ABSTRACT

Breast cancer related mortalities are currently on the rise and hence the need for model therapeutics to improve survival and quality of life in patients is a prime necessity. Much of the prognosis and tumour behaviour in patients are controlled by the cancer stem cells, which ultimately leads to chemotherapeutic resistance and disease recurrence. MiRNA are short non-coding RNAs with potential roles in carcinogenesis and mRNA silencing. miRNA-24 could be a probable regulator of epithelial to mesenchymal transition (EMT) mediated via Klf-8 and Notch-1. However, the exact mechanism for miR-24, Klf-8 and Notch-1 mediated EMT remains obscure.

EMT is a hallmark of highly metastatic and devastating tumours and reversing the process of EMT could be a potent strategy towards developing favouring therapeutics in high-grade breast carcinomas. However, it is necessary to optimally deliver miRNA inhibitors such as siRNAs (small interfering RNAs) to the afflicted cells in order to reverse or limit the process of EMT. Liposomes and alginate-chitosan gels could be potential candidates owing to their nano dimensions and the probability of using these nano particles for combinatorial therapy remains an august possibility. Nevertheless, further experiments shall be needed first to establish a concrete mechanism and then to synthesize an optimal vector for siRNA trafficking. Dual targeting of tumour cells could then be possible with two tier liposome-alginate nanostructures

Under the scope of this thesis, an attempt has been made to identify and delve one such probable EMT modulating miRNA, miR-24 in breast cancer. In addition to this liposomes and gels have been fabricated, which could in future serve as potential candidates of two tier architecture for drug delivery in breast cancers.

Keywords: Breast cancer, EMT, miR-24, Notch-1, Liposome, combinatorial therapy, nano structures

CHAPTER 1: INTRODUCTION

Breast Cancer is the most common nondermatologic cause of death among women worldwide. The year 2011 alone reported approximately 1.38 million cases of breast cancer incidence worldwide (Lamond and Younis, 2014) There were approximately 2,32,340 cases of invasive breast cancer (IBC) and 64.640 cases of ductal carcinoma in situ (DCIS), that were reported across US (United States) in the year 2013 alone. Additionally, it was surmised by the American Cancer Society that there would be close to 40,000 breast cancer related deaths in 2013 (Advani and Moreno-Aspitia, 2014). Breast cancer therefore remains the leading cause of mortality after lung cancer. The current lifetime risk of a woman developing breast cancer in the US is estimated to be one in eight (12.3%), which is a level higher as compared to the one in eleven (9.09%) lifetime risk in the 1970s. HER-2(human epidermal growth factor receptor), is a transmembrane protein which is expressed in 20-25 % of breast cancers and is associated with poor prognosis in women with both node-positive and node-negative disease. (Mally and Macrae, 2014; Sawaki, 2014).

Triple Negative Breast Cancer (TNBC) is the most deleterious form of breast cancer. These tumours lack the expression of ER (estrogen receptor), PR (progesterone receptor) and HER-2 (human epidermal growth factor receptor), thus making it the most aggressive breast tumor subtype with poor prognosis. Due to the absence of these receptors, the tumour volume is non-responsive to many conventional hormonal therapies that use drugs such as Trastuzumab, Lapatinib etc. Current modes of treating TNBC rely heavily on the use of DNA damaging agents such as Carboplatin and Taxanes but surprisingly without many benefits. Although dramatic advancement has been made towards achieving TNBC cure, drug resistance continues to baffle both scientists and clinicians (Olsauskas-Kuprys et al., 2013). Heterogeneity and drug resistance have strongly been associated with the population of stem cells existing within the tumour mass and a recent study by Azzam et al. suggested that the operation of highly active membrane transporters and an enhanced DNA repair mechanism altogether contributes towards maintaining and replenishing stem cell population following therapy. In addition, Cancer Stem Cells (CSCs) induced resistance may also be due to the misregulation of certain stem cell inherent pathways such as Hedgehog, Notch and Wnt (Aamir Ahmad, 2013).

Tremendous effort in recent years of oncology research has been directed at identifying novel mechanisms to control and/or eliminate this group of cells known as CSCs or tumour initiating cells (TICs). A panel of prognostic features such as tumour initiation, growth, metastasis, disease recurrence, resistance to conventional chemo and radio therapies, EMT (epithelial to mesenchymal transition) (Wang et al., 2013; Garner et al., 2013; Azzam et al., 2013; Han et al., 2013; Routray and Mohanty, 2014), has been strongly ascribed to this population of cells. Additionally, these cells have been held culpable of fostering heterogenic lineages and controlling numerous aspects of carcinogenesis. Nevertheless, it is strictly in agreement with the CSC hypothesis stating that; only CSCs are competent enough to initiate tumour, self renew and differentiate. (Han et al., 2013; Adamowicz et al., 2013). Tumour masses harbour cohorts of these unique constantly multiplying cells and as a result have drawn considerable interest from the research bevy in recent years in order to identify targeted therapies against CSCs. Despite, numerous advancements in this area, researchers have failed to improve the long-term life expectancy and QOI (Quality of Life) in patients. As a result, the future of such modalities hangs on morbid conjecture and fragile hopes.

Signalling is a key functional event that ultimately decides the phenotype and fate of the given cell. Notch is a highly conserved signalling pathway that has an important role in embryonic development as well as in maintenance of both normal and CSCs (Moeini et al., 2012; Espinoza et al., 2013; Giachino and Taylor, 2014). It is activated dynamically during evolution and plays a major role in cell differentiation during embryonic development (Brzozowa et al., 2013). In Cancer, Notch is misregulated and functions together with various other cancer critical proteins and pathways (Zhu et al., 2013; Da Ros et al., 2013; Buckley et al., 2013; Kim et al., 2013; Wang et al., 2013) to web out what is known as the, Notch Interactome. This interactome in turn orchestrates numerous CSC fate decisions and disease phenotypes during carcinogenesis. Aberrant Notch signalling is a hallmark of solid tumours such as that of cervix, colon, liver, lung and pancreas (Olsauskas-Kuprys et al., 2013). Interestingly, Notch pathway is cell context dependant and has widely been regarded to have both oncogenic and tumour suppressive role (Kuang et al., 2013). Emerging evidences strongly suggest that analysing and targeting critical modules in the pathway might be of therapeutic interest towards controlling the fate and existence of CSCs.

MicroRNAs (miRNAs/miRs), are 18-24 nucleotides short non-coding RNAs that control gene expression mostly at transcriptional and sometimes at translation level. These single stranded RNAs reportedly control the expression of about one-third or 30% of all protein-coding genes (Roy et al., 2012; Choi et al., 2013). Interestingly, many of those genes are key components of the Notch signalling cascade, either directly or indirectly. MiRNA coding genes are mostly located in the region of the genome most vulnerable to Cancer related mutations (Fragile sites). Therefore, it is not surprising that miRNAs control a number of oncogenic processes such as invasion, metastasis, apoptosis, differentiation, angiogenesis etc during cancer (Yan et al., 2012; Brzozowa et al., 2013).

The future of breast cancer therapies depends on the researchers ability to adjust actions to circumstances and have a clear projection relating to the deviant mechanisms that might decide numerous events during tumorigenesis. The miRNA/Notch axis presents us an interesting avenue to exploit in breast cancer samples. Impairing critical stages of the Notch pathway by means of miRNAs can solve the *"targeted therapy crisis"* problem in breast cancer.

The potential of liposomes for delivering anticancer drugs have been investigated extensively in different cancer types. Especially the chemotherapeutic drug doxorubicin has received much attention, since it is easy to load high amounts of this drug into liposomes (Drummond et al., 1999). In fact, commercially available untargeted liposomes loaded with doxorubicin, marketed under the name Caelyx®, have already been approved for clinical use in several cancers (Lammers, 2008). Untargeted liposomal formulations of doxorubicin benefits especially from the improved toxicity profile, but have also demonstrate some improvements in therapeutic efficacy compared to the free drug (Gabizon et al., 2003). So far, targeted liposomes have only been investigated in animal models of human cancers, where targeting of liposomal drugs to the cancers cells or the tumor vasculature have demonstrated a more pronounced inhibition of tumor growth than administration free drug or untargeted liposomes (Sapra and Allen, 2003). Some recent publications employ a combination of several liposome types or conjugate a number of different targeting molecules to the surface of a single liposome, in order to achieve a more efficient tumor targeting than with only one targeting agent. Several of these studies indeed demonstrated a synergistic effect compared to only using one targeting agent (Pastorino et al., 2006; Saul et al., 2006).

CHAPTER 2: BACKGROUND AND LITERATURE

2.1 Breast Cancer

Breast cancer is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk (Sariego, 2010). Cancers originating from ducts are known as ductal carcinomas, while those originating from lobules are known as lobular carcinomas. Breast cancer occurs in humans and other mammals. While the overwhelming majority of human cases are in women, breast cancer can also occur in men (US NIH: Male Breast Cancer). The balance of benefits versus harms of breast cancer screening is controversial. The characteristics of the cancer determine the treatment, which may include surgery, medications (hormonal therapy and chemotherapy), radiation and/or immunotherapy (Florescu et al., 2011). Surgery provides the single largest benefit, and to increase the likelihood of remission, several chemotherapy regimens are commonly given. In addition, radiation is used after breast-conserving surgery and substantially improves local relapse rates and in many circumstances overall survival (Buchholz, 2009).

2.1.1 Breast Cancer Statistics

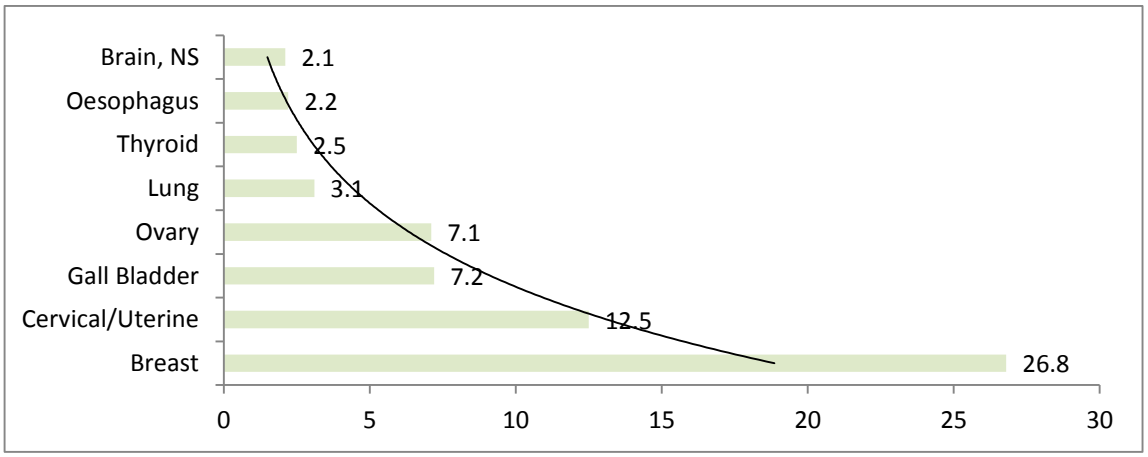


Figure 1. Incidence rates of various cancer types in New Delhi (2008-2009). *(www.breastcancer.india)*.

Table 1. Cancer deaths worldwide (2012). Source: World Health Organisation (WHO).

Cancer Type	Mortality
Lung	1.59 million deaths
Liver	7,45,000 deaths
Stomach	7,23,000 deaths
Colorectal	6,94,000 deaths
Breast	5,21,000 deaths
Oesophageal	4,00,000 deaths

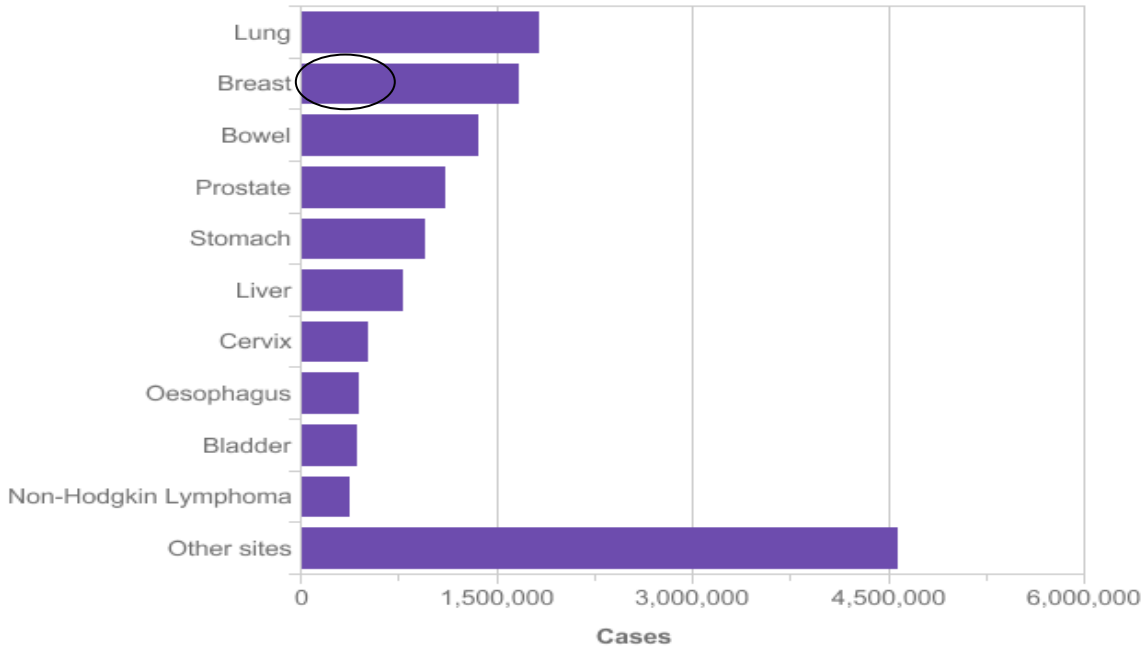


Figure 2. The 10 Most Commonly Diagnosed Cancers, World, 2012 Estimates. Original Data Source: Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F.GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide, IARC CancerBase No. 11 [Internet].Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on 16/01/2014.

