM media until the confluency reached to 60-70%. At the

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right confluency, the transfection complex (lipofectamine and siRNA mixture in Opti-MEM) prepared and added to the cell plate. Cells were incubated for 24hr with the transfection complex added media and next 24hr with the replaced DMEM growth media. Seeding to transfection step was common for all the siRNA based studies but after doing knockdown experiments in SAS cells with siOrai1 and siOrai2, cells were used for various experiments such as migration assays, colony formation assay, qPCR and western blot. The effect of siRNA stayed for 3-5days in our studies.

Table 4.2.5.1: List of siRNAs for Orai1 and Orai2 gene silencing

Target Gene	siRNA Sequences (5' to 3')	References
hOrai1 siRNA1 (A1):	5'-GGGAAGAGGAUUUUUAUAAtt-3' 5'-UUAUAAAAAUCCUCUUCCCtc-3'	(Li et al., 2011)
hOrai1 siRNA2 (B1):	5'-CCUGUUUGCGCUCAUGAUCtt-3' 5'-GAUCAUGAGCGCAAACAGGtg-3'	(Li et al., 2011)
hOrai2 siRNA1(A2):	5'-GGGCAUGGAUUACCGGGAC-3' 5'- GUCCCGGUAAUCCAUGCCC-3'	(Motiani et al., 2010)
hOrai2 siRNA2 (B2):	5'-AACCGUUUGGUUCAAUGAGG-3' 5'-CCUCAUUGAACCAAACGGUU-3'	(Peel et al., 2008)
Negative or scrambled control siRNA:	Sequence not provided	Eurogentec (Germany)

4.2.6 Store Operated Ca^{2+}-influx measurement in siRNA knockdown cells

To compare the changes in Ca^{2+}-influx efficiency of Orai1 and Orai2 knockdown oral cancer cells with respect to negative control, 50000 cells per well were seeded in triplicate in a 96 well plate (Granier) after performing knockdown experiment as mentioned above. After seeding, the silenced cells and control cells were grown in DMEM media until it forms a monolayer (~24hr). After forming the monolayer, all the wells were washed with Ca^{2+} -SBS two times. Then 2μM Fura 2-AM solution was prepared and loaded (100μL/well) using 0.02% of Pluronic F-127 in Ca^{2+} -SBS in the cell plate. After loading

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Fura-2AM, plate was incubated for 1h at room temperature in dark. After incubation with Fura-2AM, cells were washed with Ca^{2+} -SBS two times and re-incubated with Ca^{2+} -SBS for 15 minutes to cleave the AM esters group and allowed Fura-2 to enter inside the cells. After loading Fura-2, monolayer of cells were washed with zero Ca^{2+} -SBS and store of Ca^{2+} was depleted using 2µM thapsigargin for 30 min at 37°C. The SOCE inhibitor, SKF96365 (20µM) was prepared in zero Ca^{2+} -SBS which was loaded (200µl/well) in the 3 wells of untreated/untransfected SAS cells and thapsigargin solution was replaced with zero Ca^{2+} -SBS (200µl/well) just before the measuring fluorescence upon application of 3mM $CaCl_2$ in the fluorimeter. The fluorescence data were recorded and analysed in MS excel. Three independent experiments were compared for statistical significance (*) using one way ANOVA at $p < 0.05$.

4.2.7 Cell migration assay with SOCE inhibitors

To analyse the role of SOCE inhibitors in the regulation of oral cancer cell migration 4 x 10^6 SAS cells were seeded in 6 wells of a 12 well plate and grown in DMEM growth media until the monolayer formed. After formation of monolayers, cells were synchronized by using serum starvation for 8h-12h before creating the scratch or wound using p200 tips. Then cells were treated with SOCE inhibitors (100µM $LaCl_3$, 100µM 2-APB and 20µM SKF96365) with respective controls which were prepared in 0.4% serum added DMEM growth media (Liang et al., 2007). After treatment zero hour images were captured using inverted microscope attached camera. Next images were captured after 12hr or when wounds were completely healed in the control wells. Later, the captured images were compared for the migration percentage with respect to controls by analysing % area covered or inhibited using ImageJ software.

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4.2.8 Cell migration assay with siRNA knockdown cells

To study the role of Orai1 and Orai2 genes in oral cancer cell migration 4 x 10^6 SAS cells were seeded in 3 wells of a 24 well plate after performing siRNA knockdown experiment. The cells were allowed to grow in DMEM media until it forms a monolayer (~24hr). Then cell culture media was replaced with serum free DMEM media to synchronize the cells for 8-12hr before creating the scratch. Then a scratch was made by using p200 tips in each well and cells were incubated in 0.4% serum added DMEM media with for 12h. The images were captured at 0h and 12h after making the wound (Liang et al., 2007). Finally, the ImageJ software was used to analyse the captured images and obtained the % area covered or inhibited by the Orai1 and Orai2 knockdown oral cancer cells compared to scrambled control siRNA transfected oral cancer cells.

4.2.9 Colony formation assay with siRNA knockdown cells

To know the role of Orai1 and Orai2 in colony forming efficiency of oral cancer cells 250 SAS cells per well were seeded in a 12well plate by using serial dilution after performing siRNA knockdown experiment. These cells were grown in DMEM culture media and allowed the cells to form colonies in 10 days. The cell culture media was changed in every 3-4 days. After 10 days the colonies were washed with PBS 2-3 times and fixed in 70% ethanol at -20 °C for 2hr. Later, the colonies were stained with 0.5% crystal violet (w/v) for 2 min then images were captured to count the colonies using ImageJ software and survival fractions were calculated by using formula-

$$\text{Colony Efficiency} = \frac{\text{Colony count given by ImageJ}}{\text{Number of cells seeded per well}}$$

$$\text{Survival Fraction} = \frac{\text{Colony efficiency of test}}{\text{Colony efficiency of control}}$$

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4.2.10 Western blot after siRNA knockdown

To analyse the changes after silencing Orai1 and Orai2 genes 2 x 10^6 SAS cells per well were seeded in a 6 well plate for siRNA knockdown experiment as discussed in section 4.2.5. After transfection (48hr) the cells were sacrificed using the cell lysis buffer with protease and phosphatase inhibitors (20mM HEPES, 2mM EDTA, 250mM NaCl, 0.1% Triton-X, 2µg/ml aprotinin, 1mM PMSF, 0.5µg/ml of DTT, and 2µg/ml leupeptin). Then supernatant of the cell lysates were collected and stored at -80°C freezer after centrifugation at 13000 RPM for 10min at 4°C. These lysates (40µg) were used for western blot experiments with following antibodies using 8% or 12% SDS-PAGE after doing protein estimation using Bradford assay- Orai1 (abcam; cat. no. ab86748), Orai2 (abcam; cat. no. ab155216), Akt1[Cell Signaling Technology (CST); cat. no. 2938], pAkt-Ser473(CST; cat. no.4060), pAkt-Thr308(CST; cat. no. 9275), Redd1 (CST; cat. no. 2516S), MMP-9 (CST; cat. no. 13667P), VEGFA (ab46154), cyclin D1 (CST; cat. no. 2978BC), CXCR4 (ab124824), Survivin (CST; cat. no. 2808BC), mTOR (CST; cat. no. 2983T), phospho-mTOR-ser2448 (CST; cat. no. 5536T), NFkB (CST; cat. no. 8242P), pNFkB-ser536 (CST; cat. no. 3033P), β-actin (CST; cat. no. 4967S), GAPDH (CST; cat. no. 2118S) and anti-rabbit secondary antibody (abcam; cat. no. ab97080).

4.2.11 Real time PCR with knockdown cells and 20µM SKF96365 treated cells

To compare the changes at mRNA level after silencing Orai1 and Orai2 genes and treatment with 20µM SKF96365 in oral cancer cells total RNA was isolated from the siRNA transfected, SKF96365 treated and control cells using TRIzol (Sigma), after the incubation period over (Chomczynski, 1993). The RNAs (1µg/sample) were used generate cDNA using High-Capacity cDNA reverse transcription kit (Invitrogen; cat.no. 4368814) according to the manufacturer's protocol. After generation of the cDNAs and used as

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templet for qPCR. In our study we used AMP-activated protein kinase-1 (AMPK1), E-cadherin, N-cadherin, Caspase-3, Fatty acid synthase (FASN) and β – actin (as a housekeeping gene) primers for analysing the fold change using $\Delta\Delta C_T$ method, given in the table 4.2.

4.2.12 Statistical Analysis

All the experimental data were presented here is an outcome of three independent experiments. All the data were analysed in MS excel. Significance (*) of the results was considered at $p < 0.05$ by using student t-test and/or one way ANOVA.

Table 4.2: List of primers used in qPCR for comparative C_T- method based relative gene expression.

Genes	Forward(F) / Reverse(R)	Primer Sequences (5’→3’)	Amplicon Size (bp)
AMPK1 (NM_006251.5)	F	CTCAGTTCCTGGAGAAAGATGG	174
	R	CTGCCGGTTGAGTATCTTCAC	
N-cadherin (NM_001792.4)	F	CGAGCCGCCTGCGCTGCCAC	199
	R	CGCTGCTCTCCGCTCCCCGC	
E-cadherin (NM_001317186.1)	F	AAGGTGACAGAGCCTCTGGAT	223
	R	CGTCTGTGGCTGTGACCT	
Caspase 3 (NM_004346.3)	F	ACATGGCGTGTCATAAAATACC	120
	R	CACAAAGCGACTGGATGAAC	
FASN (NM_004104.4)	F	CACATGCCTTTGTGAGCACC	129
	R	TGAGGGAGGCGTAGTAGACC	
β – Actin (NM_001101.3)	F	CCCTGGCACCCAGCAC	71
	R	GCCGATCCACACGGAGTAC	

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4.3 Results and Discussion

In this chapter we have analysed the role of Orai1 and Orai2 in oral cancer progression. As our previous chapters suggested that these molecules have major role in metastasis, therefore we tried to determine the cell migration and colony formation efficiency of oral cancer cells after modulating Ca^{2+}-signals. First we have used few known SOCE inhibitors (2-APB, $LaCl_3$ and SKF96365) to find out the effect of inhibition of SOCE as well as efficiency of these inhibitors. Later, we analysed the ability of Orai1 and Orai2 to block the Ca^{2+} entry inside the cell by using siRNA knockdown. Then we studied the effect of calcium modulation on cell proliferation, cell migration, and colony formation ability. The inhibition of SOCE or I_{CRAC} has been found to regulate migration and metastasis of breast cancer cells through Orai1 and STIM1 (Yang et al., 2009). In line with the previous study, our study also suggests that Orai1 plays important role in metastasis of oral cancer cells. Additionally, we found the similar role of Orai2 in oral cancer cells where Orai2 showed negligible effect on SOCE. This is the first report where we have studied the role of Orai2 in oral cancer. Moreover, in this chapter we have also tried to decipher the mechanism behind the inhibition of cell migration and colony formation through western blot and qPCR using the signature molecules of various oncogenic pathways.

