Annexure-IX

PROJECT COMPLETION REPORT

Notes:
1. The PCR should be in bound form.
2. Cover page should include the title of the project, file number, names and addresses of the investigation.

1. Title of the project: "Studies on exosomal lipidomics and micro RNAs and their clinical utility in the management of metastatic and multiple drug resistant breast cancer"

Sanction Order: SB/EMQ-215/2014 dated 23/01/2016

2. Principal Investigator:

Dr. Anand Ramteke
Professor
Dept. of Molecular Biology and Biotechnology
Tezpur University

3. Implementing Institution(s) and other collaborating Institution(s): **TEZPUR UNIVERSITY**

4. Date of commencement: 21/01/2016

5. Planned date of completion: 20/01/2020

6. Actual date of completion: Not Applicable

7. Objectives as stated in the project proposal:

- To develop the drug resistance sublines of Breast Cancer cells.
- Isolation and Characterisation of exosomes derived from drug sensitive and resistant Breast Cancer cell lines.
- Lipidomic Analysis of exosomes derived from drug sensitive and resistant Breast Cancer cell lines.
- Study on differential expression of exosomal microRNAs derived from the drug sensitive and resistant Breast Cancer cell lines.
- Analysis of exosomal lipidomics and microRNA data for their role in drug resistance using bioinformatics and statistical tools.
- To study the role of exosomes derived from drug resistant cancer cells in proliferation, migration, invasion and drug resistance of cancer cells.
- Study of molecular mechanism of exosomes mediated drug resistance in breast cells in terms of expression of genes involved in lipid metabolism and drug resistance.

8. Deviation made from original objectives if any, while implementing the project and reasons thereof:

As cited in literatures that hypoxia in the tumor micro-environment determines disease aggressiveness and drug resistance and is considered as a prognostic indicator of poor outcome several malignancies. We have also tried to explore the possible role of exosomes derived from hypoxic MCF7 and MD-MB231 breast cancer cells and their role in drug resistance in breast cancer.

9. Experimental work giving full details of experimental set up, methods adopted, data collected supported by necessary table, charts, diagrams & photographs:

<u>Objective 1</u>: To develop the drug resistant sublines of Breast Cancer cells.

Development of drug resistant cell lines

MDA MB 231and MCF-7 cells were selected for progressive resistance to drug, epirubicin. Selection began at a drug dose (dose 1) that was 1000 fold less than concentration at which 50% of parental cells were killed (IC_{50}). The dose was then increased 1.5 fold until maximally tolerated dose is achieved. At each dose cells were maintained for atleast 3 generations and an aliquot of cells were stored before each increase in drug dose. MDA-MB-231 cells were exposed to epirubicin through 48 passages over 386 days at concentrations ranging from 0.848 to 848 nM.

Characterization of sublines

Cytotoxicity assay

The effect of the epirubicin on the proliferation of sensitive and resistant cell lines were tested in 96-well microtiter plates, using tetrazolium based semi-automated colorimetric (MTT) assay. Cells were plated and exposed to epirubicin. Cell viability was calculated in percent compared to untreated control cells. A minimum of three independent experiments was performed for each of the parental cell lines, sub-lines and final resistant cell lines. The inhibition of cell proliferation and IC_{50} values were determined for each cell line. The resistance index (RI) was determined as the ratio of the IC_{50} of the drug resistant cell line/IC_{50} of sensitive cell line. The resistance indices were evaluated according to the following expression:
R= IC_{50} resistant cell line/ IC_{50} sensitive cell line

Detection of cross-resistance to anti-cancer drugs.

Cross-resistance measurements were performed on the parental and the resistant cell lines to examine their sensitivity to a wide variety of chemotherapeutic agents. Parental and resistant sublines were seeded into 96-well plates and incubated for proper adherence. Subsequently, the drug-free medium was replaced with fully supplemented fresh medium, containing different concentrations of drugs, docetaxel and cisplatin and the plates were incubated for 72 hr at 37˚C. The suppression of cell proliferation was examined using the MTT assay.