2.2 Disease classification

Based on the expression patterns of ER (estrogen receptor), PR (progesterone receptor) and HER-2, breast cancer can be classified into luminal A, luminal B, HER-2$^+$ and basal subtypes (Olsauskas-Kuprys et al., 2013) **(see Table 2 below)**.

Table 2. Cell surface marker expression profile of Breast Cancer subtypes (Schnitt, 2010)

Subtypes	Marker Profile
Luminal A	ER+ and/or PR+, HER2−
Luminal B	ER+ and/or PR+ and HER2+ (luminal-HER2 group)
	ER+ and/or PR+, HER2−
HER2	ER−, PR−, and HER2+
Basal-like/ Triple (-ve)	ER−, PR−, HER2−, and CK5/6 and/or EGFR+

Abbreviations: CK, cytokeratin; EGFR, epidermal growth factor receptor; ER, estrogen receptor; PR, progesterone receptor.

2.3 Notch Signalling Pathway

Notch ligands Jag-1, Jag-2, Dll-1, Dll-2, Dll-3 and Dll-4 on engagement with the Notch (Notch 1, 2, 3 and 4) receptors **(Fig. 3)** triggers the cascade **(Fig. 4)**. The ligand-receptor interaction brings about a

conformational change in the receptor, prompting a metalloproteinase (ADAM-10/17) mediated cleavage and ectodomain shedding of the receptor thus generating the Notch C-terminal fragment.

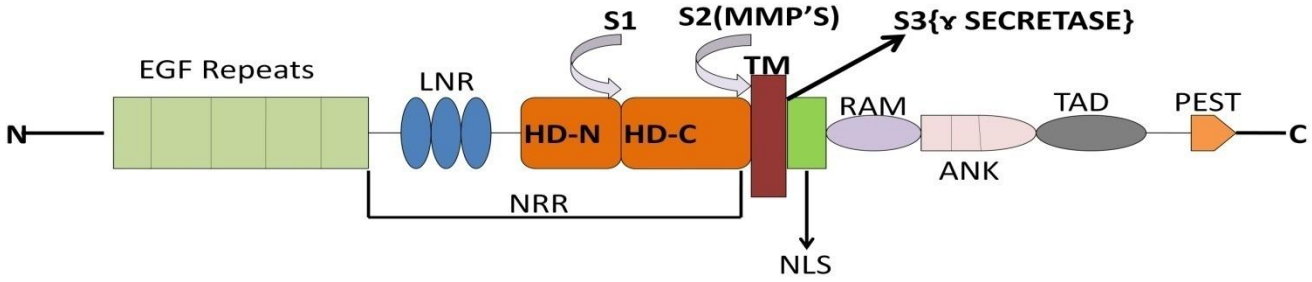


Figure 3. Structural Organisation of the Notch receptor. S2 and S3 represents two cleavage sites for Matrix metalloproteinases and gamma secretase respectively in the receptor, which marks crucial events during transmission of Notch signals. Cleavage at the S1 site generates the amino and carboxy terminal domains. LNR=Lin12/Notch repeats; NRR= Negative Regulator Region; NLS= Nuclear Localisation Sequence; RAM= region that mediates interaction with Lag-1; ANK= Ankyrin repeats; PEST= proline, glutamic acid, serine and threonine rich.

This Notch C-terminal fragment (NEXT), while still tethered to the membrane serves as the substrate for the Gamma secretase mediated second cleavage. Following this second cleavage, a membrane free and transcriptionally active Notch Intracellular Domain (NICD) is released and subsequently trafficked into the nucleus aided by import proteins. Once into the nucleus, NICD, CSL (CBF1/Su (H) /LAG1) and a representative of the mastermind family of proteins unites to form the DNA interacting complex. This complex in turn controls the expression of a panel of genes (LaVoie and Selkoe, 2003; Zhao and Lin, 2012; Purow, 2012; Murta et al., 2013; Greenwald and Kovall, 2013).

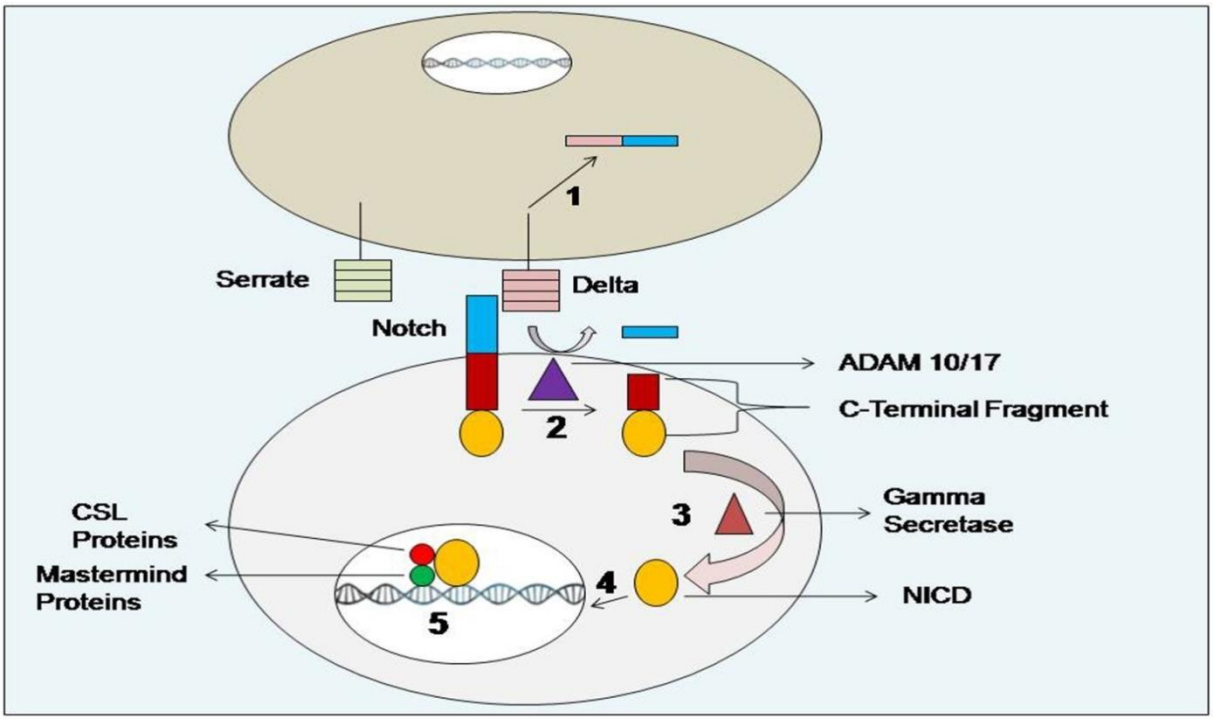


Figure 4. The Five step Notch signalling cascade. (1) Initial Receptor-Ligand interaction is followed by endocytosis of the ligand (Delta/Serrate) and a part of the extracellular domain of the Notch receptor (Blue). (2) Cleavage by ADAM 10/17 (Purple) results in shedding of the remnant extracellular receptor domain (Blue). (3) Gamma Secretase (Brown) mediated second cleavage of the Notch C-terminal fragment generates the Notch Intracellular Domain, NICD (Orange). (4) The NICD is imported into the nucleus. (5) NICD once into the nucleus forms a complex with CSL (Red) and Mastermind (Green) family of proteins and controls transcription of target genes.

2.4 Gamma Secretase: Scissors of the Cell Membrane

Gamma Secretase, are indiscriminate membrane tethered aspartyl proteases. These are crucial components of the Notch signal propagation pathway. Gamma Secretase participate in the regulated intramembrane cleavage of the Notch C-terminal fragment, in order to generate the Notch Intracellular Domain (NICD). Presenilin (PS), is the catalytic component of the Gamma Secretase protein and together with other accessory proteins such as APH-1, Nicastrin and PEN-2 forms the Gamma Secretase holoprotein (Selkoe and Wolfe, 2007; Endres and Reinhardt, 2013).

2.4.1 Gamma Secretase inhibitors of Notch signalling

It is difficult to target Notch components using inhibitors, as most of the pathway components lack or show limited enzymatic activity. However, two crucial steps in the pathway require enzyme participation and hence provide "windows for interference". One such event is the second cleavage **(Step 3, Fig.4)**, mediated by Gamma Secretase. As a result, Gamma Secretase Inhibitors (GSIs) were identified and are constantly being used as elements of Notch inhibition. Interestingly, GSIs are the only Notch inhibiting compounds that are currently in clinical trials (Purow, 2012).

2.4.2 Gamma Secretase Inhibitors: Potential drawbacks

Despite, such advancement the success of GSIs has been encumbered by adverse effects arising in the body **(Fig. 2f)** that eventually prevent GSIs from being established as the leading *"therapy of choice"*, thus prompting the need for an alternative regime. The most baffling problem to address with the usage of GSIs is the specificity issue owing to a number of other Gamma Secretase substrates such as Jagged, Delta, APP, N-cadherin, E-cadherin, CD44, Nectin-1-alpha, ErbB4, LRP, Syndecan-3, P75 NTR, ApoER2, and DCC. This makes specific targeting of Notch with GSIs a difficult proposition, as the desired specificity level is not achieved. Another crucial drawback with GSIs inhibitors are that they are unable to choose between normal and Cancer stem cells, as a result they brazenly attack Gamma Secretases in both types of cells, thus disrupting other crucial functionalities of Notch signalling in normal cells. This leads to unwarranted gastro-intestinal toxicity and immunosuppression (Selkoe and Wolfe, 2007; Purow, 2012). Most importantly, GSIs cannot impair the effect of the Notch cascade in cells with activated Sonic Hh cascade because the Notch downstream gene, Hes-1 is also a target for the Sonic Hh cascade (Andolfo et al., 2012).

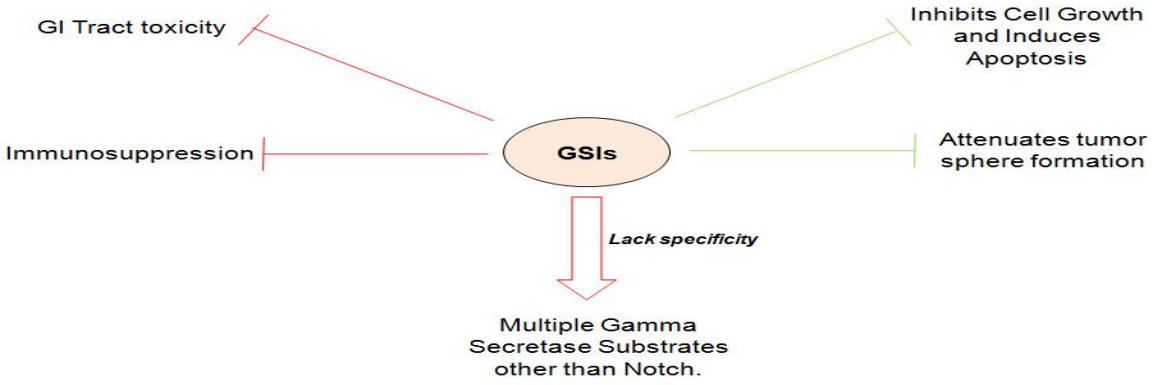


Figure 5. Role and Potential Drawbacks of GSIs (Selkoe and Wolfe 2007; Azzam et al. 2013; Groth and Fortini 2012; Ma et al. 2013). Red, Negative Role; Green, Positive role.

2.5 Notch: The cancer stem cell master switch

Notch signalling is widely regarded as a stem cell master switch. Influence of Notch signalling on a particular cell is vastly diverse and is dependent upon cell commitment, lineage commitment and timing. Activated Notch signalling is regarded as a hallmark of many aggressive Cancers (Aamir Ahmad, 2013; Mu et al., 2013). Several studies reported the misregulated status of Notch signalling across different Cancer types. The mis regulated cascade plays critical role in EMT (epithelial to mesenchymal transition), tumour neovascularisation, angiogenesis, disease progression, tumour onset, tumour migration, tumour invasion, apoptosis, colony formation, and in development, renewal and maturation of tumour stem cells (Sethi and Kang, 2012; Yan et al., 2013; Zhou et al., 2013; Zhu et al., 2013; Alamgeer et al., 2013).