4.3.1. Effect of SOCE inhibitors on oral cancer cell proliferation, cell migration and Ca^{2+}-influx inhibition efficiency

To understand the role of SOCE in oral cancer cell proliferation, first we aborted the SOCE with known SOCE inhibitors (2-APB, $LaCl_3$ and SKF96365) and performed the cell proliferation assay using MTT. Treatment with 2-APB, $LaCl_3$ and SKF96365 did not show significant effect in 24h incubation at any concentration. However, after 48h all three inhibitors showed substantial decrease in percentage proliferation of oral cancer cells. We observed that $LaCl_3$ and 2-APB were able to decrease the proliferation (~ 30%) at

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1000µM and 100µM respectively (figure 4.3.1) (Choi et al., 2010; Jan et al., 1998). 2-APB is extensively used as a tool for inhibition of SOCE ≤ 50µM concentration in most of the studies (Choi et al., 2010; DeHaven et al., 2008; Ma et al., 2002). In our study, $LaCl_3$ was found to be effective at 0.1µM, 10µM and 100µM at 24h treatment (figure 4.3.1B). Therefore, in most of our studies 100µM $LaCl_3$ was used to inhibit the Ca^{2+} influx as reported earlier in different studies (Gutierrez-Martin et al., 2005; Jan et al., 1998). Next we analysed the effect of another potent SOCE inhibitor (SKF96365) which was effective at very low concentrations and significantly inhibited the proliferation of SAS cells in a dose dependent manner (*i.e.* from 1µM to 30µM, at 48 h) (figure 4.3.1C). Since SKF96365 has shown significant inhibition on the proliferation of SAS cells at 20µM and inhibition of SOCE was also reported in literatures, we used it in our further studies (Jing et al., 2016; Yang et al., 2009; Zhang et al., 2015). With this study, we found the effective concentrations of SOCE inhibitors as- 100µM of 2-APB, 100µM of $LaCl_3$ and 20µM of SKF96365 which were used in cell migration and Ca^{2+}-influx inhibition assays.

Next we studied the role of SOCE inhibitors in oral cancer cell migration by using *in vitro* scratch assay to understand the importance of SOC in oral cancer cell migration, invasion and metastasis. In the current study we found that 20µM SKF96365 (~60%), 100µM 2-APB (~50%) and 100µM La^{3+} (10%) significantly inhibited the oral cancer cell migration (figure 4.3.2). In line with our study, the inhibition of cancer cell migration by using SOCE inhibitors were discussed in breast cancer, melanoma and colon cancer (Guéguinou et al., 2016; Umemura et al., 2014; Yang et al., 2009). Taken together, we found that when SOCE of oral cancer cells was inhibited by these inhibitors, it reduced the oral cancer cell migration efficiency and thus might serve as a key regulator of metastasis in oral cancer.

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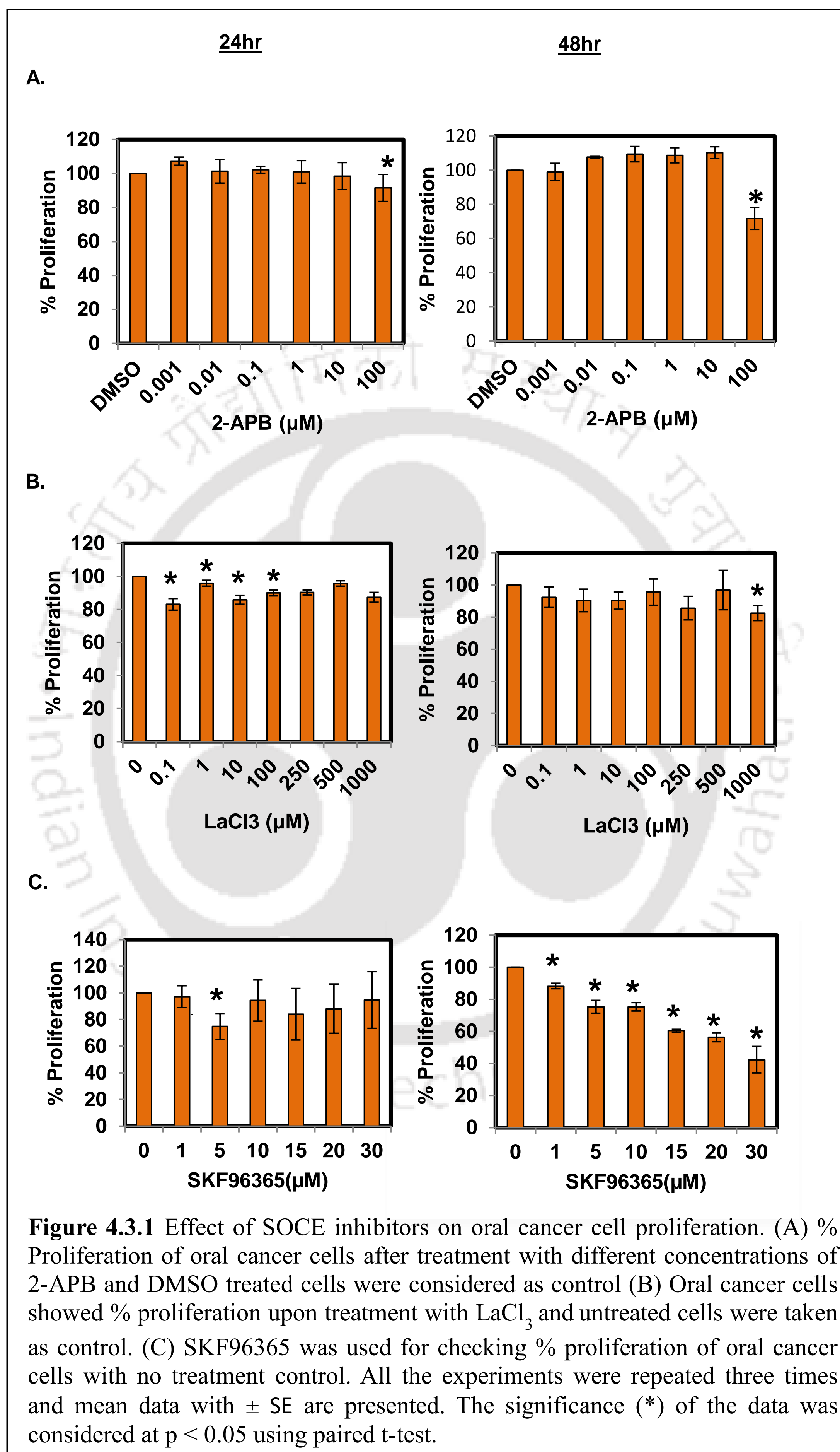

Figure 4.3.1 Effect of SOCE inhibitors on oral cancer cell proliferation. (A) % Proliferation of oral cancer cells after treatment with different concentrations of 2-APB and DMSO treated cells were considered as control (B) Oral cancer cells showed % proliferation upon treatment with $LaCl_3$ and untreated cells were taken as control. (C) SKF96365 was used for checking % proliferation of oral cancer cells with no treatment control. All the experiments were repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $p < 0.05$ using paired t-test.

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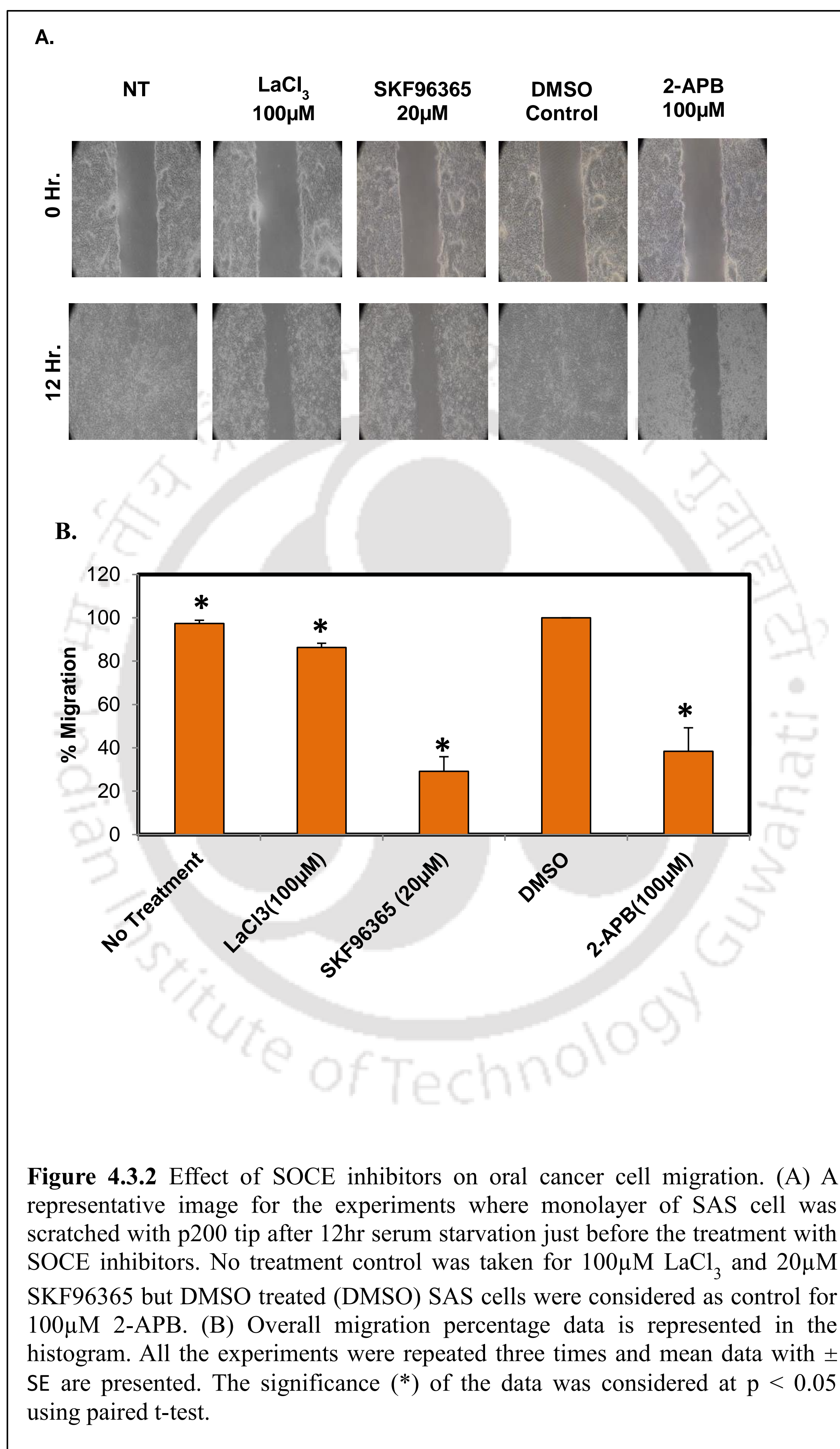