Exosomal Proteome Analysis

The proteins were extracted from the purified exosomes and treated with 100mM DTT at 95ºC for 1hr followed by 250mM IDA at room temperature in dark for 45min. The samples wer then digested with Trypsin and incubated overnight at 37˚C. The peptides were extracted in 0.1% formic acid and incubated at 37˚C for 45 minutes. The solution was then centrifuged at 10000g and the supernatant was collected into a separate tube and vacuum dried and dissolved in 20µl of 0.1% formic acid in water. 10µL injection volume was used on C18 UPLC column for separation of peptides. Liquid chromatography was performed on a ACQUITY UPLC system (Waters, UK). The separation of all samples was performed on ACQUITY UPLC BEH C18 column(Waters,UK)(150mm X 2.1mm X 1.7µm).A gradient elution program was run for the chromatographic separation with mobile phase A (0.1% Formic Acid in WATER), and mobile phase B(0.1% formic Acid in ACETONITRILE) as follows

S.No	Time	Flow	%A	%B	Curve
1	Initial	0.300	98.0	2.0	Initial
2	1.00	0.300	98.0	2.0	6
3	30.00	0.300	50.0	50.0	6
4	32.00	0.300	50.0	50.0	6
5	40.00	0.300	20.0	80.0	6

6	45.00	0.300	20.0	80.0	6
7	50.00	0.300	98.0	2.0	6
8	55.00	0.300	98.0	2.0	6
9	60.00	0.300	98.0	2.0	6

The peptides separated on the column were directed to Waters Synapt G2 Q-TOF instrument for MS and MSMS analysis. A SYNAPT G2 QTOF (Waters, UK) equipped with an electrospray ionization (ESI) source was used for mass spectrometric detection. The raw data was processed by MassLynx 4.1 WATERS. The individual peptides MSMS spectra were matched to the database sequence for protein identification on PLGS software (Protein Lynx Global Server), WATERS. The pathway were identified using UniProt ID with p and q values and the Gene Ontology was done by GOSlim.

The operation parameters were as follows

Experimental Instrument Parameters

- Polarity ES+
- Analyser Resolution Mode
- Capillary (kV) 3.5000
- Source Temperature (°C) 150
- Sampling Cone 45
- Extraction Cone 4.5
- Source Gas Flow (mL/min) 30
- Desolvation Temperature (°C) 350
- Cone Gas Flow (L/Hr) 30
- Desolvation Gas Flow (L/Hr) 80

Acquisition:

Acquisition Time

Start time : 0 min
End Time : 60 min

Source : ES

Acquisition Mode

Polarity : Positive
Analyzer Mode : Resolution

TOF MS:

Da Range

Start : 50Da
End : 1500Da

Scanning Conditions

Scan Time : 0.5 Sec
Data Format : Continuum

Collision Energy:

Function-1 Low Energy

Trap Collision Energy : On – 6V
Transfer Collision Energy : On – 6V

Function-2 High Energy

Ramp Trap Collision Energy : On – 20V to 45V
Ramp Transfer Collision Energy : Off

Cone Voltage:

Cone Voltage: 40V

Results

1. Development of drug resistant MDA MB 231 and MCF-7 sublines

Two different human breast cancer cell lines, one estrogen receptor positive and one estrogen receptor negative, MCF-7 and MDA-MB-231, which are widely used as models for hormone-dependent and independent human breast cancer respectively were cultured in the presence of increasing concentrations of epirubicin to establish a model similar to the evolution of the acquired drug resistance.