2.5.1 Role of Notch in breast cancer pathogenesis

Numerous studies have reported the role of mis regulated Notch cascade in breast Cancer Pathogenesis. Notch 1 and 4 are regarded as bonafide breast Cancer genes in addition to tumour suppressive BRCA-1. Notch 4 and Notch-1 shows four and eight fold higher expression respectively in Breast Cancer stem cells. PEA-3 (ETS family of transcription factors) controls the transcription of Notch1- and Notch-4. The action of PEA-3 on Notch-4 promoter is dependent upon c-jun and Fra-1 but negatively regulated by c-Fos. BRCA-1 can control Notch signalling by activating it. The control of BRCA-1 on Notch 1-3 is independent of p53 and may be dependent on GATA-3 whereas BRCA-1 effect on Jag-1 is mediated by p63 (Aamir Ahmad ,2013; Olsauskas-Kuprys et al., 2013; Buckley et al., 2013). This is a classic example of collaboration between Notch and BRCA-1 in deciding the fate of breast cancer cells. Role of Notch-4 in angiogenesis is unknown while the participation of Notch-1/Dll-4 axis in angiogenesis is well documented and blocking the axis leads to inhibition of tumour growth due to non-productive angiogenesis. However, as reported mammary vasculature develops normally in the absence of Notch -4 thus suggesting that the potent oncogene, Notch-4 might play a role in breast tumour angiogenesis (Costa et al., 2013).

A critical event during any malignant transformation is epithelial to mesenchymal transition, which is marked by expression of mesenchymal markers such as N-cadherin, Vimentin, fibronectin etc and loss in epithelial cell-cell junction marked by decrease in expression E-cadherin. Snail, TWIST and ZEB acts as transcriptional repressor of E-cadherin by binding to targeted consensus E boxes, thus promoting EMT. Snail activity is required for EMT initiation while Twist plays a role in EMT maintenance. Another transcription factor, Klf-8 plays a crucial role in promoting EMT. Interestingly, Klf-8 acts through miRNA-146a and Notch to exert its dominant effect on EMT in Breast Cancer (Aamir Ahmad, 2013; Jian et al., 2013; Wang et al., 2013).

Mammary stem cells continually self renew and replenish their population that results in enhanced metastatic ability of tumours and other signature phenotypes observed with CSCs. Notch again mediates critically in the process. Hyperactivated Notch 1 in $CD44^{+}CD24^{low}$ breast Cancer stem cells activates Sox-2. Sox-2 contains multiple Notch consensus target motifs and together with Notch 1 promotes self-renewal of $CD44^{+}CD24^{low}$ cells (Azzam et al., 2013). As mentioned earlier, Notch interacts with several other critical proteins and pathways in order to modulate tumorigenesis. PI3k/AKT/mTor pathway is constitutively active in Breast Cancer. It is associated with cell survival, proliferation, migration,

metabolism, angiogenesis and apoptosis. Most significantly, this pathway is associated with ER (-ve)/PR (-ve) status in triple (-ve) breast cancer. AKT pathway in turn can result in NF-κB activation via Serine phosphorylation of IKK. Notch signalling in Breast epithelial cells induces an autocrine loop that in turn activates AKT. In Triple (-ve) breast cancer cytoplasmic Notch-1 has been correlated to cytoplasmic pAKT, suggesting some kind of direct or indirect interaction (Zhu and Bhaijee et al., 2013). However, the exact mechanism by which Notch activates AKT pathway and subsequently NF-κB is still unknown. Similarly, activation of Notch-1 by Jag-1 was found to be promoting Breast Cancer progression through up regulation of uPA (urokinase -type plasminogen activator) signalling (Aamir Ahmad, 2013). Notch being a juxtracrine signalling pathway (cell-cell contact) shows relationship with the EGFR pathway. It must be noted that Notch-1 signalling activity increases following estrogen exposure in Breast Cancer stem cells and it may along with EGF act as paracrine mediators of estrogen effect on CSC activity (Harrison et al., 2013) .

The role of protein kinase cannot be ignored with respect to Breast Cancer pathogenesis. PKCα promotes steady state expression of Notch-4 protein. Thus, Tamoxifen resistance in ER (+ve) breast tumours is mediated by PKCα possibly though Notch-4. The promoter for the Notch-4 gene has AP-1 (Activator Protein) binding sites and PKCα needs to be present along with AP-1 at the promoter region. PKCα over expression causes AP-1 mediated induction of Notch-4 thus promoting Tamoxifen resistance in ER (+ve) Breast Cancer (Yun et al., 2013).

2.6 MicroRNAs: The non coding RNAs

MicroRNAs (miRs/miRNAs) are known to regulate about one-third of all protein-coding genes. Primarily, they influence gene expression by either promoting mRNA translation or degradation in a cell specific manner (Choi and Choi, 2013). The expression prototype of miRNAs is cell context dependant and can array from being tumour suppressive to oncogenic. It is interesting to know that, miRNA coding genes are housed in the region of the genome most vulnerable to cancer related mutations. Therefore, it would not be hard to imagine the tremendous capability of these short RNA stretches, in regulating a panel of cellular processes such as metastasis, invasiveness, cell cycle, apoptosis, angiogenesis differentiation and cell signalling during carcinogenesis (Yan et al., 2012; Fragoso et al., 2012; Fan et al., 2013; Tessitore et al., 2014).

2.6.1 Processing of miRNAs

Approximately, 70 nucleotides long polycistronic nascent transcripts (pri-miRNAs) are generated at the start from the Pol II transcribed miRNA gene cluster that then undergoes an initial stem-loop processing by a RNA-specific endoribonucleases, Drosha or Pasha. Drosha processing releases the truncated precursor miRNAs (pre-miRNAs) in the nucleus, which is then channelled out of the nucleus by Exportin-5. In the cytoplasm, pre-miRNAs undergoes a second processing by Dicer (double-stranded RNA-specific ribonuclease), in order to generate the double stranded mature miRNAs. One of the strands of the miRNA is then degraded and the other is loaded onto the RNA Induced Silencing Complex (RISC), where they bind to the specific 2-8 nucleotides 3’ UTR sequences of the target mRNAs and regulate gene expression. Argonaute proteins are the catalytic components of the RISC.(Lee et al., 2002; Tomankova et al., 2010; Fan et al., 2013; Vislovukh et al., 2014).

2.6.2 miRNA mediated Notch aberration

As mentioned earlier, miRNAs are unique components of the human genome due to their widespread ability to control the expression of a large panel of genes and it is not surprising that many of the genes are indeed components of the Notch signal transduction or at least influence the signal propagation in some form of the other. MicroRNAs, binds to the 3' UTR sequences of the mRNA targets and control their expression.

The question that arises now is; can miRNAs be pursued further as probable candidates of therapy in Breast Cancer? Numerous research reports supported this assumption and revealed an insight into the role of these short RNAs in modulating the Notch interactome and hence disease prognostic features.

2.6.3 The class of 34s

miRNA-34s are a family of microRNAs that are reportedly misregulated across different types of Cancer. Their tumour suppressive behaviour has been reported in Choricarcinoma (Pang et al., 2013), Prostrate Cancer (Kashat et al., 2013), Colon Cancer (Roy et al., 2012), Osteosarcoma (Yan et al., 2012), Glioma (Wu et al., 2013), Medulloblastoma (de Antonellis et al., 2011). These same studies highlighted the potential role miR-34 members modulating proliferation, invasion, apoptosis, self-renewal, growth, metastasis, and in reducing aggressiveness of tumour masses. Notch components such as Jag-1, Dll-1 Notch-1 and Notch-2 are potential targets of miRNA-34a (Pang et al., 2013).

MicroRNAs play a pivotal role in maintaining stemness of Breast Cancer stem cells (BCSCs). Mature microRNA-34 members' 34b and 34c are reportedly down regulated in BCSCs as compared to differentiated cells. Notch-4 is a target of miR-34c in BCSCs. Normal expression status of miR-34-c in BCSCs impairs EMT, self-renewal capacity and mammosphere forming ability, probably by targeting Notch-4 (Yu et al., 2012).

2.6.4 Epigenetic regulation of miRNA-34 members

DNA methylation is an epigenetic marker that undergoes dynamic modifications during stemness. Silencing of miRNA-34 members has been attributed to the methylation status of the promoter. Primarily, hypermethylation of the CpG islands near the promoter site has been the driving force behind reduced miRNA-34 expression in tumours. However, there is a strong possibility that an alternate mechanism might also be in operation (Roy et al., 2012, Kashat et al., 2013, Yu et al., 2012).

It is clear from the above discussion that understanding the collaborative action of epigenetic modification such as methylation and acetylation with other participating proteins may be helpful in reversing the aberrant miR-34 expression. This indicates towards the combined involvement of histone methyl transferases, histone deacetylases and the Polycomb group of proteins. Polycombs are functionally responsible for the remodelling of the chromatin complexes so that epigenetic silencing of the genes can take place. Polycomb proteins plays crucial role in stem cell maintenance, differentiation, proliferation and development. Emerging evidences suggests the role of EZH2 (histone methyl transferase) in Notch mediated tumorigenesis. EZH2 (Enhancer of Zeste Homolog-2) is an enhancer of the Polycomb proteins. EZH2 also play a role in angiogenesis and it is over expressed in variety of tumours. Reduced expression

of EZH2 leads to decreased cell growth, migration and clonogenicity of tumours possibly mediated by Notch-1 (Bao et al., 2012). Interestingly, Notch-1 is also a target of the miR-34 family .

Till date, no such data on the EZH2/Notch-1/miR-34 regulatory axis has been reported in Triple (-ve) Breast Cancer. Exploring the potential signalling loop in Triple (-ve) Breast Cancer may be a curious prospect towards controlling the population of BCSCs.

2.6.5 Notch and miRNAs: EMT modulators in Breast cancer

EMT (epithelial to mesenchymal transition), is a key phenotypic event during the evolution of highly invasive and metastatic tumour forms. Hence, to be able to reverse the process of EMT would be a giant stride towards limiting the metastatic behaviour of any aggressive Cancer. miRNA-200 family plays a crucial role in EMT, assisted by ZEB-1 and Notch. ZEB-1 is a mesenchymal marker that operates in a feedback loop and indirectly activates Notch by down regulating miR-200 expression (Brabletz et al. 2011). This is in parallel to the earlier discussion wherein the role of miRNA-146 and Klf-8 was discussed as possible mediators of EMT in Breast Cancer. Both studies have one thing in common though that Notch indeed is a key regulator of EMT in highly metastatic Breast tumours.

2.7 E3 Ligase and Notch functionality

E3 ligases are widely being studied in order to identify and validate their position as diagnostic markers in Cancer. Ubiquitination is a mechanism of tagging proteins for identification and degradation by the Proteasome. The ubiquitin proteasome system comprises of three distinct enzymes that tags ubiquitin molecules to lysine residues of the target proteins in order for them to be engulfed and degraded into small peptide residues by the 26s proteasome E3 ligases are ligating enzymes that comprises of two binding domains, one each for substrate and E2 (Ubiquitin conjugating enzymes) respectively. These ligases transfers Ubiquitin molecules from E2 and bonds them to the protein substrate. A repetition of the process generates a polyubiquitin chain (Kumar et al., 2013).

E3 ligase plays a crucial role in Notch signalling by assisting in the degradation of both receptor sand ligands. The C-terminus of the Notch receptor architecture comprises of PEST domains. PEST contains degradation signals called Degrons, that stabilises the Notch intracellular domain (NICD) in the nucleus and hence degradation by E3 ligases. Cdk-8 and GSK3β initiate phosphorylation of the PEST domain. Polyubiquitination of PEST is then carried out by E3 ligases such as Itch-3, Numb and FBW7 (Olsauskas-Kuprys et al., 2013).

FBW7 is a component of SCF (Skp-1, Cul-1, F-Box) type E3 ligases. FWB7 might play a crucial role in lateral inhibition. Notch, c-Myc, Cyclin E are common targets of SCF type E3 ligases. It is interesting to note here that FBW7 plays a crucial role in Intestinal tumour formation. FBW7 heterozygosity results in human colorectal tumours possibly via Notch (Sancho et al., 2013). The effect of lateral inhibition in conjunction with FBW7 sounds like an interesting domain for further research in TNBC cells. Surprisingly, not much work has been reported from that area in Breast Cancer.

2.8 Liposomes as nano vehicles for drug delivery

A liposome (**Fig. 6**) is defined as a self-forming structure consisting of one or more concentric spheres of lipid bilayers separated by water or aqueous buffer compartments (Lieberman et al., 1998). Phospholipids are the backbone of these structures. Phosphatidylcholine (PC), also called lecithin, is a biocompatible phospholipid that exists in plants and animals and used frequently in liposomal preparation. Moreover, there are other molecules widely used in combination with phospholipids, such as cholesterol (Weiner et al., 1989; Torchilin and Weissig, 2003). The exact location of a drug in liposomes depends upon its physicochemical characteristics and the composition of the lipids (Weiner et al., 1989). However, as a rule, the hydrophilic drug molecules can be encapsulated in the aqueous space whereas the hydrophobic and amphiphilic molecules can be incorporated into the lipid bilayer (Hupfeld et al. 2006).

Numerous evidences have demonstrated the ability of liposomes to enhance the efficiency of drug delivery via several routes of administration (Egbaria and Weiner, 1990). One of the major effect of liposomes as drug carriers is altering the pharmacokinetics of drug. It is known that pharmacological response is dependent upon the concentration of the drug in the target cell. The drug concentration in the target site is governed by absorption, distribution and elimination. These processes may influence the pharmacokinetics of the drug and lead to inefficient utilization of the therapeutic agent. Thus, higher doses need to be administered. Furthermore, higher drug doses often lead to resistance and undesirable immunological and toxicological effects (Fendler and Romero, 1977).