Figure 4.3.2 Effect of SOCE inhibitors on oral cancer cell migration. (A) A representative image for the experiments where monolayer of SAS cell was scratched with p200 tip after 12hr serum starvation just before the treatment with SOCE inhibitors. No treatment control was taken for 100µM $LaCl_3$ and 20µM SKF96365 but DMSO treated (DMSO) SAS cells were considered as control for 100µM 2-APB. (B) Overall migration percentage data is represented in the histogram. All the experiments were repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $p < 0.05$ using paired t-test.

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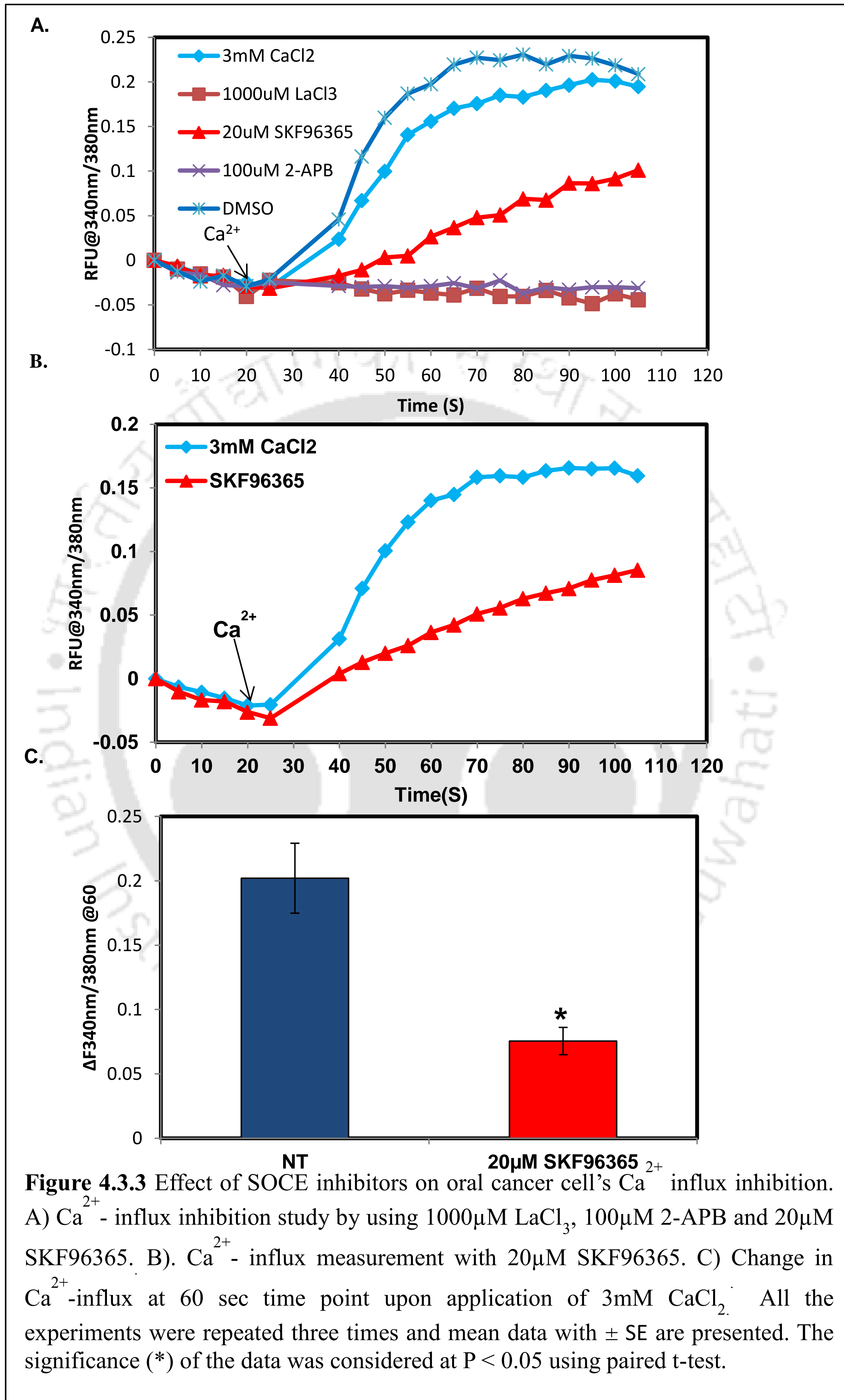

Figure 4.3.3 Effect of SOCE inhibitors on oral cancer cell's Ca^{2+} influx inhibition. A) Ca^{2+} - influx inhibition study by using 1000µM $LaCl_3$, 100µM 2-APB and 20µM SKF96365. B). Ca^{2+} - influx measurement with 20µM SKF96365. C) Change in Ca^{2+}-influx at 60 sec time point upon application of 3mM $CaCl_2$. All the experiments were repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $P < 0.05$ using paired t-test.

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Furthermore, we were interested to know the Ca^{2+}-inhibition efficiency of these SOCE inhibitors. To understand that, we performed Ca^{2+}-influx measurement experiment after depleting the store by using 2µM thapsigargin and applied SOCE inhibitors. We found that Ca^{2+}-entry in oral cancer cells was inhibited differently by all these inhibitors. In our study, we observed that Ca^{2+} influx was completely blocked by 100µM 2-APB and 100µM La^{3+} (figure 4.3.3 A) but 20µM SKF96365 was able to inhibit SOCE ~80% (figure 4.3.3 A & B) which clearly suggests that SKF96365 targets only SOCCs or Orai1 but other inhibitors are blocking all the Ca^{2+}-entry route at 100µM concentration. In addition, 20µM SKF96365 was found to be statistically significant when the 60th second data was compared with untreated control (figure 4.3.3 C). Recent studies suggested that SKF96365 was used as a control for SOCE inhibition while comparing with other studies in cancer research (Jing et al., 2016; 2010; Song et al., 2014; Zhang et al., 2015). Therefore, we considered 20µM SKF96365 as positive control for Ca^{2+}-influx assays in knockdown cells' based studies.

4.3.2 Confirmation of Orai1 and Orai2 knockdown

To understand the role of Orai1 and Orai2 in SOCE and their role in oral cancer progression, we silenced these genes using siRNAs. After knockdown, we confirmed the gene expression at mRNA level as well as protein level (figure 4.3.4). After confirming the knockdown, we performed Ca^{2+}-influx measurement assay, cell migration assay and colony formation assay to understand the role of Orai1 and Orai2 in oral cancer.

4.3.3 Orai1 and Orai2 knockdown in oral cancer cells decreased SOCE, inhibited cell migration and reduced colony forming efficiency

We confirmed earlier in this chapter that inhibition of SOCE can inhibit the migration of oral cancer cells through SKF96365 but the approach was non-specific. Now we first

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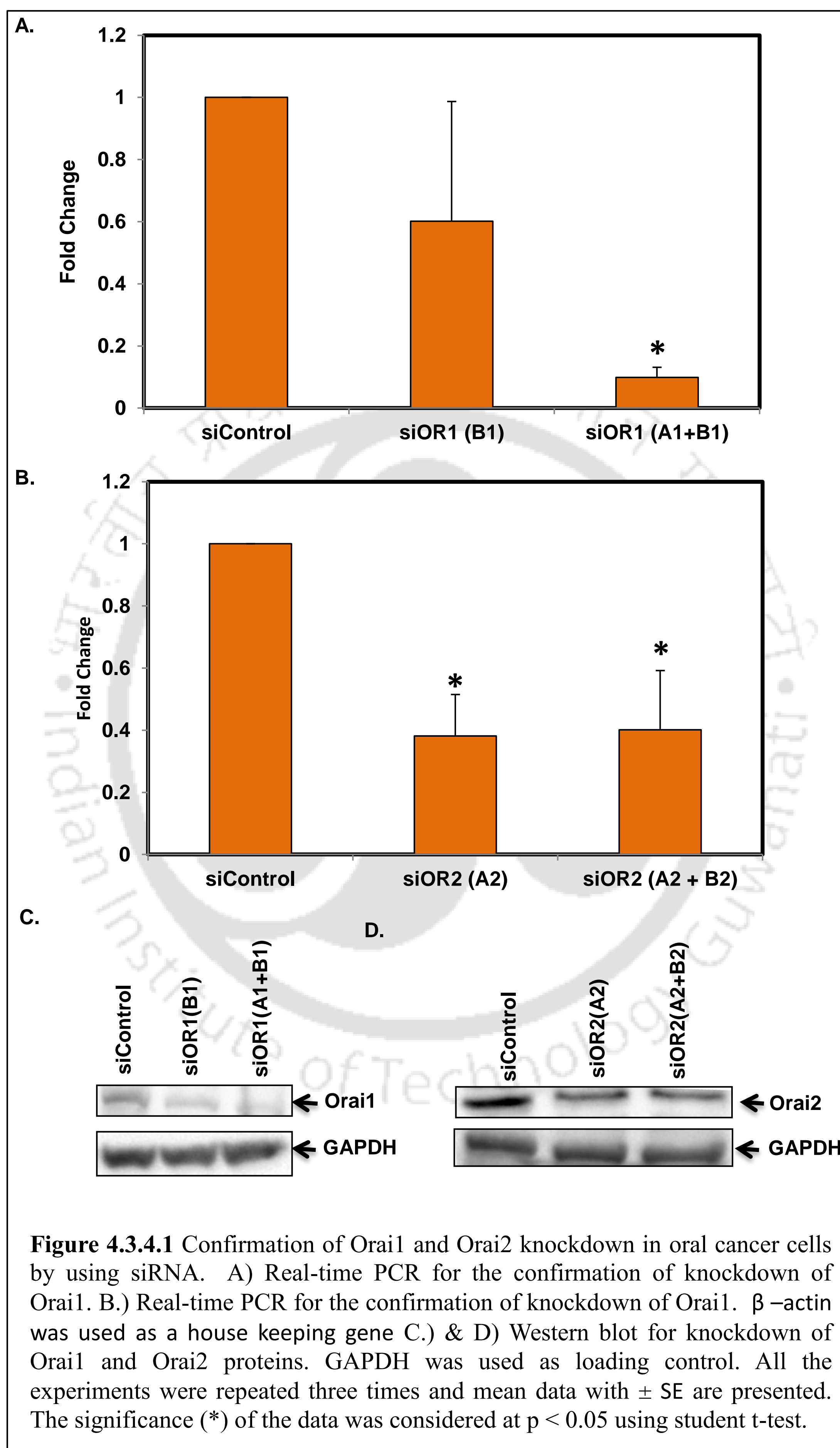

