INHIBITION OF CD146 IN BREAST CANCER STEM CELLS AND TUMORIGENESIS TO CONTROL BREAST CANCER METASTASIS

BY

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UNDER THE SUPERVISION OF

DR. INDUMATHI SOMASUNDARAM

THESIS SUBMITTED TO

FOR THE DEGREE OF

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A THESIS SUBMITTED TO

D. Y. PATIL EDUCATION SOCIETY
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IN
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BY

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DECLARATION

I, hereby declare that the work presented in this thesis entitled "**INHIBITION OF CD146 IN BREAST CANCER STEM CELLS AND TUMORIGENESIS TO CONTROL BREAST CANCER METASTASIS**" is an original work and was carried out by me independently in D. Y. Patil Education Society, Kolhapur under the supervision of **Dr. Indumathi Somasundaram** (former Assistant professor at Department of Stem Cell and Regenerative Medicine) in collaboration with **Prof. Marcel Blot Chabaud** (Research Director at Aix- Marseille University), INSERM, C2VN Lab, Aix- Marseille University, Marseille, France.

I further declare that present work has not formed the basis for award of any other degree, fellowship or associateship or any similar title of any other University. The extent of information derived from the existing literature has been indicated in the body of thesis at appropriate places giving the references.

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CERTIFICATE OF GUIDE

This is to certify that the work incorporated in this thesis entitled "**INHIBITION OF CD146 IN BREAST CANCER STEM CELLS AND TUMORIGENESIS TO CONTROL BREAST CANCER METASTASIS**" is submitted herewith for the degree of Doctor of Philosophy in Biotechnology by **Akshita Sharma,** D.Y. Patil Education Society (Deemed to be University), Kolhapur, Maharashtra. The work was carried out under my supervision. This thesis or any part of it was not submitted for any Degree/ Diploma or any other academic award elsewhere.

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Place: Kolhapur

LIST OF PUBLICATIONS AND CONFERENCES

PUBLICATIONS-

1) Pankaj Kaingade, Indumathi Somasundaram, **Akshita Sharma**, Darshan Patel and Dhanasekaran Marappagounder. Cellular Components, Including Stem-Like Cells, of Preterm Mother's Mature Milk as Compared with Those in Her Colostrum: A Pilot Study Breastfeed Med 2017 Sep;12(7):446-449. doi: 10.1089/bfm.2017.0063. Epub 2017 Jun 22. (I.F.0.836)

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5) **Akshita Sharma**, Indumathi Somasundaram1, Marcel Blot Chabaud. CD146 as a prognostic marker in breast cancer: A meta-analysis. (Communicated in Journal of Cancer Research and Therapeutics).

CONFERENCES-

1) Attended International conference on **Nanotechnology addressing the convergence of material science, biotechnology and medical science- 2017 (IC-**

NACMBM 2017) organized by Centre for interdisciplinary research, D. Y. Patil Education Society (Deemed to be University) Kolhapur on 9-11th November 2017.

2) Attended Second national conference on **REGEERATIVE MEDICINE AND STEMC ELL RESEARCH organized by DHANALAKSHMI SRRINIVASAN MEDICAL COLLEGE AND HOSPITAL, TRICHY on 6-7th April 2018.**

3) Attended two days training program on **FLOW CYTOMETRY** held on 18th - 19th September 2019 at D.Y. Patil Medical College, Kolhapur, jointly organized by Luminex and CIR, D. Y. Patil Education Society (Deemed to be University) Kolhapur.

4) Attended one day International webinar on **CHALLENGES AND PROSPECTS OF CANCER THERAPEUTICS** organized BY Dept of Zoology, Diamond Harbor Women's University, West Bengal on 29th Sept 2020.

5) Participated in Webinar on **LIFE CELL BY CELL: UNDERSTANDING BIOLOGY AT TRUE RESOLUTION** on 16th August 2020 organized by GENOMIX.

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LIST OF ABBREVIATIONS

NCRP	National Cancer Registry Programme
VEGF	Vascular Endothelial Growth Factors,
IL-8	Interleukin-8
FGF	Fibroblast Growth Factor
HGF	Hepatocyte Growth Factor
MCP-1	Monocyte Chemo-Attractant Protein
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
HIF-1	Hypoxia-inducible factor 1
EC	Endothelial Cells
TSP	Thrombospondin
INF	Interferron
IL	Interleukin
CD	Cluster of Differentiation
Wnt-5a	Wingless/integrase
BCSC	Breast Cancer Stem Cell
EMT	Epithelial to Mesenchymal Transition
MET	Mesenchymal to Epithelial transition
AAR	Adjusted Incidence Rate
APC	Annual Percentage Change
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma
TNBC	Triple Negative Breast Cancer
DNMT1	DNA (cytosine-5)-methyltransferase 1

MMP	Matrix Mettalo Protein
OPN	Osteopontin
PDGF	Placental Derived Growth Factor
EPCAM	Epithelial Cellular Adhesion Molecule
sCD146	soluble Cluster of Differentiation 146
HUVECs	Human Umbilical Vein Endothelial Cells
FSC	Forward Scatter
SSC	Side Scatter
MTT	(3-(4,5-Dimethylthazolk-2-Yl)-2,5-Diphenyl Tetrazolium Bromide)
XTT	(2,3-Bis-(2-**Methoxy-4-Nitro-5-Sulfophenyl**)-2 H
CFSE	5(6)-Carboxyfluorescein Diacetate N-Succinimidyl Ester
PAGE	Poly- Acrylamide Gel Electrophoresis
PVDF	Polyvinylidine Difluoride
HRP	Horseradish Peroxidase
AP	Alkaline Phosphatase
ELISA	Enzyme Linked Immune-Absorbent Assay
H&E	Hematoxylin and Eosin
IgG	Immunoglobulin G

CHAPTER-1

INTRODUCTION

1. INTRODUCTION-

1.1Epidemiology-

Cancer is the leading cause of death worldwide (1). Breast cancer contributes to be the most common malignancy among the women around the world with 70 to 80 % cases being cured as they were detected early and were non-metastatic. Metastatic breast cancer is considered incurable with ongoing treatment options like hormonal therapy, chemotherapy (2). Cancer cases are rising rapidly worldwide with exponential population growth and aging contributing majorly to its rise (3). The data recorded from 2006- 2015 reported stable cases in women while a 2% increase in men was recorded. Additionally, reports of decline in mortality by 1.4% - 1.8% in women and men respectively has also been documented. The death rate declined by 27% from 1991- 2016 contributing to around 2.6 million lesser deaths around the world (4). The decline in death rate was reported mainly in the leading cancers like lung, breast, prostate and colorectal cancer. During 2008-2017, there was reduction of female breast and colorectal cancers and a halt in prostate cancer mortality. Also mortality rate of lung cancer declined from 3% in 2008 to 5% in 2013- 2017 in men while 2% -5% in women. Even with such a declining rate of mortality, lung cancer alone contributes to the most numbers of deaths in 2017 than breast, prostate, colorectal and brain cancer altogether (5).

In 2020, female breast cancer emerged as the most commonly diagnosed cancer surpassing lung cancer with total 2.3 million (11.7%) new recorded cases around the world with lung (11.4%), colorectal (10%), prostate (7.3%) and stomach (5.6%) cancers. Lung cancer is still the leading cause of cancer mortality with 1.8 million deaths, colorectal (9.4%), liver (8.3%), stomach (7.7%) and female breast cancer (6.9%). The global cancer burden is expected to increase by 47% by 2040 due to demographic changes (6). Survival rate of breast cancer patients for at least 5 years after diagnosis range from more than 90% in high-income countries, to 66% in India and 40% in South Africa (7). According to a published report of NCRP, the number of breast cancer deaths in India will climb to 1,06,124 in 2015 and 1,23,634 in 2020

respectively (8).In 2018, Breast cancer statistics reported 1,62,468 new breast cancer cases along with 87,090 deaths. Patients with higher stages of cancer growth find it difficult to survive. In India, approximately 50% cases of breast cancer are of higher stages i.e stage 3 or 4, post cancer survival rate of women in India is around 60% in comparison to 80% in the US. According to NPBC reports published in 2020, there have been 57% of advanced stage breast cancer cases among total cancer cases (9).

1.2 History of Breast cancer research development-

Women have been suffering from breast cancer for a very long time. The earliest record of breast cancer by Edwin Smith Papyrus dates back to 3,000–2,500 B.C.E. During 1st Century AD, tumors were removed by surgical incision giving the theory of association between cancer, menstruation and older age. **John Hunter**, Scottish father of Investigative surgery first identified lymph as the cause of breast cancer giving rise to lumpectomy as the surgical technique for treating patients (10). In 400BC, Hypocrates, "Father of Medicine" gave the term of cancer derived from 'KARKINOS' meaning crab in Greek because of its vascularising nature and invasiveness (11). During 2nd Century Galen; a Greek doctor gave the term 'Onco' to describe the malignant disease because of imbalance in humors and systemic nature of breast cancer caused by black bile in blood (12). At the beginning of 17th Century, Galenic humoral theory was challenged and Lymphatic theory for the origin of breast cancer was given by Olof Rudbeck in 1652 contradicting the previous theory (13). Franciscus Sylvius in 1680 challenged Galen's theory of black bile of blood (14). John Hunter, Astley Cooper, and John Warren advocated removal of cancer with lymphoid tissue (15) marking the next 100 years of surgery as "Century of Surgeon" with the discovery of anesthesia in 1846 with William Halsted introducing radical mastectomy in 1882 as next major milestone in history of breast cancer (16). The 20th century marked remarkable developments in breast cancer treatment ranging from tamoxifen being FDA approved drug for treatment to HER2 gene being discovered and in 2006 Raloxifene being used for reducing risk of breast cancer in post menopausal women (9). In 1967 Elwood Jensen described relationship between sexual hormones and breast cancer. This discovery made the way for the

development of various hormones modulating drug (17). Pivotal research of Bernard Fisher, USA (18) and Gianni Bonadonna, Italy (19) of cytotoxic drugs function in improving breast cancer paved the way for adjuvant therapy. He gave the evidence for the efficacy of cyclophosphamide, methotrexate and fluorouracil (CMF) in adjuvant therapy (20). During 1990's further developments in chemotherapy took place from methotrexate combinations to anthracyclines and taxanes for treating breast cancer (21). Conventional histological evaluation have identified several subtypes (22) but development of microarray and gene expression profiling further identified breast cancer intrinsic subtypes (23).

1.3Angiogenesis and breast cancer-

Angiogenesis process has dual function i.e it functions in normal cellular processes like wound repair, growth and embryonic development and also in tumor development and its metastasis (24). Father of angiogenesis research Judah Folkman, has outstanding achievements in the field of angiogenesis (25) which revolutionized biomedical research and drug development. He discovered an unknown family of angiogenesis regulatory molecules which controls the growth of almost all tumors, showing that continuous endothelial replication and neovascularization is the governing rule for the growth of neoplastic and non-neoplastic tumors (26). Judah Folkman in 1971 hypothesized that tumor growth is angiogenesis dependent. He gave primary evidence that without the support of new capillary blood vessels, the tumor could not grow beyond a millimeter size. According to him, there are few substances secreted by tumors which further stimulate the proliferative capacity of endothelial cells in host blood vessels (27). Folkman also suggested that certain angiogenesis dependent diseases like psoriasis, rheumatoid arthritis and ocular neovascularization can be treated with angiogenesis inhibitors involved in angiogenesis based cancer therapy (28).

After Folkman's hypothesis, tumor employed microvascular endothelial cells became the second target with ***cancer cell per se***as primary cancer therapy target (26). Bortezomib was reported to have potent anti-angiogenic activity before its approval for treating multiple myeloma by FDA in 2003 (29). Since 2003, FDA has approved

10 drugs for anti-angiogenic treatment; eight drugs for treating cancer while two for age related macular degeneration (30).

Breast cancer is the most prevalent and commonly diagnosed cancer in women with largely unknown etiology along with several risk factors involved like reproductive factors, hormones, environmental factors (obesity, limited physical activity) and heredity (31,32). Alike other solid tumors, breast cancer has various growth factors involved in its growth and progression like vascular endothelial growth factors (VEGF), interleukin-8 (IL-8), fibroblast growth factor (FGF) (33), hepatocyte growth factor (HGF), monocyte chemo-attractant protein (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES), further identified to be negative for prognostic markers of breast cancer subtype (34). These pro-angiogenic factors are major players in induction of angiogenesis; however some cancer cells like breast cancer cells produce angiogenesis inhibitors like thrombospondin, angiostatin or endostatin (35). The balance of negative and positive regulation of tubule formation results in the angiogenesis role in cancer growth and progression, also known as **"Angiogenic switch"**. Pro and anti angiogenic proteins regulate the activation and inhibition of the angiogenesis process as per the requirement (36). It is evident that decrease in anti-angiogenic proteins results in the activation of tumorigenesis switch to assist growth and metastasis of tumor (37,38). Activation of tumorigenesis and formation of endothelial tubes is a complex multistep process being strictly controlled by heterodimeric transcription factor HIF-1α which creates hypoxia (39). Under hypoxic conditions, HIF-1α forms heterodimer with HIF-1β to create a hypoxic tumor microenvironment for tumor cells (39,40). HIF pathway is key regulator of angiogenesis by controlling almost all features of angiogenesis through HIF-1α and other members of HIF family; making HIF as an important therapy target with HIF-1α and HIF-2α being associated with poor prognosis and cancer metastasis (41,42,43).

Judah Folkman proposed to treat cancers by inhibiting the proliferation capacity of cancer cells, which later laid the foundation of treatment using anti-angiogenic factors. Through this, researchers formulated VEGF pathway inhibition as an advanced way of angiogenic therapy by using antibodies against VEGF/R,

sVEGF/VEGFR hybrids and inhibitors against tyrosine kinase (27,44,45). Endogenous stimulators and activators found in or circulation as protein, glycoprotein, proteoglycans, regulate angiogenesis by meddling with functioning of endothelial cells (EC) like gene down regulation, cell formation and migration, and tube morphogenesis (46). Some of the angio- inhibitors are as follows-

- Endogenous inhibitors. (47)
  - ✓ Thrombospondins (TSP)
  - ✓ Endostatin
  - ✓ Interferon (INFs)
  - ✓ Interleukins (ILs)
  - ✓ Angiostatin
  - ✓ Decorin

- Pharmaceutical inhibitors.
  - ✓ Axitinib (Inlyta®)
  - ✓ Bevacizumab (Avastin®)
  - ✓ Cabozantinib (Cometriq®)
  - ✓ Everolimus (Afinitor®)
  - ✓ Lenalidomide (Revlimid®)
  - ✓ Lenvatinib mesylate (Lenvima®)
  - ✓ Pazopanib (Votrient®)
  - ✓ Ramucirumab (Cyramza®)
  - ✓ Regorafenib (Stivarga®)
  - ✓ Sorafenib (Nexavar®)
  - ✓ Sunitinib (Sutent®)
  - ✓ Temsirolimus
  - ✓ Thalidomide (Synovir, Thalomid®)
  - ✓ Vandetanib (Caprelsa®)
  - ✓ Ziv-aflibercept (Zaltrap®) (221)
  - ✓ Anti-VEGFR antibodies

Although, the discovered anti-angiogenic drugs have not proven to be very effective against breast cancer but have certainly paved a way to suitable targets which can help in treating breast cancer. Presently, the best way of treating breast cancer can be to understand the mechanism of action of angiogenic agents and redirecting treatment in similar fashion.

1.4 CD146 and breast cancer stem cells-

Recent evidences suggest that an endothelial junction molecule, CD146 (Cluster of differentiation) plays a key role in angiogenesis. Two types of isoforms of CD146 have been discovered- long and short; on different cell types, thus making it a novel angiogenesis therapy target (48). Prof. J. P. Johnson first identified CD146, a 113kDa transmembrane glycoprotein as melanoma progression marker by using MUC18 antibody generated through mouse immunization with lysate of metastasizing melanoma (49). Prof. F. Dignat-George *et. al.* identified S-endo-1 antigen as marker of circulating endothelial cells in blood by flow cytometry (50). CD146 observed in different tumors show that their over-expression increases the invasiveness, metastatic potential and regulates growth and dissemination of tumors (51). In ovarian carcinoma, decrease in RhGTPase activity is due to absence of CD146 which further inhibits invasion and increased cancer cell apoptosis (52). CD146 acts as tumor suppressor in breast cancer (53) while few other evidences state that it is a poor prognostic marker (54). Indeed, elevated CD146 expression induces increased migration and tumorigenicity in breast cancer cells (55). Also recently, in gastric cancer, CD146 was found to regulate expression of EMT markers (56). Also in triple negative breast cancer cases, elevated CD146 expression in epithelial cells was found to be linked to EMT wherein reduced epithelial markers expression to increased mesenchymal markers expression results in increased migration, mammosphere formation and elevated CD44 expression; which conclusively shows the formation of cancer stem cells (57).

CD146 is highly expressed on vessel cells and cancer cells, giving higher probability of correlation between CD146 and cancer metastasis by interveningin homophillic

adhesion between cancer cells and vascular endothelial. However, there is still no evidence showing self interaction of CD146 (58,59). Possibly, CD146 directed adhesion between cancer cells with vascular endothelia and surrounding elements through bidirectional heterophilic interaction between CD146 and its ligand but not homophillic interaction with itself (60). Jiang T. *et. al.* (2012) found that CD146 binds to VEGFR2 as co-receptor for activation by VEGF-A which is a potent pro-angiogenic factor thereby stating the role of CD146 in tumorigenesis (61). Later, subsequent pro-angiogenic factors as ligand for CD146 were found as Wingless/integrase (Wnt)5a (62), Netrin-1 (63), fibroblast growth factor-4 (FGF4) (64), VEGF-C (65). Later in 2017, PDGFR-β on pericytes was found to be another ligand for CD146 for PDGF-β induced PDGFR-β activation (66), found to play role in recruiting pericytes to endothelial thereby making CD146 as important molecule for vessel integrity.

Wicha *et al.* in 2003 demonstrated that some of the breast cancer cells with stem cell surface markers have the capacity to regenerate tumor in the immune-compromised mice; given the name BREAST CANCER STEM CELLS (BCSC) (67). These BCSCs have the ability of self-renewal; can also regulate the tumor micro-environment and thus it can withstand chemo and radiotherapy (68). With such abilities, BCSCs proves to be a potent therapeutic target, making survival of patients' disease free (69).

Evidence for breast cancer metastasis by BCSC is given by EMT (Epithelial to Mesenchymal Transition) coupled with BCSC formation and MET (Mesenchymal to Epithelial transition) as last step for metastasis process; also inducing stem cell features in cancer cells (70).

Thus, aim of the present study was to evaluate the role of CD146 in angiogenesis and breast cancer metastasis. Both *in-vitro* and *In-vivo* experiments have been carried out to substantiate our findings. In this study, breast cancer stem cells form triple negative breast cancer cell line MDA MB 231 was used.

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CHAPTER- 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Breast cancer is the second most common cause of cancer death (1). It is the most prevalent cancer among women with 25% of new cases each year and 15% of total deaths of cancer causing extensive morbidity and physiological distress around the world (2). The world witness variable number of cases of breast cancer (3) with 27 per 100,000 in middle Africa and East Asia to 92 per 100,000 in Northern America (4). With such serious rise in cases, it is estimated that the cases of breast cancer might reach up to 3.2 million by 2050 year with increased number of older women affected (5,6). In the span of 1988-2006, there has been 24% contribution in total cases by Asian- pacific countries like Japan, China, Indonesia (7,8) and Korea of with highest number of cases (9). In 2012, there were about 2,77,054 breast cancer reported in East Asia, 223,889 cases in south- central Asia and 107,545 cases in Southeast Asia (10). Later in 2017, 252,710 and 6341 cases were reported for invasive and in–situ breast cancer in US (6).

Breast cancer is the fastest growing and most common cancer among Indian women with most cases reported in the urban areas (11). Major metropolitan cities like Delhi, Chennai, Bangalore, Mumbai and Kolkata have age adjusted incidence rate (AAR) of 41.0, 37.9, 34.4, 33.6 and 25.5 cases per 1,00,000 population respectively. Breast cancer has rapidly amplified in past two decades with increased annual percentage change (APC) from 0.91% to 5.31% in Delhi over the period 1998-2012 (12) with an assumed number of breast cancer cases reported to approximately 179,790 in India including 10% of other cancers (12).

Breast cancer is the most commonly diagnosed and researched cancer around the world. The first documented records of breast cancer date back to 1600 BC in Egypt by Edwin Smith Papyrus, mentioned it as "*tumors or ulcers of breast*" (13). Until the 19th century, breast cancer was not considered as a serious problem, but with improvement in sanitation and disease control it became a matter of concern. Breast cancer was first surgically treated by French surgeon Jean Louis Petit and Scottish surgeon Benjamin Bell independently by removing lymph node, breast tissue and chest muscles. Around 1882, surgeon William Stewart Halsted started performing

mastectomy. But this method involved invasiveness, long term pain and disability to the patient. By 1970s, lumpectomy was introduced along with radiotherapy, which was less invasive and less painful. Chemotherapy was introduced for the treatment after World War II., as a treatment method for metastatic cancer. Hormonal therapy was then concluded by report from Nurses' Health study (1995) and Women's health initiative report (2002) to be an insignificant method of treatment as it leads to the relapse of breast cancer. Late 1990s witnessed the emergence of evidences showing BRCA1 and BRCA2 gene variants as the major cause of breast cancer risk (3). During the time when hormonal dependency of breast cancer was not proved, Beatson revolutionized endocrine surgery in 1906, even before estrogen receptor being introduced by Jensen 1967 (14,15). Modified radical mastectomy with preservation of pectoralis major was encouraged by Patey and Handley from London and Auchincloss Jr, of New York. Advances in cancer treatment and management by killing cancer cells through chemotherapy and targeted mutation tumor therapy and early cancer detection of lesion by mammography added new milestones in the cancer management (16).