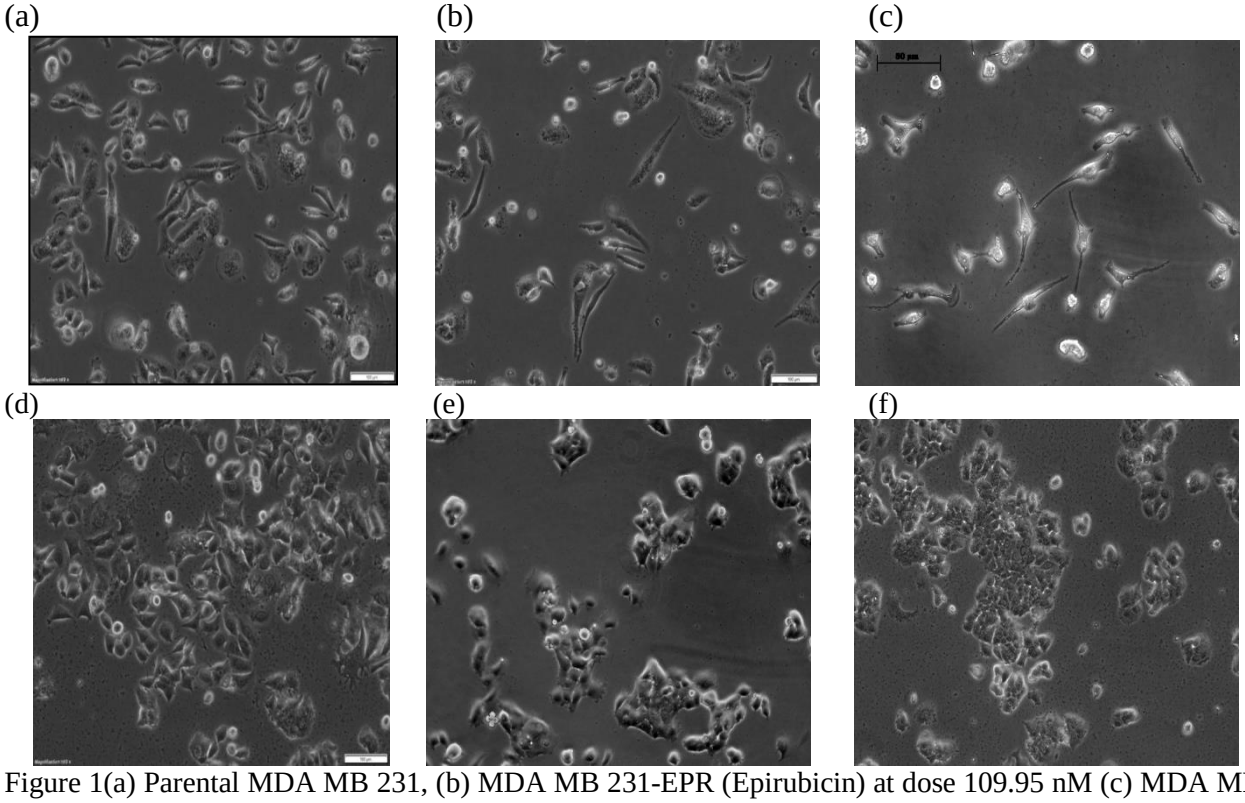
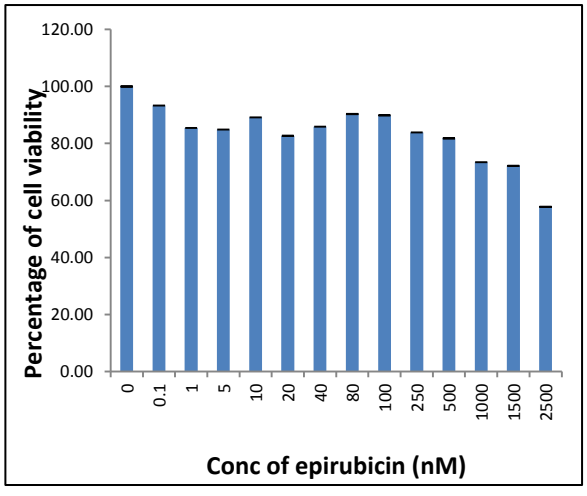


Figure 1(a) Parental MDA MB 231, (b) MDA MB 231-EPR (Epirubicin) at dose 109.95 nM (c) MDA MB 231-EPR (Epirubicin) at dose 556 nM , (d)Parental MCF-7, (e) MCF-7 -EPR (Epirubicin) at dose 191.67 nM, (f) MCF-7 -EPR (Epirubicin) at dose 431 nM