Figure 4.3.4.1 Confirmation of Orai1 and Orai2 knockdown in oral cancer cells by using siRNA. A) Real-time PCR for the confirmation of knockdown of Orai1. B.) Real-time PCR for the confirmation of knockdown of Orai1. β –actin was used as a house keeping gene C.) & D) Western blot for knockdown of Orai1 and Orai2 proteins. GAPDH was used as loading control. All the experiments were repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $p < 0.05$ using student t-test.

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analysed the effect of Orai1 and Orai2 knockdown on inhibition of SOCE and we found that silencing of Orai1 showed inhibition of SOCE equivalent to 20µM SKF96365 compared to siRNA negative control and untreated control. But Orai2 knockdown cells showed negligible effect on SOCE inhibition compared to siRNA negative control (figure 4.3.5.1A). Further, we analysed the effect of Orai1 and Orai2 knockdown on cell migration and colony forming efficiency of oral cancer cells and found that silenced cells showed significant inhibition of cell migration (figure 4.3.5.1B & C) and reduced colony forming efficiency (figure 4.3.5.2). In similar studies, Orai1 was found to regulate cell migration and metastasis in colon cancer and breast cancer (Guéguinou et al., 2016; Yang et al., 2009). This is the first report where we found that Orai2 significantly regulated cell migration and colony formation in oral cancer cells which showed negligible downregulation of SOCE upon silencing. In line with our study, the role of Orai2 was explored in human leukaemia where cell migration and phosphorylation of focal adhesion kinase (FAK) were regulated by Orai1 and Orai2 dependent SOC (Diez-Bello et al., 2017). Since, Orai1 and Orai2 showed their role in regulating cell migration and colonization, we wanted to elucidate the mechanism behind it and analyse the changes at molecular level.

4.3.4. Knockdown of Orai1 and Orai2 in oral cancer cells brought molecular level changes in proteins of Akt/mTOR pathway and NF-κB regulated CXCR4

To unveil the mechanism behind disruptions of cell migration and colonization in oral cancer cells after silencing Orai1 and Orai2, we first performed Western blot experiment with Akt/mTOR pathway mediators as it was reported to regulate multiple hallmarks of cancer (Dennis et al., 2014; O'Donnell et al., 2018). Later, we also analysed NF-κB/phospho- NF-κB and tried to elucidate the role of SOCs in oral cancer (Jana et al., 2017). We found that silencing of Orai1 and Orai2 also downregulated the expression of phosphorylated Akt1 (both pAkt-ser473 and pAkt-thr308) which could be a reason behind

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the upregulation of REDD1 or vice versa (Jia et al., 2014). Further, we observed downregulation of MMP-9, phospho-mTOR, and VEGFA in the silenced oral cancer cells (figure 4.3.4). In line of our study, Orai1 and CRAC channel were found to be associated with VEGF where silencing of Orai1 not only reduced the Ca^{2+}-entry, it also disrupted the tube formation in human blood endothelial progenitor cells (Li et al., 2011). Earlier MMP-9 expression was found to be associated with extracellular calcium where its expression was regulated by ERK pathway (Mukhopadhyay et al., 2004) but in melanoma it was found that matrix metalloproteinase (i.e. MT1-MMP) was entrapped and inhibited the ECM degradation when Orai1-mediated SOCE was blocked (Sun et al., 2014). The growing evidences suggested that Akt/mTOR pathway is regulated through SOCE and it further takes part in cancer angiogenesis, invasion and metastasis (Chetty et al., 2010; Ogawa et al., 2012; Schmidt et al., 2014). Additionally, we also analysed the expression of NF-κB /phospho- NF-κB, CXCR4, survivin and cyclin D1 to understand the role of Orai1 and Orai2 in oral cancer metastasis in the knockdown oral cancer cells. We found that CXCR4 and phospho- NF-κB were downregulated in the knockdown cells compared to control. The enhanced cell migration and metastasis was known to be regulated by NF-κB mediated overexpression of CXCR4 in breast cancer cells (Helbig et al., 2003). However, the correlation between SOCE and NF-κB / CXCR4 has not been reported so far but the calcium and calcineurin signaling were found to be regulating CXCR4 expression in human T lymphocytes (Cristillo and Bierer, 2003). Here we are reporting for the first time that Orai1 and Orai2 regulate oral cancer cells' migration through Akt/mTOR pathway and NF-κB / CXCR4 pathways. Since, we found unaffected expression of cyclin D1 and survivin in the knockdown oral cancer cells in our study, therefore targeting Orai1 and Orai2 might regulate metastasis but not proliferation of oral cancer cells.

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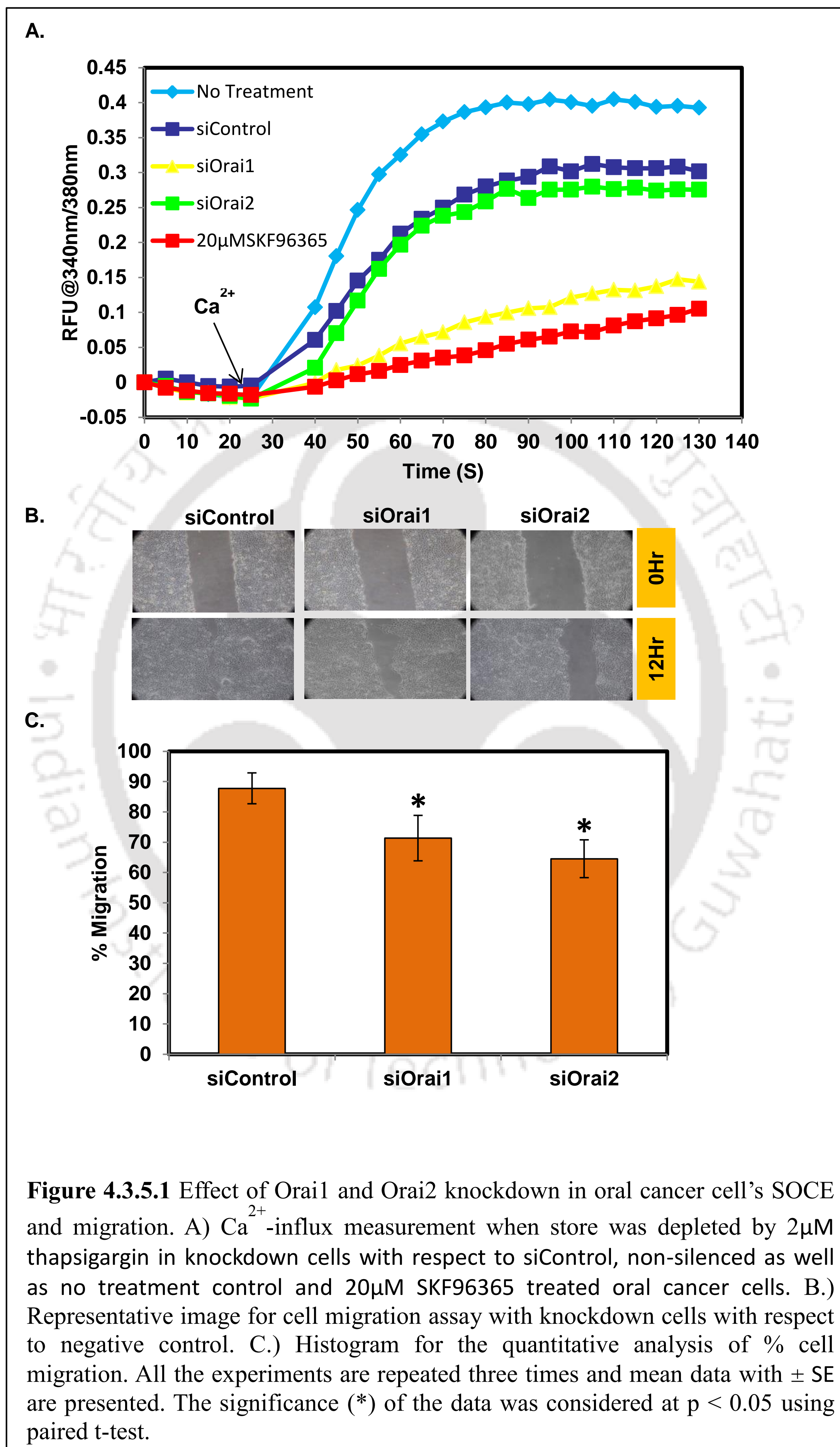

Figure 4.3.5.1 Effect of Orai1 and Orai2 knockdown in oral cancer cell's SOCE and migration. A) Ca^{2+}-influx measurement when store was depleted by 2µM thapsigargin in knockdown cells with respect to siControl, non-silenced as well as no treatment control and 20µM SKF96365 treated oral cancer cells. B.) Representative image for cell migration assay with knockdown cells with respect to negative control. C.) Histogram for the quantitative analysis of % cell migration. All the experiments are repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $p < 0.05$ using paired t-test.