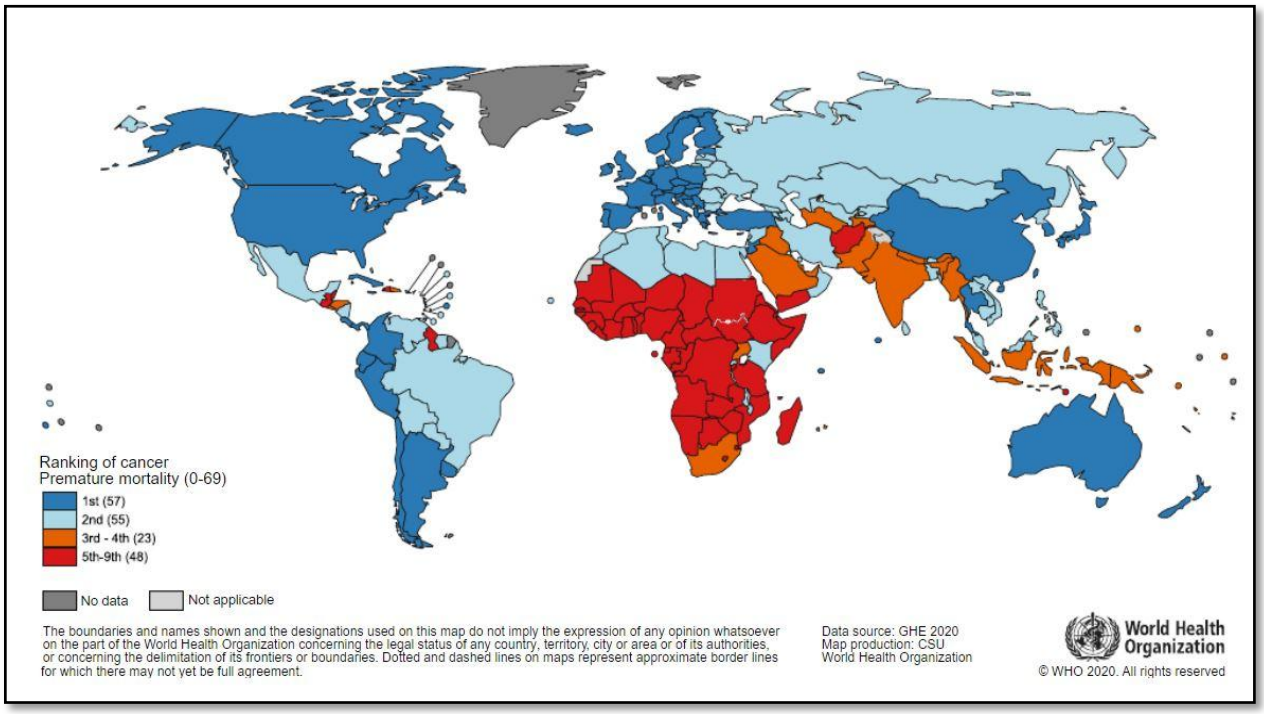

Fig 2.1: National ranking of cancer as cause of death at age ≤70 years in 2019. The numbers of countries included represented in each ranking group are included in legend. Source-World Health Organization

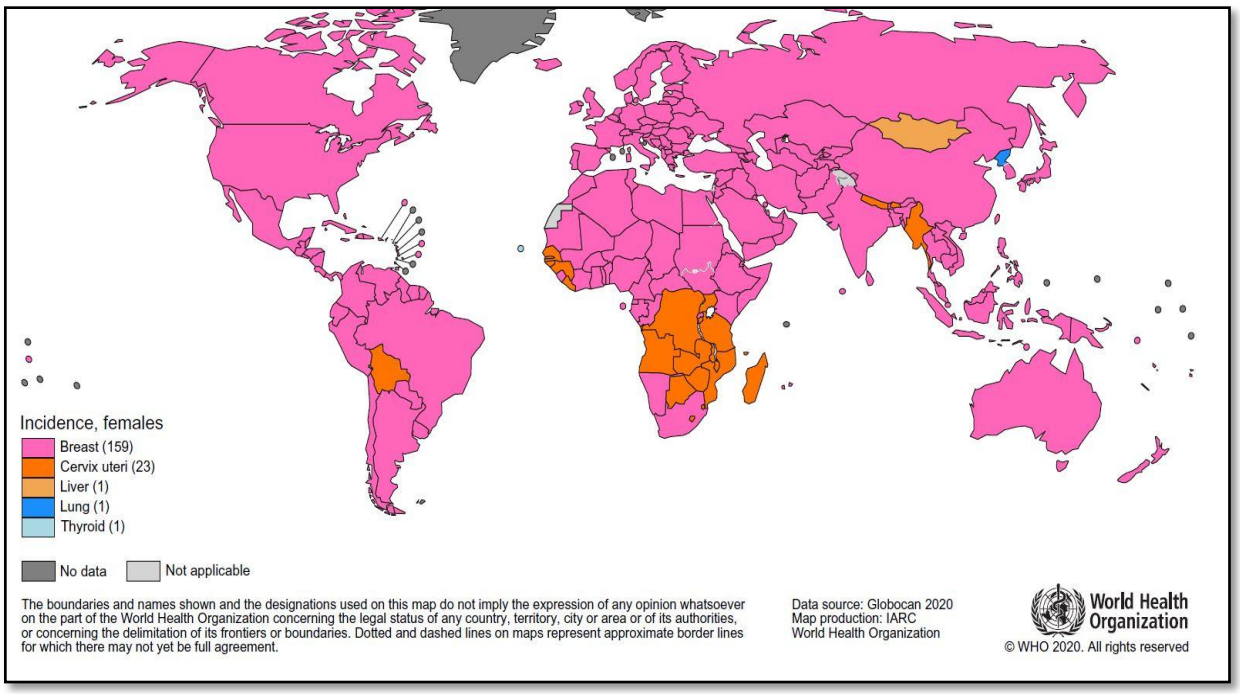

Figure 2.2- Most common type of cancer incidence in 2020. However, non-melanoma skin cancer (excluding basal cell carcinoma), the most common type of cancer in Australia and New Zealand among men and women and in the United States among men, was excluded when constructing the global maps. Source: GLOBOCAN 2020.

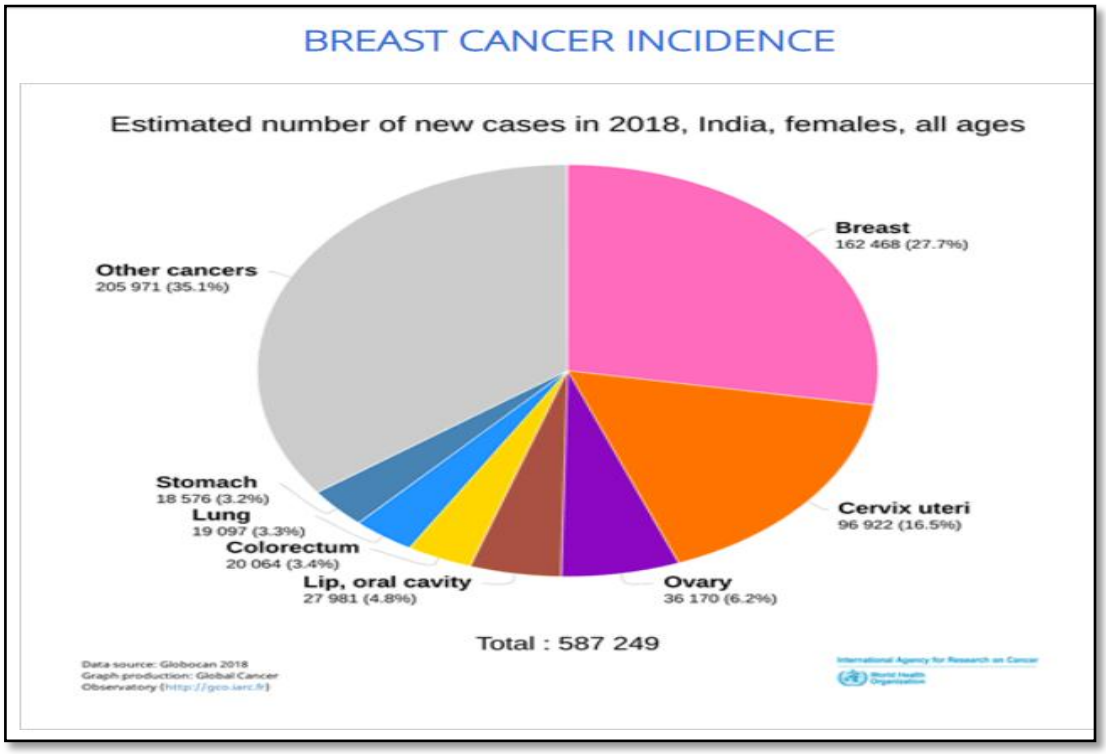

Fig 2.3: For the year 2018, 162,468 women were newly detected with breast Cancer in India. Also, Breast Cancer accounted for 27.7% of all newly detected cancers in women. Which means, roughly, one in four newly detected cancers in women in India was breast cancer. Source: GLOBOCAN 2018. Graph production: Global Cancer Observatory.

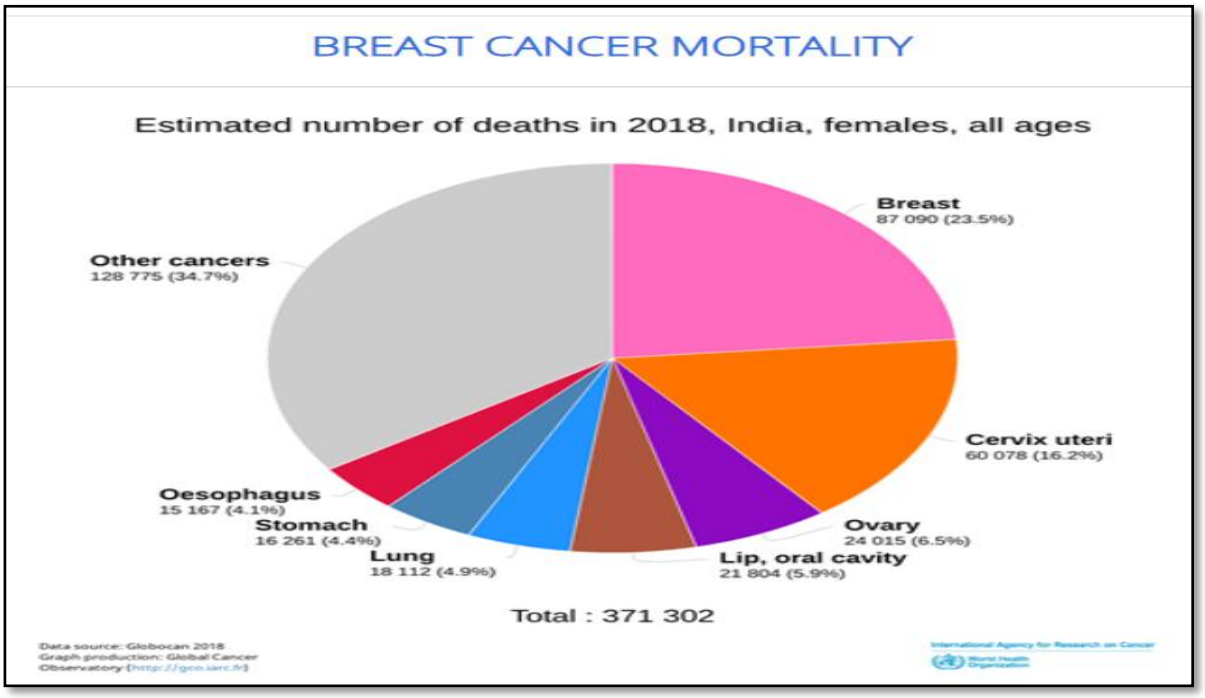

Fig 2.4: For the year 2018, there were 87,090 death of women due to breast Cancer in India, accounting a total of 23.5% deaths of all cancer related deaths. This shows that out of every four deaths by cancer one is of breast cancer death. Source: GLOBOCAN 2018. Graph production: Global Cancer Observatory.

With constant evolution in breast cancer surgery and treatment methods, Verneuli, French surgeon in 1887 (17), tried to restore cosmesis through minimal extirpation, which later lead to the use of synthetic materials for usage like muscles (18), myocutaneous flap (19), lipomas (20), omentum (21), *transverse rectus abdominis myocutaneous flap* (introduced in 1979) (22) as natural source and petroleum jelly, glass balls, ivory, rubber, polyvinyl alcohol sponge (23) and silicone (24) as synthetic source. Modern surgery evolution has helped in cancer treatment and management by giving new techniques which are patient friendly.

Breast cancer has been divided into various subtypes to find prognostic information related to tumor behaviour like propensity for metastasis, response towards various therapies. Subtypes have been formed by using standard methods like histopathology, molecular profiling, genetic analysis and gene expression profiling. Currently, several techniques are used in order to study subtypes from patient to patient like tumor morphology, grade classification, tumor size, presence of lymph node metastases and

expression of hormones like ER, PR and HER. Along with the information from these factors, there is a need for understanding both prognostic and predictive markers which will further help in planning treatment strategies (25).

Breast cancer can be classified in two ways-

a) Histological Classification, and

b) Molecular Classification

a) **Histological Classification**- This can be used to study the morphology of the tumor. Several characteristic features can be used to classify breast tumor histology like cell type, number, location of secretion, immune-histochemical nature, and the architecture of tumor helps in describing its ductal or lobular nature (26). Breast cancer can either be invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC); can be divided into following subtypes-

1. Invasive ductal carcinoma
2. Medullary carcinoma,
3. Metaplastic carcinoma,
4. Apocrine carcinoma,
5. Mucinous carcinoma,
6. Cribriform carcinoma,
7. Tubular carcinoma,
8. Neuroendocrine carcinoma,
9. Classic lobular carcinoma, and
10. Pleomorphic lobular carcinoma.

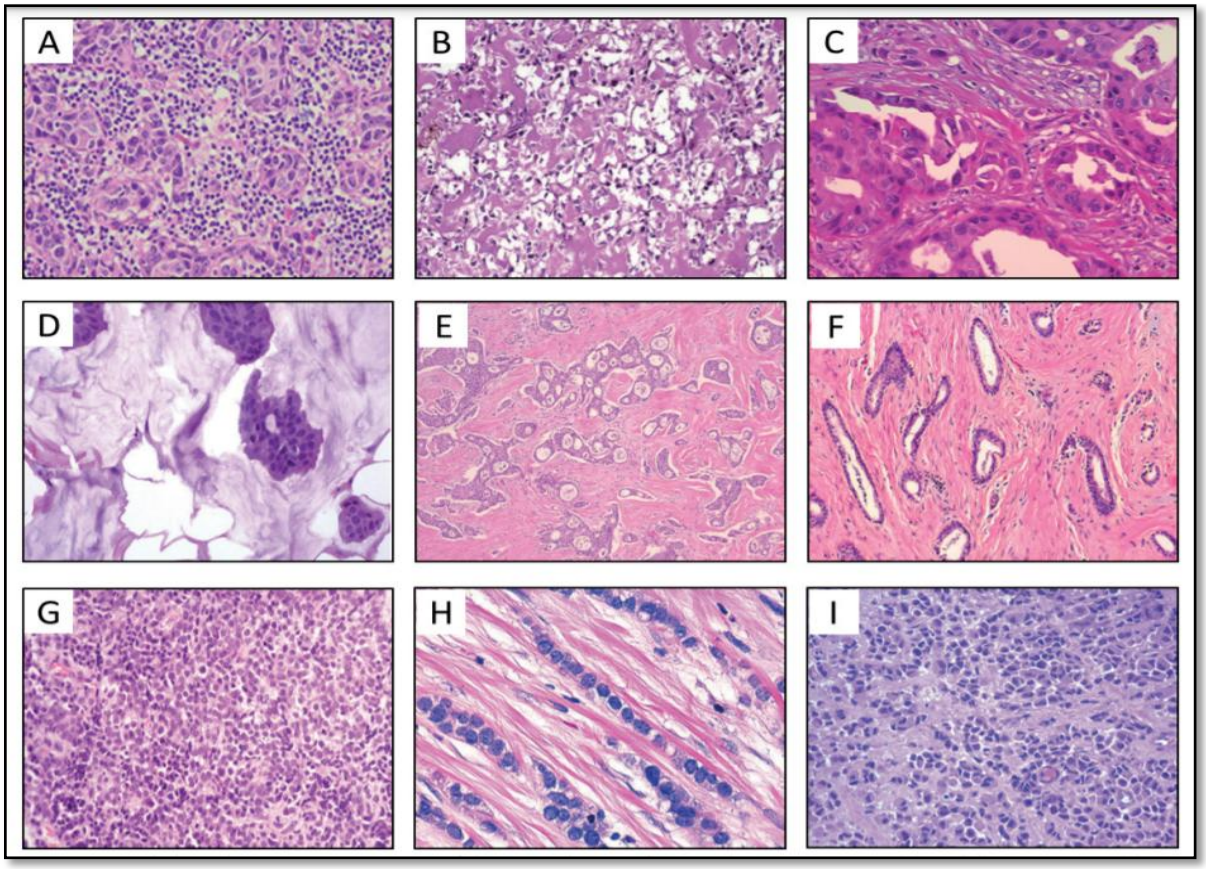

Fig. 2.5- Morphological variants representative of the main subtypes of invasive breast carcinomas. a) medullary carcinoma, b) metaplastic carcinoma, c) apocrine carcinoma, d) mucinous carcinoma, e) cribriform carcinoma, f) tubular carcinoma, g) neuro-endocrine carcinoma, h) classic tubular carcinoma, and i) pleomorphic lobular carcinoma. (27)

b) <u>Molecular Classification</u>- Perou, Sorlie *et. al.* gave molecular classification of breast cancer on the basis of similarities in gene expression profile` by using cDNA microarray technique. They gave 4 molecular subtypes of breast cancer on the basis of expression of estrogen, progesterone, HER2 and cell proliferation regulator Ki-67 – (28-30)

- Luminal A
- Luminal B
- Enriched HER2 (Human epidermal growth factor receptor 2, and
- Triple Negative (TNBC) (28)

Angiogenesis is recognized as the key player in tumor growth and metastasis as it provides continuous blood and oxygen for growth and progression of tumor beyond $2mm^3$ of size (31). Several signalling pathways along with the angiogenic molecules like vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), or the platelet-derived growth factor (PDGF) and phenomena such as hypoxia, immune cell activity, tumor-microenvironment contribute to the angiogenesis process (32,33). In angiogenesis, blood capillaries proliferate when endothelial cells present on their lining multiplies under the influence of low O2 levels triggering various transcriptional responses regulated by Hypoxic inducible factors (HIFs). These HIFs are highly conserved transcription factors which control genes regulating physiological processes like angiogenesis, metabolism and cell division (34).

Breast cancer is the most invasive and commonly occurring cancer among women with higher chances of relapse due to drug resistance and tumor failure (35). Earlier, EMT and MET were considered as binary process but later they were found to be related to cancer progression by giving phenotypic expression of epithelial and mesenchymal features thereby giving stem cell like property to the cancer cells (36-38). BCSCs have characteristic features like slow cell cycle, chemo-resistance, anti-oxidative, tumorosphere formation ability (39,40), surface markers CD44(+)/CD24(−/low) with cancer initiation property, pleuripotency genes SOX2, Nanog, OCT4, DNA (cytosine-5)-methyltransferase 1 (DNMT1) (41,42). CD44 is a stemness marker (43) which binds to hyaluronic acid and intervene in the cell to cell and cell to matrix protein interaction; for example- Matrix metallo protein (MMP) and osteopontin (OPN) (44,45). CD24 is the glycocylated cell surface protein which regulates metastasis and proliferation of BCSCs negatively by controlling CXCR4 (46). BCSCs have several other surface markers like EpCAM (initiate cell adhesion, proliferation and invasion of BCSC) (47), ALDH1 (therapeutic target regulating BCSC malignancy) (48), epithelial specific antigen and E-cadherin. Presence of several other surface markers like CD133, CD131, ALDH1, ESE along with CD44 and CD24 is required to characterise cancer cells with stem like properties (49). Breast cancer stem cells helps in tumor growth by maintaining resistant clones and by

acquiring resistance related traits to help withstand treatment therapies (50) and help in maintaining tumor mass (51).

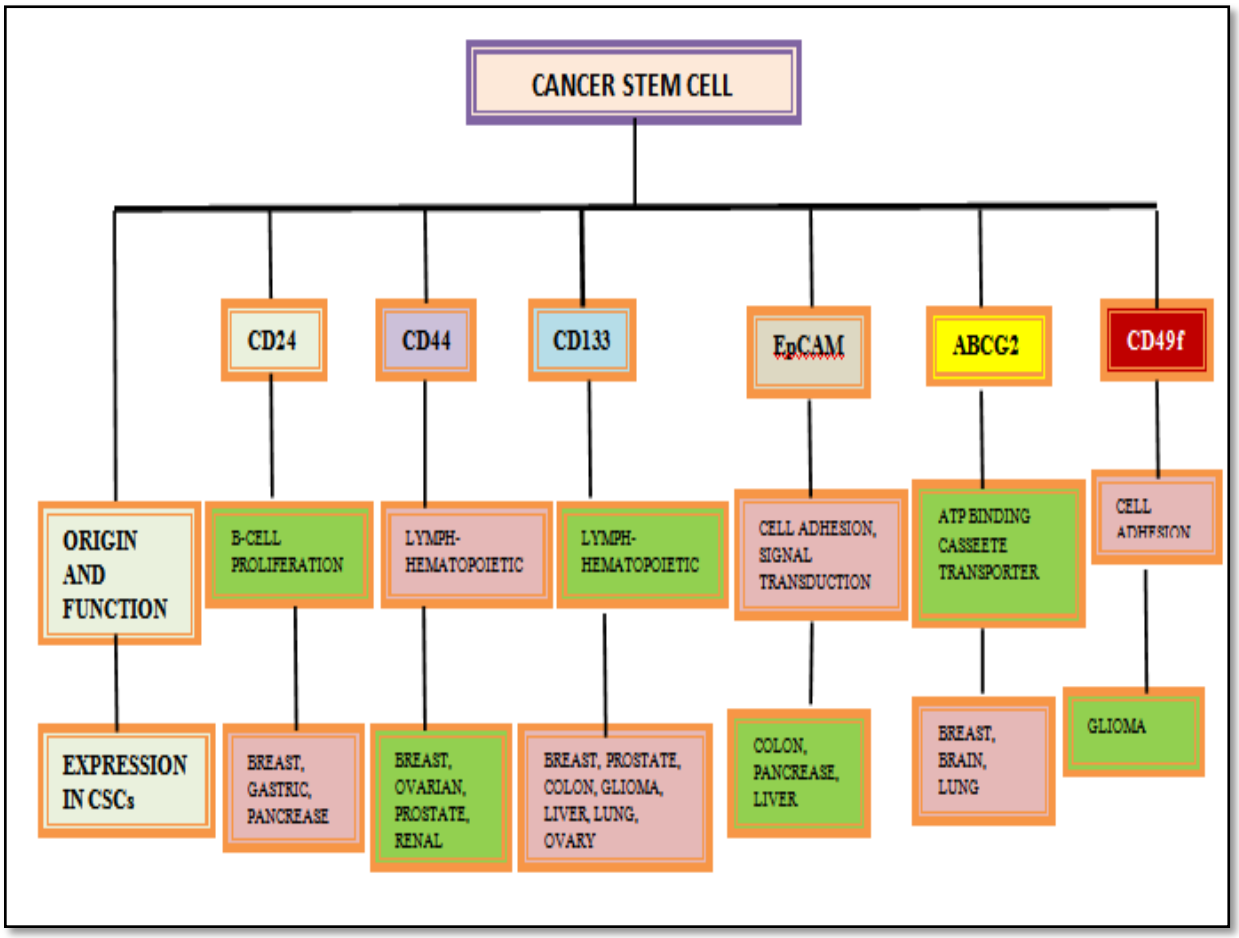

Fig 2.6- Cancer stem cell markers with their origin, function and expression in various cancers.

CD146 known by several names like MUC18, P1H12, MUC18, A32 antigen, S-Endo-1, Mel-CAM, MET-CAM, HEMCAM, or CD146 (52), was first discovered as melanoma cell adhesion molecule (53) by Prof. Johnson in 1987, on metastatic melanoma lesions and rarely on benign lesions (54). Cell adhesion molecules are the proteins present on the surface of cells and function to mediate contacting and binding cell to cell and cell to extracellular matrix (55). CD146 is sequence homology to cell adhesion molecule thus named as Melanoma Cell Adhesion Molecule (MCAM) (56). CD146 has been found to be a tumorigenesis marker and also plays a vital function in normal vascular development, tumor progression of cancers like

prostate (57) ovarian (58) and breast (59). CD146 has been found to be the unique activator of epithelial to mesenchymal transition (EMT) which is an important phenomenon for cancer progression and metastasis, also promotes breast cancer progression; thus making it an important therapeutic target for breast cancer treatment (60). Three different forms of CD146 have been discovered in mouse, human and chicken. cd146 gene encodes two membrane anchored forms and proteolytic cleavage of membrane bound form generates soluble form of CD146. Soluble form of CD146 can be detected in cell culture supernatant, serum and interstitial fluids from normal or diseased samples (61-62). sCD146 is not a very potent cell adhesion molecule as it lacks trans-membrane or cytoplasmic regions but still is a potential target in tumor microenvironment of invasive tumors (63). Membrane CD146 has been shown to act as receptor for various ligands to stimulate signal transduction without adhesion feature (64).