Liposomes are thought to shield all or most of the drug molecules resulting in decreasing the direct contact of drug with biological environment, thus the pharmacokinetic profile of the drug will be determined by the physiochemical properties of liposomes, rather than the drug itself (Sætern, 2004). Incorporating the drug into a vehicle capable of delivering it intact would overcome many of the disadvantages of the free drug administration.

Improving the pharmacokinetics of the drug by this method could lead to beneficial effects such as reduced dosages, increased cellular permeability and delayed drug elimination (Fendler and Romero, 1977). It is worth mentioning that liposomes are also non-toxic, biodegradable and can be manufactured on large scales (Washington et al., 2001).

The potentials of liposomes to serve as delivery systems have been proven by the numberof liposomal formulations already approved by FDA for clinical use such as AmBisome® (amphothericin B) and DaunoXome® (Daunorubicin). These formulations were clinically compared with conventional drug formulations and proved superiority of liposomal delivery (Allison, 2007).

The use of liposome as a drug carrier system were proposed several decades ago and since then it has been one of the most extensively investigated drug carrier systems. Modication of the basic liposome structure and optimization of methods for preparation of liposomes have overcome many of the initial obstacles encountered, when applying liposomes in vivo. Today liposomes can be tailor-made in many aspects to meet the demands needed for a specific in vivo application.

Liposomes are lipid bilayer vesicles within the 50-1000 nm range, containing an aqueous core. Unilammellar liposomes consist of a single lipid vesicle with an aqueous core, whereas multilammeller

liposomes contain several lipid vesicles inside each other. Lipids can be made from various mixtures of natural and synthetic lipids, however the specific lipid composition is an important determinant of the liposomal in vitro and in vivo characteristics of the liposomes (Lasch et al., 2003).

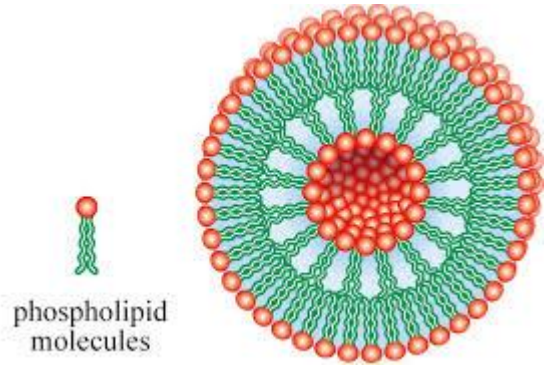


Figure 6. Schematics of Liposome architecture.

Liposomes are well suited for the use as drug carriers, since they have the ability to transport many different types of drugs at high concentrations. Hydrophilic and amphiphilic drugs can be encapsulated into the aqueous core, whereas hydrophobic drugs can be incorporated into the lipid bilayer (Drummond et al. ,2009).

To control the cellular fate of liposomes, they can be modified with various surface molecules, such as polymer-coatings and targeting agents. Based on such modifications, unilammellar liposomes have been divided into several types. The first liposomes to be investigated were unilammellar liposomes between 100-500 nm without any surface modifications, named conventional liposomes.

Investigating various formulations of conventional liposomes revealed that in vivo circulation time and other pharmacokinetic parameters were closely related to size, lipid composition and charge of these liposomes (Drummond et al., 1999). For example, small liposomes (< 100 nm) containing gel-state phospholipids and cholesterol had a significantly longer circulation time than larger liposomes of the same lipid composition (Senior et al. 1985). In addition, neutral liposomes were generally found to have a longer circulation time than electrostatically charged liposomes (Drummond et al., 1999). The fast removal of conventional liposomes from the circulation is predominantly caused by a rapid liposomal uptake by the macrophages of the reticuloendothelial system (RES) in the liver and spleen (Drummond et al., 1999). To overcome this problem liposomes coated with linear polyethylene glycol (PEG) molecules was developed, limiting the rapid uptake by the RES and significantly prolonging the half life of circulating liposomes to about 20 hours in mice and rats (Allen et al., 1991) and up to 45 hours in humans (Allen, 1997). PEG are commonly grafted to the liposomes by modifi-cation of the PEG molecules with a hydrophobic residue at one end, typically a phospholipid, which then can be incorporated into the bilayer of the liposomal membrane **(Fig. 6)**. As for conventional liposomes, liposome size was also found to be an important factor in liposomal clearance for PEGylated liposomes after intravenous injection (Drummond et al., 1999). Prolonged circulation was especially observed for small liposomes with a size of 70-200 nm and a high degree of homogeneity (Liu et al., 1992). In contrast, 300-400 nm PEGylated liposomes avoided significant entrapment in the liver, but not of the red pulp and marginal zone of the spleen (Litzinger, 1994). Thus, the biodistribution pattern of liposomes can significantly be altered by changing their basic structure of both conventional and PEGylated liposomes.

The next step in the development of liposomes as drug carriers involved preparation of liposomes that were conjugated with a targeting ligand. This enabled direction of the liposomes towards specific molecules expressed by the target cell population, promoting close interaction of the liposomes with the target cell and possibly cellular internalization. Two different approaches was investigated for conjugation of liposomes; 1) coupling the targeting ligand directly to the lipid bilayer, which was explored for both conventional and PEGylated liposomes. 2) coupling of the targeting ligand to the distal end of surface grafted PEG-chains of PEGylated liposomes (Sapra and Allen, 2003). The simplest approach is method one and worked well for conjugation of conventional liposomes. However, for conjugation to PEGylated liposomes a major drawback was that the flexible PEG-coat could both prevent interaction of the conjugated ligand with its target, as well as the reaction for conjugation of ligands to reactive groups on the liposomal surface (Hansen et al., 1995; Klibanov, 1991). Due to these constraints, methods for coupling ligands to the distal end of PEG-chains were developed, restoring the availability of the conjugated ligand for interaction with its target.

The most commonly used types of targeting ligands used for conjugation to liposomes are anti-bodies or fragments hereof and peptide ligands (Sapra and Allen 2003). When choosing a targeting ligand for liposomal conjugation, it is not only important to ensure that the targeting ligand binds specifically to a target sufficiently expressed by the target cell, but several other considerations must also be taken into account to choose the best type of targeting ligand and chemical method for attaching of the ligand to the liposome.

First, conjugating a ligand to the surface of a liposome may increase the rate of liposome uptake by the RES, despite the presence of PEGylation. Next, the immune system might attack the ligand or other parts of ligand-conjugated liposomes and cause an unwanted immune reaction. (Sapra and Allen, 2003, Park, 2002) The extent of antibody development depends both of the type of ligand, e.g. a small peptid or Fv fragment is usually less immunogenic that a complete foreign antibody, and the lipid composition of the liposome (Park et al., 2001, Harding et al., 1997). Based on these principles, it is understandable why the amount of conjugated ligand must be balanced to establish successful binding to its target, while maintaining a long circulation time. Using liposomes with a lower ligand-density may extend the liposome circulation time and improve the chance of interaction with targets located in tissues with limited blood flow, whereas such liposomes might not bind most efficiently to the same target in vitro compared to liposomes maximally conjugated with ligand.

2.8.1 Liposome Classification

Liposomes are usually classified according to their lamellarity and size. The following categories show the major types of liposomes (New 1990; Philippot and Schuber 1995): Multilamellar vesicles (MLV): This population has a broad range of size distribution that occurs in a range of 100-1000 nm. The lipid composition may influence the lamellarity of these MLVs. However, the lamellarity typically varies between 5 and 20 concentric lamellae and liposomes can be classified as below:

- Large unilamellar vesicles (LUV): The size of these vesicles is normally up to 1000 nm and the structure consists of a single lamellae.

- Small unilamellar vesicles (SUV): The structure normally consists of single lamellae and the diameter of this population is below 100 nm.

2.8.2 *In vivo* properties of Liposomes

The behaviour of the various formulations of liposomes has been extensively studied at the cellular level both in vitro and in vivo, as well as at whole organism level in vivo. As mentioned above, the fate of liposomes in vivo is determined by the structure and single components of the liposome, such as size, lipid composition, PEGylation, and the presence of targeting ligands on the surface.

The predominant administration route for liposomes is by intravenous injection, after which the liposomes circulate in the blood. Due to the large size of liposomes, the lining of the blood vessels act as a barrier and the liposomes can only extravasate at sites with discontinuous or fenestrated endothelium (Drummond et al., 1999). Both the liver and spleen have fenestrated endothelium, and therefore liposomes not ex-ceeding the size of the fenestrae are prone to accumulate in these organs. However, accumulation of liposomes within these organs has been demonstrated not to depend solely on the size of the liposomes, but also their lipid composition. For example, liposomes with a significant larger size than the pore size of the liver fenestrae, but having a high fluidity, were found to accumulate in the liver in a high degree. Accumulation of liposomes in the liver and the spleen promote the interaction with the large quantity of inherent macrophages of the RES, which are the main source of liposome elimination from the body. Depending on the size and lipid composition of the liposomes, liver hepatocytes may also play an important role in removing liposomes from the blood. No convincing evidence have demonstrated a significant uptake of liposomes by other cell types easily accessible from the blood, such as cell of the endothelium or circulating blood cells.(Kamps and Scherphof, 2003).

Several theories exist for the mechanisms of liposome removal by the RES, and why PEGylation of the liposomes slow down this process. In the circulation, liposomes of various compositions unspecifically adsorb proteins present in serum, and one theory is that the opsonization of liposomes pro-motes the clearance by macrophages through receptor-mediated endocytosis by the means of some of the liposome bound proteins (Drummond et al., 1999). Some of the identified proteins that opsonise liposomes include fibronectin, complement factors, β2-glycoprotein, C-reactive protein, and α2-macroglobulin (Maruyama et al., 1995; Allen et al., 1995; Zalipsky et al., 1995; Zalipsky et al., 1994; Zalipsky, 1993). After receptor-mediated endocytosis of the liposomes, lysosomal digestion of the liposome membrane releases the liposomal content into the cytoplasm. Several theories have been proposed to explain why PEGylation increase the circulation times of liposomes. One of the most popular views suggests that the PEG-chains act as a steric barrier, preventing macromolecules such as the mentioned opsonins from interacting with the liposomal membrane. Due to the lack of liposome opsonization, the rate of macrophage uptake is significantly reduced. (Drummond et al., 1999)

Long-circulating liposomes have a higher probability for encountering their target cells, especially if located in an area with limited blood supply, and a longer circulation also increase the probability of extravasation and accumulation at sites with increased vascular permeability, such as in tumours (Kamps and Scherphof, 2003). Increasing the circulation time will increase the number of times a liposome passes through a specific tissue, and thereby increasing the probability of interaction with its target cell or increasing the probability of intratumoral extravasation through the EPR effect. Several possible mechanisms for interaction of liposomes with cells, after accumulation in the target tissue have been described. First of all, unspecific binding or absorption of liposomes to cells can occur, and untargeted liposomes can deliver their drug load by this mechanism. When in close contact with the cell, lipophilic drugs encap-sulated in the liposomal membrane can be transferred to the cell membrane, while

encapsulated hydrophilic drugs in some circumstances can be released from absorbed liposomes and taken up into the cells. Both these processes are not fully understood, but it is clear that the lipid composition of the liposomal membrane and the physiochemical characteristics of the drug in question are of great importance. Another suggested mechanism by which liposomes can deliver their content to the cellular compartment are through fusion of the liposomal lipid membrane with the cellular membrane, there-by inducing cytoplasmic release of the liposome content. However, no evidence indicate that cellular fusion occur when using standard conventional or PEGylated liposome and in general specific fusion-inducing agents are believed to be required for stimulation of this process (Kamps and Scherphof, 2003).

Two primary mechanisms have been proposed for how targeted liposomes can increase the cellular uptake of liposomes, depending on both the properties of the target molecule and the targeting ligand. When targeting the liposomes to a cell surface receptor at a site where binding induce internalization of the whole complex, the liposomes can be internalized through receptor-mediated endocytosis into the cellular compartment of the cancer cells. Like described for receptor-mediated endocytosis of liposomes by macrophages, the liposomes can now degraded by lysosomal enzymes and the drug released into the cytoplasm. However, even liposomes targeted to a non-internalizing molecule have demonstrated a higher intracellular accumulation of the transported drug. A likely mechanism is probably that binding of the liposome to its target keep the liposomes in close contact with target cells, facilitating diffusion of the encapsulated drug into the cells degradation of the bound liposome (Sapra and Allen, 2003, Park et al., 1997).

2.8.3 Liposomes in Cancer Therapeutics

Pathological conditions inducing an inflammatory state of the vasculature, such as infections, ischemic heart disease, and cancer, the vasculature may become leaky, permitting the liposomes to extravasate at the lesion site (Kamps and Scherphof, 2003). In tumours, this is known as the EPR effect and can be exploited to passively target liposomes to the tumor tissue (Maeda, 2001). However, to achieve significant liposomal accumulation in the tumor, the liposomes must circulate repeatedly through the tumor vessels to increase the change of extravasation. Furthermore, the pore size found in the leaky parts of the tumor vasculature vary widely according to the type and location of tumor (Hobbs et al., 1998). Therefore, the liposome size must be carefully chosen from the experimental model used for studying the therapeutic efficacy of the liposomes. Both untargeted and targeted liposomes accumulate in tumor tissue through the EPR effect to a similar extent. The superior anticancer effects observed when using targeted liposomes loaded with chemotherapeutic drugs do therefore not stem from a higher intratumoral accumulation of the liposomes, but rather from differences in the intratumoral localization at the cellular level. After intratumoral accumulation, untargeted liposomes have been observed to reside predominantly in the interstitium, whereas targeted liposomes and especially liposomes targeted to an internalizing receptor are found intracellularly in a much higher degree (Sapra and Allen, 2003).