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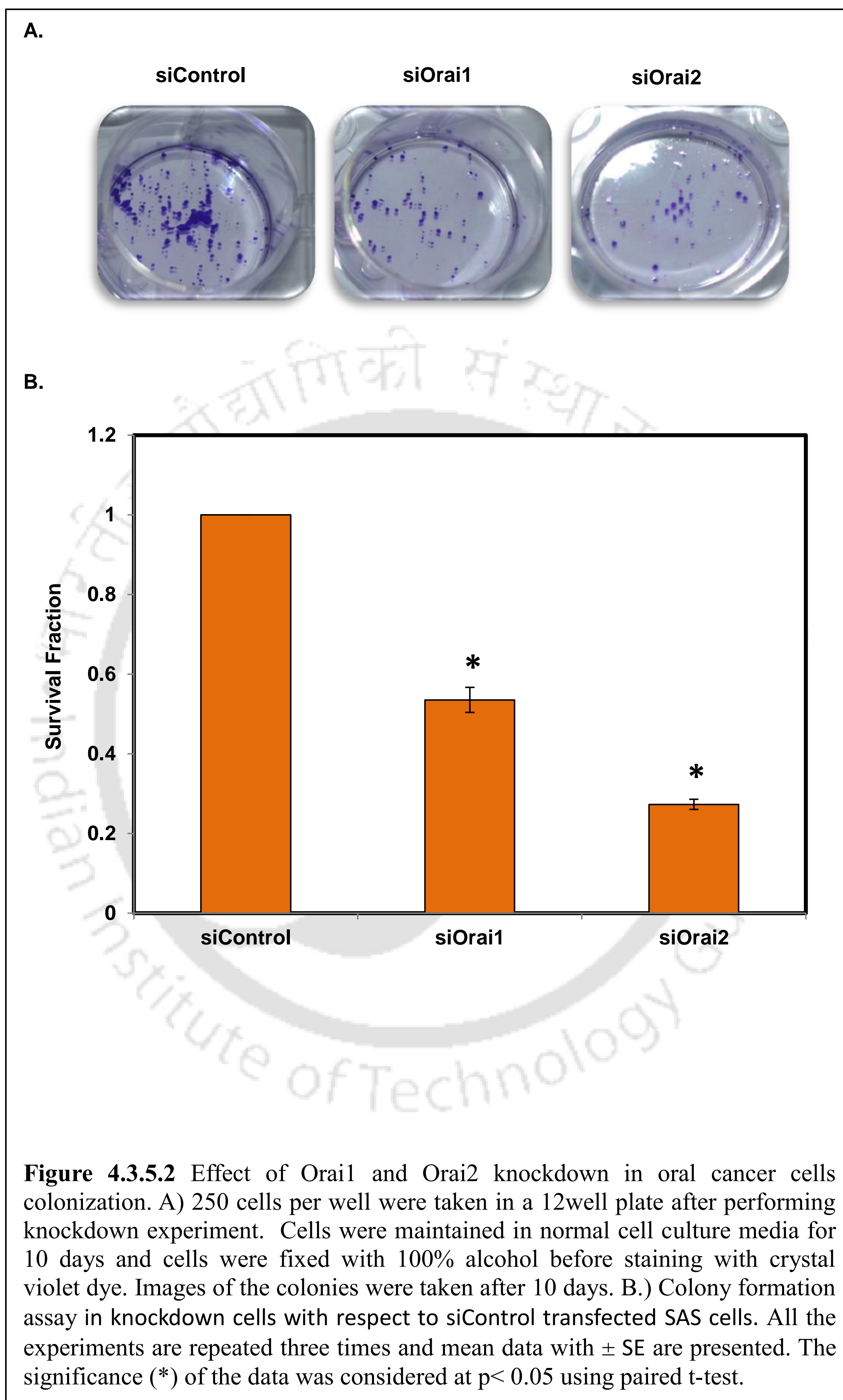

Figure 4.3.5.2 Effect of Orai1 and Orai2 knockdown in oral cancer cells colonization. A) 250 cells per well were taken in a 12well plate after performing knockdown experiment. Cells were maintained in normal cell culture media for 10 days and cells were fixed with 100% alcohol before staining with crystal violet dye. Images of the colonies were taken after 10 days. B.) Colony formation assay in knockdown cells with respect to siControl transfected SAS cells. All the experiments are repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $p < 0.05$ using paired t-test.

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4.3.5 Modulation of SOCE in oral cancer cells altered the expression of AMPK1, cadherins, fatty acid synthase and caspases 3

To extend our study further, we analysed the expression of AMP-activated protein kinase 1 (AMPK1), cadherins, fatty acid synthase (FASN) and caspases 3 to understand the mechanism behind Orai1 and Orai2 mediated Ca^{2+}-entry in oral cancer. We analysed SKF96365 treated oral cancer cells along with Orai1 and Orai2 knockdown cells for these genes at mRNA level using qPCR. We found that expression of AMPK1 was increased 2 to 3 fold with respect to control. In recent studies, a mediator of cellular energy generating system (AMPK) was reported for its tumor suppressing properties in various type of cancers (Li et al., 2015). Recently, knockdown of AMPKα1 showed SOCE augmentation through inhibition of STIM1 phosphorylation in endothelial cells which clearly showed the expression of AMPK1 is closely associated with SOCE (Sundivakkam et al., 2013). Next, we analysed the expression of cadherins (i.e. E-cadherin as well as N-cadherin), FASN and caspases 3 in our study and found that SKF96365 treated cells showed decrease in expression of E-cadherin (~0.5 fold) and very minor increase in N-cadherin (1.2 fold) compared to untreated control. However, the expression of E-cadherin in Orai1 and Orai2 silenced cells was high (1.7 fold and 2 fold respectively) but N-cadherin expression was almost negligible in Orai1 knockdown cells and remained unchanged in Orai2 knockdown cells. Switching of E-cadherin to N-cadherin is directly associated with epithelial to mesenchymal transition (EMT) and metastasis (Gravdal et al., 2007; Li et al., 2016). Our data supported the hypothesis that EMT is promoted by calcium signaling as it was shown in breast cancer and prostate cancer through TRPM7 and STIM1 respectively (Davis et al., 2014; Xu et al., 2015). Recent studies suggested that in tumor cells, alteration of the micro environment may occur due to dysregulated lipogenic pathway which is regulated by one of the key enzymes, FASN and is upregulated in many cancers (Flavin et al., 2010; Kuhajda, 2006; Menendez and Lupu, 2017). Therefore, we observed the expression of

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FASN expression in SKF96365 treated cells and knockdown cells. We found 0.8 fold change (*i.e.* 20% decrease) in expression of FASN in SKF96365 treated cells but we observed its significantly inhibited expression in Orai1 (99%) and Orai2 (50%) knockdown cells. In a recent study, the NFκB-Orai1 was found to be regulated through FASN but this is the first report where we found that Orai2 also regulate FASN and *de novo* lipogenesis (Zhang et al., 2018). Overall, our results show that SOCE is involved in the regulation of FASN expression and *de novo* lipogenesis through Orai1 and Orai2. Furthermore, we wanted to analyse the expression of caspase 3 in cells treated with SKF96365 and knockdown cells because it was reported in many cancers that caspase 3 regulates cell migration apart from its role in regulation of apoptosis (Cheng et al., 2008b; Gdynia et al., 2007; Zhou et al., 2018). We found that caspase-3 expression was inhibited by SKF96365 (80%) and Orai1 silencing (35%) but not by Orai2 knockdown. Similar to our finding, caspase-3 activation was found to be regulated through SOCE in cervical cancer and prostate cancer but SOCE mediated caspase-3 regulation was first time shown in our study which might be associated with oral cancer cells' migration and colony formation (Chiu et al., 2018; Wertz and Dixit, 2000).

4.4 Conclusion

In this chapter, we extended our study at molecular level to understand the role of SOCE through Orai1 and Orai2. We found that knockdown of Orai1 in oral cancer cells decreased the SOCE significantly but Orai2 silencing showed negligible change in SOCE of oral cancer cells. However, we found that cell migration and colony formation were aborted in both Orai1 and Orai2 silenced oral cancer cells. We further investigated the mechanism behind inhibition of cell migration and decreased colony formation efficiency in SOCE modulated cells at molecular level. Overall the study suggested that Orai1 can modulate store dependent Ca^{2+} concentration in the cytoplasm similar to 20μM SKF96365

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but Orai2 does not regulate store dependent Ca^{2+} as Orai1. However, silencing of these genes affected the total cellular calcium which regulates oral cancer cell migration and colonization through Akt/mTOR pathway or NF-κB pathway. In the present study we observed the downregulation of pAkt, p-mTOR and pNF-κB in Orai1 and Orai2 knockdown oral cancer cells. These proteins are associated with the expression of MMP9 and CXCR4 which are known to regulate invasion and metastasis in most of the cancer cells by modulating FAK and other cytoskeletons. Additionally, we also investigated energy generation regulatory protein, AMPK1 which was upregulated in SKF96365 treated cells and knockdown cells. In many cancers, AMPK1 activation helps in tumor suppression. Moreover, we also investigated lipogenic pathway key regulator FASN and found its strong association with SOCE when we observed downregulation in SKF96365 treated cells, Orai1 and Orai2 knockdown oral cancer cells. The EMT in most of the cancer cases was considered as a cascade event of metastasis and it totally depends on switching of E- cadherin to N-cadherin. Moreover, we also found that switching of E- cadherin to N-cadherin was regulated by Orai1-mediated Ca^{2+} in oral cancer cells. Recently, caspase-3 was found to be involved in the regulation of cell migration and metastasis. Therefore, we investigated the role of SOCE in caspase activation and found that caspase-3 expression was strongly associated with Orai1-regulated SOCE. Overall, in this chapter we conclude that cell migration and colonization of oral cancer cells are regulated by Orai1/Akt/mTOR/MMP9 pathway or AMPK/ NF-κB/CXCR4 pathway.