CD146 acts as the co-receptor for various pro-angiogenic factors like VEGF, PDGF, FGF. Angiogenesis is a highly ordered process requiring a series of signalling inside and between endothelial cells, mural cells and other cell types like immune cells (65,66). Platelet derived growth factor (PDGF) comes in function after vessel formation to recruit pericytes and vascular smooth muscle cells to the surface of endothelial cells (67). Zhaoqing *et. al.* reported that CD146 has the ability to promote tumorigenesis and plays a vital role in cerebrovascular development by directly interacting with VEGFR-2 and PDGF-β respectively (64). It functions as a co-receptor for VEGFR2 in the process of tumorigenesis. There are five members of VEGF family namely; VEGF- A,B, C, and Placental derived growth factor (PDGF) (67). Jiang T. et al in 2012 found that CD146 interacts with VEGFR2 on endothelia and acts as a co-receptor independent of VEGF in extracellular protein domain of CD146 (68). Phosphorylation of VEGFR2 by VEGF-A is inhibited when CD146 is blocked by antibody AA98 or CD146 siRNA in human umbilical vein endothelial cells (HUVECs), making CD146 as an important molecule for VEGFR2 signalling (69). It has been reported that during angiogenesis, PDGF-β secreted by endothelial cells induces the recruitment of pericytes over the surface of endothelial cells. (68).

Chen J *et. al.* gave the evidence for the role of CD146 in recruiting pericytes and maintenance of vessels stability through interaction with PDGFR-β in protecting central nervous system. The pericytes on the surface of endothelial surface secretes TGF-β1 to down-regulate the expression of CD146 and promote BBB maturation. As CD146 along with PDGF-β functions in growth and survival of different types of cells (including cancer cells), so further investigation of functions of CD146 and PDGFR-β interaction in cancers may help deeply understand the dysregulation of PDGF-β induced signaling during tumor development and progression, especially tumor angiogenesis and lymphangiogenesis (66).

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CHAPTER- 3

AIMS AND OBJECTIVES

I. Choice of the topic with reasoning:

Breast Cancer is the second most prevalent cancer after lung cancer. About 15% of cancer death is due to breast cancer. It can be treated through different therapeutic methods like surgery, radiotherapy or targeted treatments. Angiogenesis plays an important role in growth and metastasis of cancer. By targeting angiogenic factors like VEGF, FGF, PDGF, we can treat breast cancer by anti-angiogenic therapy. EMT also plays a major role in tumor angiogenesis and cancer metastasis. EMT can be regulated by the help of various transcription factors like SIP1, Snail, Slug, and Twist thereby inhibiting epithelial phenotype and thereby repressing E-cadherin transcription. CD146, also known as MCAM; is a membrane glycoprotein of the mucin family and a key cell adhesion protein highly expressed by cells of vascular endothelial cell and pericytes, which plays a vital role in angiogenesis. Besides, CD146 and its soluble forms were also found to play a key role in regulating monocyte- transendothelial migration and a unique activator of EMT in hBC cells [9]. However, mechanism underlying CD146 in breast cancer progression and their role in promoting cancer stem cells is yet to be explored. This forms the basis of the study.

II. Statement of the problem:

Breast cancer is the most common cancer in women both in the developed and under-developed countries and is expected to account for 29% all new cancer diagnoses in women. Much lower incidence of breast cancer have been reported in India as compared to Western Countries because of their lifestyle, reproductive and dietary factors and lack of population screening also contribute to the given statistics. In India, breast cancer is the most common cancer among women with age adjusted rate of 25.8 per 1, 00,000 women and mortality rate of 12.7 per 1,00,000 women. The breast cancer has been classically treated with surgery, hormonal therapy, radiation and recently targeted therapy using several drugs like herceptin, perjeta, Lapatinib is been under practice. CD146 has been shown to be associated with high grade and triple negative breast cancer and is included in stromal gene cluster enriched in

mesenchymal genes. However, the mechanistic approach of how CD146 regulates breast cancer tumorigenesis is uncertain.

Besides, along with the tumor angiogenesis, further investigation of relapsed cases has shown that there are some cancer cells with stem like properties which are resistant to the classical treatments. These cancer stem cells are the culprit found to cause a major role in relapse and metastasis.

Thus, combining these problems, the study is focused on two hypotheses:

1. Understanding the role of CD146 in tumor angiogenesis and breast cancer stem cells.
2. Inhibition of CD146 to control breast cancer metastasis.

III. Hypothesis of the study:

CD146 is a surface marker playing key role in breast cancer for adhesion, migration and invasion. It is recently found to possess a major role in regulating tumor angiogenesis. However, its correlation with angiogenesis and stem cell mediated pathways and their functioning; especially in breast cancer stem cells is yet to be explored. Thus, the major hypothesis of the project is to understand the role of CD146 in tumor angiogenesis and breast cancer stem cells and to inhibit CD146 to control breast cancer metastasis.

AIMS AND OBJECTIVE

AIM-The aim of our study was to justify the dual role of CD146 in tumor angiogenesis and breast cancer metastasis, in order to make CD146 a potent therapeutic target for breast cancer treatment.

OBJECTIVES-

- **Flow cytometric characterisation of breast cancer stem cells of MDA MB 231 and sorting of CSC population on the basis of differential CD146 expression.**

In this objective, the breast cancer stem cells were characterised for the presence of cancer stem cell markers CD24, CD44, CD133, EpCAM and also for CD146 surface marker. The BCSCs were then sorted for CD146 expression as CD146high and CD146low cells.

- **Comparison of differential CD146 expression in sorted BCSCs of MDA MB 231 at protein and mRNA level (Western BLOT and ELISA).**

  The BCSCs CD146high and CD146low cell population were studied for the differential expression of CD146 in the respective population at mRNA and protein level.

- **Comparison of differential CD146 expression in CD146high/low sorted cell population *in-vitro* with respect to the anti-sCD146 antibody M2J-1.**

  The two sorted cell populations were then studied for their *in-vitro* characteristics and then the expression of CD146 was inhibited simultaneously with M2J-1 to show the effect of CD146 on BCSCs properties.

- **Comparison of differential CD146 expression in CD146high/low sorted cell population *in-vitro* with respect to the anti-sCD146 antibody M2J-1.**

  The two sorted cell populations were then studied for their *In-vivo* characteristics and then the expression of CD146 was inhibited simultaneously with M2J-1 to show the effect of CD146 on BCSCs properties.

- **Biostatistical analysis of CD146 expression in breast cancer molecular subtypes and correlation of CD146 expression with lymph node metastasis and overall survival of breast cancer patients.**

  CD146 expression in various breast cancer subtypes, also the correlation of CD146 expression to overall patient survival and lymph node metastasis was studied using various biostatistical softwares.

CHAPTER- 4

MATERIAL AND METHOD

4.1MATERIAL

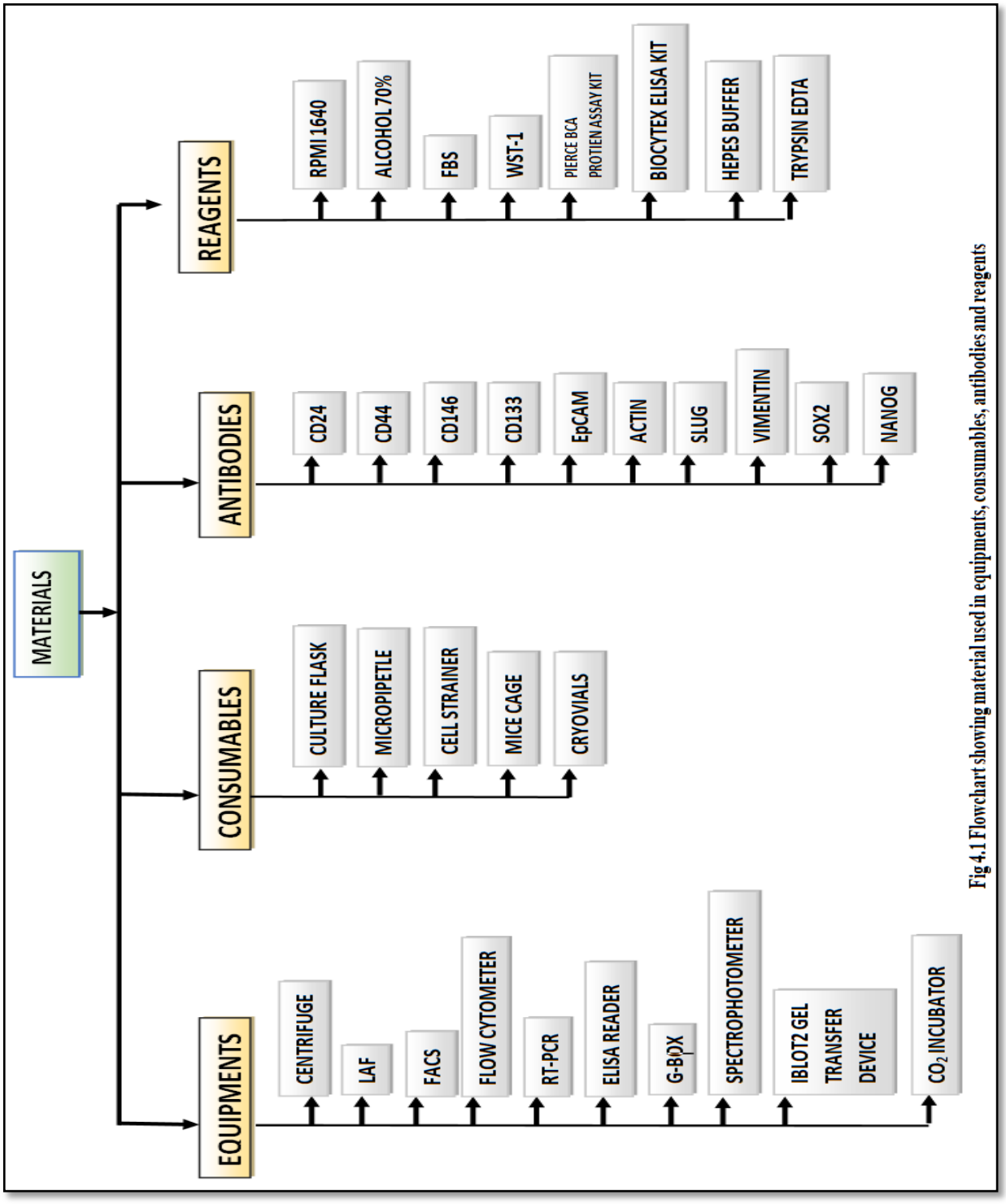

Fig 4.1 Flowchart showing material used in equipments, consumables, antibodies and reagents

4.2 TECHNIQUES USED

4.2.1 Flow cytometry and FACS

Flow cytometry is a very advanced laser based biology technique used to count, examine and sort the microscopic particles being suspended in a fluid. It is a rapid quantitative method by which multi-parametric analysis of the physical and chemical properties of thousands of particles in a fraction of seconds can be done. Also, phenotypic study, counting and sorting of live cells can be done (1).

Fluorescence activated cell sorting (FACS) is an advanced flow cytometry technique which further adds functionality by utilizing highly specific antibodies with fluorescent conjugates against the targets. Data collection and sorting can be done simultaneously in FACS using limitless different parameters. (2). It is enriched for subset of cells studied in details using flow cytometry or other analytical technique. On the other hand, flow cytometry is used for analyzing cells and mainly focused on measuring protein expression in a certain cell population. (1). Flow cytometry has its use in various fields like molecular biology, marine biology, protein engineering, pathology, plant biology and immunology (4).

- **Principle working of Flow cytometer-**

The instrument uses beam of light of single wavelength directed on hydrodnamically focused fluid stream. A number of detectors aim at the junction point of fluid stream and light beam; one detector in line with light beam (know as forward scatter FSC) and several detectors in perpendicular to it (Side scatter SSC) and few fluorescence detectors. Every fluorescence labeled particle in the fluid passes through the beam of light which excites the fluorescence at lower frequency than light beam. This is then detected by the detectors and by analyzing the brightness at each detector, detailed information can be extracted about the physical and chemical structure of each individual particle. FSC depends on cell volume while SSC depends on inner complexity of particles. FSC corresponds to cell volume while SSC corresponds to inner characteristics of the cells (3).

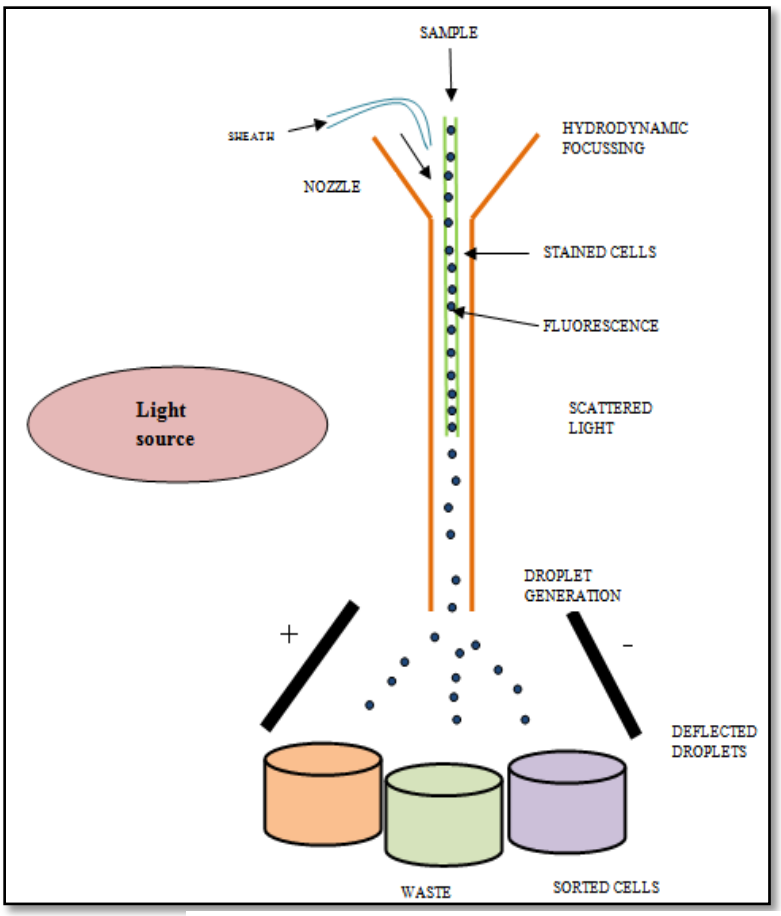

Fig 4.2: Working principle of flow cytometer

4.2.2 Cell Proliferation Assay-

Cell proliferation assay is widely used in various scientific research processes like drug testing for growth factors, cytotoxicity effect on cell functioning, changes in cell population. There are four types of proliferation assay- ATP concentration assay, cell proliferation marker assay, metabolic activity assay and DNA synthesis assay. Choice of proliferation assay depends upon the requirement and information to be extracted from the experiment.

a) DNA synthesis assay- This assay includes BrdU proliferation assay and EdU proliferation assay. The BrdU proliferation assay uses radioactive isotope which binds to thymidine followed by autoradiography. BrdU assays can be used instead of thymidine where monoclonal antibody against BrdU for detection along with the secondary antibody is used (5). EdU proliferation assay helps in monitoring

replicating DNA through fluorescence. Modified nucleotide when added to live cells, it then incorporates into replicating DNA. Copper induction allows the easy detection of fluorescent probe to EdU.

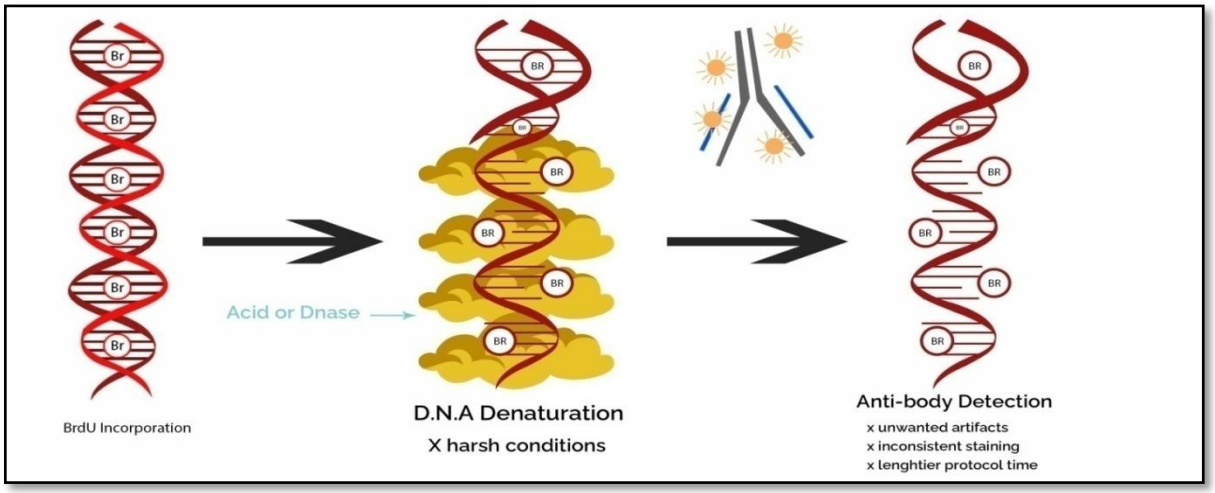

Fig 4.3: Working mechanism of BrdU protocol.

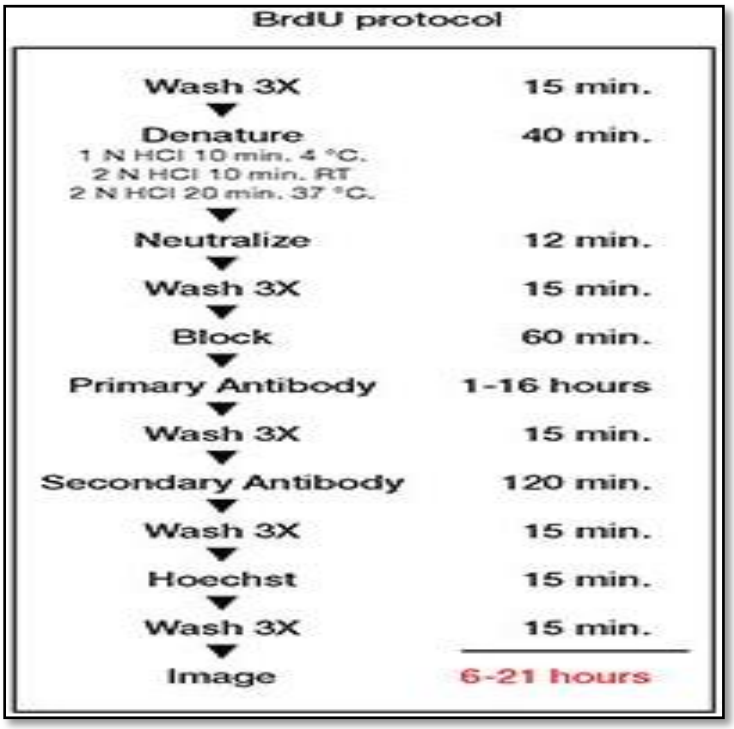

Fig 4.4: BrdU protocol. Source-Thermo Fisher Scientific.

b) Metabolic Proliferation assay- This assay measures the metabolic activity of cells and is used for analyzing proliferation, viability and cytotoxicity of cells. In this, the

tetrazolium salts like MTT, XTT and WST-1 are reduced to colored formazon and the coloration is measured through spectrophotometer.

In our thesis, we have used WST-1 as the tetrazolium salt to measure the expression of CD146 and its inhibition effect.

c) Luminescence Proliferation assay- It includes CFSE labeling and live/ dead cell double staining. 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) is used to measure number of cell divisions occurred in cell population while live/ dead cell double staining measures number of live and dead cells in a cell population through green fluorescence.

4.2.3 Migration assay-

Migration is a significant property of live cells and is important for the normal development, immune response, disease processes and various other processes. It is mainly used in biomedical research to examine cancer biology features, immunology, vascular biology, cell and development biology (6). Cell wound closure is the most common migration assay procedure used to determine migration ability of cell masses. Furthermore, it can be used to observe cell morphology during migration. Migration assay can reveal a variety of information like migration changes, lamellipodium formation, tail retraction, directional movement (7).

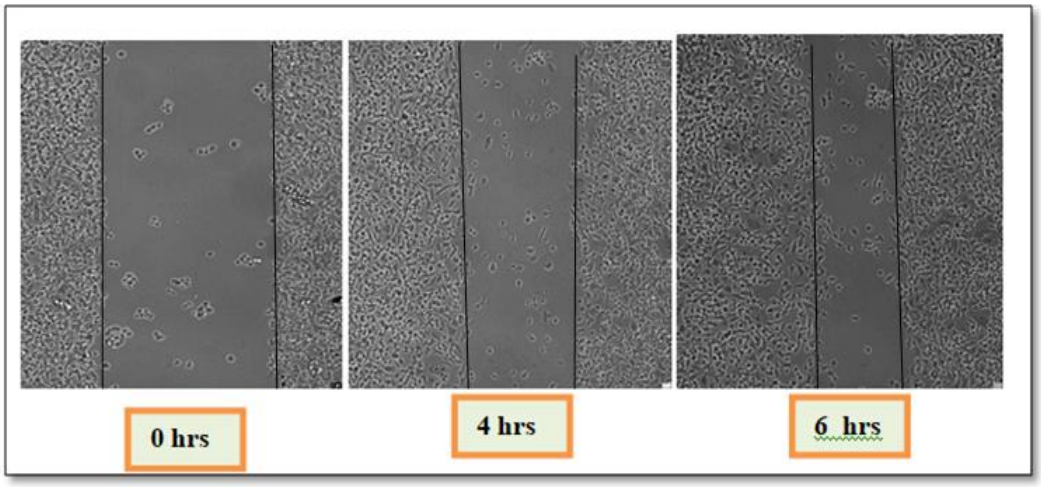

Fig 4.5: Migration of Breast cancer stem cells at 0hrs, 4hrs and 6hrs.

4.2.4 Invasion Assay-

From pathological point of view, penetration of tissue barriers like basement membrane is called Invasion. Directional response of a single cell to various chemo-attractants like chemokines, growth factors, lipids, and nucleotide can be studied using this method (8). Over expression of receptors might lead to differential migratory ability. This assay can be used to assess identity and characteristic features of key regulators of migration like Rho family of GTPase2. Mode of cell migration and invasion ability of a cell in 3-D matrix can also be determined.

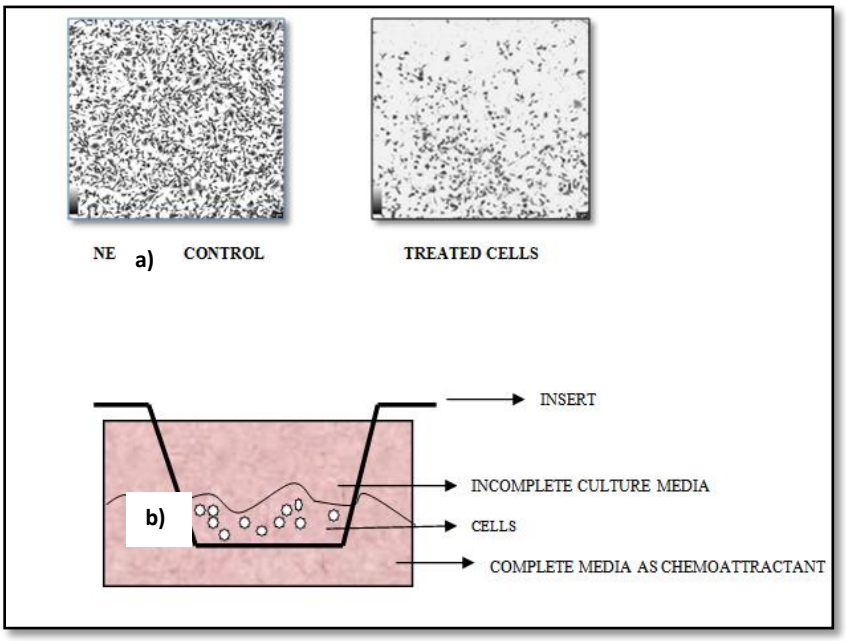

Fig 4.6: a) Transwell migration of breast cancer stem cells- negative versus control.
b) Diagrammatic representation of Transwell migration of cells

4.2.5 Western Blot-

Western blot is a protein assay used to identify and separate proteins on the basis of molecular weight through gel electrophoresis. The bands of protein are transferred to the membrane to fetch bands of each type of protein. Protein specific antibodies are then incubated with the membrane and then detected by G-

Box. Thickness of the band is directly proportional to the amount of protein present in the sample (9).