2.8.4 Liposomal system for cancer targeting

The liposome behaviour in vitro and in vivo depends on the overall structure and the characteristics of its single components. A long circulation time in vivo is essential for intratumoral accumulation of liposomes via the EPR effect, and therefore small PEGylated liposomes conjugated with a moderate amount of antibody remains the preferred choice for cancer targeting applications. Small unilammellar liposomes of a defined size can be prepared by a variety of different techniques. A simple technique

widely used for preparation of such liposomes is the lipid film-hydration technique, followed by extrusion of the liposomes until researching to the desired size. A dry lipid film of the lipids desired for liposome preparation is prepared by measuring out lipids dissolved in an organic solvent such as chloroform and evaporating the solvent, dispersing a thin lipid film on the inside of the flask. Addition of an excess aqueous solution followed by mechanical agitation, result in spontaneously formation of a suspension of heterogenous large multilammellar liposomes within the 0.5 to 10 μm range. In order to prepare a more homogeneous suspension of small unilammellar liposomes, the multilamellar liposomes can be sequentially extruded through polycarbonate membranes with decreasing pore size until the liposomes have the desired size. During extrusion, the large multilammellar liposomes are forced through the pores of the polycarbonate membrane by application of pres-sure, resulting in transient rupture of the lipid membrane and resealing at a size closer to the pore size of the membrane. Typically, passing a suspension of large multilammellar liposomes 10 times through a membrane with a pore size of 100 nm produces a homogeneous liposome suspension with a mean diameter of approximately 100 nm. (Mui et al., 2003)

In order to direct the liposomes to either the cancer cells or the tumor endothelial cells, the chosen targeting antibodies must be conjugated to the liposomes. Coupling of the antibodies to the distal ends of the surface grafted PEG-chains have demonstrated to be superior in retaining antigen binding ability. PEG-lipids with various reactive groups at their distal end have been developed for the purpose of chemical conjugation with targeting ligands. One highly efficient and widely used method for protein conjugation involves the formation of a thioether bond between a maleimide group on the end of the PEG-chains to a protein thiol group (Derksen and Scherphof, 1985). To use this conjugation method, a free thiol group must be available in the protein for chemical reaction. Free thiol groups can be generated either by breaking endogenous disulfide bridges in the protein, if this can be done without destroying the desired proper-ties of the targeting molecule, e.g. its binding properties, or exogenous thiols can be introduced into the protein by the use of one out of several available thiolating agents (Duncan et al., 1983). One of the most advantageous thiolating agents is succinimidyl-S-acetyl thioacetate (SATA). Reacting proteins with SATA, randomly introduces thioester groups into the proteins and free thiol groups for reaction with the PEG-coupled maleimide can easily be generated by deacetylation of the thioester using hydroxylamine. The free thiol can now react with maleimide to generate a thioether bond between the liposome and the conjugated protein. The thioether bond between maleimide and thiol group of the protein is very sta-ble in a biological environment, and this method is therefore suitable, when conjugation for a longer period of time is desirable (Derksen and Scherphof, 1985).

Traditionally, functionalized PEG-lipids have been incorporated into liposomes by including it in the initial lipid mixture used for liposome preparation, followed by conjugated of preformed lipo-somes. However, drawbacks of this method include the random orientation of the functionalized PEG-molecules and PEG-chains localized in the inner leaflet of the lipid bilayer will not be accessible for ligand conjugation. To circumvent this problem, a method for post-insertion of PEG-lipids already conjugated with ligand, known as the post insertion technique (Ishida et al., 1999). Here, micelles consisting of the functional PEG-lipids are initially formed and the targeting ligand conjugated to the micelles. Afterwards the micelles can be inserted into preformed liposomes, by incubating above the phase transition temperature of the lipids. Liposomes prepared by the post insertion technique have been demonstrated to have ligand densities and in vivo performance characteristics similar to liposomes prepared by the traditional approach (Iden and Allen, 2001).

2.8.5 Liposome: Methods of Preparation

There are various methods to produce different types of liposomes. However, all preparation methods can be simplified as to involve three basic steps: 1) Preparation of the dispersion of the lipids in aqueous media 2) purification of prepared liposomes, and 3) analysis of final product (New, 1990). The following is a brief description of methods thatare considered among the most widely used in liposome preparation:

Hand-shaking, (MLV)

The principle of this method is to dissolve the lipid in efficient solvent. Lipophilic drugs can be dissolved in compatible solvent which can be added later to the lipid solution. This mixture will be dried down under pressure till it forms lipid films. Hydrophilic drugs can be dissolved in aqueous solution which will be added to the lipid films under shaking. This will produce swelling and peeling of the lipid films and gives a milky suspension of MLVs. Using this technique, one can entrap as high as 100% of the lipid soluble molecules, whereas hydrophilic compounds are often encapsulated in amount of 5-10% (New, 1990).

Sonication, (SUV)

Sonication is the most widely used method for producing small vesicles. It is usually used to convert MLVs to SUVs through the employment of energy at high levels by exposure of MLVs to ultrasonic irradiation. Probe-sonication and bath sonication are frequently used techniques to reduce the size of liposomes. The probe has the most efficient transfer of energy to the liposomal dispersion. However, it is associated with metal particle shedding from its probe and therefore one must be aware of the potential contamination. Bath sonication is much milder than probe sonication, but it is time-consuming and may result in low yield of smaller liposomes (New, 1990).

Extrusion, (LUV)

The principle in extrusion technique is based on employment of moderate pressure to force MLVs through polycarbonate filters with defined pore size. At low pressures (100 psi), MLVs display a reduced-size while maintaining their multi-lamillarity, whereas at higher pressure the liposomes are broken down as they pass though the membrane filter resulting in reorganizing of the phospholipid bilayer giving rise to unilamellar vesicles (Philippot and Schuber, 1995). It is simple method and easy to use and there are several products available on the market. Among the equipments are the Hamilton® syringes. These are two syringes connected by a filter holder allowing the samples to pass back-and-forth through the polycarbonate filter. However, this method can be limited by production of small volumes of LUVs and the back pressure that can be tolerated by the syringe and filter holder is limited (Gregoriadis, 2007).

Unprocessed MLVs have limited uses in in vivo studies because of their large diameter and heterogeneity of size (Gregoriadis, 2007). However, the techniques used to change these parameters may influence physical properties of liposomes. The conversion to SUVs from MLVs may result in vesicles with very low trapped volumes. Furthermore, SUVs can be unstable and prone to fusion process due to the high curvature of the lipid bilayer (New, 1990). Extrusion technique used to produce LUVs may result in rupturing and resealing which leads further to leakage of the entrapped drug and the final vesicles may have lower amount of entrapped material, depending on the lipophilicity of the drug (Gregoriadis, 2007).

CHAPTER 3: MATERIALS AND METHODS

3.1 In silico analysis

Computational analysis of probable targets for drug targeting in breast cancer was performed using various bioinformatics tools as discussed below.

3.1.1 Predicting functional Protein-protein interaction (ppi)

In molecular biology, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) is a biological database and web resource of known and predicted protein-protein interactions. The STRING database contains information from numerous sources, including experimental data, computational prediction methods and public text collections. It is freely accessible and it is regularly updated. The latest version 9.0 contains information on about 5.2 millions proteins from 1133 species. STRING has been developed by a consortium of academic institutions including CPR, EMBL, KU, SIB, TUD and UZH.

Protein-protein interaction networks are an important ingredient for the system-level understanding of cellular processes. Such networks can be used for filtering and assessing functional genomics data and for providing an intuitive platform for annotating structural, functional and evolutionary properties of proteins. Exploring the predicted interaction networks can suggest new directions for future experimental research and provide cross-species predictions for efficient interaction mapping.

The data is weighted and integrated and a confidence score is calculated for all protein interactions. Results of the various computational predictions can be inspected from different designated views. There are two modes of STRING: Protein-mode and COG-mode. Predicted interactions are propagated to proteins in other organisms for which interaction has been described by inference of orthology. A web interface is available to access the data and to give a fast overview of the proteins and their interactions. A plug-in for cytoscape to use STRING data is available. Another possibility to access data STRING is to use the application programming interface (API) by constructing a URL that contain the request.

Like many other database that store protein association knowledge STRING imports data from experimentally derived protein-protein interactions through literature curation. Furthermore, STRING also store computationally predicted interactions from: (i) text mining of scientific texts, (ii) interactions computed from genomic features, and (iii) interactions transferred from model organisms based on orthology.

All predicted or imported interactions are benchmarked against a common reference of functional partnership as annotated done by KEGG (Kyoto Encyclopedia of Genes and Genomes).

Predicted Data

Neighbourhood*:* Similar genomic context in different species suggest a similar function of the proteins.

Fusion-fission events*:* Proteins that are fused in some genomes are very likely to be functionally linked (as in other genomes where the genes are not fused).

Occurrence*:* Proteins that have a similar function or an occurrence in the same metabolic pathway must be expressed together and have similar phylogenetic profiles.

Coexpression*:* Predicted association between genes based on observed patterns of simultaneous expression of genes.

3.1.2 Predicting miRNA and mRNA interaction

In order to establish Notch interactome associated miRNA misregulation, prospective protein mRNA-miRNA interaction was initially predicted using *TargetScan (release version 6.2, http://www.targetscan.org/)*. TargetScan predicts biological targets of miRNAs by searching for the presence of sites that match the seed sequences (presence in the 3' UTR) of each miRNA transcript. In flies and nematodes, predictions are ranked based on the probability of their evolutionary conservation. In zebrafish, predictions are ranked based on site number, site type, and site context, which includes factors that influence target-site accessibility. In mammals, the user can choose whether the predictions should be ranked based on the probability of their conservation or on site number, type, and context. In mammals and nematodes, the user can chose to extend the predictions beyond conserved sites and consider all sites. TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA (Lewis et al., 2005). As an option, nonconserved sites are also predicted. Also identified are sites with mismatches in the seed region that are compensated by conserved 3' pairing (Friedman et al., 2009). In mammals, predictions are ranked based on the predicted efficacy of targeting as calculated using the context+ scores of the sites (Grimson et al., 2007; Garcia et al., 2011). As an option, predictions are also ranked by their probability of conserved targeting (PCT, Friedman et al., 2009). TargetScanHuman considers matches to annotated human UTRs and their orthologs, as defined by UCSC whole-genome alignments. Conserved targeting has also been detected within open reading frames (ORFs).

TargetScan Release 6.2 retrieves predicted regulatory targets of mammalian microRNAs. Compared to previous releases, Release 6 extends context score contributions to include seed-pairing stability and target-site abundance (Garcia et al., 2011).

3.1.3 CpG island prediction

CpG sites or CG sites are regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence of bases along its length. "CpG" is shorthand for "—C—phosphate—G—", that is, cytosine and guanine separated by only one phosphate; phosphate links any two nucleosides together in DNA. The "CpG" notation is used to distinguish this linear sequence from the CG base-pairing of cytosine and guanine.

CpG island prediction and CpG plot generation was done using *EMBOSS CpGplot.*

Plot parameters

Window Size*:* The percentage CG content and the Observed frequency of CG is calculated within a window whose size is set by this parameter. The window moves down the sequence and statistics are calculated at each position that the window moves (Default value, 100).

Minimum length of an island*:* Refers to the minimum length that a CpG island has to be before it is reported (Default value, 200)

Minimum observed/expected*:* This sets the minimum average observed to expected ratio of C plus G to CpG in a set of 10 windows that are required before a CpG island is reported (Default value is: 0.6).

Minimum percentage: This sets the minimum average percentage of G plus C a set of 10 windows that are required before a CpG island is reported.

3.1.4 Protein active site prediction (druggable vs. non druggable)

DogSiteScorer is an automated pocket detection and analysis tool, which was used for protein druggability assessment.

Predictions with DoGSiteScorer are based on calculated size, shape and chemical features of automatically predicted pockets, incorporated into a support vector machine for druggability estimation. Based on the 3D coordinates of a protein, its potential active sites on the protein surface are calculated with DoGSite. DoGSite is a grid-based function prediction method which uses a Difference of Gaussian filter to detect potential pockets on the protein surface and splits them into subpockets. Subsequently, global properties, describing the size, shape and chemical features of the predicted pockets are calculated. Examples for these descriptors are volume, depth, surface, ellipsoid main axes, site lining atoms and residues, as well as functional groups present in the pockets.

Per default, a SimpleScore is provided for each pocket, based on a linear combination on the three descriptors describing volume, hydrophobicity and enclosure. For the discrimination of the druggability, a subset of meaningful descriptors is used in a support vector maschine (libsvm). For each queried input structure, a druggability score between zero and one is returned. The higher the score the more druggable the pocket is estimated to be.

3.1.5 Pharmacophore prediction

ZINCPharmer (http://zincpharmer.csb.pitt.edu) is an online interface for searching the purchasable compounds of the ZINC database using the Pharmer pharmacophore search technology. A pharmacophore describes the spatial arrangement of the essential features of an interaction. Compounds that match a well-defined pharmacophore serve as potential lead compounds for drug discovery. ZINCPharmer provides tools for constructing and refining pharmacophore hypotheses directly from molecular structure. A search of 176 million conformers of 18.3 million compounds typically takes less than a minute. The results can be immediately viewed, or the aligned structures may be downloaded for off-line analysis. ZINCPharmer enables the rapid and interactive search of purchasable chemical space.