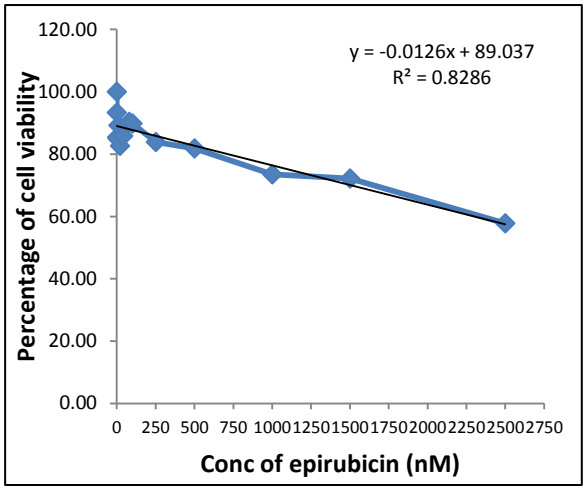
2. CHARACTERIZATION OF RESISTANT SUB- LINES

MCF-7-EPR-(431nM) and MDA-MB231-EPR-(556nM), their respective parental cell lines and additional sub-lines, isolated during development of resistance, were further characterized. Culturing of cells with continuous exposure to increasing concentrations of epirubicin induced resistance to the drug. Both MCF-7 and MDA-MB-231 cells showed a biphasic response pattern when exposed to epirubicin, with their resistant sub-lines being significantly more resistant both in a first phase at lower epirubicin concentrations, and in a second phase at higher concentrations. (Figure 2 and 3)

(a)



(b)



2. (A) Determination of inhibitory concentration (IC50) of epirubicin against MCF 7$_{(sub\text{-}431\ nm)}$ cell line

Figure.2: Effects of epirubicin on viability of (a) MCF 7 (sub-431nM) cells. Cells were treated with different concentration of drug for 72 h. (b) Dose response curves with epirubicin for cell line MCF 7 (sub-431nM)

TABLE 1: Comparison of antiproliferative effects of epirubicin on sensitive and resistant MCF 7 sub lines

Drug	Cell Lines	IC50 (µM)	Resistance Index
Epirubicin	MCF 7/S	2.216	1.00
Epirubicin	MCF 7/EPR (sub-431 nM)	3.252	1.46

The resistance index of MCF 7 sub line maintained at 431nm was calculated to determine the degree of acquired resistance of resistant sub line to its selective drug.

2.(B) Determination of inhibitory concentration (ic50) of epirubicin against MDA MB 231 (sub-556nm) cell line