This technique is based upon the principle of immunochromatography, in which proteins are separated in poly-acrylamide gel (PAGE) on the basis of their molecular weight. It is a rapid and sensitive test for detecting characterizing proteins. The proteins separated in PAGE are transferred onto nitrocellulose membrane and detected using specific antibodies.

Firstly, the macromolecules of the sample are separated by using gel electrophoresis. Then, these separated molecules are transferred on nitrocellulose or polyvinylidine difluoride (PVDF) membrane. The membranes are blocked using blocking buffer to inhibit non-specific binding of antibodies to the membrane surface. Two antibodies are used in this process- primary antibody to bind to the protein and secondary antibody is enzyme linked to produce effective signals for detection of protein target. Labels like biotin and fluorescent probes like as Invitrogen Alexa Flour or DyLight fluorophores, and enzyme conjugates such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) can be used. The direct method requires only one antibody and thus avoids cross-reactivity of secondary antibody, but the labels of the antibody can interfere with target binding, requires high potential background for weak target signals, costly and limited conjugate primary antibodies. The indirect methods are advantageous over direct method as signals for detection are amplified, vast variety of conjugated secondary antibodies, useful for various primary antibodies, no interference with primary antibody binding and it provides various options for signal detection but this also has some disadvantages as the non- specific staining might increase background and additional steps are required for binding of secondary antibodies (10).

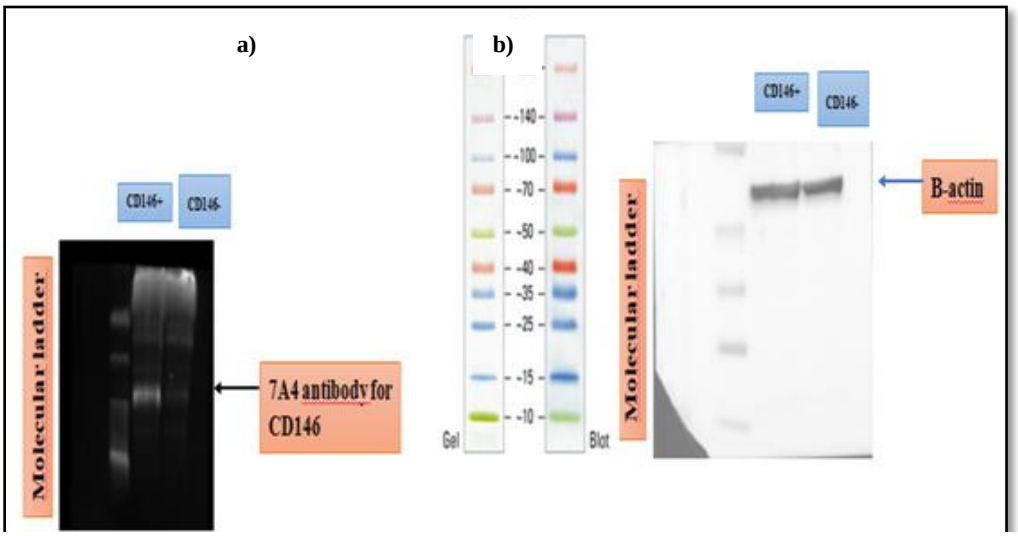

Fig 4.7: a) Bands on gel of Western blot. b) Molecular ladder and bands on the gel

4.2.6 ELISA Assay-

Enzyme Linked Immune-Absorbent Assay (ELISA) is similar to other immunoassays and works in 96 well late. It is used for detection and quantification of peptides, proteins, antibodies and hormones. In this assay, specific antibody binds to the target antigen and generation of signals and its amount indicates the presence and quantity of antigen binding. Antigen is mobilised on the solid surface and coupled with antibody linked to enzyme. Signals are detected by measuring enzymatic activity in presence of substrate. This antigen-antibody interaction is highly specific and is performed in a 96 well plate which passively binds antibody to protein. Sensitivity and precision of the assay can be increased by carefully coating wells with highly specific antibodies (11). There are 4 types of ELISA- Direct, indirect, sandwich and competitive ELISA.

a) Direct ELISA

b) Indirect ELISA

c) Sandwich ELISA,

d) Competitive ELISA

We have performed sandwich ELISA. In Sandwich ELISA, matched antibody pairs are used in which both antibodies are specific to different epitope of an antigen. Wells of the plate are coated with primary antibody known as capture antibody and then sample solution is added to the well. Thereafter, secondary antibody; also known as detection antibody is added to measure the concentration of sample. This has several advantages over other types of ELISA as there is high specificity for antigen, not purification of antigen is required to perform this method, flexible and sensitive method.

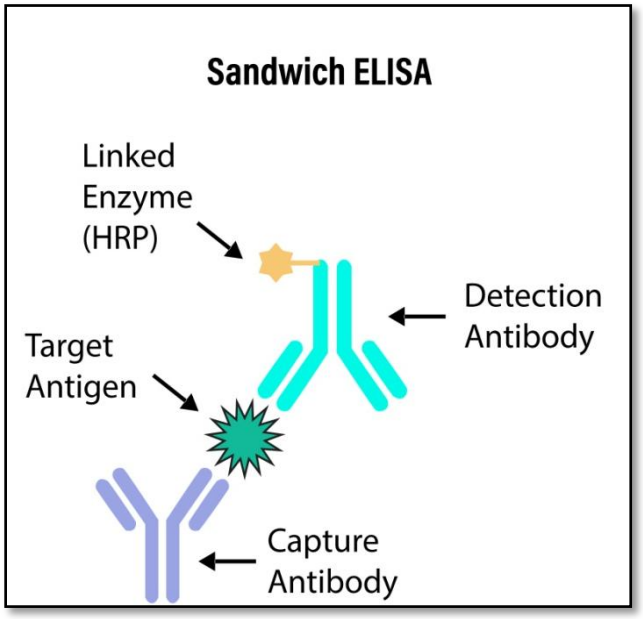

Fig 4.8: Sandwich ELISA

4.2.7 Hematoxylin and Eosin (H&E) staining-

Hematoxylin and eosin staining is a basic tissue staining method being used as it is for over a decade. It is basically used to detect cancer cells from biopsy of patients. The stain was invented by A. Wissowzky in 1976, where nuclei were stained as blue and cytoplasm and extracellular matrix (ECM) as pink. Amount of coloration is directly proportional to the amount of DNA and amount of time spent in hematoxylin stain. Hematoxylin dye is extracted from *Haematoxylum campechianum* and oxidation of hematoxylin produces hematein. Hematein binds to the anionic or

negatively charged components of the tissue and this binding can be enhanced by addition of mordant (12).

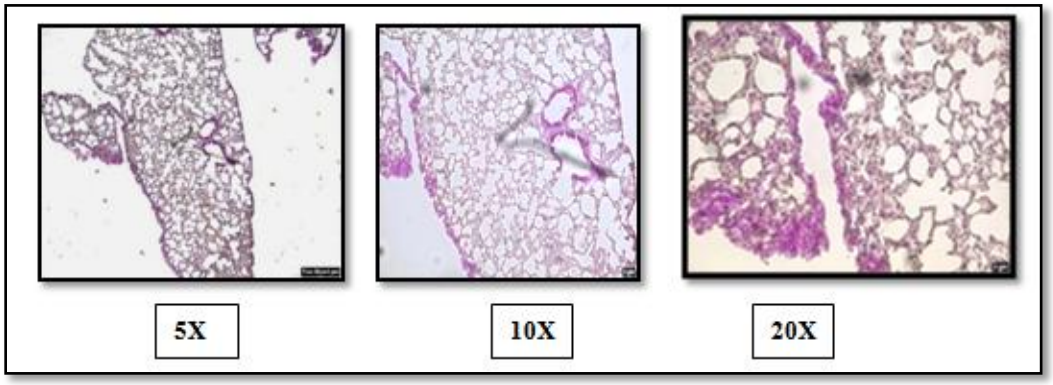

Fig 4.9: Haematoxylin stained lungs of mice at 5X, 10X ad 20X.

4.3. METHOD

4.3.1 Revival of frozen breast cancer cell lines-

The cryovials of breast cancer stem cells (BCSCs) of breast cancer cell line MDA MB 231 were thawed in water bath maintained at 37°C. The vials were then sterilized and opened in laminar air flow to transfer the cells in centrifuge tube and then centrifuged at 350rpm for 5min at 4°C. After the centrifuge, supernatant was discarded and pellet was resuspended in warm cell culture media RPMI 1640.

4.3.2 Cell culture of breast cancer cell line MDA MB 231-

Cells were culture in culture flasks- 25ml, 75ml and 100ml respectively. The cells suspended in culture media were transferred to the culture flask and appropriate amount of culture media was added to the flask to provide nutrients to the growing cells. The flask were kept in incubator maintained at 37°C, 5% CO_2, 95% humidity and 7.2 - 7.5 pH; for the cells to grow. When flasks were confluent for 90%, they were sub-cultured.

4.3.3 Sub-culturing of breast cancer cell lines MDA MB 231-

After the flask are 90% confluent, the cells were tyrosinised with Trypsin- EDTA for 3min and then FBS was added to stop its activity. The cells were then centrifuged and pellet was suspended in culture media. The cells were then transferred to fresh flask and allowed to grow in incubator until confluency of 90%.

4.3.4 Flow cytometry characterization of cancer stem cell and CD146 markers

Cells growing in the culture flask were tryspinised and studied for the cancer stem cell markers through flow cytometry. The cultured cells were trypsinised and centrifuged as previously mentioned in 4.3.3. Approximately 20,000 cells were then suspended in PBS-/- and antibodies for cancer stem cell markers- CD24, CD44, CD133, EpCAM and surface marker CD146 were added. After staining cells with antibodies, the cells were centrifuged and suspended in PBS-/-. The cells were then studied in Beckman Coulter Flow cytometer.

4.3.5 FACS sorting

Cells in the culture flask were trypsinized for sorting on the basis of two cell population of CD146 in MDA MB 231 breast cancer cell line. ˜36, 00,000 Cells were stained for CD146 and incubated for 1 hr. Cells were then centrifuged and collected in tube for sorting. The cells after sorting were collected separately as CD146high and CD146low cells.

4.3.6 Proliferation assay of Sorted Cell population

CD146high and CD146low cells sorted from CSCs of MDA MB 231 were cultured and studied for proliferation capacity of each type. Around 5000 cells from each population were cultured in 96 well plates for 24 to 48 hrs until confluency of 70%. Then WST-1 was added to each well and incubated for 1 hr. Using spectrophotometer, OD was calculated at 450nm. The same experiment was repeated

for 5 times for sorted cell population to confirm the results and then combined mean of each result was obtained using Mean- Whitteny test.

❖ **Inhibition of CD146 in CD146high cells-**

CD146high cells sorted from the cell line MDA MB 231 were taken to observe the effect on proliferation capacity of BCSCs when CD146 was inhibited. Around 5000 sorted CD146high cells were taken. Four wells for each category i.e. Control and treated were separated (4 wells for control and 4 wells for treated). The controls were treated with IgG and other set were treated with monoclonal ant- CD146 antibody M2J-1. After 24hrs, 10µl of WST-1 was added and OD was measured at 450nm using spectrophotometer. The same experiment was repeated for 5 times for sorted cell population to confirm the results and then combined mean of each result was obtained using Mean- Whiteny test.

4.3.7 Migration assay of Sorted Cell population-

CD146high and CD146low cells were cultured and studied for their migration capacity of each type. Around 10,000 cells of CD146high/low were cultured in 24 well plates for 24 to 48 hrs until 70% confluency. Scratch was made using 10µl tip at the centre of well and allowed the cells to grow further. The width of scratch was measured at 0hr and 8hr using imageJ software and mean was obtained. The same experiment was repeated for 5 times for sorted cell population to confirm the results and then combined mean of each result was obtained using Mean- Whiteny test in Graphpad Prism Software.

❖ **Inhibition of CD146 in CD146high cells from both cell population-**

CD146high cells from the cell lines MDA MB 231 were taken to observe the effect on migration capacity of CSCs when CD146 was inhibited. Around 10,000 sorted CD146high cells were taken each for control and treatment and seeded in 24 wells plate. One set of 10,000 CD146high cells were treated with IgG as control, while other set was treated with humanized monoclonal anti-CD146 antibody M2J-1.

Fresh media was added to the wells and a scratch was made in the centre of wells perpendicularly using a 10µl tip. Images were captured for each well using confocal microscope at 0hrs and 8hrs of scratch. The width of scratch was measured using imageJ software and mean was obtained. The same experiment was repeated for 5 times to confirm the results and then combined mean of each result was obtained using Mean- Whitteny test.

4.3.8 Invasion assay of Sorted Cell population

CD146high and CD146low cells were cultured and studied for their invasion capacity. Inserts of diameter 0.8µm were activated by using PBS-/- 1ml and incubated at 37°C for 1hr.100µl of prepared matrigel was poured on inserts and allowed to solidify in incubator for 1 hr. Around 20,000 cells of CD146high and CD146low cells were seeded into 24 wells plate using incomplete media and complete media was added to the wells. After 48hrs, inserts were then transferred to 4% PFA for 20min and then to crystal violet for 30min. Inserts were then washed in PBS-/- and then with distill water. Images were taken under the microscope and analyzed through ImageJ software. The same experiment was repeated for 5 times for sorted cell population to confirm the results and then combined mean of each result was obtained using Mean- Whitteny test.

- **Inhibition of CD146 in CD146high cells from both cell population-**

  CD146high cells from the cell line MDA MB 231 were taken to observe the effect on invasive capacity of CSCs when CD146 was inhibited. Inserts of diameter 0.8µm were activated by using PBS-/- 1ml and incubated at 37°C for 1hr. 100µl of prepared matrigel was poured on inserts and allowed to solidify in incubator for 1 hr. Around 20,000 cells of CD146high for each category of control and treated were seeded into 24 wells plate using incomplete media and complete media was added to the wells. 4 wells for control IgG and 4 wells for treatment with humanized monoclonal anti-CD146 antibody M2J-1were seeded with CD146high cells and incubated for 48 hrs. After 48hrs, inserts were then transferred to 4% PFA for 20min and then to crystal violet for 30min and then washed in PBS-/- and then with

distill water. Images were taken under the microscope and analyzed through ImageJ software. The same experiment was repeated for 5 times for sorted cell population to confirm the results and then combined mean of each result was obtained using Mean- Whitteny test.

4.3.9 RT-PCR of Sorted Cell population to study the mRNA expression of embryonic and EMT genes

❖ **Preparation of cell lysates-**

Approximately 5×10^5cells were taken for the experiment. The BL+TG buffer was added to the pellet of cells as per the requirement (given in table 4.2). 35 µl of 100% isopropenol was added to the tubes and mixed for 5sec. The cell lysates were transferred to minicolumns and centrifuged at 12,000–14,000 × g for 30 seconds at 20–25°C. The pellet was placed on Reliaprep minicolumn and centrifuged. 500 µl RNA wash solution was added and centrifuged at 12,000–14,000 × g for 30 seconds. DNAse1 enzyme was added and stored in ice. 20µl of Column wash solution was added and centrifuged at 12,000–14,000 × g for 30 seconds. 500 µl of RNA wash solution was added and centrifuged at 12,000–14,000 × g for 30 seconds. Reliaprep minicolumn was then transferred to new collection tube and 300µl RNA wash solution was added and centrifuged at high speed for 2 min. Minicolumn was then transferred to elution tube and nuclease free water was added to the membrane and centrifuged at 12,000 × g for 1 minute. The elution tube was sealed and stored at -70°C.

Cell number	BL + TG Buffer	100% Isopropanol
1×10^2 to 5×10^5	100 µl	35 µl
$>5 \times 10^5$ to 2×10^6	250 µl	85 µl
$>2 \times 10^6$ to 5×10^6	500 µl	170 µl

Table 4.1: Table shows the amount of BL+TG buffer and !00% Isopropanol to be used for number of cells in sample.

Cell Input Range	Nuclease-Free Water
1×10^2 to 5×10^5	15µl
$>5 \times 10^5$ to 2×10^6	30µl
$>2 \times 10^6$ to 5×10^6	50µl

Table 4.2: RNA Elution Volume per Number of Cells.

❖ RT-PCR of extracted RNA from cell population of MDA MB 231–

RNA Extraction–

Freshly prepared 350µl of buffer was added to each well and walls were scraped. Cell lysates were recovered in an eppendorf tube. Cells were passed from syringe for lysis and collected in tube with 70% alcohol then homogenized for enhanced RNA binding to column. 700µl sample was centrifuged at 10,000 rpm, 30sec and RNA was then passed on to the column. 350 µl of RNA wash buffer was added to the column and centrifuged twice at 10000rpm, 30sec, then washed. Then 500µl of Buffer RPE was added to the column and centrifuged at 10000rpm for 30sec, washed and centrifuged for 1min. Column was then placed in a new tube and 50µl of RNase free water was added and centrifuged at 10000rpm for 1min. Extracted RNA was then assessed with Nanodrop. The 260/280 ratio must be more than 2.2 (non-contaminated).

RNA	100µg
RT Buffer	2µl
DNTPs	0.8µl-
Primer	3µl
Multiscribe Reverse Transcriptase	1µl
Water	3.2µl
Total volume	10µl

Table 4.3: PCR master mix

❖ **For RT**

10ml of PCR mixture was added in special PCR tube with concentration of 1µg in 20µl and mixed thoroughly. Master Mix of PCR and samples were then prepared. The tubes were transferred in thermocycler avoiding edges and the RT-PCR machine was programmed as 25°C 10min, 37°C 2hrs, 85°C 5min and 4° rest end.

❖ **For PCR**

PCR mixture was prepared- 250 µl of sybergreen, 100 µl right primer, 100 µl of left primer, 30 µl of water. For no template control water was used instead of cDNA. 1 µl of cDNA and 24 µl of master mix were added at the bottom of the PCR tube. Program was set at- 10min 95°C, 30 sec 95°C, 1min 60°C. Add RNA from the cells of respective cell population, sybergreen and no reference dye for 40 cycles.

4.3.10 Western Blot of Sorted Cell population

❖ **Protein dosage for MDA MB 231 sorted cell population-**

Eppendorf tubes were prepared for protein dosage for both CD146high and CD146low cell population.

- ✓ Protein sample 1- 1 µl sample + 4µl DW- 24 µl water (to make volume 25 µl).
- ✓ Protein sample 2- 1 µl sample + 4µl DW- 24 µl water (to make volume 25 µl).

20 µl of freshly prepared coloring agent was added and incubated for 15 min in incubator at 37°C. Then OD was recorded at 550nm. The final concentration of protein was then calculated.

Approximately 1,80,000 to 2,00,000 CD146high and CD146low of cell line MDA MB 231 were taken and centrifuged for 5min at 300g.The supernatant was discarded and the pellet was resuspended in 20 µl of RIPA buffer then stored in 4°C. For western blot, the cell lysates were thawed naturally on ice and processed with concentration derived from protein dosage.

❖ Western blotting

TBST, BSA 4%, SBX4 and MOPS were freshly prepared. Protein sample (concentration derived from protein dosage) of CD146high/low cells were then loaded onto the gel. Proteins were loaded on the gel along with the molecular ladder. After the electrophoretic gel run is complete, it is then transferred onto the nitrocellulose membrane and membrane is then washed with Ponceau S solution for 5 min on shaker. Membrane is then cut washed with TBST buffer twice for 10min each, then stained with 7A4 antibody for CD146 and β-actin for control. Membranes are packed air tight and incubated overnight over shaker at 4°C. Membranes were then washed with TBST buffer for 3 times 10min each and 10ml of each Anti- rabbit and anti-mouse antibodies against CD146 were prepared at concentration of 1/2000 dilution. Membranes were then stained with secondary antibodies and incubated for 1hr. Membranes were washed with TBST buffer 4 times 10 min each further washed with ECL revelation buffer and incubated in dark for 5min. Revelation of bands was performed in G-BOX.

4.3.11 ELISA of Sorted Cell population

Snap freezed CD146high and CD146low cells from cell line MDA MB 231 were prepared for ELISA. 6 tubes were prepared from D1 to D6 and dilutions were performed in all the tubes as given below-

Dilutions	R2 VOLUME (µl)	R4 VOLUME (µl)	CONCENTRATION (ng/ml)
D1	1000	*	1600
D2	500 of D1	500 µl	800
D3	500 of D2	500 µl	400
D4	500 of D3	500 µl	200
D5	500 of D4	500 µl	100
D6	500 of D5	500 µl	0

******* CONCENTRATION ALREADY DILUTED TO 1:10 DILUTION**,

Table 4.4: Serial dilution to perform ELISA.

Pipette into each precoated well		
ANTIGEN IMMOBILIZATION	Diluted test sample	**200 µL**
	Incubate for **30 minutes** at room temperature	
Wash all wells **5 times** with diluted Reagent 5 then add immediately :		
IMMOBILIZATION OF IMMUNO-CONJUGATE	Reagent 6	**200 µL**
	Cover the wells and incubate **30 minutes** at room temperature	
Wash all wells **5 times** with diluted Reagent 5 then add :		
COLOR DEVELOPMENT	OPD/ H_2O_2 Substrate (Reagents 3a + 3b)	**200 µL**
	Incubate at room temperature for **5 minutes* in the dark** for each sample then add:	
	H_2SO_4 3M	**50 µL**
	Incubate for **20 minutes in the dark** at room temperature	
LECTURE	Measure the absorbance at 492 nm (adjust reader to zero on blank Reagent)	

* the best absorbance at 492nm is located between 1 and 1.5 for the highest calibration point. To achieve this, adjust the time for hydrolysis.

Table 4.5: Protocol for ELISA as per the kit. Source- CY-QUANTä ELISA sCD146 kit used for ELISA.

4.3.12 Intra-cardiac and sub-cutaneous injecton of sorted cell population into NOD-SCID mice

Two models were used for conducting animal experiment on the sorted cell population CD146high/low cells of cell line MDA MB 231. Also the same models were used when inhibition of CD146 was studied by inhibiting CD146high cells using humanized monoclonal antibody M2J-1.

a) Intra-cardiac, and
b) Sub-cutaneous.

a) Intra cardiac injection of cells-

4 mice for each category were taken

CD146high - 4 mice

CD146low – 4 mice

Cells treated with humanized monoclonal antibody. 4 mice were taken for each category

CD146high control IgG- 4 mice

CD146high cells mAb M2J-1 – 4 mice

˜1, 00,000 cells were taken for CD146high and CD146low cells and CD146high cells for control versus treated. The cells were injected into mice under sterile animal facility and rested for 15days. The mice were then sacrificed and lungs and bones were extracted. The isolated lungs and bones were fixed in paraffin wax after 70% alcohol bath.

b) Sub-cutaneous injection

4 mice for each category were taken

CD146high - 4 mice

CD146low – 4 mice

Cells treated with humanized monoclonal antibody

3 mice for each category were taken

CD146high control IgG- 4 mice

CD146high cells mAb M2J-1- 4 mice

˜5, 00,000 to 10, 00,000 cells were taken for CD146high and CD146low cells and also CD146high cells for control versus treated. The cells were injected into the mice and then mice in sterile animal facility for 1 month to 2 months. After the tumor grew into the mice, they were sacrificed tumors were isolated to study the CD146 and EMT gene expression.

4.3.13 Tissue preservation and section cutting using microtome

Tissue preservation and section cutting procedure includes FIXATION, PROCESSING AND EMBEDDING OF SPECIMEN.

a) Fixation-

The lungs isolated from CD146high/low and CD146 control/M2J-1 treated lungs of mice were submerged in 10% neutral buffered formalin for 24-48hrs. Tissues were then placed on cassette after fixation.

b) Processing-

The lungs were submerged in 70% Ethanol for 24 hrs, then transferred to 95 % Ethanol bath two times for 30min and then in 100% ethanol for three times 45min each. Lungs were then transferred to xylene bath for three times 45min each. Lungs were transferred in paraffin for two times 1hr and 15min each.

c) Embedding-

Tissues were removed from paraffin and oriented on cassette to solidify at room temperature. After solidification, thin sections of diameter 0.8µm were cut using microtome.