3.2 Experimental Methods

3.2.1 Human breast samples

The patients examined in this study underwent surgery at the Meharbai Tata Cancer Hospital between 2013 and 2014. Normal and cancerous breast tissue were obtained from. Cancerous tissue (n=2) and normal breast tissue (n=1), were obtained from three different parients who belonged to the same age group (45-50).

3.2.2 MicroRNA isolation

Phenol based isolation procedure was used to isolate miRNAs. Phenol-based isolation procedures can recover RNA species in the 10–200-nucleotide range (e.g., the miRNAs, 5S rRNA, 5.8S rRNA, and U1 snRNA). It utilizes the powerful guanidine isothiocyanate–phenol:chloroform extraction method which facilitates rapid isolation of RNA. This then separates proteins and DNA into the organic layer of the

biphasic solution interface, while retaining RNA in the upper aqueous layer. The aqueous phase is removed to a second tube, and RNA is precipitated with an equal volume of isopropanol. High yields of pure, undegraded total RNA can be recovered from even small quantities of tissue or cells. Large numbers of samples may be performed simultaneously, because of the simplicity of the technique.

3.2.3 Primer designing

The primer pair found to be most suitable was: 5'-CACTACCCTCCTCCCTCAGA-3' (Forward Primer): 5'-GCTTGGAGCCAGACTCAGA-3' (Reverse Primer)**.** The primer pair so designed was made in such a way such that it could amplify a 68 bp stretch of DNA. The primer pair was designed in such a way that the 68 base amplicon contained 2 sites of CCGG in the sequence. The first, found at the 10^{th} position from the 5' end. Another CCGG site was purposefully chosen at 55^{th} position from the 5' end of the amplicon.

3.2.4 RT-PCR

Total RNA amplification was carried out using RT-PCR. Primers for miR-24 amplification, both sense and antisense were designed using Sigma-Aldrich primer designing platform. PCR cocktail were obtained by mixing the sense primer (CTCCGGTGCCTACTG) (Sigma-Aldrich, Bangalore), antisense primer (CTCCTGTTCCTGCTGAA) (Sigma-Aldrich, Bangalore), dNTPs, Taq polymerases, nuclease free water, $MgCl_2$ and the extracted total RNA. Following this RT-PCR was carried out using standard procedure and as per manufacturer's instructions.

3.2.5 Immunohistochemical analysis

Paraformaldehyde fixed and sectioned cells were incubated with rabbit IgG primary antibodies followed by incubation with cy-2 conjugated goat anti-rabbit secondary antibodies as per standard immunohistochemical procedures. The cells were then visualized under a fluorescence microscope at 200X resolution.

3.2.6 Liposome Synthesis

Liposomes were synthesized using the Lipid Hydration Method. L-α-Phosphatidylethanolamine, dioleoyl (Sigma-Aldrich) and cholesterol (Hi-Media) were dissolved in chloroform followed by solvent drying and subsequent hydration using distilled water. The samples were then characterized using SEM.

CHAPTER 4: RESULTS & DISCUSSION

4.1 Breast cancer statistics

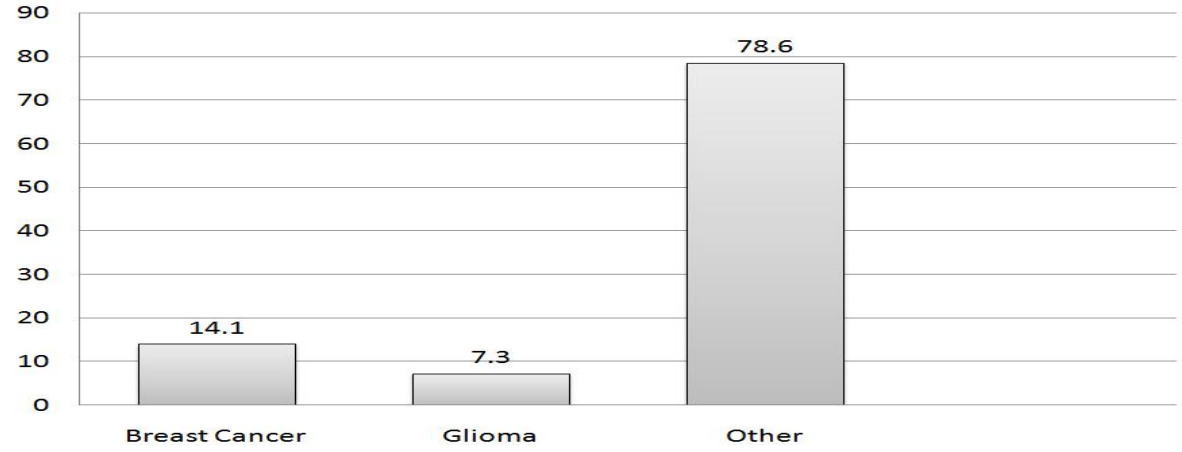


Figure 7. Cancer incidences worldwide in the year 2013. Breast cancer and glioma occupies approximately 22 % of all cancer related incidences. Data source: National Cancer Institute (NIH, USA).

4.2 Breast cancer gene expression profiles

A wide range of genes is reportedly misregulated during breast carcinogenesis. Using gene expression atlas, the expression characteristics of a handful of genes were analysed. In addition to our knowledge on Notch-1 being up regulated in breast carcinomas we observed an over expression in Klf-8 levels. Notch-1 and Klf-8 together potentially can mediate EMT in aggressive breast tumours.

PROTEIN	GENE ID	Expression Data	SIGNATURE EXPRESSION
AP-1/ c-jun	JUN	1. Breast Cancer Cell Lines- OE(5), UE(2) 2. MDA468 breast cancer (96 groups) - UE(1) 3. Invasive ductal carcinoma (Histology)- UE(2)	Non Differentially Expressed
protein kinase C, alpha	PRKCA	1. Breast Cancer Cell Lines- OE(2),UE(4) 2. MDA468 breast cancer(96 groups)- UE(1) 3. Invasive ductal carcinoma(Histology)- UE(2)	Underexpressed
TBX 3	TBX3	1. Breast Cancer Cell lines- OE(5), UE(1) 2. MDA468 breast cancer(96 groups)- UE(1) 3. Invasive ductal carcinoma (Histology)- UE(2)	Underexpressed
ΔNp63	TP63	1. Breast Cancer Cell Lines- OE(4), UE(2) 2. Breast Cancer(Disease)- UE(1) 3. BT474 breast cancer(96 groups)- OE(1) 4. invasive ductal carcinoma(Histology)- UE(2)	Non Differentially Expressed
Hes 1	HES1	1. Breast Cancer Cell Lines-OE(7), UE(1) 2. MDA-MB-231 breast cancer(96 groups)- UE(1)	Overexpression
Klf 8	KLF8	1. Breast Cancer Cell Lines- OE(5), UE(2) 2. Lymph node-negative breast carcinoma(Disease)- OE(1) 3. MDA-MB-231 breast cancer(96 groups)-UE(1) 4. Invasive ductal carcinoma(Histology)-UE(2)	Overexpression

Figure 8. Expression profiles of breast cancer vulnerable genes. Klf-8 (Blue shading), shows over expression in breast cell lines and human cancerous breast tissue.

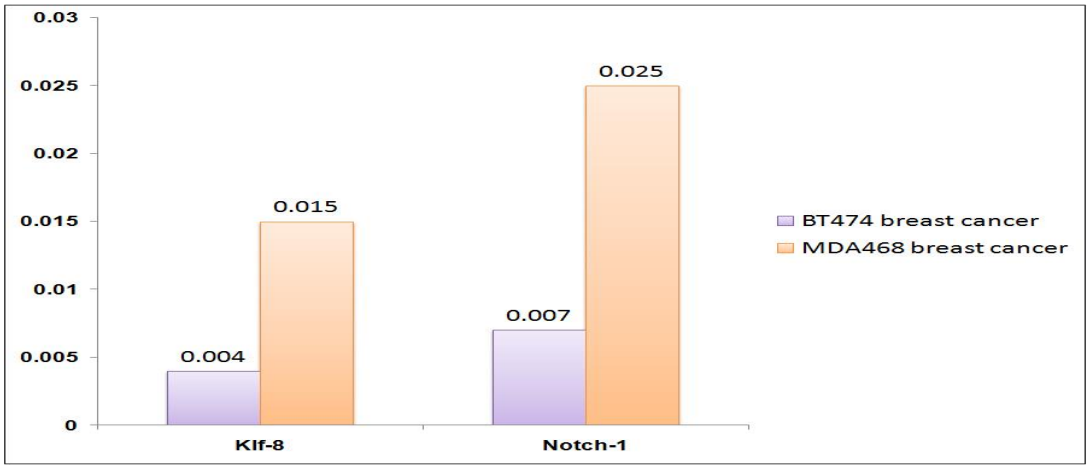


Figure 9. Comparative p-value statistics of Klf-8 and Notch-1 expression in BT474 and MDA468 cell lines.

4.3 Molecular structure of the Klf-8 protein

The molecular structure of Klf-8 was determined as below using Hex. Hex is essentially a docking tool but it can also provide an idea about the formal appearance of the molecule as depicted below. In order for Hex to be able to generate a structure the .pdb file corresponding to the Klf-8 protein was obtained from protein data bank.

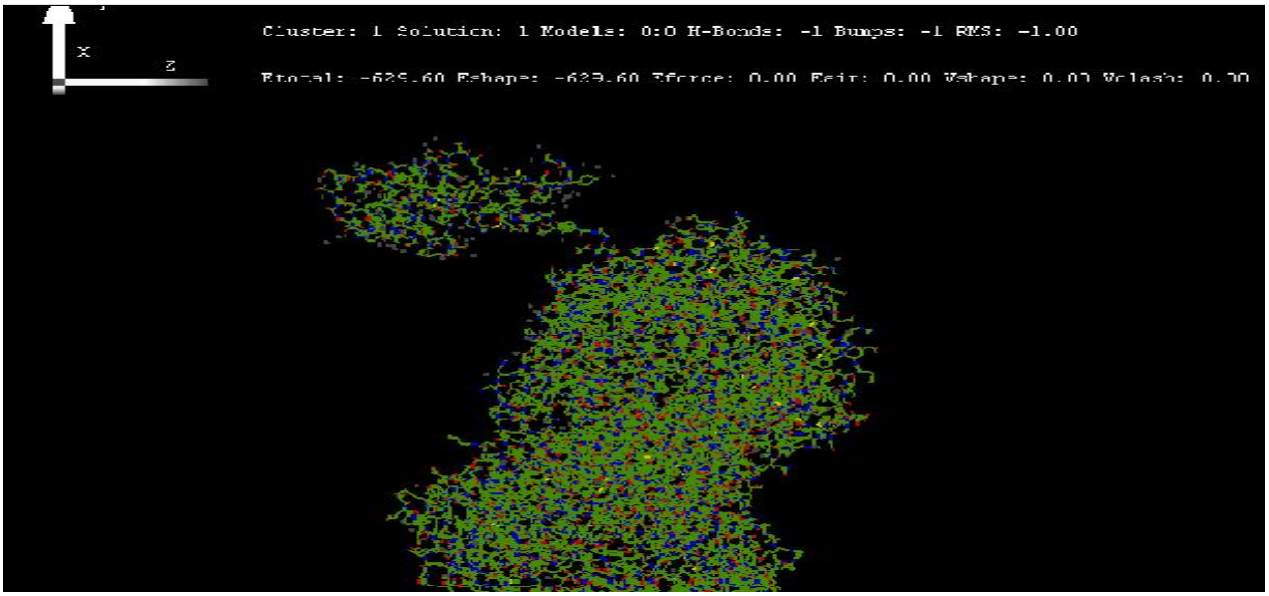


Figure 10. Molecular structure of Klf-8 protein.

4.4 Notch-1 interacting components

Notch-1 signalling pathway interacts with the several other disease critical proteins and pathways to control carcinogenesis.. Using the bioinformatics tool STRING (see Fig. 5e below), we were able to predict the known Notch-1 interaction partners in order to have an insight into the cellular processes that Notch-1 might be regulating.

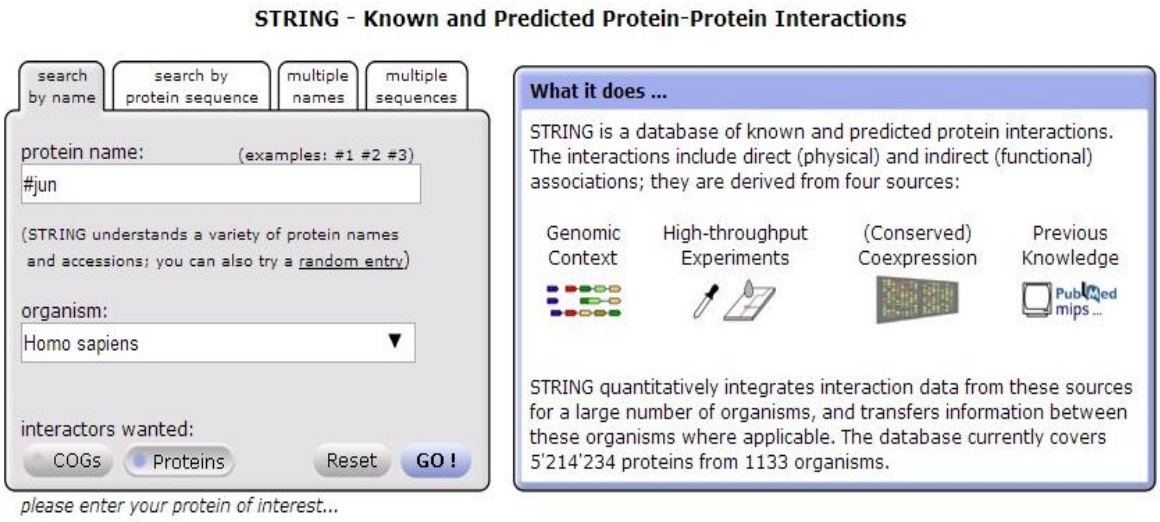


Figure 11. STRING interface for protein-protein interaction network prediction. The input protein name has to be entered in #proteinname format, followed by selecting the target organism. The search returns a protein interaction network highlighting all probable interactions.