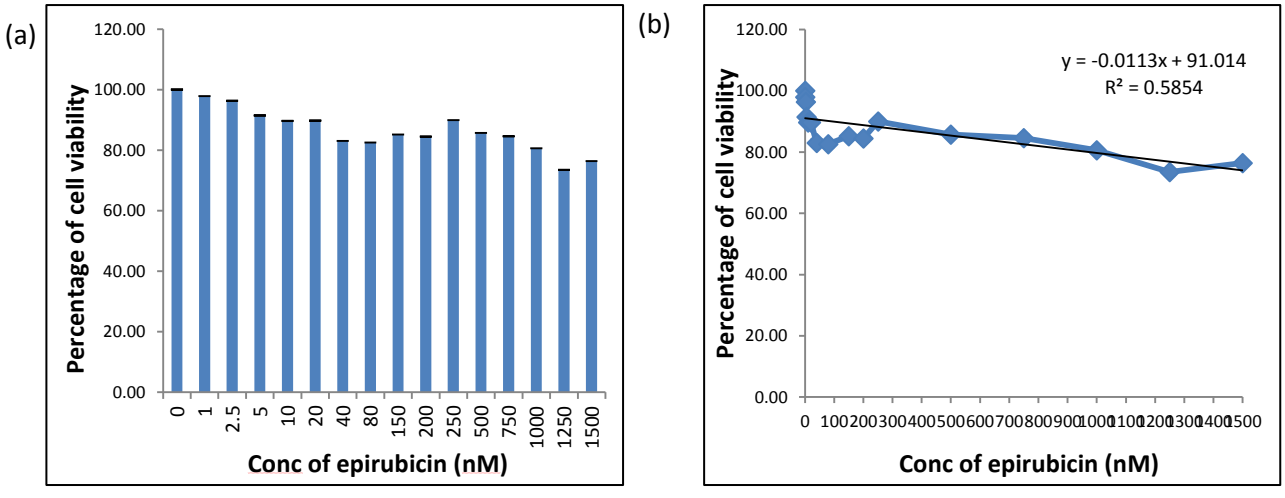


Figure.3: Effects of epirubicin on viability of (a) MDA MB 231 (sub-556nm) cells. Cells were treated with different concentration of drug for 72 h. Dose response curves with epirubicin for cell line (b) MDA MB 231 (sub-556nm)

TABLE 2: Comparison of antiproliferative effects of epirubicin on sensitive and resistant MDA MB 231 sub lines

Drug	Cell Lines	IC50 (µM)	Resistance Index
Epirubicin	MDA MB 231/S	0.848	1.00
Epirubicin	MDA MB 231/EPR (sub-48 nM)	2.639	3.11
Epirubicin	MDA MB 231/EPR (sub-556 nM)	3.728	4.39

The resistance index of MDA MB 231 sub line maintained at 48nm and 556nm was calculated to determine the degree of acquired resistance of resistant sub lines to its selective drug.

TABLE 3. Evolution of epirubicin resistance in MDA MB 231 cells

Epirubicin concentration	Passage numbers	Days at given concentration
0 (Parental cells)	———	———
0.848 nM	1–3	07
1.272nM	4–5	06

1.908 nM	6–7	08
2.862 nM	8–9	09
4.293 nM	10–12	15
6.439 nM	13–14	07
9.659 nM	15-17	12
14.488nM	18-20	15
21.733nM	21-23	29
32.599nM	24-26	34
48.899nM	27-29	44
73.349nM	30-32	22
109.95nM	33-35	37
164.925nM	36-38	42
247.387nM	39-41	35
371.081nM	42-44	24
556.621nM	45-47	40
848.930nM	48-	

2. (c) Drug cytotoxicity for MDA MB 231 subline

The sensitivity of selected sub-lines isolated during development of resistance was investigated to a range of epirubicin concentration and the survival of each of the sub-lines was calculated as the ratio of surviving cells in each sub-line compared to the corresponding parental cell line. The results are depicted in Figure 4 (a) and (b)