4.3.14 Hematoxylin and Eosin (H&E) staining of tissues

After section cutting of the tissue, the sections were stained by Hematoxylin and eosin stain and whole procedure is carried out at room temperature. Slides of each section of lung tissues were labeled for both CD146high and CD146low, CD146high control and CD146high treated. Slides were placed in three different pools of xylene sequentially for 2min each and then transferred to two different pools of 100% Ethanol sequentially for 2min each. Slides were then transferred to 95% Ethanol pool for 1min and then to 75% Ethanol pool for 1 min and then washed with distill water for 2 min to drain excess of alcohol from the tissues. The slides were then transferred to the pool of Haematoxylin for 2 min and then washed with water for 2 min to drain

excess of haematoxylin stain. Slides were transferred to Eosin pool for 1 min and then washed with distill water for 2 min to remove excess stain then transferred to 95% Ethanol for 30 sec and then to 100% Ethanol 2 times for 1 min each. The slides were then transferred to two different pools of xylene for 2 each and dried.

4.3.15 Statistical analysis

The statistical analysis was performed using Review Manager 5.4.1 (RavMan 5.4.1) software. Using data of each study, two by two tables were prepared and pooled odd ratios (OR) for molecular subtype and pooled risk ratio (RR) for lymph node metastasis along with their 95% confidence intervals were calculated by Inverse Variance method. Generic inverse variance analysis was performed for overall survival and pooled hazard ratio (HR) was calculated using log hazard ratios from individual studies. Fixed effect model was used for all analyses.For each indicator, homogeneity was examined by Breslow-Day test. Further, I2 -test was used to assess homogeneity using I2 –value of 50% as threshold. Forest plots and funnel plots were prepared to visualize data by RavMan 5.4.1 software.

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CHAPTER-5

RESULTS

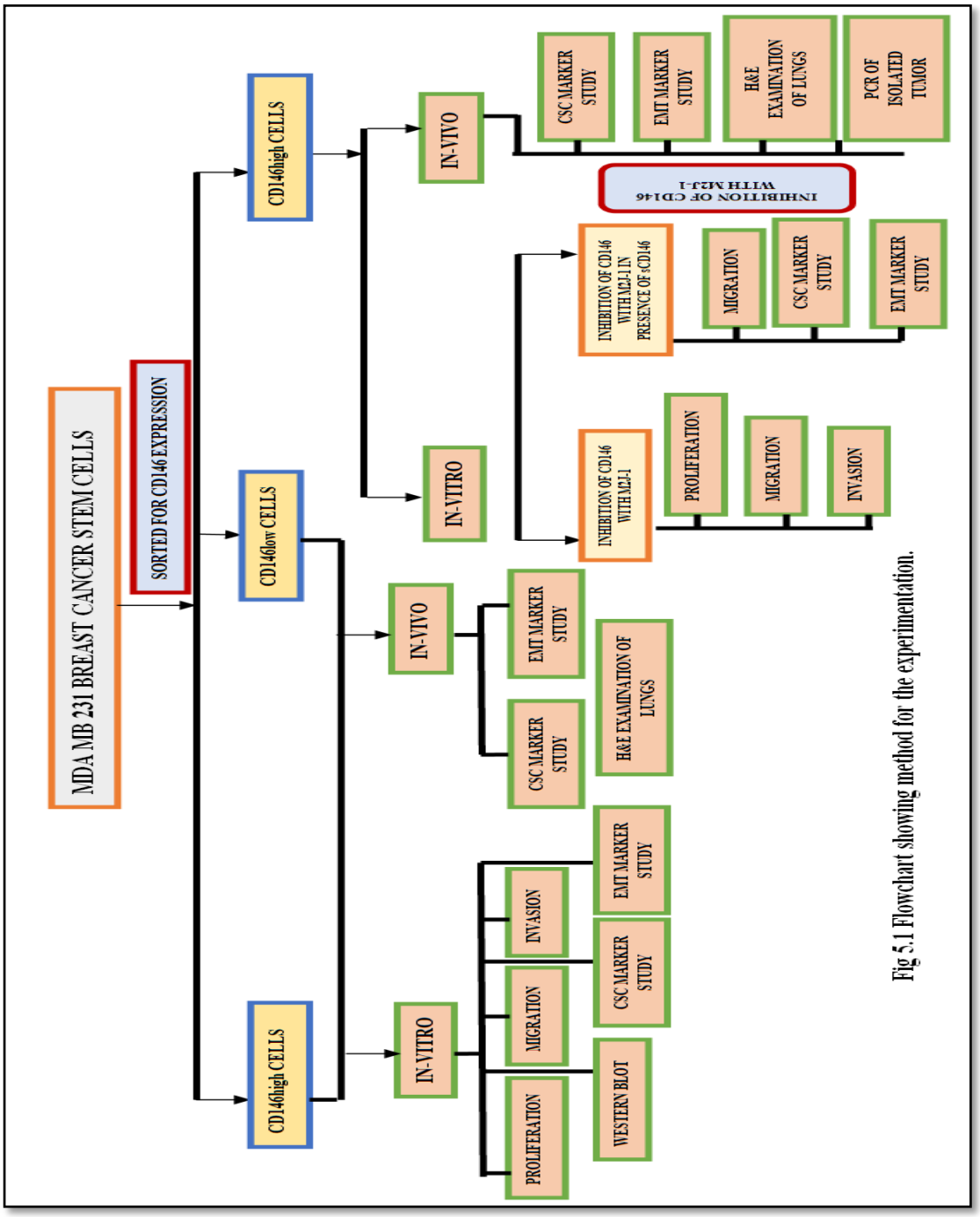


Fig 5.1 Flowchart showing method for the experimentation.

5.1 Culture and characterization of cancer stem cells from breast cancer cell line MDA MB 231

- **Flow cytometry and sorting of cultured MDA MB 231 triple negative breast cancer cell line-**

In this study, we have used Becton Coulter flow cytometer to characterise the cultured cell population for cancer stem cell markers and CD146 surface marker. The cultured cells were stained with **CD146 (APC)** and cancer stem cell markers **CD133 (APC), EPCAM (PE), CD24 (Vioblue), CD44 (FITC)**.

The cultured BCSCs when characterised were found to have low CD24 expression with high CD44 expression, CD133 was found to be highly expressing and cells were found to have two populations of CD146. The two population of CD146 were named as CD146high and CD146low, wherein CD146high cells had higher CD146 expression as compared to CD146low, as shown in **Figure 5.15**. The two population of CD146 breast cancer stem cells were sorted by Florescence assisted flow cytometry (FACS) and studied for cancer stem cell markers as shown in **Figure 5.16**. These two cell populations were further cultured for the *in-vitro* and *In-vivo* experiments.

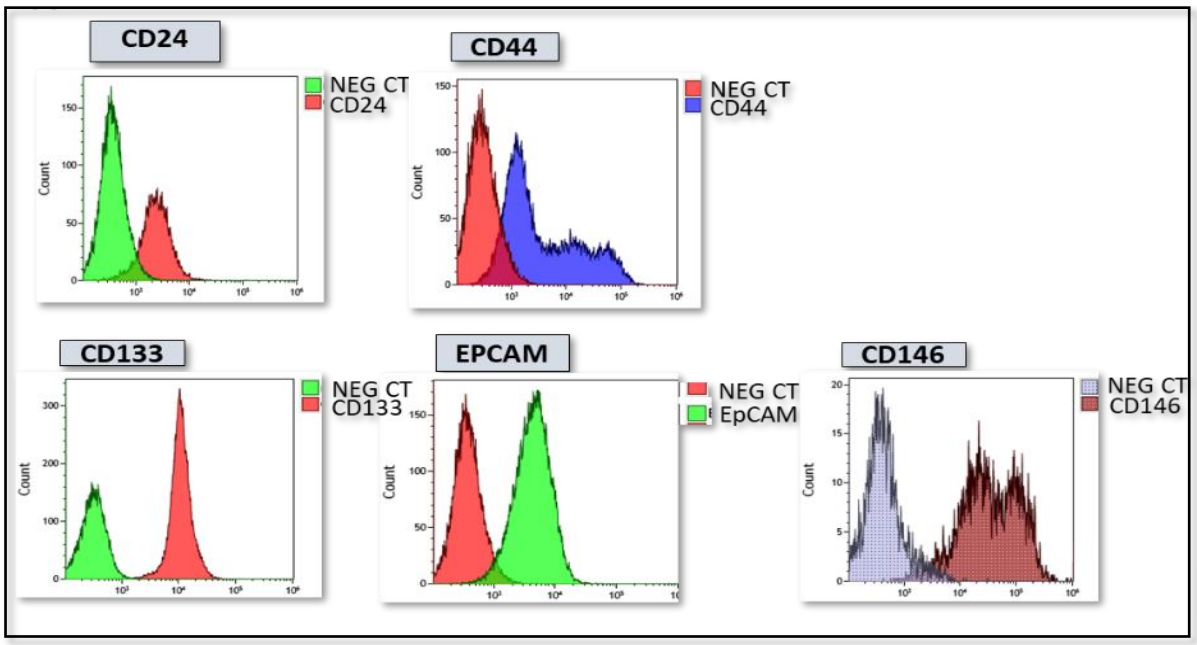


Fig 5.2-The expression of CD24, CD44, CD133, EpCAM and CD146 in MDA MB 231 BCSCs.

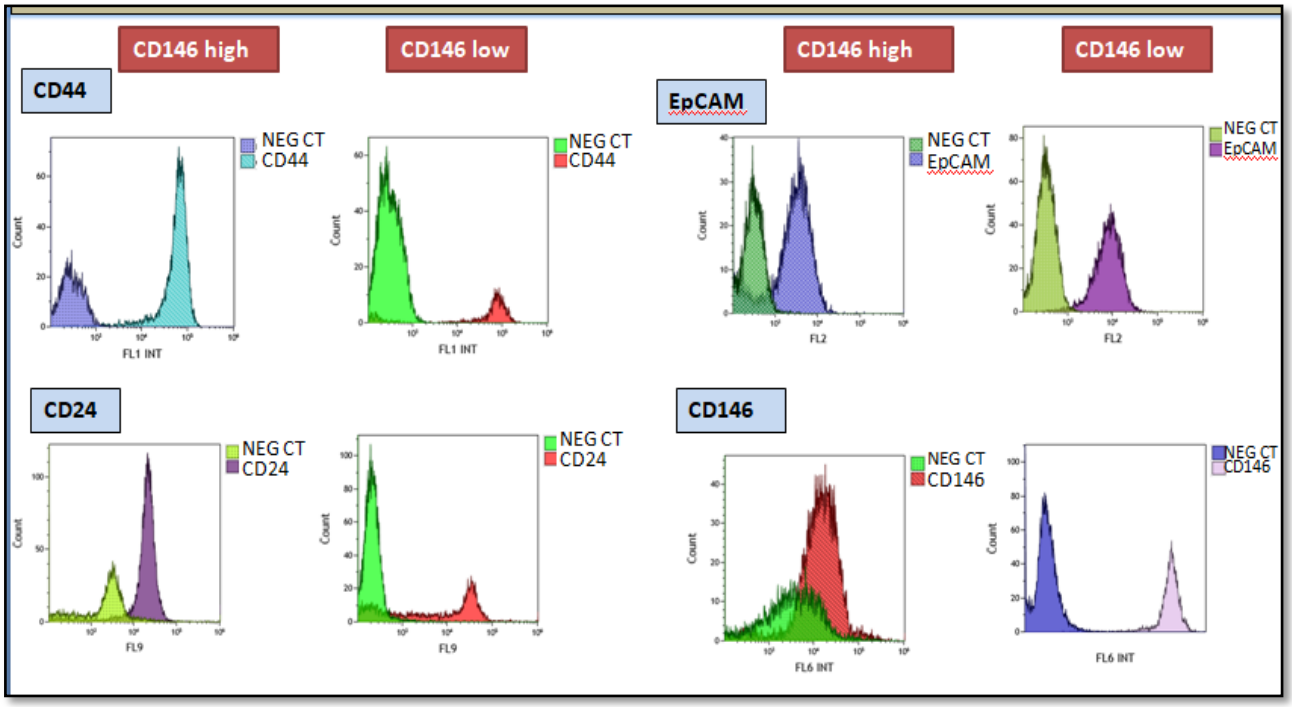


Fig 5.3- The expression of different cancer stem cell markers in CD146high and CD146low cell population.

The ratio of differential expression of CD44 and CD24 was calculated from percentage of CD24 and CD44 subpopulation in flow cytometry analysis. It was

found that the ration CD44/CD24 was significantly higher in CD146high cell population as compared to CD146low sorted cell population as shown in **Figure 5.4 graph a)**. Also, the ratio of EpCAM expression was found to be higher in CD146high sorted cell population as compared to CD146low sorted cell population as shown in **Figure 5.4 graph b).**

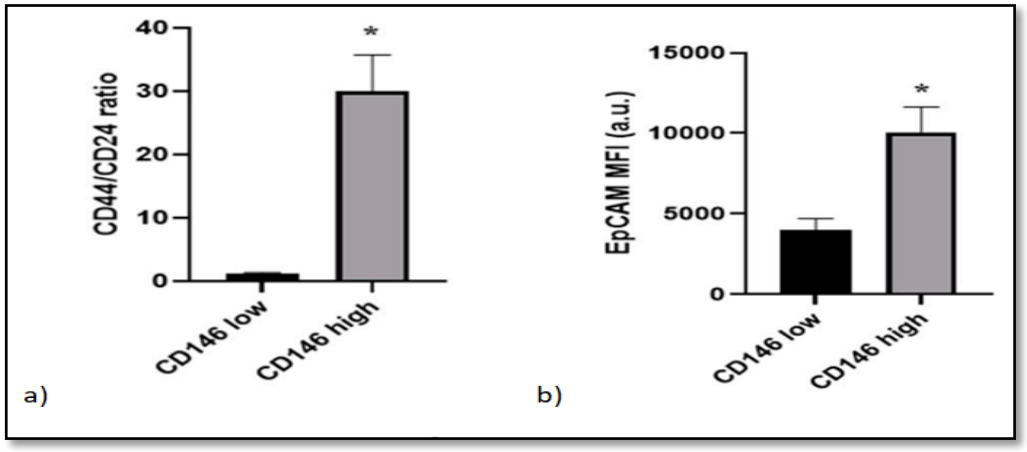


Fig 5.4: a) Average CD44/CD24 ratio in CD146high and CD146low cell cells. b) The average Mean Fluorescent Intensities (MFI) of EpCAM in CD146high vs CD146low. *: p < 0.05

The two populations of cancer stem cells CD146high and CD146low were cultured for several passages to monitor their differentiation ability. It was found that CD146high cells remained only CD146high while CD146low cells again differentiated into CD146high and CD146low cells at passage 1 only as shown in **Figure 5.5**. This change in differentiation ability of both populations justifies the role of CD146 in maintaining the stemness of breast cancer stem cells.

In **Figure 5.5 graph a)** shows that CD146low cells when further cultured differentiates into two cell populations with differential CD146 expression. **Figure 5.5 Graph b)** shows that CD146high cells when further cultured did not differentiate into two cell population. **Figure 5.5 Graph c)** and **graph d)** shows that CD146high cells maintained their phenotype even at passage 4 and passage 9 respectively.

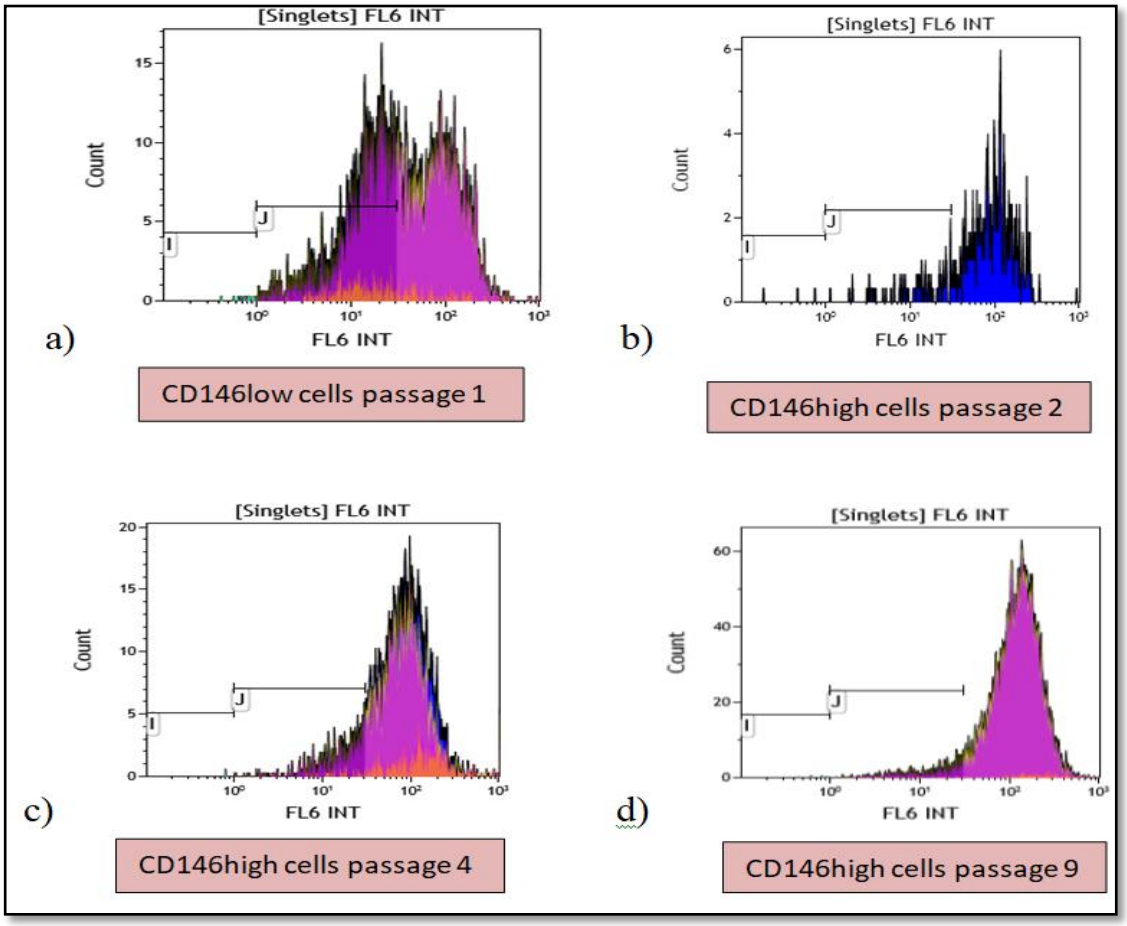


Fig 5.5: a) CD146low cell population after culturing at passage 1.
b), c,) d) CD146high cells after culturing at passage 2, 4 and 9 respectively.

5.2 *In-vitro* examination of CD146 expression in sorted cell population CD146high and CD146low-

The CD146 sorted cell population from breast cancer cell line MDA MB 231 – CD146high and CD146low were studied for their proliferation, migration and invasion capacity. Series of experiments from each category were performed and average was calculated by using Graphpad Prism Software.

The BCSCs CD146high sorted cell population of MDA MB 231 were found to have high proliferation, migration and invasion capacity as compared to the CD146low cell population. The comparative graphs for proliferation, migration

and invasion for CD146high and CD146low and their respective images are given below in **Figure 5.6 a) and b).**

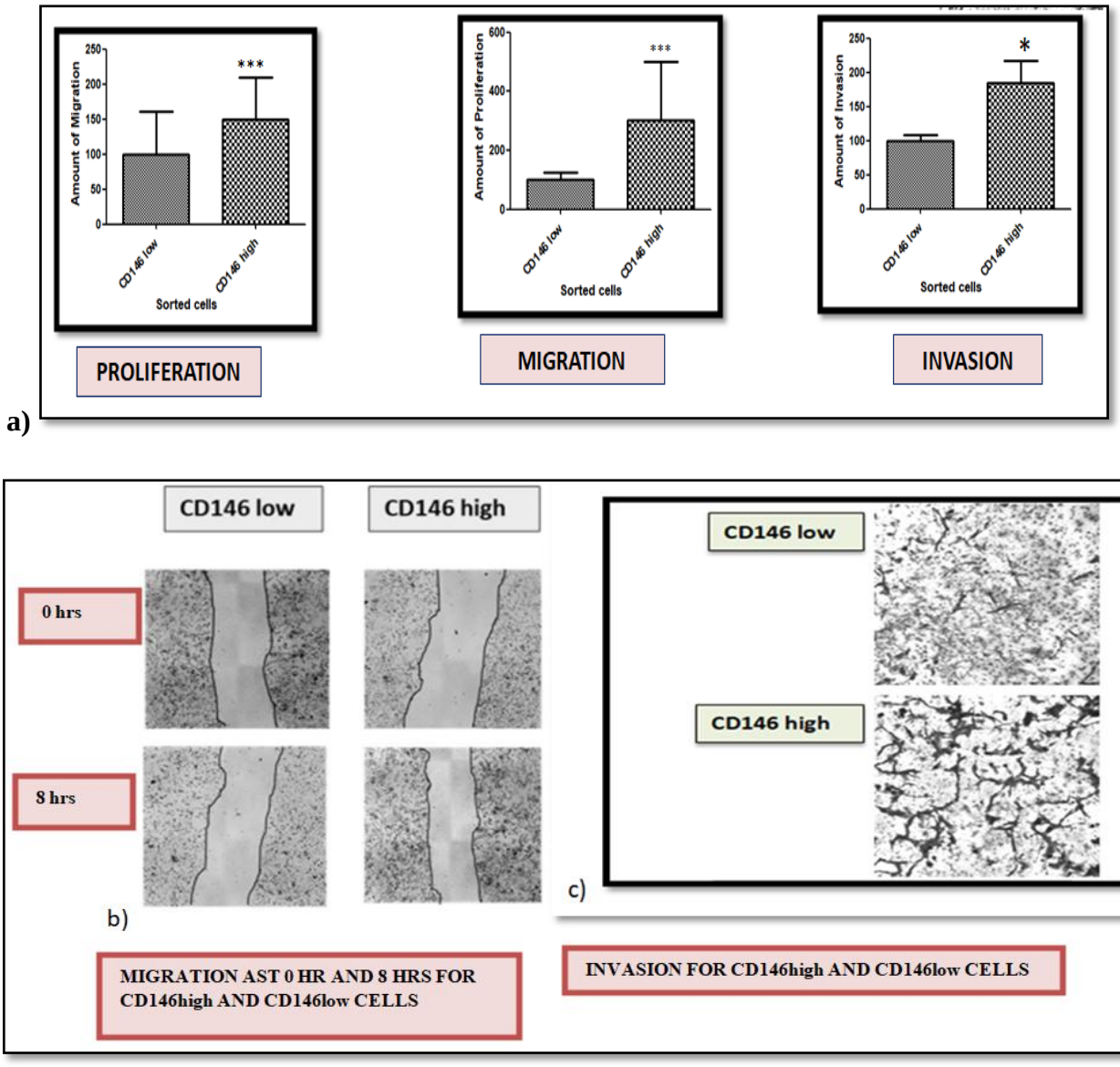


Fig 5.6: a) Quantitative analysis of CD146high and CD146low for Proliferation, Migration and Invasion respectively.

b) CD146high and CD146low for Migration at 0hr and 6 hrs.

c) CD146high and CD146low for Invasion.

An average of 5 experiments is given. *, ***: $p < 0.05$, $p < 0.001$, CD146 high vs. CD146 low.