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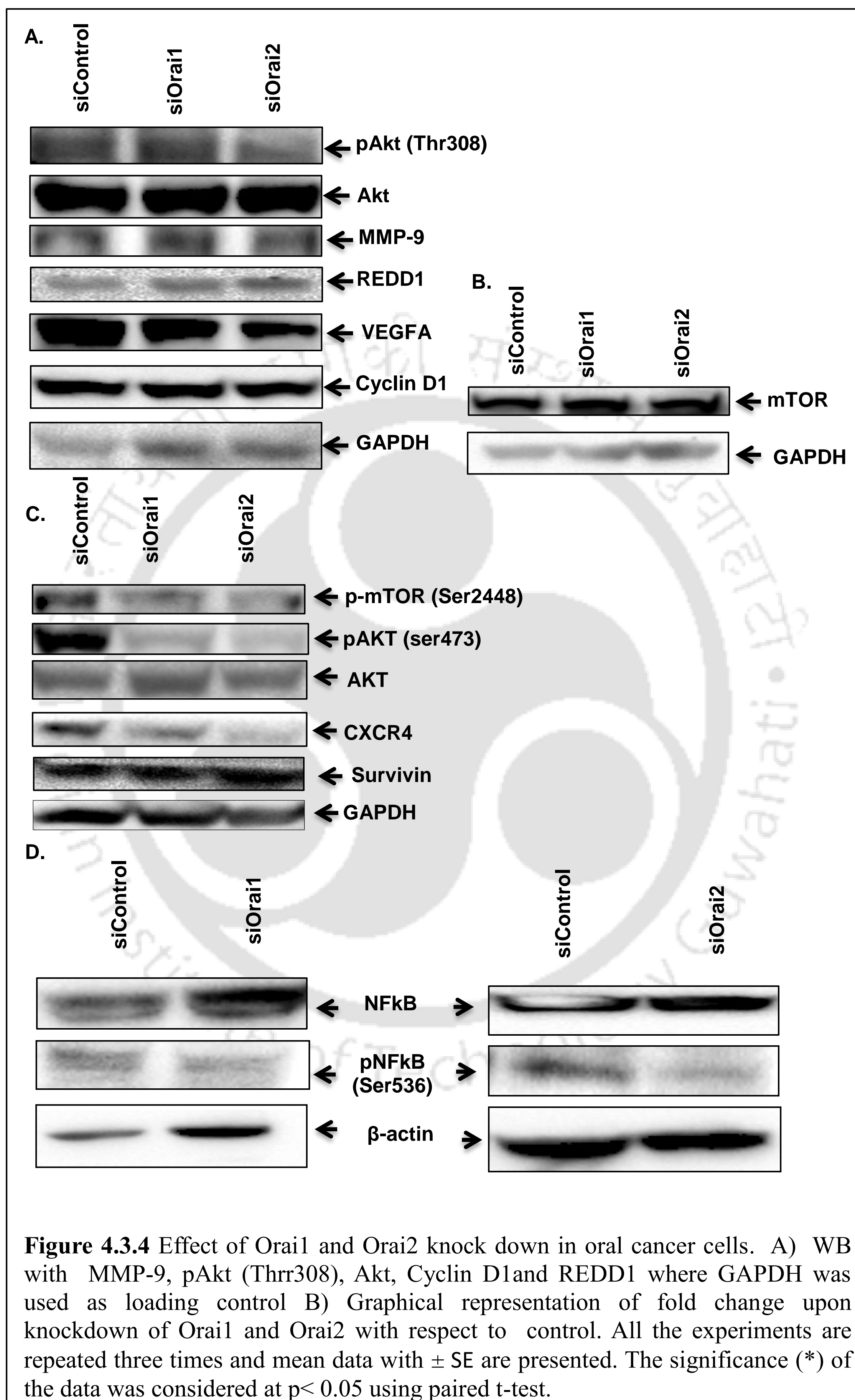

Figure 4.3.4 Effect of Orai1 and Orai2 knock down in oral cancer cells. A) WB with MMP-9, pAkt (Thrr308), Akt, Cyclin D1and REDD1 where GAPDH was used as loading control B) Graphical representation of fold change upon knockdown of Orai1 and Orai2 with respect to control. All the experiments are repeated three times and mean data with ± SE are presented. The significance (*) of the data was considered at $p < 0.05$ using paired t-test.

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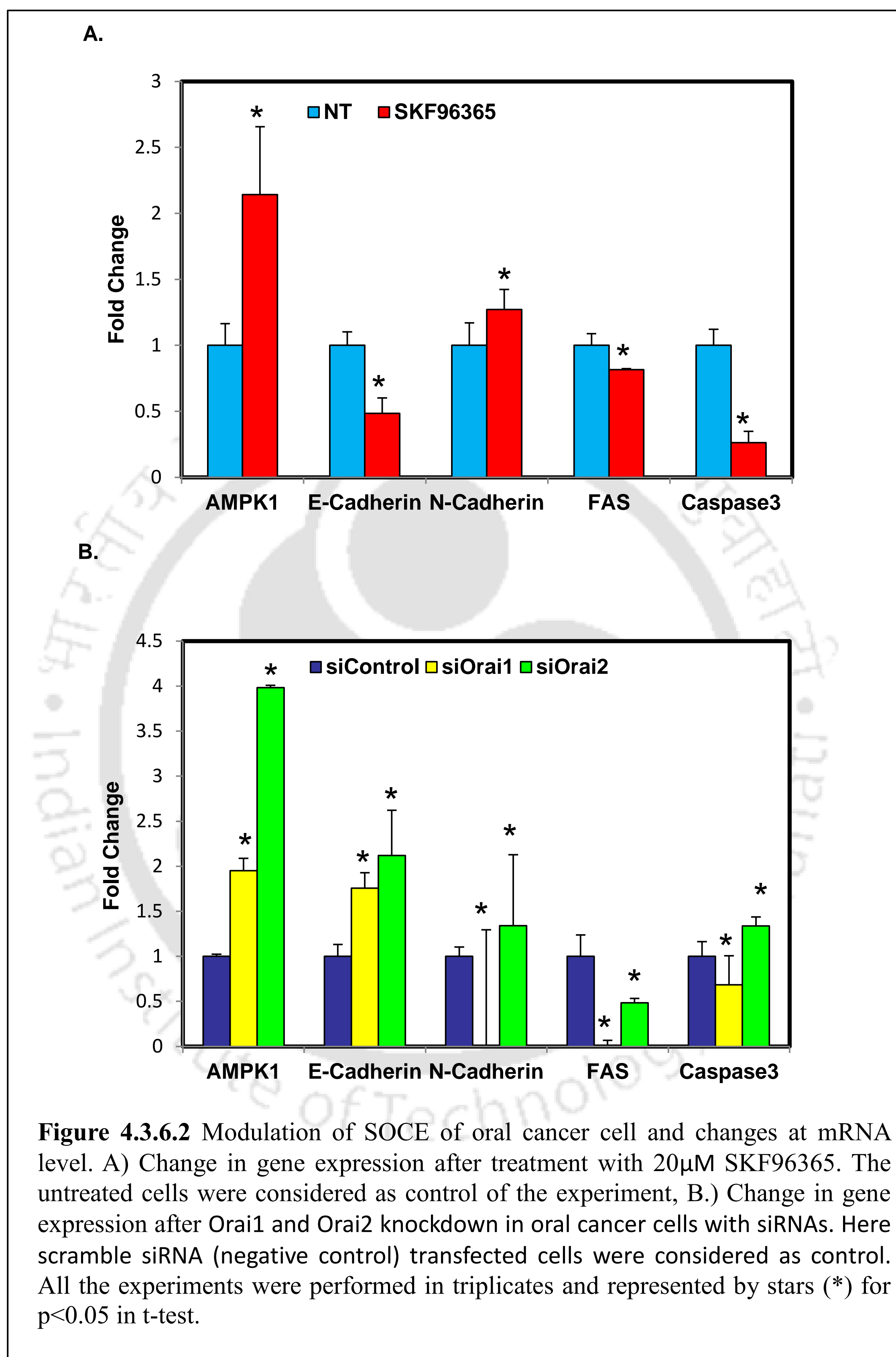

Figure 4.3.6.2 Modulation of SOCE of oral cancer cell and changes at mRNA level. A) Change in gene expression after treatment with 20µM SKF96365. The untreated cells were considered as control of the experiment, B.) Change in gene expression after Orai1 and Orai2 knockdown in oral cancer cells with siRNAs. Here scramble siRNA (negative control) transfected cells were considered as control. All the experiments were performed in triplicates and represented by stars (*) for $p<0.05$ in t-test.