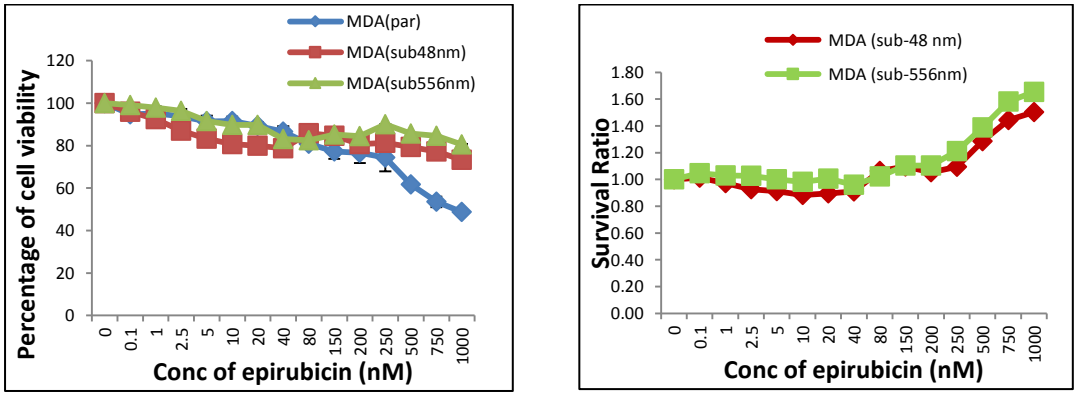


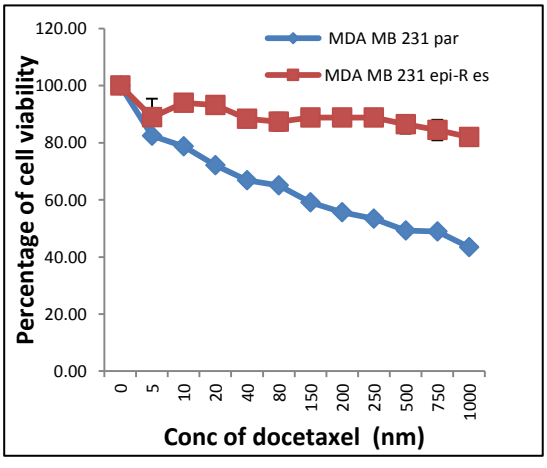
Figure 4(a) Sensitivity of parental and resistant sub lines to epirubicin. The survival ratio of (b) MDA MB 231-EPR(sub-48 nM and sub-556 nM)) compared to corresponding parental cell line MDA(par). Cells were exposed to epirubicin at the indicated concentrations for 72 h and survival was estimated using an MTT

assay. Values are expressed as relative values compared to the parental cell lines. Mean values of triplicate samples ± SEM are shown.

2. (D) Assessment for cross resistance of MDA MB 231 resistant sublines

The resistant cell lines were assessed for cross-resistance to the taxane docetaxel (Figure 5) and the platinum cisplatin (Fig.5.b). With respect to their parental cell lines, MDA MB231 EPI-Res had developed a significant level of cross-resistance to docetaxel, but could not developed cross-resistance to cisplatin suggesting that resistance to epirubicin, an anthracycline has the capacity to generate cells highly resistant to taxanes

(a)



(b)

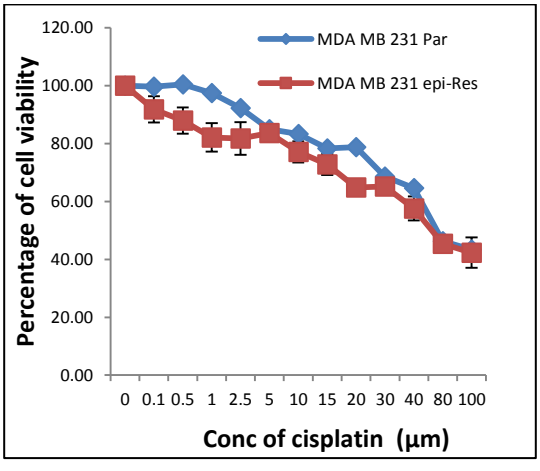


Figure 5 Effects of docetaxel and cisplatin on viability of epirubicin-resistant and parental MDA MB 231 cell lines. Cells were treated with docetaxel and cisplatin for 72 h and cell viability assessed by MTT assay. Response of epirubicin-resistant and parental cell lines to (a) docetaxel and (b) cisplatin. Error bars represent the standard deviation of triplicate samples.

We have developed the epirubicin resistant MCF7-EPR and MDA-MB231-EPR sublines that exhibited resistance index of 1.46 and 4.39 respectively. For further studies we have selected MDA-MB231-EPR subline but unfortunately we lost the cells due to severe contamination in the laboratory. Accordingly we have modified the objectives and tried to explore the possible role of exosomes derived from hypoxic MCF7 and MD-MB231 breast cancer cells in drug resistance. This was done as per the discussion in the progress presentation at KIIT, Bhubaneshwar.

Hypoxia and Drug resistance

Several studies have shown that hypoxia in the tumor micro-environment determines disease aggressiveness and drug resistance and is considered as a prognostic indicator of poor outcome in several malignancies. However, the precise mechanisms through which hypoxic condition promotes disease aggressiveness and drug resistance are not well understood.