Effect of CD146 on the proliferation, migration and invasion was studied by inhibiting CD146 using humanised monoclonal antibody M2J-1. CD146high cells were cultured and inhibited with M2J-1. 0

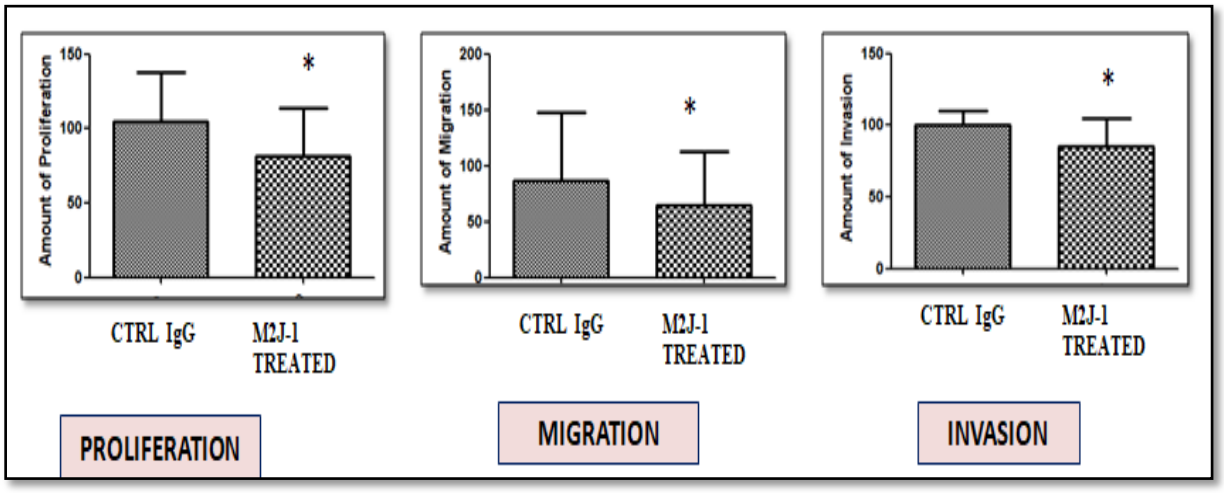
One section of CD146high cells treated with IgG was taken as negative control and other section of CD146high cells were treated with M2J-1.

There was decrease in proliferation, migration and invasion capacity of CD146high sorted cell population when they were treated with M2J-1. Series of experiments for proliferation, migration and invasion were performed for inhibition and an average of each experiment results was taken and graphs were plotted using GraphPad PRISM software. The representative images are shown in **Figure 5.7.**

Figure 5.7 a) shows the quantification graphs of CD146high cells control versus treated cells for proliferation, migration and invasion. The CD146high cells have decreased proliferation, migration and invasion capacity when CD146 was inhibited by humanised monoclonal antibody M2J-1.

Figure 5.7 b) and c) shows the images for the migration and invasion assay for inhibited expression of CD146high cells when treated with M2J-1.

a)



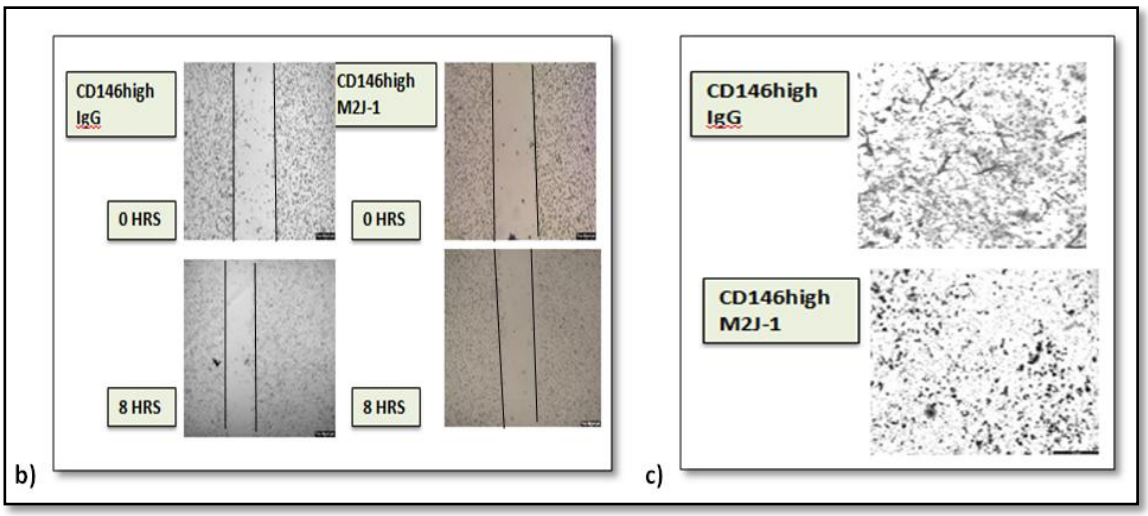


Fig 5.7: a) Quantitative analysis of effect of M2J-1 in Proliferation, Migration and Invasion assay.

b) Inhibition of CD146 by M2J-1 for Migration at 0hr and 6 hrs.

*c) Inhibition of CD146 by M2J-1 for Invasion. n=5. *: p < 0.05, M2J-1 vs. Control IgG*

Migration ability of CD146high cells stimulated by sCD146 was also studied. It was found that CD146high sorted cell population was treated with 100ng/ml sCD146 for 24hrs, then migration ability of the cells increased significantly. The representative images are shown in **Figure 5.8.**

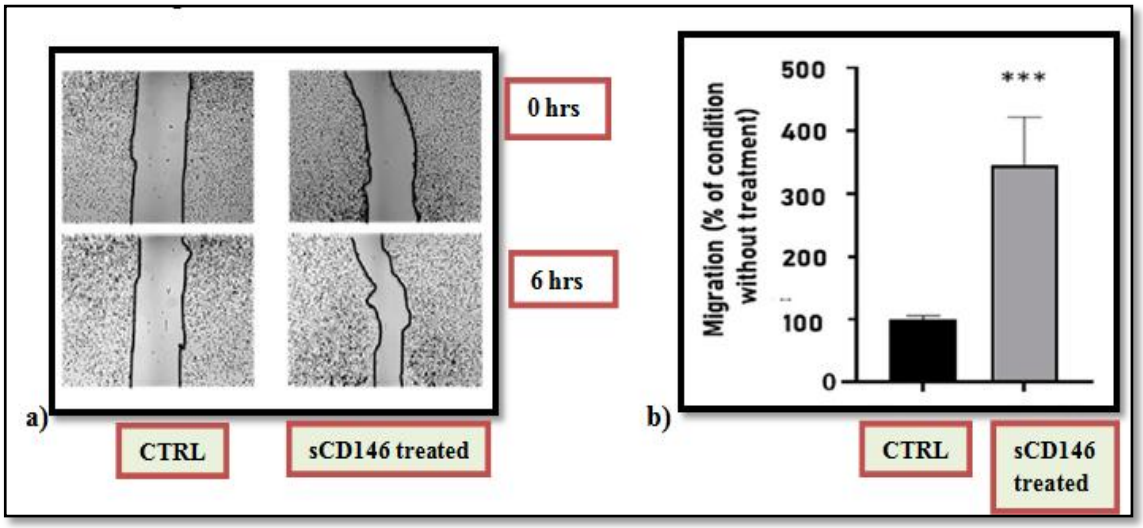


Fig 5.8: a) sCD146 treatment on CD146high cells versus control.

b) Graph representing the effect on migration ability of CD146high cells when treated with sCD146. (n=5), **, ***: p < 0.05, p < 0.01, p < 0.001 experimental vs. no treatment (No trt).*

5.3 *In-vitro* examination of CD146 expression in CD146high and CD146low BCSC cell population of MDA MB 231 breast cancer cell line-

The CD146 sorted BCSC cell population - CD146high and CD146low were studied for the different expression level of CD146 through Western BLOT and ELISA Assay, as shown in **Figure** 5.9.

In Western BLOT, β-actin was used as control and A74 antibody was used against CD146. CD146 expression and sCD146 expression was studied through Western BLOT and ELISA.

In Western BLOT, the bands were thick in CD146high cells while in CD146low cell population bands were comparatively thin as shown in **Figure 5.9 a).**

Also in ELISA, CD146low cell population was found to have lower expression of CD46 as compared to CD146high cell population as shown in **Figure 5.9 b).**

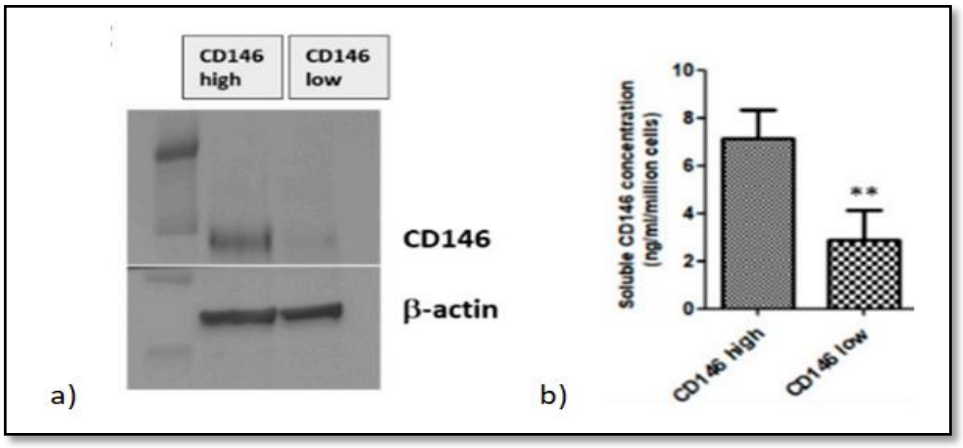


Fig 5.9: a) Western BLOT of CD146high and CD146low cell population.
b) ELISA of CD146high and CD146low.
(n=6) **: p < 0.01, CD146 low vs. CD146 high.

5.4 *In-vitro* examination of CSC markers expression at mRNA and protein level for CD146high cells when treated with sCD146 and its effect when inhibited using anti-sCD146 antibody M2J-1-

Effect of sCD146 on the expression of cancer stem cell markers Sox2 and Nanog was studied at both protein and mRNA level. A series of 4 experiments were performed and an average was deduced by using GraphPad PRISM software.
CD146high sorted cell population when treated with 100ng/ml sCD146 for 24hrs and studied for its effect on the expression of cancer stem cell (CSC) markers Sox2 and Nanog at both mRNA (qPCR) and protein levels as compared to the cells with no treatment. Representative images of mRNA and protein level expression of CD146high cells are shown in **Figure 5.10.**

Figure 5.10 a) represents the graph for comparative expression of cancer stem cell markers Sox2 and Nanog at mRNA level for CD146high cells treated with sCD146 as compared to CD146high cells with no treatment of sCD146. It was found that Sox2 and Nanog were significantly higher as compared to non-treated cells.

Figure 5.10 b) represents the bands of Sox2 and Nanog at protein levels when CD146high cells were treated with sCD146.

Figure 5.10 c) represents the graph for comparative expression of cancer stem cell markers Sox2 and Nanog at protein level for CD146high cells treated with sCD146 as compared to CD146high cells with no treatment of sCD146. It was found that Sox2 and Nanog were significantly higher as compared to non-treated cells.

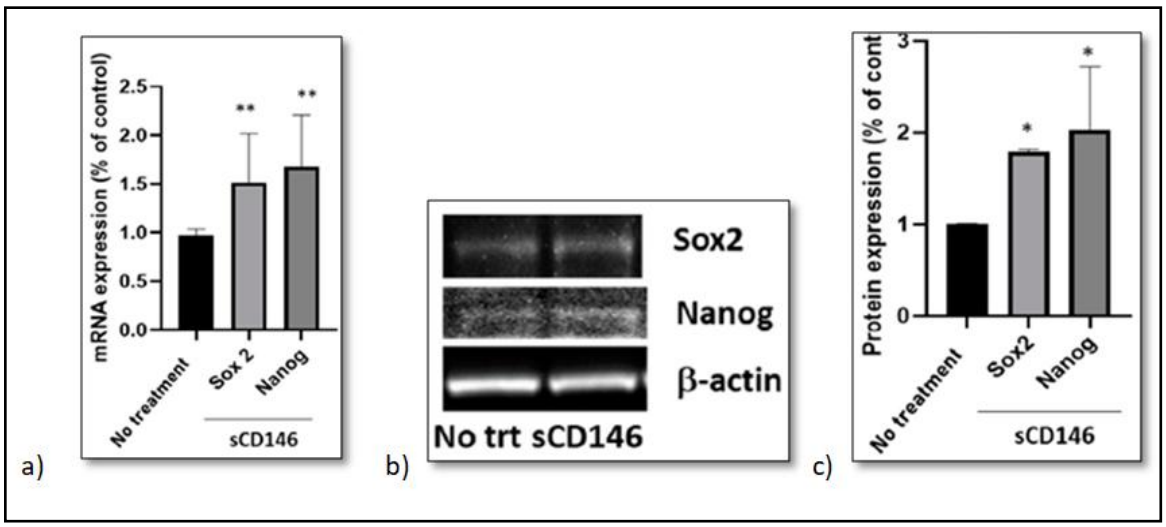


Fig 5.10: Effect of sCD146 100ng/ml on the expression of CSC markers Sox2 and Nanog at mRNA(qPCR) and protein (Western Blot) levels in comparison to the absence of treatment. An average of 5 experiments was given. *, **, ***: $p < 0.05$, $p < 0.01$, $p < 0.001$ experimental vs. no treatment (No trt).

Effect of anti-sCD146 antibody M2J-1 was studied in CD146high sorted cell population at both mRNA and protein level. CD146high cells were treated with anti-sCD146 M2J-1 50 µg/ml for 24hrs and compared with control IgG 100ng/mL sCD146 induced increase in expression of CSC markers Sox2 and Nanog at both mRNA and protein level. An average of 4 experiments was deduced and graphs were plotted using Graphpad PRISM software. Representative images are given in **Figure 5.11.**

Figure 5.11 a) represents the graphs for comparative expression of CSC markers Sox2 and Nanog at mRNA level in CD146high cells when treated with anti-sCD146 antibody M2J-1 as compared to CD146high cells treated with sCD146 and IgG taken as control. There was significant decrease in Sox2 and NAnog expression in presence of anti-sCD146 antibody M2J-1 as compared to control CD146high cells treated with sCD146 and IgG.

Figure 5.11 b) represents the protein bands of CSC markers Sox2 and Nanog at protein level in CD146high cells when treated with anti-sCD146 antibody M2J-1 as compared to CD146high cells treated with sCD146 and IgG taken as control.There was slight decrease in band size of Sox2 and Nanog expression in presence of anti-sCD146 antibody M2J-1 as compared to control CD146high cells treated with sCD146 and IgG.

Figure 5.11 c) represents the graphs for comparative expression of CSC markers Sox2 and Nanog at mRNA level in CD146high cells when treated with anti-sCD146 antibody M2J-1 as compared to CD146high cells treated with sCD146 and IgG taken as control. There was significant decrease in Sox2 and Nanog expression in presence of anti-sCD146 antibody M2J-1 as compared to control CD146high cells treated with sCD146 and IgG.

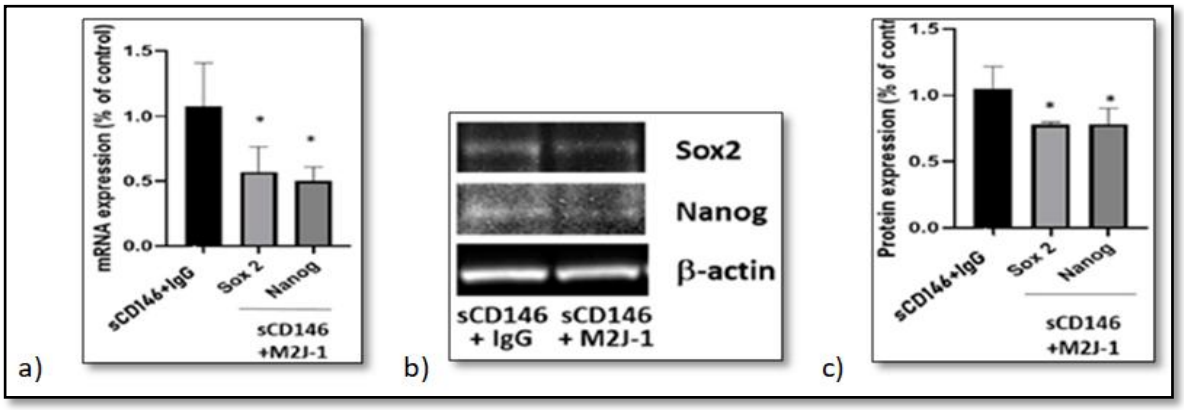


Fig 5.11: Effect of anti-sCD146 antibody M2J- 1 50 µg/mL on CD146high cells for 24 h on the expression of CSC markers Sox2 and Nanog in CD146high cell population at mRNA and protein level in comparison to control IgG on 100 ng/mL sCD146 induced increase CSC markers expression. n=5. *: p < 0.05, sCD146 + M2J-1 vs. sCD146 + IgG.

5.5 *In-vitro* examination of EMT markers expression at mRNA and protein level for CD146high cells when treated with sCD146 and its effect when inhibited using anti-sCD146 antibody M2J-1-

Effect of sCD146 on EMT markers Vimentin and Slug was studied on CD146high sorted cell population. A series of 5 experiments were conducted and an average was deduced. Graphs were plotted using GrpahPad PRISM.

CD146high sorted population was treated with sCD146 to study the differential expression of EMT markers Vimentin and Slug at protein (Western BLOT) and mRNA (qPCR) level. Representative image and graphs are given below in **Figure** 5.12.

Figure 5.12 a) represents the expression of Vimentin and Slug at mRNA level, when CD146high sorted cell population was treated with sCD146 versus control. The expression level of EMT markers was found to have significantly enhanced in comparison to control.

Figure 5.12 b) represents the protein bands of Vimentin and slug when CD146high sorted cell population were treated with sCD146. The bands were comparatively thicker for CD146high cell treated with sCD146 as compared to the cells in control.

Figure 5.12 c) represents the expression of Vimentin and Slug at protein level, when cD1446high cells were treated with sCD146. As sCD146 increases the expression of CEMT markers, thus EMT markers expression was found to be significantly enhanced as compared to the control CD146high sorted cells.

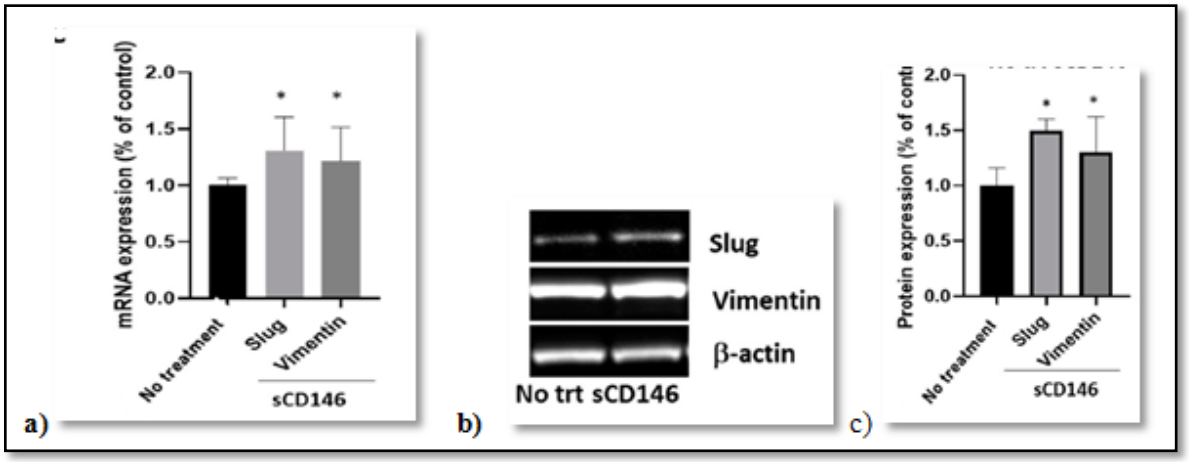


Fig 5.12: Expression of EMT markers Vimentin and Slug in CD146high cells treated with scD146 at mRNA and Protein levels. n=5, *, **, ***: p < 0.05, p < 0.01, p < 0.001

Anti-sCD146 antibody (500µg/ml) M2J-1 was used against CD146 to study its effect on CSCs markers Slug and VIMENTIN expression.

CD146high sorted cell population was treated with anti-sCD146 antibody M2J-1 50µg/ml for 24hrs and compared with CD146high sorted cells induced with sCD146 and IgG to study the differential expression of EMT markers Vimentin and Slug at both protein and mRNA level. Series of 4 experiments were performed and an average was deduced. Representative images are given in **Figure 5.13.**

Figure 5.13 a) represents the graph of differential expression of EMT markers Vimentin and Slug at mRNA level when CD146 was inhibited by using anti-sCD146 antibody M2J-1 for 24hrs. The expression of Vimentin and slug was found to have reduced significantly as compared to control CD146high cells treated with sCD146.

Figure 5.13 b) represents the bands of Vimentin and slug at protein level, when CD146high cells were treated with anti-sCD146 M2J-1 for 24hrs. Bands were reduced significantly for CD146high sorted cell population treated with antisCD146 as compared to control CD146high cells treated with sCD146 and IgG.

Figure 5.13 c) represents the graph of differential expression of EMT markers Vmentin and Slug at protein level when CD146 was inhibited by using anti-sCD146 antibody M2J-1 for 24hrs. The expression of Vimentin and slug was found to have reduced significantly as compared to control CD146high cells treated with sCD146.

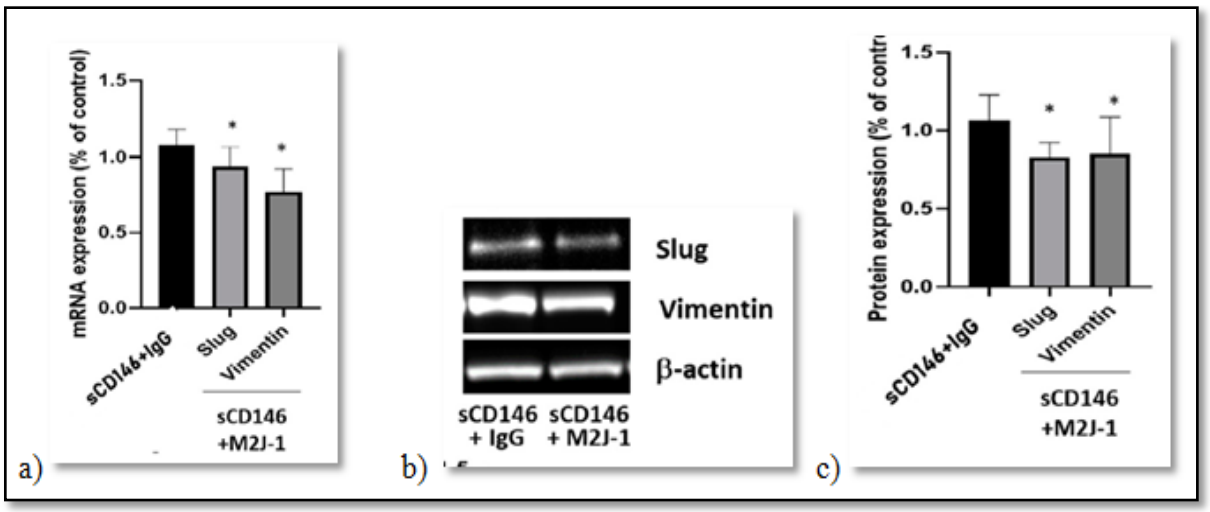


Fig 5.13: Effect of anti-sCD146 antibody on the expression of EMT markers Vimentin and Slug in CD146high cell population at mRNA and protein level.
n=5 *: p < 0.05, sCD146 + M2J-1 vs. sCD146 + IgG.

5.6 *In-vivo* examination of CD146 expression and effect of its inhibition on metastasis-

During *In-vivo* study, 16 NOD-SCID mice were used to study the expression of CD146 when BCSCs were injected and when CD146 was inhibited using humanised monoclonal antibody against CD146 M2J-1. Both intra-cardiac and sub-cutaneous model of injections were performed. It was found that bones did not have significant metastasis/ tumor generation while lungs had higher tumor metastasis. There were several clustered, disfigured patches on the periphery of lungs section of mice with CD146high cells while many disfigured, clustered patches were found on alveolar area of the section. So, images at 5X, 10X and 40X were taken and metastasis was measured using imageJ software.

Figure 5.14 shows the NOD-SCID mice with intra-cardiac injection of CD146high and CD146low cell and isolation of lungs and bones from mice after sacrificing it.