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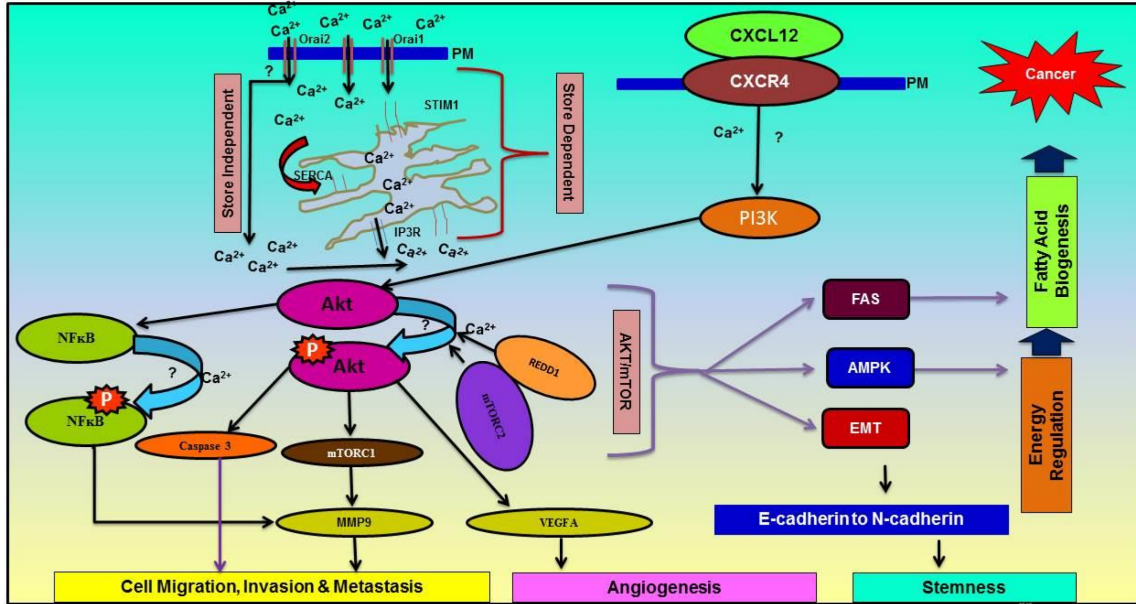

Figure 4.4: Role of Orai1 and Orai2 in oral cancer progression

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CHAPTER-5

Discussion and Conclusion

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5.1 Discussion and conclusion

Oral cancer is a major health concern in India and other developing countries (Coelho and Coelho, 2012) as the rate of new incidences and death due to oral cancer is increasing remarkably every year despite the recent advances in the diagnostics and therapeutics in the field of oncotherapy (Gupta et al., 2016). Most favourable subsites for oral cancer are gingiva, alveolar ridge, buccal mucosa, anterior two third of tongue, mandible, and maxilla. The major risk factors for oral cancer are tobacco, alcohol, and viruses like HPV which helps in the development of oral cancer (Iype et al., 2001; Sharma et al., 2018). Development of oral cancer is a long and multi-step process which starts with the influence of carcinogens where normal cells become neoplastic due to change in some of the key signaling machinery which might have a role in regulating cell division, apoptosis or metastasis (Krishna et al., 2015). On the basis of oral tissue histology, the multi-step processes are divided into three main categories as the disease progress- precancerous lesions (initialization), precancerous condition (intermediate), and oral neoplasms (Watanabe et al., 2015). Aforementioned, cancer is the disease condition where multiple alterations take place in normal cells. All these alterations are confined to two large categories of genes- tumor suppressor genes and oncogenes. These genes regulate many growth factors, cell surface receptors, signaling pathways and many transcription factors (Krishna et al., 2015). The basic treatment modalities for oral cancer are- surgery, radiation therapy and chemotherapy. However, the growing evidences of chemoresistance and tumor recurrence suggest the need for new drug target for oral cancer (Tsai et al., 2012). Consequently, there is an urgent need to develop new biomarkers for early diagnosis and treatment of oral cancer.

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Recent reports on the role of SOCE and the key players of store operated calcium channels in cancers brought a hope that it could be a potential target for oral cancer and other cancers (Pan and Ma, 2015). The major SOCCs are Orai1, Orai2, and Orai3; which are known to present on the plasma membrane which open and close through a kind of sensing proteins of endoplasmic reticulum, STIM1 and STIM2 (Parekh and Putney, 2005). The Orai-STIM regulated CRAC channel gets activated when phospholipase C enzyme breaks phospholipid, PIP_2 into DAG and IP_3. Then IP_3 acts as a secondary messenger to bind with the IP_3 receptor, present on ER membrane, allows the stored Ca^{2+} to be released out. Later, the Ca^{2+}-store depletion was sensed by STIM protein of ER membrane which leads to the conformational changes in STIM and puncta formation in combination with Orai proteins and facilitates the Ca^{2+}-entry in the cytoplasm (Parekh and Putney, 2005; Putney, 1986, 2010). The refilling of the Ca^{2+}-store, ER lumen, is done by SERCA pump present on ER membrane (Stathopulos and Ikura, 2013). Additionally, some TRP proteins were also found to be involved in SOCC mainly TRPC1, TRPC5 and TRPC6 (Ong et al., 2007; Salido et al., 2009; Wen et al., 2016) Since most of the studies were focused on Orai1-STIM1 mediated SOCE, it is considered a potential target (Lacruz and Feske, 2015; Yang et al., 2009a) but Orai2 and Orai3 are considered as neglected players of SOCC (Hoth and Niemeyer, 2013). The role of SOCs was evidenced in different cancers such as breast cancer, cervical cancer and hepatocellular carcinoma (Chen et al., 2011; Huang and Jan, 2014; Yang et al., 2013, 2009b). These SOCs were found to cause enhanced invasion, migration, metastases, angiogenesis and also upregulated proliferation and prolonged cancer cell survival in various cancer types (Bose et al., 2015). Therefore, we hypothesized that targeting these proteins might prove beneficial to treat oral cancer patients.

We first analysed the SOCE in oral cancer cells and non-cancerous (normal) cells and found that Ca^{2+}-influx was upregulated in oral cancer cells compared to normal cells. Further, we

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examined the gene expression of the associated channel proteins (Orai1, Orai2, Orai3, STIM1, and STIM2) in both oral cancer cells and normal cells. Interestingly, we found that Orai1 and Orai2 expression levels were significantly higher in oral cancer cells compared to normal cells. Later, we confirmed our results with oral cancer patient tissue samples (present on tissue microarray slides) using Orai1 and Orai2 antibodies for IHC. The overall expression scores were compared for malignant tissues and normal tissues where we found that Orai1 and Orai2 were significantly overexpressed in oral cancer tissues compared to normal tissues. We also found that both Orai1 and Orai2 were significantly upregulated in metastatic tissues, male tissues and tissues with advanced stages of the disease compared to normal tissues. Recently, the similar study was performed by Lee et al where Orai1 was found to be overexpressed in oral cancer patients and shown to be regulated through NFAT singling pathway which controls dysregulated stem cell properties for oral cancer progression (Lee et al., 2016). The Orai1 upregulation was also reported in many other cancers (Guéguinou et al., 2016; McAndrew et al., 2011; Pla et al., 2016), but upregulation of Orai2 in oral cancer cells is shown here for the first time. However, in human leukemia cells, both Orai1 and Orai2 were found to regulate phosphorylation of focal adhesion kinase (FAK), which is known for regulating cell migration in cancer cells (Diez-Bello et al., 2017). Notably, the Ca^{2+}-influx rate varies for all three Orai proteins although Orai3 does not show any detectable calcium current (DeHaven et al., 2007). Later, Orai1 and STIM1 were also found to regulate FAK and calcium-dependent protease calpain in breast cancer which further regulates focal adhesion turn over-mediated cell migration (Yang et al., 2009b).

Next, we hypothesized that high SOCE in oral cancer is probably due to consumption of tobacco in either form; smokeless or smoking. Several studies suggested that tobacco is the risk factor of oral cancer (Johnson, 2001; Rodu and Jansson, 2004; Sadri and Mahjub, 2007). Additionally, higher tobacco consumption in males compared to females could be a reason

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for the growing number of incidences of oral cancer among males than females (Asthana et al., 2016). Therefore, we first analysed the effect of crude tobacco extract which was found to induce high Ca^{2+}-influx at very low concentrations (10^{-4} to 10^{-3} μg/ml) and Orai2 showed minor upregulation (~1.2 fold) but a transient receptor potential channel, TRPC1, was found to be significantly upregulated (1.5 fold) at mRNA level. Further, we studied the effect of tobacco-associated carcinogens (B[a]P, NNN & NNK) and 4NQO (a synthetic carcinogen) on SOCE and relative expression of SOCC genes and TRPC1 gene. The study revealed that after 24hr treatment, these carcinogens induced SOCE at very low concentration and resulted in upregulation of Orai2 and TRPC1 genes. From our results, it can be concluded that upregulation of SOCE through tobacco and associated carcinogens affect oral cancer cells proliferation and lead to overexpression of the SOCC genes.

Now we know that SOCE is high in oral cancer. Additionally, Orai1 and Orai2 were found to be overexpressed in oral cancer cells as well as in patients' tissue samples. Therefore, we further attempted to understand the role of SOCE and their regulators, Orai1 and Orai2, in oral cancer progression through modulation of Ca^{2+}-influx by either SOCE inhibitors or knockdown of Orai1 and Orai2 in oral cancer cells. Among three SOCE inhibitors (2-APB, LaCl3, and SKF96365), we found that 20μM SKF96365 was the best to significantly decrease the proliferation, SOCE and inhibit the migration of oral cancer cells. The similar studies with SKF96365 were discussed in other cancers such as breast cancer, melanoma, and colon cancer (Guéguinou et al., 2016; Umemura et al., 2014; Yang et al., 2009a). Since a number of studies based on SOCE and cancer used SKF96365 as a control, we also decided to use it in our Orai1 and Orai2 knockdown based Ca^{2+}-influx assays (Jing et al., 2016; 2010; Song et al., 2014; Zhang et al., 2015). Later, we silenced Orai1 and Orai2 using siRNAs which were confirmed by qPCR and Western blot experiments. Next, we found that Orai1 silencing led to the downregulation of the SOCE which was equivalent to 20μM SKF96365,

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but Orai2 silencing showed a negligible decrease in SOCE of oral cancer cells. However, we found that Orai1 and Orai2 knockdown inhibited the migration and decreased the colony forming efficiency of oral cancer cells. Overall, it can be concluded that Orai1 and Orai2 play very important role in oral cancer cells' migration and metastasis. This is the first report which shows the vital role of Orai1 and Orai2 in migration and colonization of oral cancer cells. Nevertheless, the importance of Orai1 in cell migration and metastasis in case of breast and colon cancer has already been reported (Guéguinou et al., 2016; Yang et al., 2009a). Recently, the role of Orai2 was analysed in human leukaemia along with Orai1 and found that SOC regulates the focal adhesion kinase phosphorylation and cell migration (Diez-Bello et al., 2017). We also tried to unravel the molecular mechanism(s) behind this inhibited cell migration and decreased colonizing efficiency. To elucidate the mechanism, we first performed Western blot experiments with the key molecules of Akt/mTOR pathway because of its role in regulating numerous cellular functions and cancer hallmarks (Dennis et al., 2014; O'Donnell et al., 2018). We also tried to decipher the association between NF-κB pathway and Orai1 and Orai2 in oral cancer (Jana et al., 2017). Overall our study suggested that Orai1 and Orai2 knockdown downregulated the expression of phosphorylated form of Akt (both pAkt-ser473 and pAkt-thr308), phospho-mTOR, phospho- NF-κB, MMP-9, VEGFA, and CXCR4. Additionally, we also analysed the expression of REDD1, cyclin D1, and survivin and observed that REDD1 was overexpressed but cyclin D1 and survivin were unaffected with the knockdown. Taken together, we understand that the key molecules regulating cell migration or colonization are MMP-9 and CXCR4 which could be the downstream of either Akt/mTOR pathway (Chen et al., 2009; Kou et al., 2016; Zhu et al., 2015) or NFκB pathway (Guarneri et al., 2017; Shi et al., 2015) are affected by knockdown of Orai1 and Orai2 in oral cancer cells. Additionally, the overexpression of REDD1 in Orai1 and Orai2 knockdown oral cancer cells were inversely associated with phospho Akt