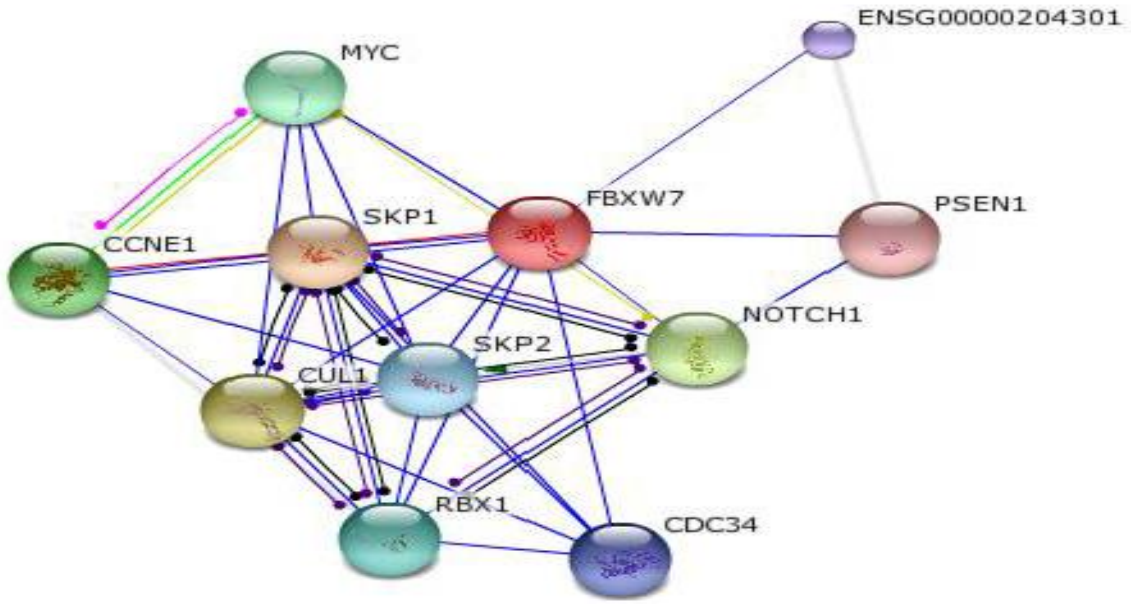
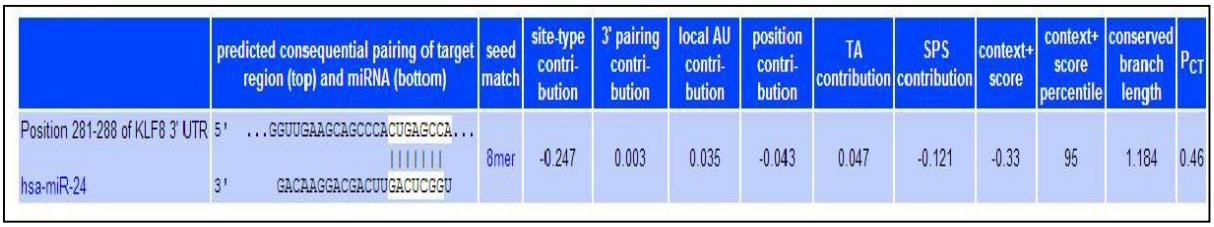


Figure 12. Protein interaction network showing Notch-1 interaction components. Interaction type= Binding; Evidence= Experiments and Text mining; Interaction score= 0.999, Confidence level= Highest (0.900).

4.5 Predicting Klf-8 and microRNA-24 interaction

In order to predict for miRNAs having binding seed sequences in the 3'-UTR region of Klf-8 mRNA. Investigating through Target Scan data, we were to establish Klf-8 mRNA to be a probable target of miR-24. As per literary reports Klf-8 is up regulated in breast tumours, in that aspect miR-24 should then be under expressed.



	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	P_{CT}
Position 281-288 of KLF8 3' UTR	5' ...GGUUGAAGCAGCCCACUGAGCCA...	8mer	-0.247	0.003	0.035	-0.043	0.047	-0.121	-0.33	95	1.184	0.46
hsa-miR-24	3' GACAAGGACGACUUGACUCGGU											

Figure 13. In silico data showing miR-24 as binding partner for Klf-8 mRNA. Data reveals miR-24 to have binding sequences in 3'UTR region of Klf-8 mRNA

Figure 14. TargetScan interface for miRNA target prediction.

4.6 Klf-8 druggable site prediction

In addition to the below analysis, pharmacokinetic features of the most druggable site (green) were also determined **(Table 4)**. This shall help in specific drug targeting and development of tailor made drugs for targeting the Klf-8 molecule.

Table 3. In silico investigation showing Klf-8 druggable probability. The predicted volume, area, depth and corresponding drug score is also shown. Green indicates the site with highest druggable capability as compared to other sites (orange, brown and red).

Name	Volume [Å³]	Surface [Å²]	Depth [Å]	Drug Score
P0	1546.69	1228.07	926.23	0.82
P2	340.41	572.77	352.69	0.63
P8	197.67	305.9	203.45	0.27
P18	101.83	209.43	184.79	0.16

Table 4. Pharmacokinetic properties of the most druggable PO pocket

Descriptors	Value
Hydrogen bond donors	23
Hydrogen bond acceptors	70
Metals	0
Hydrophobic interactions	50
Hydrophobicity ratio	0.35
Pocket atoms	331
Carbons ©	249
Nitrogens (N)	37
Oxygens	40
Sulfors (S)	5
Other elements	0

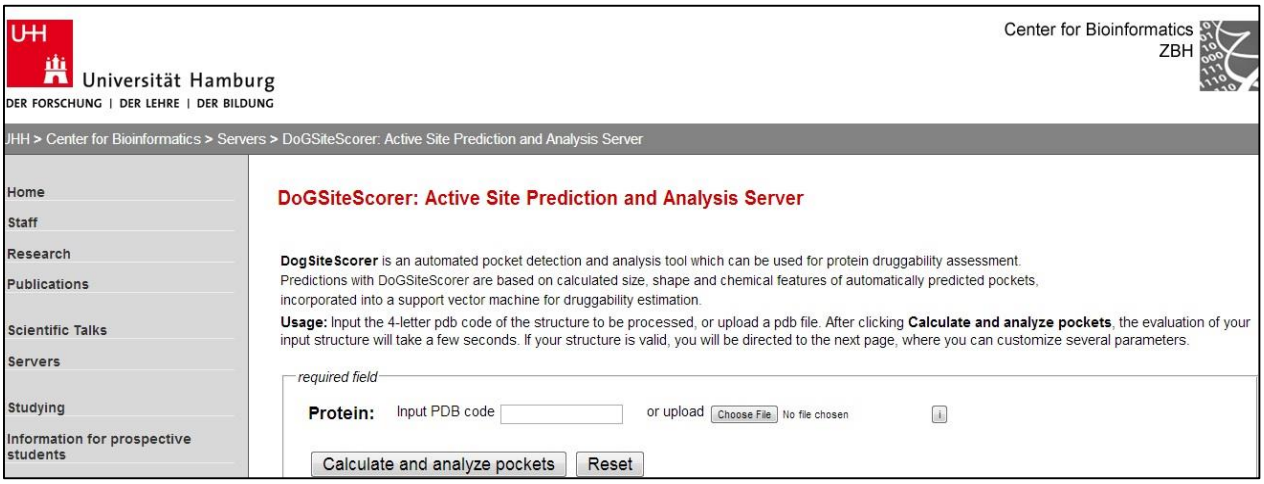


Figure 15. DogGSitescorer interface for active sites and druggability prediction

4.7 Pharmacophore analysis of 2-Furan-2-yl-thiazolidine.

Structure of 2-Furan-2-yl-thiazolidine was taken from PubChem for further pharmacophore analysis using Zinc Pharmer.

Figure 16. ZincPharmer interface for drug molecule pharamcophore prediction.

Table 5. Pharmacaphore properties of 2-Furan-2-yl-thiazolidine.

Property	2-Furan-2-yl-thiazolidine
Hydrogen Donor	6
Hydrogen Acceptor	10
Negative Ion	2
Hydrophobic	3
Aromatic	1

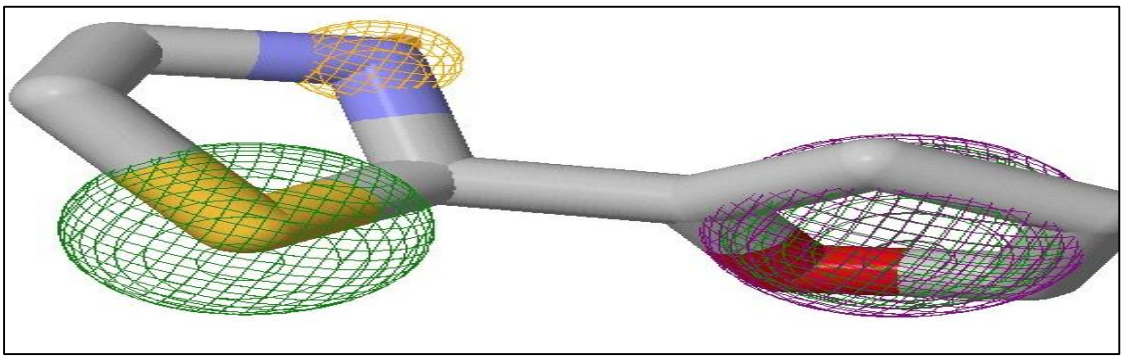


Figure 17. Pharmacophore diagram for 2-Furan-2-yl-thiazolidine. Rings indicates hydrogen donor, acceptor and aromatic sites in the drug molecule.

4.8 CpG island prediction for the miR-24 gene

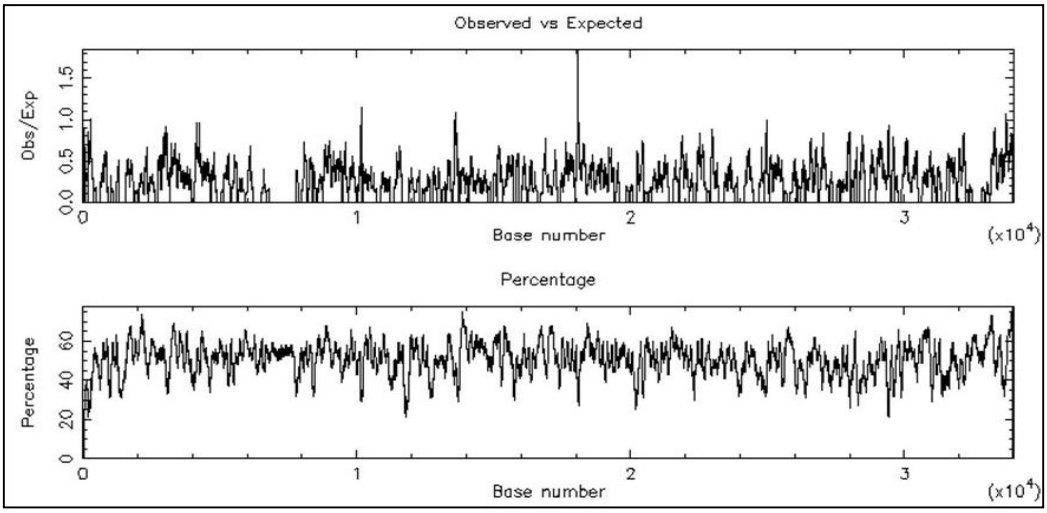


Figure 18. CpG island prediction for miR-24 gene using EMBOSS Cpg Plot. Window size= 100; Minimum Length=200; Minimum Observed= 0.6; Minimum Percentage=50.

4.9 miRNA-24 amplification

miRNA-24 was amplified using primers (Sense: CTCCGGTGCCTACTG and Antisense: CTCCTGTTCCTGCTGAA), following total RNA extraction using phenol extraction method. Fresh breast cancerous tissue, fresh breast cancerous tissue kept @ 4ºC for 12 hrs and normal breast tissue were used for miRNA-24 amplification and analysis.