Figure 5.15 shows the lungs and bones isolated from respective mice injected with CD146high and CD146low cells.

Figure 5.16 shows the H&E images of isolated lungs at 5X, 10X and 20X and also the quantification of metastasis in lungs.

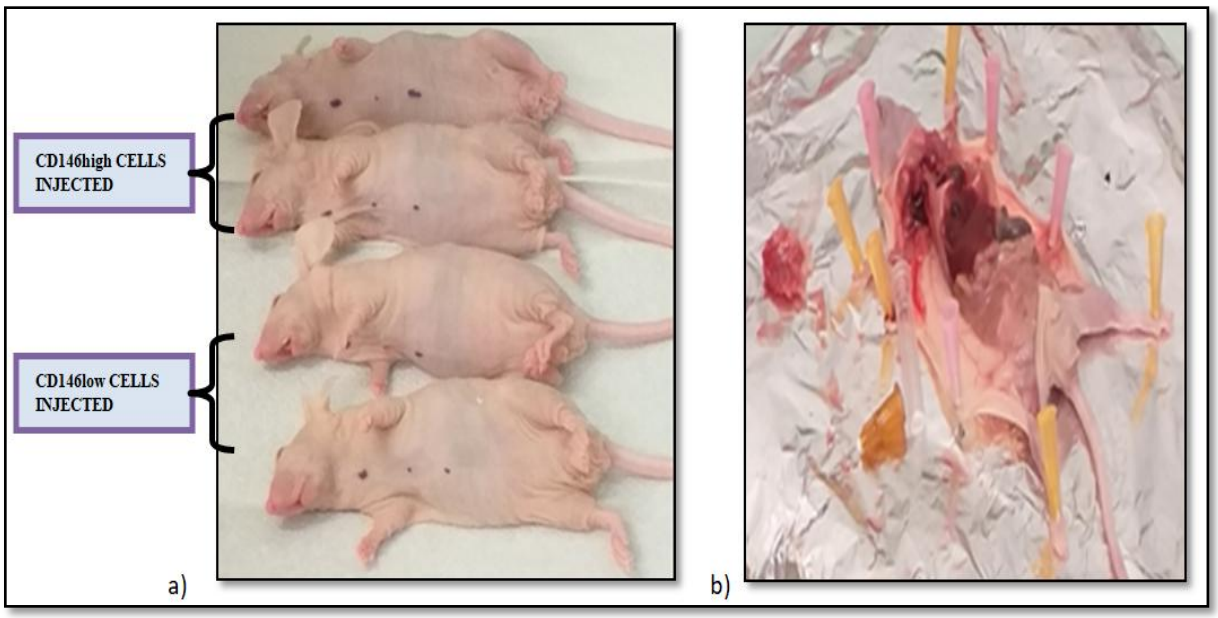


Fig 5.14: a) Intra-cardiac injection of CD146 high and CD146low cells.
b) Isolation of lungs and bones from mice.

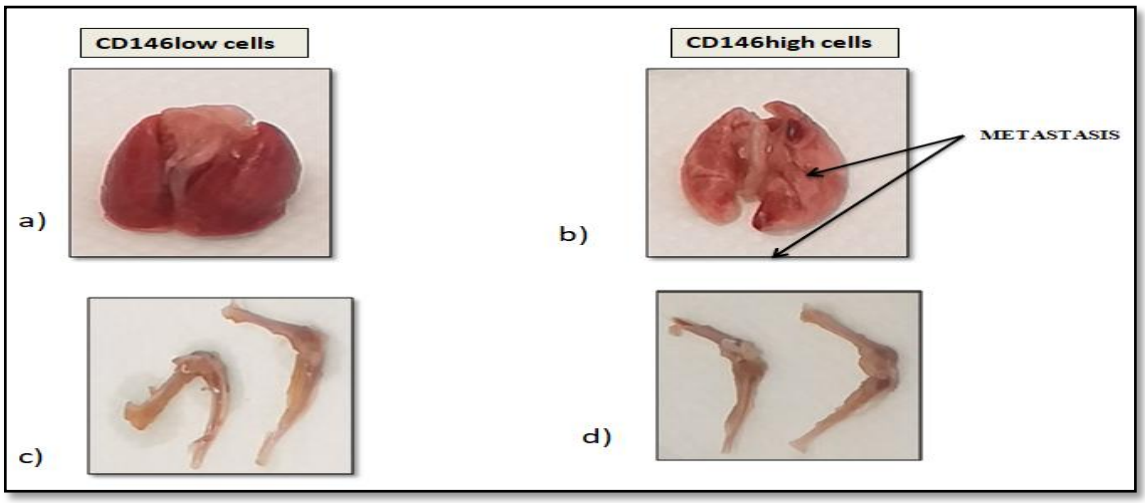


Fig 5.15: a) Lung of mice injected with CD146low cells with no visible metastasis.
b) Lung of mice injected with CD146high cells with visible metastasis.
c) Bones of mice injected with CD146low cells.
d) Bones of mice injected with CD146high cells.

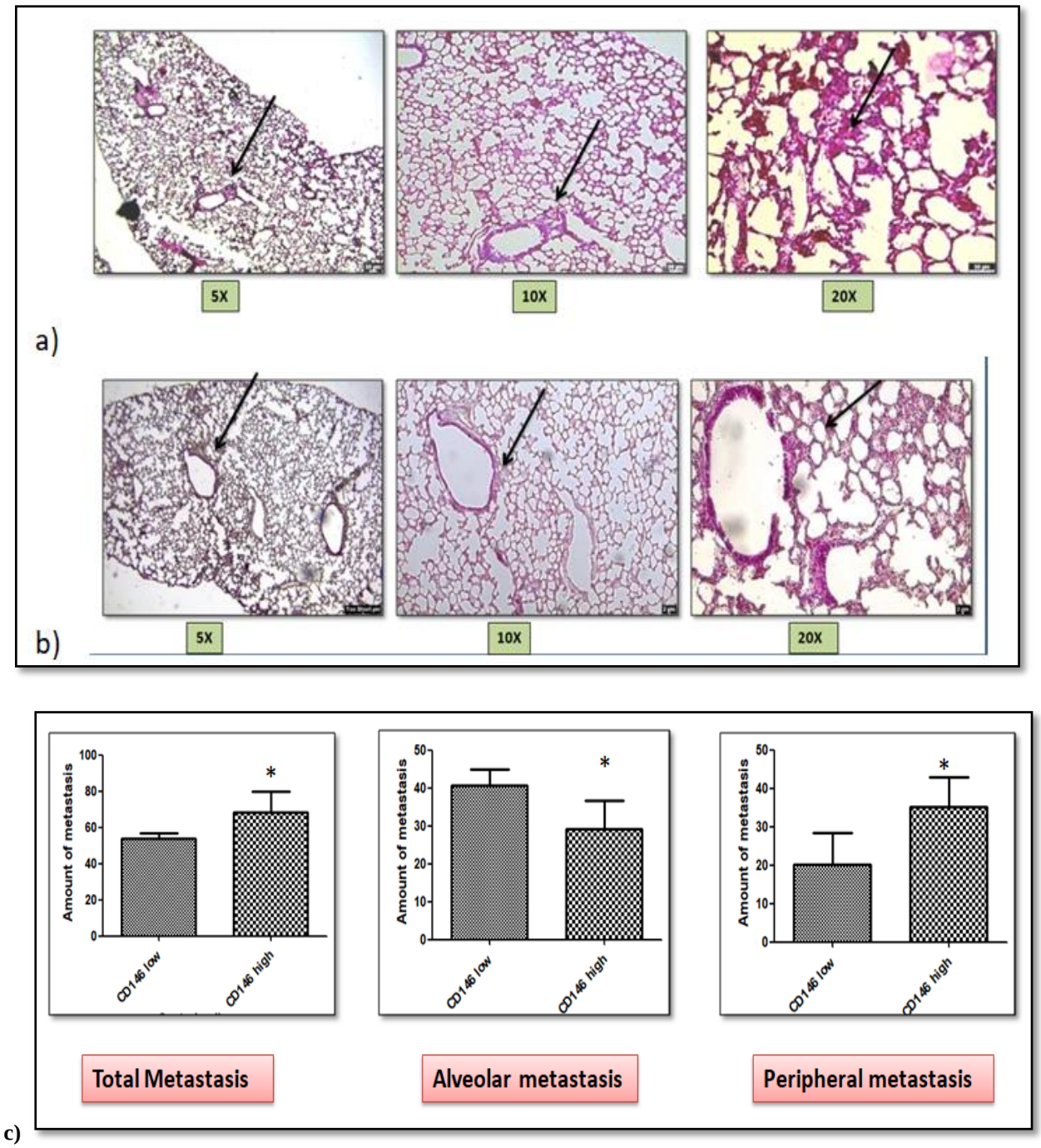


Fig 5.16: a) Representative images of lungs of mice injected with CD146low cells

b) Representative images of lungs of mice injected with CD146high cells

c) Quantification of total, alveolar and peripheral lung metastasis.

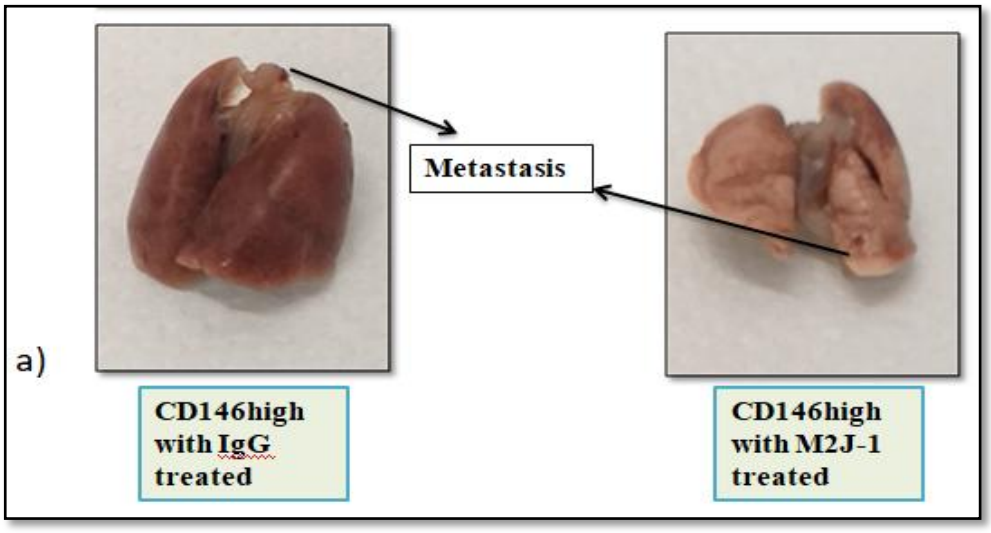
Mice injected with CD146high cells treated with anti-sCD146 antibody M2J-1 and sCD146 and IgG as control were sacrificed and lungs were isolated. Lungs from the treated mice showed many clustered and disfigured patches corresponding to

metastasis while lungs from control mice had only a few clustered disfigured patches of metastasis on the surface.

In **Figure 5.17 a)** surface metastasis is shown by arrows. Surface of lungs have dark coloured patches in both the lungs of mice injected with CD146high cells with and with mAB M2J-1.

In **Figure 5.17 b)**, the H&E images shows the metastasis in lungs of mice injected with CD146high cells and treated with IgG as control. The sections have dark clustered patches and visibly higher metastasis.

In **Figure 5.17 c),** the H&E images shows the lungs of mice injected with CD146high cells and treated with M2J-1 mAB. The lungs have lower metastasis with declined clustered patches.



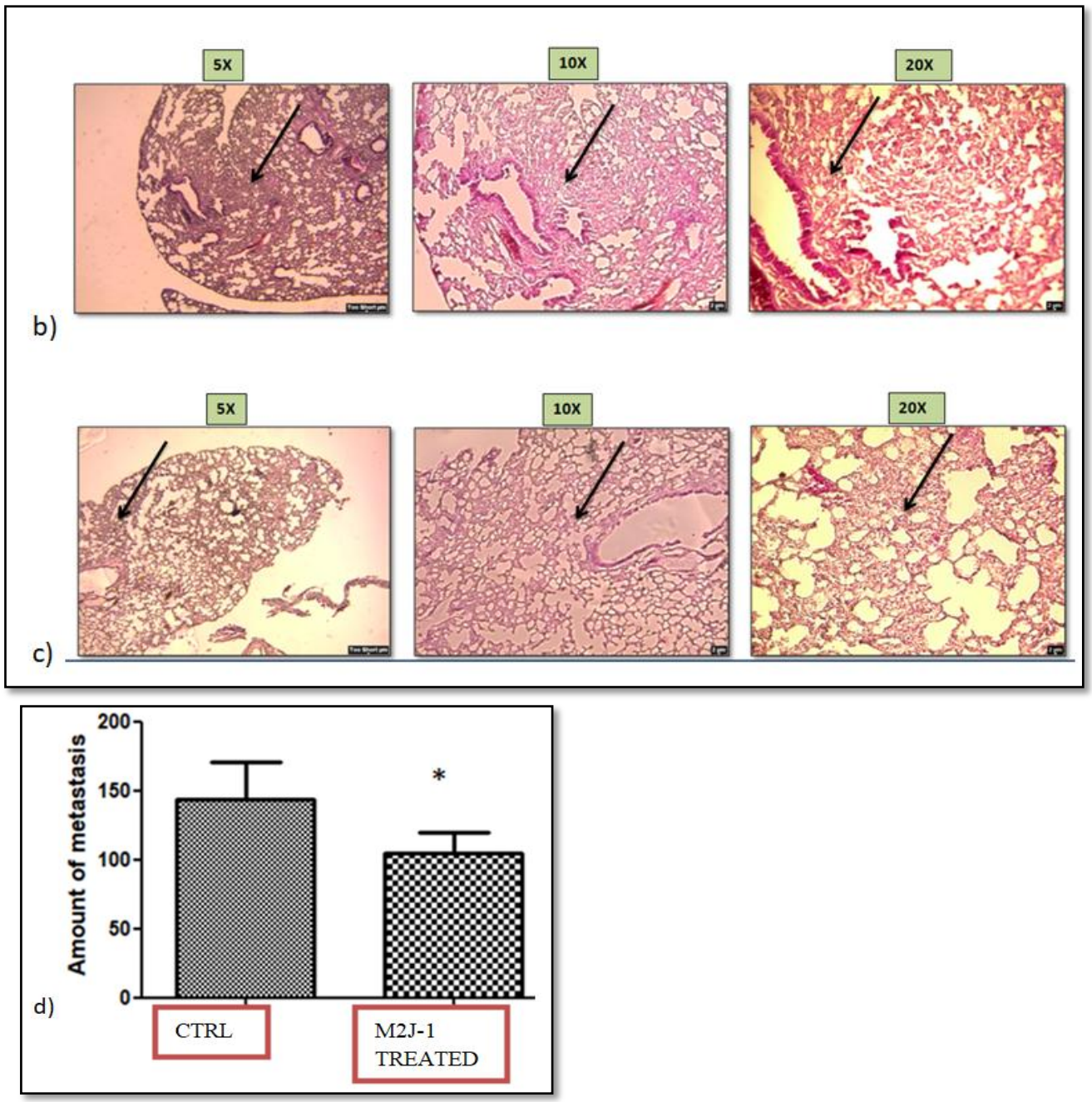


Fig 5.17: a) Lungs of mice injected with CD146high cells and treated with IgG set as control and lungs of mice injected with CD146high cells and treated with anti- sCD146 M2J-1 respectively.

b) H&E images of lungs of mice injected with CD146high cells treated with IgG, at 5X, 10X and 20X respectively.

c) H&E images of lungs of mice injected with CD146high cells treated with M2J-1, at 5X, 10X and 20X respectively.

d) Quantification of lung metastasis.

Mice injected sub-cutaneously with CD146high cells were found to have tumour bulging on the surface while and mice injected with CD146low cells did not had any tumor on the surface (as shown in the image given below). Tumours isolated from these mice were studied for CD146 expression through PCR and it was found that there was higher CD146 expression in mice injected with CD146high sorted cell population.

Figure 5.18 a) shows the NOD-SCID mice injected with CD146high/low cells subcutaneously and mice with CD146high cells have tumor outgrowth on the surface, highlighted by circle.

Figure 5.18 b) shows the quantification of CD146 expression in both the mice with CD146high and CD146low cells injected into them. The mice with CD146high cells have been found to have higher CD146 expression in comparison to mice injected with CD146low cells

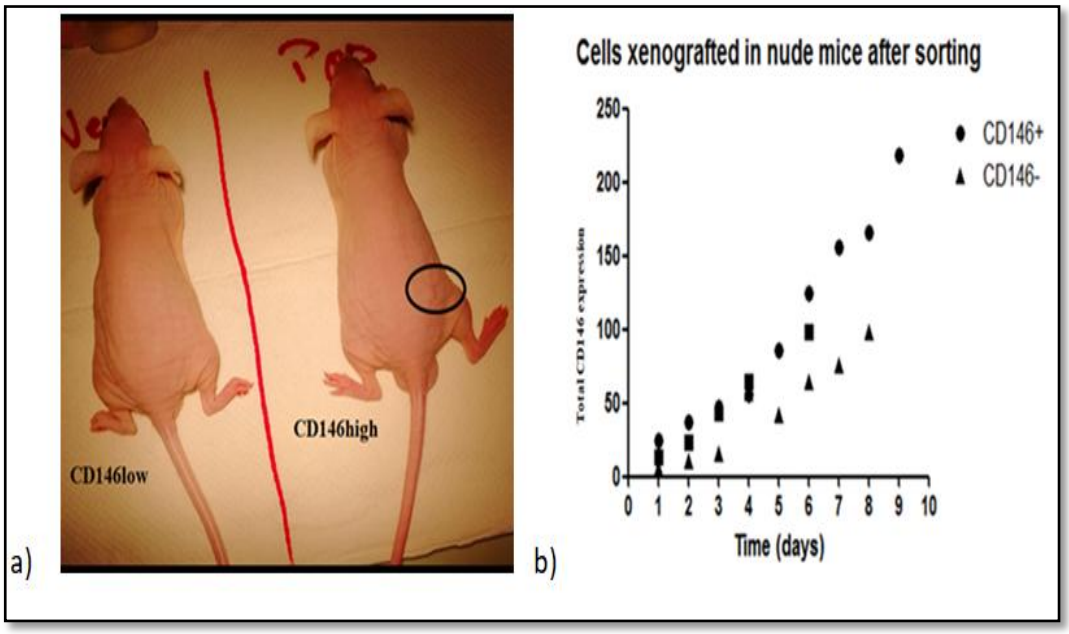


Fig 5.18: a) Sub-cutaneous injection of CD146high/low cells in NOD-SCID mice. The tumor outgrowth is highlighted by circle.
b) Quantification of CD146 expression after tumor generation.

Mice injected sub-cutaneously with CD146high cells were studied for CD146 expression in comparison to the mice injected with CD146high cells treated with

IgG as control and treated with M2J-1. It was found that treated mice have lower CD146 expression as compared to control mice.

Also the tumors isolated from these mice were studied for the expression of EMT and CSC markers as shown in **figure 5.19**. It was found that the expression of CSC and EMT markers Sox2, Nanog, Slug and Vimentin was significantly lower in the cells treated with M2J-1 as compared to the control IgG treated CD146high cells.

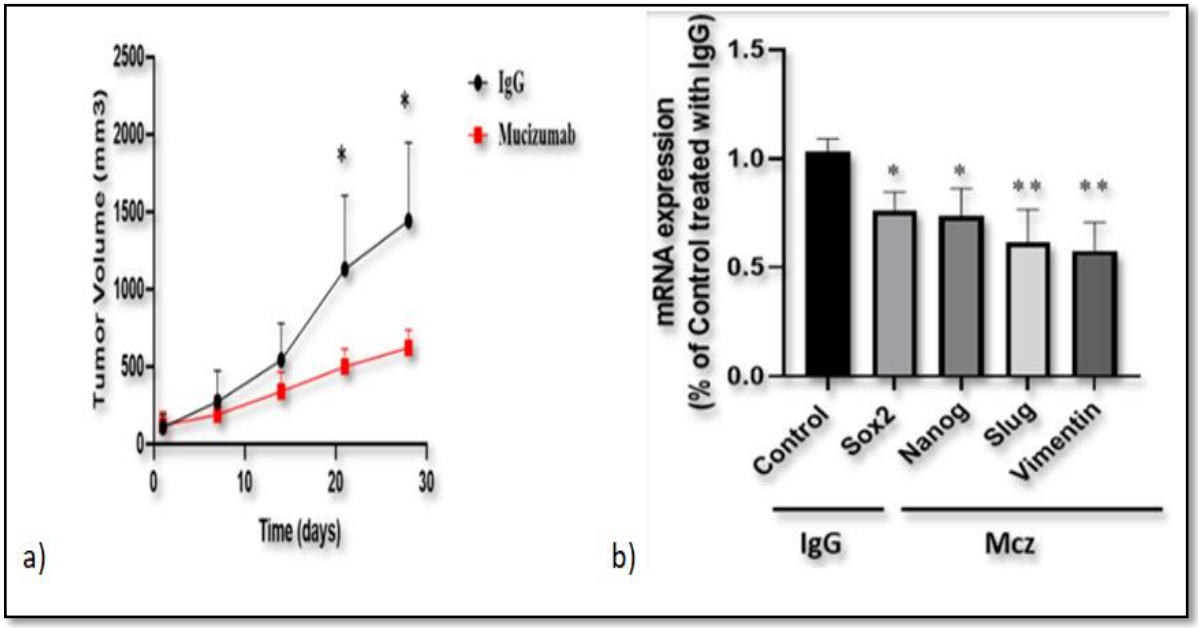


Fig 5.19: a) Effect of anti-sCD146 antibody M2J-1 500µg/kg in comparison to IgG, on tumor volume in In-vivo mouse model of CD146high cells from MDA MB 231 cell line.

b) Expression of CSC (Sox2 and Nanog) and EMT (Slug and Vimentin) markers at mRNA level (qPCR) in tumors isolated from sub-cutaneously injected mice treated with M2J-1.

5.7 Biostatical analysis of CD146 expression in molecular breast cancer subtypes and its correlation with lymph node metastasis and overall survival of breast cancer-

The correlation of CD146 expression with overall survival of BC patient and lymph node positivity was studied using forest plot. It was revealed that breast cancer patients with high expression of CD146 displayed significantly higher risk of lymph node metastasis with pooled risk ratio of 1.64 (95% CI = 1.44–1.87, P < 0.00001) as shown in **figure 5.20**.