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expression (Dennis et al., 2014; Katiyar et al., 2009). We further analysed the expression of AMPK1, fatty acid synthase, E-cadherin, N-cadherin, and caspase-3 and found that AMPK1 and E-cadherin were overexpressed in Orai1 and Orai2 knockdown cells. However, N-cadherin and caspase-3 were downregulated in Orai1 silenced cells but remained unchanged in Orai2 knockdown cells. Besides, fatty acid synthase expression was downregulated in both Orai1 (99%) and Orai2 (50%) silenced oral cancer cells. A recent study showed that AMPKα1 was silenced in endothelial cells and found that SOCE was augmented due to inhibition of STIM1 phosphorylation which showed a close association between AMPKα1 and SOCE (Sundivakkam et al., 2013). Overexpression of E-cadherin and downregulation of N-cadherin indicate inhibition of EMT which is directly associated with metastasis (Gravdal et al., 2007; Li et al., 2016). Recently, EMT was found to be promoted by calcium signaling through TRPM7 in breast cancer and STIM1 in prostate cancer (Davis et al., 2014; Xu et al., 2015). The expression of FASN was found to be upregulated in many cancers (Flavin et al., 2010; Kuhajda, 2006; Menendez and Lupu, 2017). In line with our study, Zhang and group showed that expression of FASN is regulated through NFκB-Orai1 mediated SOCE (Zhang et al., 2018). However, ours is the first report which shows that Orai2 also regulates FASN and *de novo* lipogenesis. Similar to our results, SOCE was reported to regulate caspase-3 activation in cervical cancer and prostate cancer (Chiu et al., 2018; Wertz and Dixit, 2000). Additionally, it was observed in many cancers that the apoptosis-regulating caspase-3 also plays a key role in cell migration (Cheng et al., 2008b; Gdynia et al., 2007; Zhou et al., 2018). Altogether, tumor suppressing AMPK1 expression and lipogenesis through FASN were regulated by Orai1 and Orai2-mediated SOCE but EMT and caspase-3 activation were regulated by only Orai1 mediated SOCE. Therefore, it was concluded that oral cancer cell migration and colonization might be regulated either through Orai1/Akt/mTOR/MMP9 pathway or AMPK/ NFκB/CXCR4 pathway.

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5.2. Limitations of the studies

During this study, we explored SOCC in oral cancer and revealed the importance of Orai1 and Orai2 in the pathogenesis of the disease. In our study, we performed IHC with 80 tissue samples from different subsites of the oral cavity and showed different expression score for Orai1 and Orai2. The highest expression score was in lymph node tissues than tongue tissues but we had only SAS cell line which is of tongue origin. Studies with cell lines of different origins would have been better. Second, we wanted to explore oral cancer tissues from the local populations but the difficulties in ethical clearance limited us to do IHC with commercially available tissue microarray only which is not from India. Additionally, in the Ca^{2+}-influx efficiency and SOCC gene expression studies, we compared a normal skin epithelial cell with oral cancer cell because we were unable to get normal oral epithelial cells or develop primary cells of oral origin. Moreover, we understand that flow Ca^{2+} through SOCC is associated with I_{CRAC} and it would be important information if we would have performed patch clamp experiments. But due to the scarcity of the equipment we were limited to use of Ca^{2+}-sensing probes only. Further, we were interested to do animal studies based on our findings to validate our outcome but we could not perform the same due to the lack of animal facility.

5.3. Future Direction

Ca^{2+} is a secondary messenger required in many normal cellular processes as well as in cancer progression which can be controlled through various types of channels, pumps, and exchangers. During our study, we explored SOCC and found the role of Orai1 and Orai2 in oral cancer progression, the role of tobacco in the regulation of SOCE and the molecular changes associated with Orai1 and Orai2. The suggestions for future research in continuation of this study might be as follows-

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A) Tobacco is a well-known carcinogen for causing cancer in mouth, lung, larynx, throat, bladder, liver, stomach, kidney, pancreas, colon and rectum, and cervix, as well as acute myeloid leukaemia as reported in many studies. In our analysis, we explored the role of tobacco and associated carcinogens in the regulation of SOCE and we also observed overexpression of TRPC1 genes at mRNA level but we did not pursue any further studies with TRPC1. Therefore, TRPC1 must have some role in oral cancer progression because its role was found in many other cancers (Cheng et al., 2008a; Guéguinou et al., 2016; Ong et al., 2007). Additionally, other TRP channels like TRPC5, TRPC6, and TRPM8 which were also found to play role in regulating calcium homeostasis in many studies (Okamoto et al., 2012; Wen et al., 2016; Xu et al., 2005) can be studied further.

B) The north-eastern part of India is well known for betel nut chewing habit along with high tobacco and tuibur consumption which is directly associated with tobacco-related cancers. Therefore, the SOCE must be explored in the cancer patients of the northeast and specifically among tobacco product users.

C) We faced difficulty in procuring oral cancer cell lines in India and for a good research one can work in the development of new and Indian origin oral cancer cell line.

D) The current research work supports the role of SOCE in cell migration and metastasis *in vitro* which must be validated *in vivo* as well

E) During this study, we desired to explore for natural compounds which may work as an inhibitor of SOCE and could show some beneficial role against oral cancer. To understand that, one can perform bio-informatics based docking study for natural compounds and available chemotherapeutic agents against Orai1 and Orai2 proteins. Additionally, the well-suited ones can be used for wet lab-based validation with and without chemotherapeutic drugs.

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ABBREVIATIONS

µg : Microgram
µM : Micromolar
ANOVA : Analysis of variance
ATP : Adenosine triphosphate
B[a] : Benzo[a]pyrene
CAT : Cancer adjacent tissues
CRAC : Ca^{2+} release activated Ca^{2+} channels
CXCR4 : Chemokine receptor type 4
C*IS* : Carcinoma *in situ*
CL2 : Cytosolic loop 2
CTD : Cytosolic C-terminal domain
DAB : 3,3'-diaminobenzidine
DAG : Diacylglycerol
DFS : Disease free survival
DMEM : Dulbecco's Modified Eagle Medium
DMSO : Dimethyl sulfoxide
DPX : distyrene, plasticizer (tricresyl phosphate), and xylene
DTT : Dithiothreitol
EBRT : External beam radiotherapy
EBV : Epstein barr virus
EDTA : Ethylenediaminetetraacetic acid
EGFR : Epidermal growth factor receptor
EL1 : Extracellular loop1
EMT : Epithelial-mesenchymal transition
ER : Endoplasmic reticulum
ERM : Ezrin-radixinmoesin
EL3 : Extracellular loop3
FACS : Fluorescence-activated cell sorting
FASN : Fatty acid synthase
FAK : Focal adhesion kinase
FBS : Fetal bovine serum
GAPDH : Glyceraldehyde-3-Phosphate Dehydrogenase
GLOBOCAN : Global cancer statistics database of IACR
GPCR : G protein-coupled receptor
HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV : Human papillomavirus
HSV : Herpes simplex viruses
IHC : Immunohistochemistry
IACR : International agency for research on cancer
LGCC : Ligand-gated Ca^{2+} channels
ml : Milliliter
mM : Millimolar
mRNA : Messenger RNA
mTOR : Mammalian target of rapamycin
MTT : (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide)

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nAChR	: Nicotinic acetylcholine receptors
NF-κB	: Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	: National Institutes of Health
nm	: Nanometer
nM	: Nanomolar
NNK	: 4 [methylnitrosoamino]-1-[3- pyridyl]-1-butanone
NNN	: N-nitrosonornicotine
4NQO	: 4-Nitroquinoline 1-oxide
NTD	: N-terminal domain
OSCC	: Oral squamous cell carcinoma
PAH	: Polycyclic aromatic hydrocarbons
PBS	: Phosphate Buffer Saline
PDB	: Protein Data Bank
PFS	: Progression free survival
pH	: potential of hydrogen
PI	: Propidium iodide
PI3K	: Phosphoinositide 3-kinase
PIP2	: Phosphatidylinositol (3,4)-bisphosphate
PIP3	: Phosphatidylinositol (3,4,5)-trisphosphate
IP3	: Inositol trisphosphate
PKB	: Protein kinase B
PLC	: Phospholipase C
PMSF	: Phenylmethane sulfonyl fluoride
pRb	: Retinoblastoma protein
PRAD1	: Parathyroid neoplasia gene on 11q13(Cyclin D1)
PTEN	: Phosphatase and tensin homolog
Ras	: Rat Sarcoma
RGCB	: Rajiv Gandhi Centre for Biotechnology
ROS	: Reactive oxygen species
SCC	: Squamous cell carcinomas
SDS	: Sodium dodecyl sulfate
SERCA	: Sarcoplasmic/endoplasmic reticulum ATPasa
siRNA	: Short interference RNA
SOC	: Store operated calcium
SOCC	: Store operated calcium channel
SOCE	: Store operated calcium entry
STIM	: Stromal interaction molecule
SAM	: Sterile α motif
TBS	: Tris buffer saline
TBST	: Tris-buffered saline (TBS) and Polysorbate 20 (also known as Tween 20)
TRP	: Transient receptor potential
TRPC	: Transient receptor potential canonical
TE	: Tobacco crude extract
TM	: Transmembrane domains
TMA	: Tissue microarray
TSNA	: Tobacco-specific-nitrosamines

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VEGF : Vascular endothelial growth factor
VGCC : Voltage-gated Ca^{2+} channels
WHO : World health organization

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