Figure 19(A). Agarose gel showing miRNA-24 expression. The bands were visualised following RT-PCR using standard procedures. Lane 1: Normal breast tissue in 10 % neutral buffered formalin; Lane 2: Normal breast tissue in 40 % formalin; Lane 3: Normal breast tissue in PBS; Lane 4: Fresh breast cancerous tissue from patient 1; Lane 5: Breast cancerous tissue from patient 1 stored @ 4^{o}C for 12 hrs; Lane 6: Fresh breast cancerous tissue from patient 2; Lane 7: Breast cancerous tissue from patient 1 stored @ 4^{o}C for 12 hrs

Figure 19(B). Agarose gel showing miRNA-24 expression under differential conditions of storage. Lane 1: Patient 1 breast cancerous tissue stored in 70 % ethanol; Lane 2: Patient 2 breast cancerous tissue stored in 70 % ethanol; Lane 3 and 4: Normal breast tissue; Lane 5: Breast cancerous tissue from patient 1 stored @ 4^{o}C for 12 hrs

4.91 Immunohistochemical analysis

In order to study the expression levels of Notch-1 in cancerous as compared to normal breast tissue immunohistochemical analysis was done using cy-2 secondary antibodies(Excitation: 489 nm; Emission: 506 nm) by observing using a fluorescence microscope 200 X magnification.

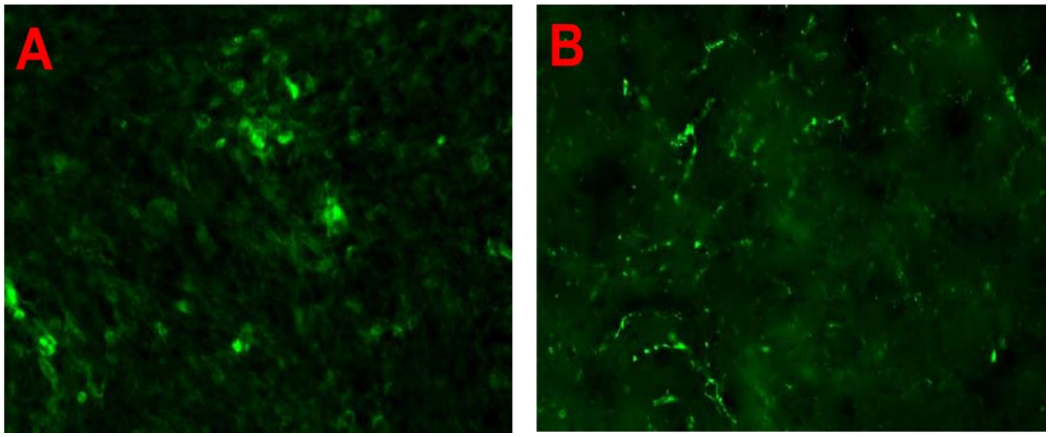


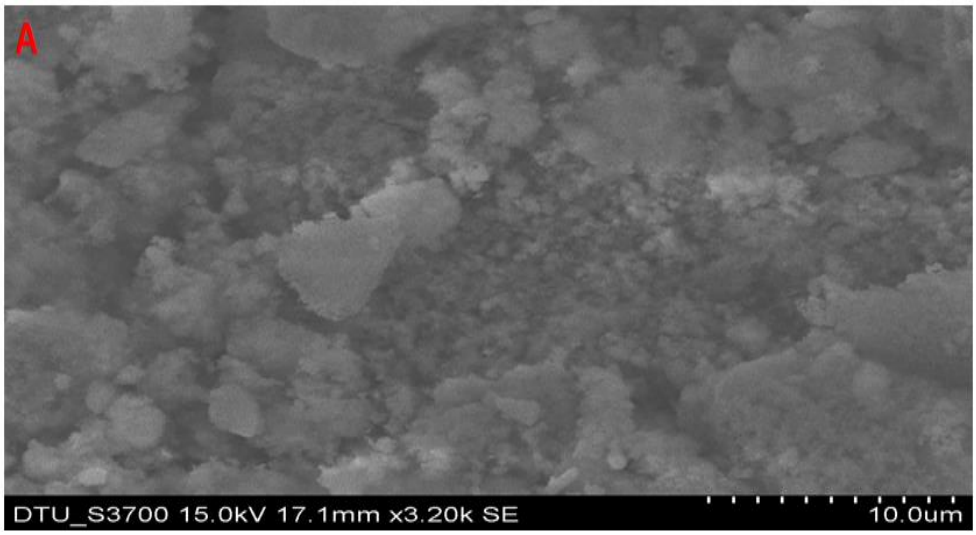
Figure 20. Accumulation of Notch-1 in breast cancerous tissue from patient 1 as compared to normal breast tissue. (A). Immunohisto-chemical staining of Notch-1 vaguely showing a diffuse distribution in the tumor (A), and a (B) diminished pattern in the normal breast tissue (B). A) Tumor tissue at high magnification. B) Normal breast tissue at high magnification. All images: 200x magnification

4.91 Liposome synthesis parameters

Table 6. Experimental sets for liposome synthesis.

Set No.	Phospholipids (mg)	Cholesterol (mg)	(PL+Ch) (mg)	Solvent (ml)
A	1.5	0.5	2	0.5
B	0.375	0.125	0.5	0.005
C	3.75	1.25	5	0.5

4.92 Characterization of Liposomes



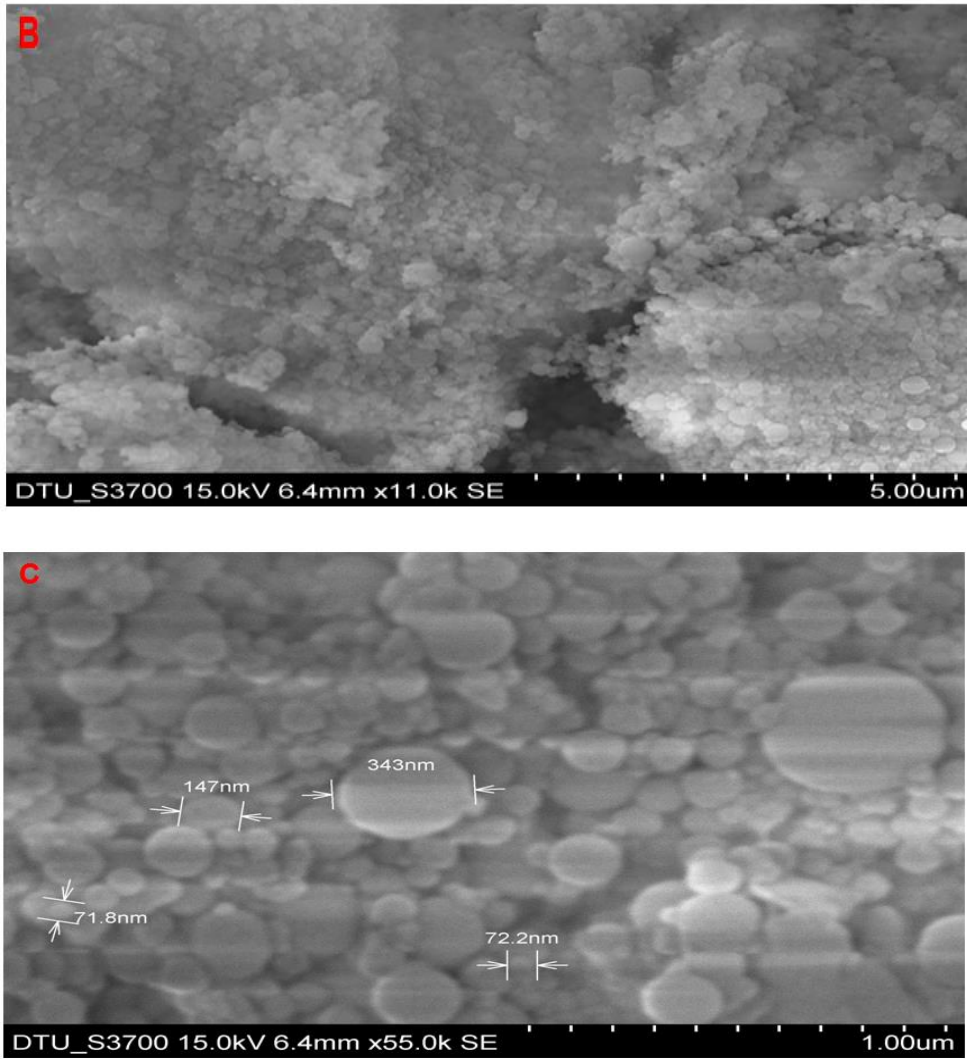


Figure 21. SEM images showing spherical nano dimensional liposome architecture. A, B and C indicates characterization images for different sets of experiment as indicated in Table 6 above. Liposome with sizes less than 100 nm can be clearly seen in 21(C) among other polydispersed sizes.

CHAPTER 5: CONCLUSION

The broader aim of this project is to establish the regulatory Notch-1/miRNA axis, which might be contributing towards aggressive and metastatic behaviours of breast neoplasm. However, it was evident from literary surveys and in silico analysis that a number of key intermediate components could also be involved thus building up the entire Notch/miRNA mediated EMT signalling loop. Klf-8 and Zeb-1 are known EMT regulators but not much is understood about the mechanism that leads to these components participating directly or indirectly in the EMT signalling loop. An attempt has been made in this project to study the probable role of miRNA-24 and Notch-1 in modulating EMT in breast carcinomas. The critical inferences drawn from the study are enlisted below.

Klf-8 expression levels

The Krüppel-type zinc finger transcription factors comprise a conserved family of DNA binding proteins that are important in developmental regulation. The Krüppel zinc finger transcription factor was initially identified in Drosophila as a segmentation gene. Krüppel-like factor 8 (KLF8), also called basic Krüppellike factor 3 and zinc finger protein 741, is a 359 amino acid transcriptional repressor that binds CACCC elements in DNA and activates or represses their target genes in a context-dependent manner. KLF8 is expressed ubiquitously in the nucleus of many cell types and its expression is elevated in several human cancers. KLF8 is post-translationally modified and negatively regulated by sumoylation via SUMO-1, SUMO-2 or SUMO-3. Mutation of the sumoylation site, Lysine 67, to Arginine 67 enhances the ability of KLF8 to repress or activate its target promoters (van Vliet et al., 200; Lossi et al., 2002; Zhao and Bian et al., 2003).

Expression data obtained from Gene expression atlas revealed that Klf-8 is mostly over expressed in breast carcinomas. Klf-8 can mediate EMT via miR-146a and Notch-1 as discussed earlier so the fact that Klf-8 is over regulated signifies that Klf-8 might be involved in some kind of interaction thus leading to poor prognosis in breast cancer patients.

miR-24 and Klf-8 interaction

It is evident from in silico analysis that Klf-8 3'UTR region consists of seed sequences for miR-24 binding. Therefore, it can be expected that miRNA-24 controls Klf-8 expression by direct binding interactions. However, under conditions of Klf-8 expression miR-24 expression should be diminished thus leading to Klf-8 mediated effects during breast carcinogenesis.

Hypermethylation of miRNA-24 gene promoters

Hypermethylation of miRNA gene promoters is the most prominent cause of miRNA aberrations observed in cancerous tissues. CpG islands present in and around the miRNA gene promoters are the active sites of methylation by histone methyl transferases. The CpG plot as revealed through in silico studies points at the high density of CpG sites in an around the miRNA-24 gene thus supporting the fact that hypermethylation of this densely populated CpG sites could be a probabilistic theory supporting miRNA-24 aberrations in breast tumours.

miRNA-24 expression levels

In order to estimate the miR-24 expression status in breast cancerous and normal tissues, RT-PCR was performed using pre designed primers (T_m=58.1^o). PCR analysis revealed that miRNA-24 is under expressed in cancerous tissues obtained from two patients as compared to normal control. It could then possibly indicate that miRNA-24 could be tumour suppressive in nature. Furthermore, based on the PCR data it can be suggested that due to its under expression, miRNA-24 is unable to bind to KLf-8 3'UTRs as a result leading to an augmented Klf-8 expression as was observed earlier by the Klf-8 studying gene expression data.

Increased Notch-1 levels

Notch-1 levels were found to be increased in cancerous breast tissue as compared to normal breast tissue although not significantly. This indicates that Notch-1 signalling is up regulated in breast cancerous tissue as compared to normal breast tissue.

miR-24/Klf-8/Notch-1

A probable correlation therefore can be suggested at this point which vaguely supports our initial hypothesis that Klf-8 and Notch-1 are probable EMT modulators. In addition, our recent experiments on miR-24 suggests that it could be tumour suppressive by nature and in addition could be a key regulatory component of the Klf-8/Notch-1 loop Additional experiments are however required and a statistical inference be reached at before a concrete establishment for the miR-24/Klf-8/Notch-1 axis can be laid in breast neoplasm.

Liposome as dual drug carriers

Liposomes synthesized here shall be used as a siRNA transfer vector. Although, liposomes has long been used as drug delivery modules but in contrast only a limited number of liposome drug formulatios are currently in operation. In recent years, different modification techniques such as PEGylation and PASalytaion has been used to make the drug loaded liposomes more bioavailable. A plethora of polymers exist and can be used for liposome modifaction before systemic release.

The basic idea behind synthesizing liposomes in our project was to lay a platform for liposome-gel two tier drug delivery platform. Alginate-chitosan gels have nano meter range pore sizes and has been individually used as drug carriers. Therefore, modifying the liposmes with alginate-chitosan gel will allow a couple of benefits; Firstly, for combinatorial drug (simultaneous delivery of two drugs) delivery as well as make the system more bioavailable.

However, it looks amateurish at this stage and further experiments and animal model study needs to be performed before the assumption becomes a blatant reality.

CHAPTER 6: REFERENCES

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