CD146 expression in various breast cancer subtypes and pooled odd ratio were calculated. Low level of CD146 expression was found in Estrogen Receptor (ER) (pooled OR = 0.18, 95% CI = 0.13–0.24, P < 0.00001), progesterone receptor (PR) (pooled OR = 0.27, 95% CI = 0.20–0.36, P < 0.00001) and human epidermal growth factor receptor 2 (HER2) (pooled OR = 0.59, 95% CI = 0.41–0.84, P = 0.003) -positive breast cancers as evidenced by their respective pooled odd ratios. However, pooled odd ratio for triple negative breast cancer (TNBC) exhibited significantly high expression of CD146 (pooled OR = 2.98, 95% CI = 2.19–4.05, P < 0.00001) as shown in **figure 5.21.**

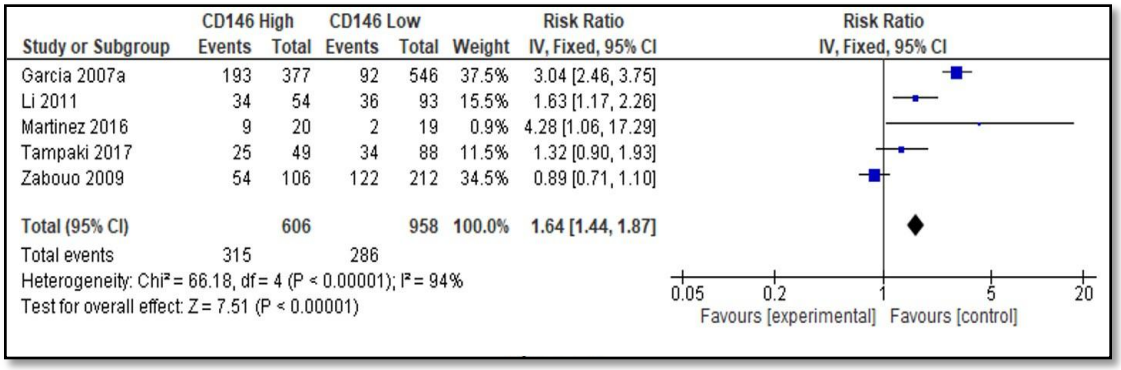


Fig 5.20 Correlation of CD146 expression with lymph node metastasis in breast cancer

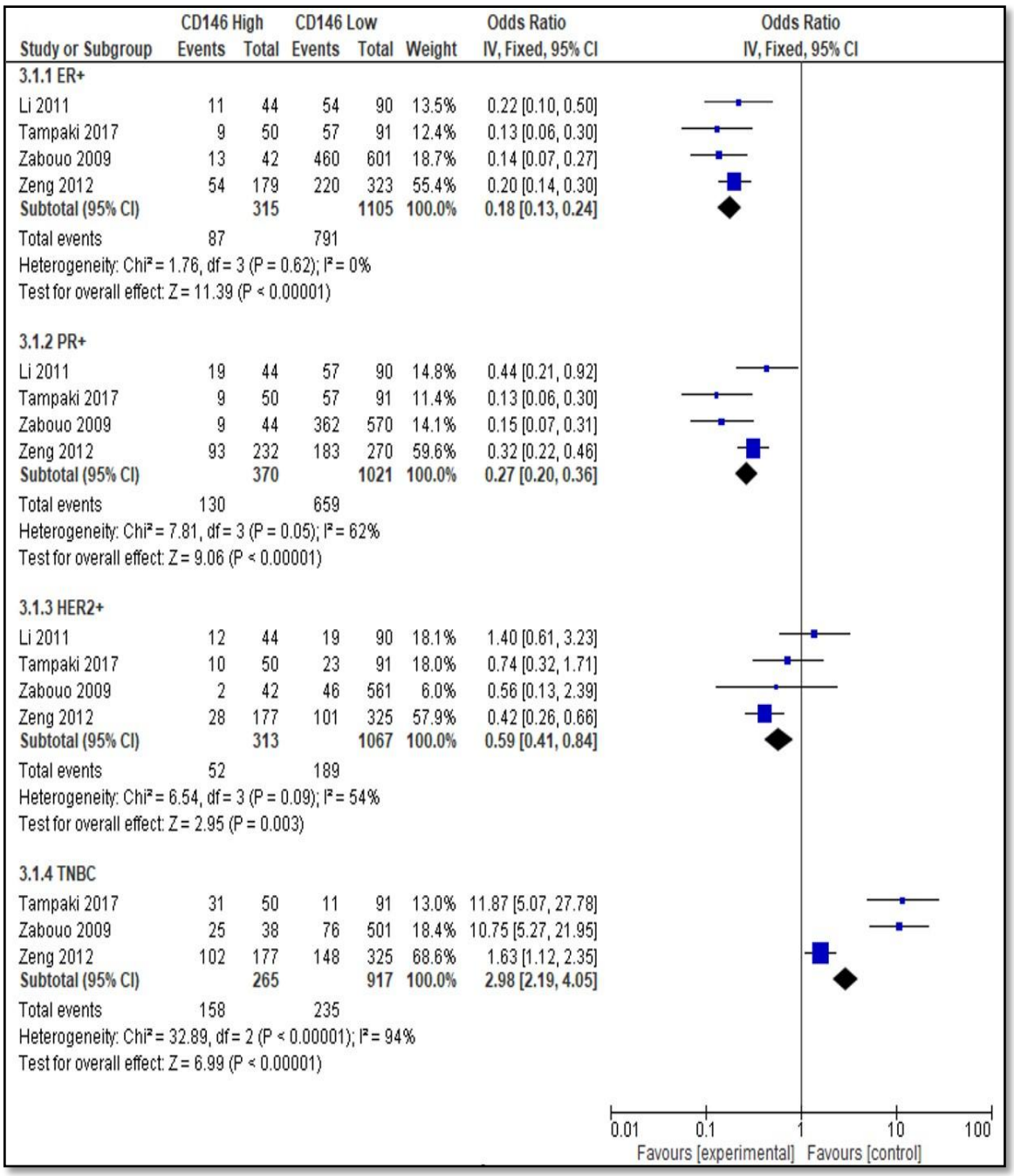


Fig 5.21: The correlation between CD146 expression and breast cancer molecular subtypes.`

CHAPTER-6

DISCUSSION

DISCUSSION-

Among all the cancers, breast cancer is second most prevalent cancer and most common among women worldwide. Also, in subtypes of breast cancer, Triple Negative Breast Cancer (TNBC) is the most aggressive and there are only few therapeutic options for its treatment. CD146 is expressed in both vascular compartments and epithelial cells of malignant breast, thus signifies its involvement in tumor aggressiveness by enhancing tumor cell motility. Zabouo *et. al.* showed that patients with CD146high had significantly lower OS in 5 years. Also downregulation of CD146 expression lead to various changes in several biological processes like EMT and phenomenon related to the metastasis of cancer cells (1). In this thesis, we have emphasized on the role of CD146 in breast cancer metastasis and tumorigenesis, specifically in triple negative breast cancer. Fascinatingly, CD146 when inhibited by anti-sCD146 antibody M2J-1 lead to the decrease in tumor growth and metastasis thereby making it new therapeutic target for treating breast cancer.

CD146 is a double edged sword playing role in both angiogenesis and tumor growth and dissemination. Several *In-vivo* research studies have shown that over-expression of CD146 results in the increase of metastatic ability of cancer cells (2).Tsuchiya *et. al.* and Tsukamoto *et. al.* used chicken model (3,4) and mouse carcinoma models respectively to show the increase in metastasis of lymphoma cells. It has also been found to play role in advanced tumor stage and results in poor prognosis for tumor relapse in ovarian cancer (5), also when over-expressed results in the increased metastasis in prostate cancer *In-vivo* (6).

It has been shown that sCD146 was significantly higher in NSLC patients in comparison to the normal subjects. sCD146 has been found in human blood with its changed levels in different pathological conditions like inflammatory bowel diseases, vasculitis, pathologic pregnancies and chronic renal failure as shown by Bardin *et. al.* (2003), Pasquier et. al (2005), Bardin *et. al.* (2006) (7-9). M. Ilie *et. al.* in 2005 showed positive correlation between circulating endothelial cell and sCD146 suggesting both biomarkers as key players in tumor vasculature with supporting evidence from Monestiroli *et al*, 2001; Harhouri *et al*, 2010 individually (10,11).

sCD146 has been found to play role in inflammation by specifically binding to monocytes thus stimulates transendothelial migration and angiogenesis concluding its chemotactic effect on different cell types involved in vessel formation (12,13). Qiqun *et. al.* in 2012 showed that CD146 plays key regulator of EMT and thus helps in breast cancer progression (14). Tripathi *et. al.* in 2017 proposed CD146 as a novel escape route to treat breast cancer over chemoresistence in breast cancer (15).

sCD146 is secreted in any tumor cells (14) and acts as a major role player in tumor growth and dissemination (16). In this study, we have used MDA MB 231 breast cancer line with high expression of CD146 and gave the evidence for the effect on sCD146 on the expression of CSC markers which provides resistance from various treatment therapies to cancer cells after acquiring stem like properties. Due to sCD146, the expression of cancer stem cell markers like SOX2 and Nanog was elevated. We have shown that BCSCs when sorted on the basis of CD146 expression, then there were two populations of CD146, named as CD146high and CD146low CSC population. These two populations were also observed by Mostert *et. al.*(17) When these sorted cells were characterized, then CD146high cell population found to have higher CD44, EPCAM, CD133 and lower CD24 expression; which are characteristic features of breast cancer stem cells (BCSCs). Also, when these CSC sorted cell population CD146high and CD146low cells were again cultured and characterised showed fascinating results. The CD146high cells were found to have maintained their higher CD146 expression while CD146low cells differentiated into two CD146high/low cells. CD146high cells were most important cell population in the study of MDA Mb 231, the results were further supporting the fact that high expression of CD146 leads to the growth and dissemination of cancer cells. Along with this, CD146high sorted cells showed high proliferation, migration and invasion capacity in comparison to the CD146low sorted cell population. Also, in presence of sCD146, the CD146high cells had higher proliferation, migration and invasion capacity along with the elevated CSC and EMT marker expression by CD146high cells. To estimate the expression of CD146 on breast cancer cell properties and overall patient survival, public database was used. This comparison showed

significantly higher CD146 expression in TNBC in comparison to other breast cancer types like HER2, luminal breast cancer.

The comparison of CD146 expression of CD146 and overall patient survival through public database confirms the fact that CD146/ sCD146 are important markers for tumor growth and dissemination, thereby a novel potential target for therapy. Also, confirmed through the review of Dr. Kruijff in univariable analysis showed CD146 expression as a key molecule for prognostic factor of metastatic free survival and overall survival of patients (18). However, in cancers especially TNBC, it is difficult to target CD146 as it is highly expressed in surrounding of cancer cells like whole vascular system (19). Therefore, we hypothesized that targeting sCD146 can be a novel therapeutic target for treating breast cancer.

This hypothesis was justified by effect of sCD146 in CD146high cells sorted from MDA MB 231 cell line. Also, newly generated antibody specifically targeting sCD146 namely M2J-1 mAb was used to counteract the effect of sCD146 on proliferation, migration, invasion, CSC and EMT marker expression of CD146high cells both *in-vitro* and *In-vivo*. Also, in two experimental models- intra-cardiac and sub-cutaneous model of injections the growth and dissemination was significantly reduced for CD146high cells MDA MB 231.

Up until today, effects of M2J-1 are major breakthrough as there are only few therapeutic options to treat TNBC. Immunotherapy has shown to be of recent interest, particularly in combination of atezolizumab (anti-PDL1 antibody) with chemotherapy (20).With this; we speculate that breast cancer can be prevented by targeting CD146 through newly anti-sCD146 mAb M2J-1 to increase the overall survival of women suffering from breast cancer.

The expression level of both the cell population was studied *in-vitro* through Western Blot and ELISA technique. CD146low cell population had lower expression band in western blot as compared to CD146high cell population. Also, in ELISA technique CD146high cell population had higher expression of CD146 then CD146low cell population.

The two sorted cell population CD146high and CD146low cells were examined *in-vitro* for CD146 expression on CSC markers Sox2 and Nanog at mRNA and protein

level. CD146high cells were found to have higher expression of CD146 with thicker band as compared to CD146low cell population. When CD146 was inhibited by using M2J-1, then the expression of Sox2 and Nanog also decreased. This shows that expression of CD146 correlates to the expression of cancer stem cells.

The Epithelial to mesenchymal transition (EMT) markers Vimentin and Slug were also studied on CD146high and CD146low cell population. The CD146high cells had higher effect on EMT markers as compared to CD146low cells. There are several evidences suggesting role of CD146 in cancer cells motility (21). *In-vitro* studies of breast cancer cells have revealed that ectopic expression of CD146 induces EMT process and *In-vivo* expression of CD146 is triggered by upstream signalling thereby inducing EMT. Also, CD146 gene expression is highly observed in TNBC (13).

Also shown by Imbert *et. al.* (21) CD146 affects several molecules and directly or indirectly it induces Slug expression thereby contributing to EMT. There is the possibility that CD146 interacts with Erb by modifying surface expression of Erb83 and Erb84 thus increases chemotherapy. Also the effect of CD146 inhibition by M2J-1 on EMT markers was studied. It was found CD146 inhibition had decreased the expression of EMT markers.

In-vivo study was also performed for the expression of CD146 and its inhibition effect. Intra-cardiac and subcutaneous injections on NOD-SCID mice were used for the study. Lungs from injected intra-cardiac with CD146high and low cells were cut into thin sections and stained with H&E. It was found that CD146high cells had higher peripheral metastasis as compared to CD146low cells, which had higher alveolar metastasis. CD146high cells were injected into mice and treated with M2J-1, when examined were found to have lower metastasis. This concludes that CD146high cells have capacity for metastasis as compared to CD146low cells.

In mice injected with CD146high and CD146low cells subcutaneously, tumors were isolated and examined for CD146 expression and CD146 was found to be highly expressed in CD146high BCSCs, which when inhibited were found to have lower metastasis.

We have studied the correlation of CD146 expression with lymph node metastasis and overall patient survival along with its expression in various breast cancer molecular subtypes. TNBC was found o have higher expression of CD146 in comparison to other subtypes. Lymph node metastasisis is commonly the earliest detectable clinical manifestation of breast cancer before distant metastasis. With CD146 involvement in breast cancer metastasis, its correlation of lymph node metastasis was studied. It was found that breast cancer patients with higher CD146 expression were at greater risk of lymph node metastasis and also there was opoor overall survival of patients with higher cD146 expression.

All the experimental results both *in-vitro* and *In-vivo* shows that CD146 plays major role in both angiogenic processes and cancer stem cell metastasis. Thus, it concludes that CD146 when targeted by using anti-sCD146 in combination with other immunotherapeutic techniques can prevent breast cancer cases and its relapse.

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CHAPTER-7

CONCLUSION

CONCLUSION-

Breast cancer is the most common cancer among women at national and global level, with classical treatment strategies like hormonal, radiation, chemotherapy and advanced therapy like target therapy. With the advanced treatment therapies, there is rise in relapse cases due to several reasons. One such reason is the presence of cancer cells with stem like property called cancer stem cells. This further stimulates the requirement to find better treatment therapy. Targeted angiogenic therapy is the recent advancement in the therapy, where angiogenic factors are targeted through various ways to inhibit angiogenesis and thereby inhibiting tumor growth and metastasis. The CD146 surface molecule is a glycoprotein which plays an important role in angiogenesis, EMT and cancer stemness. CD146 also plays major role in adhesion, migration and invasion of breast cancer cells. Thus, targeting CD146 can inhibit tumorigenesis, epithelial to mesenchymal transition (EMT) and also the stemness of breast cancer stem cells.

Breast cancer stem cells (BCSCs) of MDA MB 231 breast cancer cell line were characterized and found to have varied expression of CD146 as some CSCs had higher CD146 expression while some had lower CD146 expression. So, the BCSCs were sorted on the basis of CD146 expression as CD146low and CD146high. These two populations were then studied for their proliferation, migration and invasion capacity and also *In-vivo*. The BCSCs with higher CD146 expression were highly proliferative, migrating and invasive. Also, the proliferation, migration and invasion capacity was found to reduce when CD146 expression was inhibited by using anti-CD146 monoclonal antibody M2J-1. On the other hand, when CD146 expression was enhanced using sCD146 then the migration capacity was found to enhance greatly and also the expression of EMT markers (Slug and Vimentin), CSC markers (Sox2 and Nanog) were found increased at protein and mRNA levels. When expression of CD146 was inhibited in BCSCs with higher CD146 expression, then there was reduced EMT and CSC expression at protein and mRNA level.

The effect of CD146 was studied *In-vivo* also. The results were found to be similar to that of *in-vitro* study. The CD146high and CD146low BCSCs were found to have varied metastatic ability with CD146low BCSCs having more of alveolar metastasis while CD146high BCSCs had higher peripheral metastasis. This proves that higher expression of CD146 results in rapid metastasis of cancer cells. Also, when CD146 expression was inhibited by using anti-sCD146 monoclonal antibody M2J-1, then the expression of CSC and EMT markers was found to reduce.

These findings suggest that CD146 plays a major role in cancer metastasis by regulating the expression of EMT and CSC markers expression, also the proliferation, migration and invasion of BCSCs which leads to the metastasis of cancer. Higher the migration, invasion and proliferation capacity; higher is the metastatic ability of breast cancer stem cells.

Therefore, inhibition of CD146 can lead to the regression of breast cancer and can also prevent the relapse of breast cancer cases when used in combination to other therapies like chemotherapy or radiation therapy.

CHAPTER-8

LIMITATIONS AND FUTURE PERSPECTIVE

This was an original approach which was supported by observing effects of sCD146 on CD146high MDA MB 231 BCSC cells. In this work, we have taken the advantage of recently generated antibody M2J-1, which specifically target CD146. We have witnessed the counter effect of M2J-1 against CD146 on *in-vitro* assays like proliferation, migration and invasion as well as on CSC and EMT markers both *in-vitro* and *In-vivo*. Also, in two animal models (sub-cutaneous and intra-cardiac), M2J-1 was able to reduce growth and dissemination of CD146high MDA MB 231 BCSC cells. With reported effects of M2J-1 on TNBC, we speculate that targeting CD146 in combination of therapies with newly developed mAB M2J-1 can prove to be of therapeutic benefit to prevent TNBC dissemination and overall patient survival.

Like with many benefits, this study comes with some drawbacks. We have conducted this study only on one breast cancer cell line i.e. MDA MB 231; with higher CD146 expression. Also, in order to implement this targeting approach at clinical level, we need to first confirm the results on primary tissues isolated from patients with breast cancer subtypes. We recommend carrying out same experimentation on different breast cancer cell lines like MDA MB 157, SUM 221, CAL51.

This study of targeted approach to treat breast cancer has shown favorable outcomes for treatment strategy of breast cancer subtypes. With increase in breast cancer relapse cases, this targeted approach towards targeting CD146 in combination of other treatment therapies can prove to be a milestone to save patients.

CHAPTER-9

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RECOMMENDATION

9.1 RECOMMENDATION-

Breast cancer is the leading cause of cancer death among women around the world and second most prevalent cancer worldwide. This alarming increase in cases of breast has raised the concern regarding treatment strategies of breast cancer around the globe. Several treatment methods are being used for breast cancer like radiation therapy, chemotherapy, hormonal therapy, targeted drug therapy etc. But all these treatment methods are ineffective and lead to the relapse of breast cancer. This relapse is leading to poor patient survival rate and thus raises concerns over evolution of treatment methods. In subsequent years of research by several scientists around the world have found new target approach to treat breast cancer like correlation of angiogenesis with breast cancer metastasis and the molecules involved in regulating both the processes. Also, presence of cancer cells with stem like properties in tumor microenvironment has lead to the opening of new possibilities of treatment of BC.

CD146, a transmembrane glycoprotein has been found to be involved in both angiogenesis and cancer stem cell metastasis. CD146 is found in two forms i.e. soluble and membrane bound. Membrane bound CD146 can be targeted easily but targeting soluble CD146 (sCD146) is a daunting task. Recently a new humanized monoclonal antibody was developed against sCD146 named M2J-1 which targets specifically sCD146 and leads to the decrease in proliferation, migration and invasion capacity of BCSCs.

In this study, we have used triple negative breast cancer (TNBC) cell line MDA MB 231 as CD146 is highly expressed in TNBC. Also, therapeutic approach lacks in case of CD146 in TNBC which makes it more of interest to work on MDA MB 231 TNBC cell line. Upon flow cytometric characterization, the breast cancer stem cells (BCSCs) were found to have varied expression of CD146 as high and low. We have sorted the BCSCs of MDA MB 231 on the basis of CD146 expression as CD146high and CD146low cells. These sorted CD146high/low cell population is studied for their proliferation, migration and invasion capacity. Also, the effect of CD146 on proliferating, migrating and invasive properties was studied further by inhibiting the expression of CD146 using humanized monoclonal anti-sCD146 antibody M2J-1.

This resulted in the decrease in proliferation, migration and invasion capacity of the BCSCs. *In-vivo* models also showed similar results for CD146high and CD146low cell population and when inhibited using M2J-1 mAb. Also, EMT and CSC markers were studied for both *In-vivo* ad *in-vitro* and showed the relation between angiogenesis and breast cancer stem cells.

These finding suggest the significant role of CD146 in tumorigenesis and breast cancer metastasis as it regulates several properties involved in maintain stemness of breast cancer.

8.2 Summary of the research work

In our thesis, we have given various experimental evidences showing C146 as a link between tumorigenesis and metastasis of breast cancer. The chapter wise conclusions of the research work carried out and summarized below are

Chapter 1 and Chapter 2-

These chapters produce the introduction and review of literature for breast cancer and its epidemiology at global and national level. Then, a brief history of breast cancer is given dated since 3,000–2,500 B.C.E, the time of earliest recorded case of breast cancer by Adwin Smith Papyrus. A brief research work of various scientist in linking breast cancer and angiogenesis is given and then the various scientific finding related to CD146 in breast cancer stem cell is also given. Thus, brief introduction of contribution of various scientists towards CD146, breast cancer, angiogenesis, breast cancer stem cell and breast cancer subtype is given.

Chapter 3-

It provides the aim and objective of the study. There are four objectives of the study. First objective deals with the flow cytomteric characterisation of BCSCs of MDA MB 231 for cancer stem cell (CSC) markers and CD146 expression. The second objective deals with comparison culture, characterization and sorting of CD146

expressing BCSCs from MDA MB 231. The second objective deals with the study of differential expression of CD146high and CD146low cells at RNA and mRNA level. The third objective deals with the *in-vitro* study of CD146high/low cells and effect of inhibition of CD146. The fourth objective deals with *In-vivo* study of CD146high/low cells along with the effect of inhibition of CD146. The fifth objective includes biostatistical analysis of CD146 expression with overall patient survival and breast cancer subtypes.

Chapter 4

It gives an insight to the material used for performing the experiments and the techniques used in performing the experiments. The technique section includes the various assays and processes used to get the results. The techniques give a brief glance on their respective principle and mechanism to give a better understanding for the results. It also gives an insight into steps taken to perform the experiments. This chapter gives detailed steps for the culture, characterisation, sorting, *in-vitro* assay and *In-vivo* assays. This chapter also includes the detailed steps showcasing the inhibition of CD146 in *in-vitro* and *In-vivo* models of experiment.

Chapter 5-

It includes the results of both *in-vitro* and *In-vivo* models. The BCSCs were studied for CD146 expression and the effect on various properties with varied CD146 expression. The BCSCs with higher CD146 expression had higher proliferative, migrating and invasiveness. Also, the cancer stem cell markers (Sox2 and Nanog) and EMT (Vimentin and Slug) markers were highly elevated with higher CD146 expression and thus implying the significant role of CD146 in regulating CSC and EMT markers expression in maintain cancer stemness of breast cancer cells. The role of CD146 in maintaining cancer stemness was further confirmed by inhibiting CD146 expression by using humanised monoclonal antibody M2J-1. IT was found to have decreased proliferative, migratory and invasive properties along with lower EMT and CSC markers expression of BCSCs.

The role of CD146 was further studied *In-vivo*. In mice injected with CD146high and CD146low cells subcutaneously, tumors were isolated and examined for CD146 expression and CD146 was found to be highly expressed in CD146high BCSCs, which when inhibited were found to have lower metastasis. Also, the EMT and CSC markers expression was higher in CD146high cells injected mice.

All the experimental results both *in-vitro* and *In-vivo* shows that CD146 plays major role in both angiogenic processes and cancer stem cell metastasis. Thus, making CD146 a potential target for treating breast cancer via angiogenesis therapy and metastatic targeting too.

Chapter 6-

It discusses the outcome of the results obtained from various experiments. It also covers the previous findings in relation to the CD146 and tumorigenesis in breast cancer stem cells. The previous findings were found to be in support of the results obtained for CD146high/low cells for tumorigenesis and breast cancer metastasis.

8.3 Future scope

Our study has found CD146 to play a major role in regulating cancer stemness and tumorigenesis in TNBC. The same study can also be carried out primary breast tumor tissues, wherein breast tumor tissues can be isolated from the patients and processed to isolate cancer stem cells. These isolated cancer stem cells could be studied for *in-vitro* and *In-vivo* examinations. The same study can also be carried out on other breast cancer cell lines like CAL51, SUM229. We have started work on MDA MB 157 breast cancer cell line and some of the *in-vitro* results are similar to the findings of MDA MB 231 breast cancer cell line.