To study the effect of hypoxia on cell proliferation and morphology human breast cancer cell lines – MDA-MB 231 and MCF7 were treated for 72h under hypoxic (2-5% oxygen) and normoxic conditions, and cell viability was studied using trypan blue exclusion method (Figure 6). The data showed 1.46 and 2.16 time more cells in normoxic than hypoxic conditions for MDA-MB 231 and MCF-7 cells respectively.

The number of normoxic MDA-MB 231 cells was found to be 1.46 times more than that of hypoxic ones and that for MCF7 was found to be 2.16 times more than the hypoxic ones. Lower proliferation rate in hypoxic condition has been accredited to hypoxia-associated deficiencies in other nutrients such as glucose which causes cells to stop or slow their rate of progression**.** Lower cell density in hypoxic conditions is also observed in the microscopic images in Figure 6 (scale bar- 100□m).

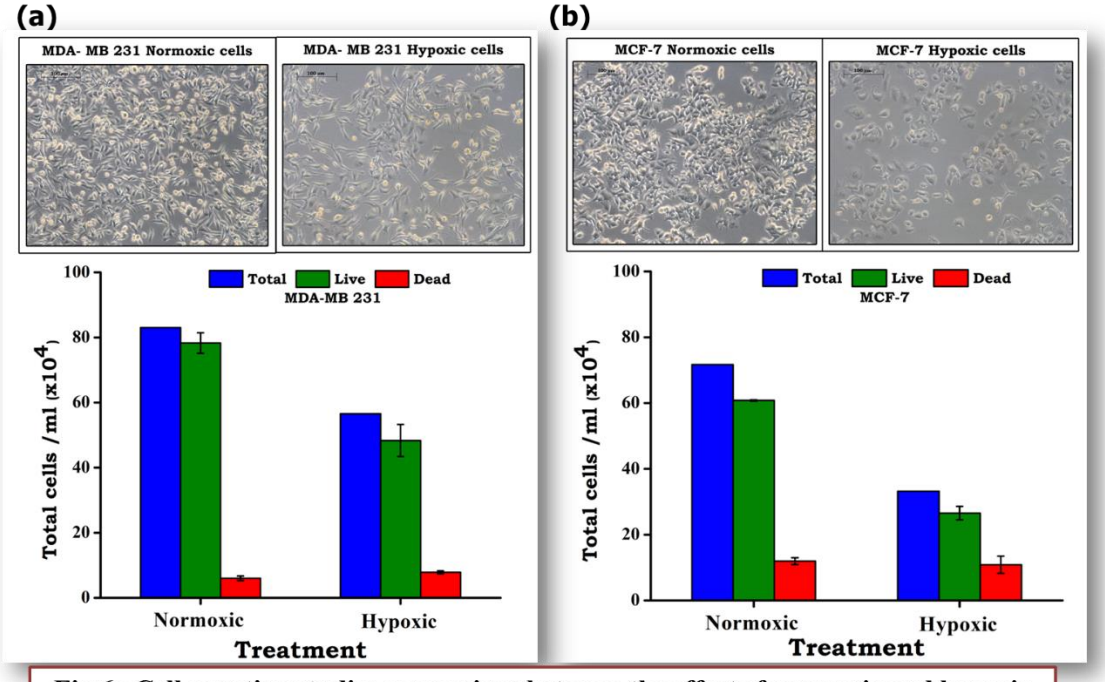


Fig.6 : Cell counting studies comparison between the effect of normoxic and hypoxic conditions on (a) MDA-MB 231 cells, (b) MCF7 cells.

The morphological changes normoxic and hypoxic MDA-MB-231 and MCF7 cells were evaluated by microscopy (scale bar- 20 μm). Hypoxia induced changes in structure and morphology, with smaller size, more elongation of the hypoxic structures (Figure 7) a well-known trait for hypoxic conditioned cells but cannot accredit this trait to less proliferation. More processing and analysis regarding the shape factor of the cells from the microscopic images is required to illuminate us on the morphological variations. Further insight about the cell morphology can be gained by Scanning Electron Microscopy (SEM) analysis.

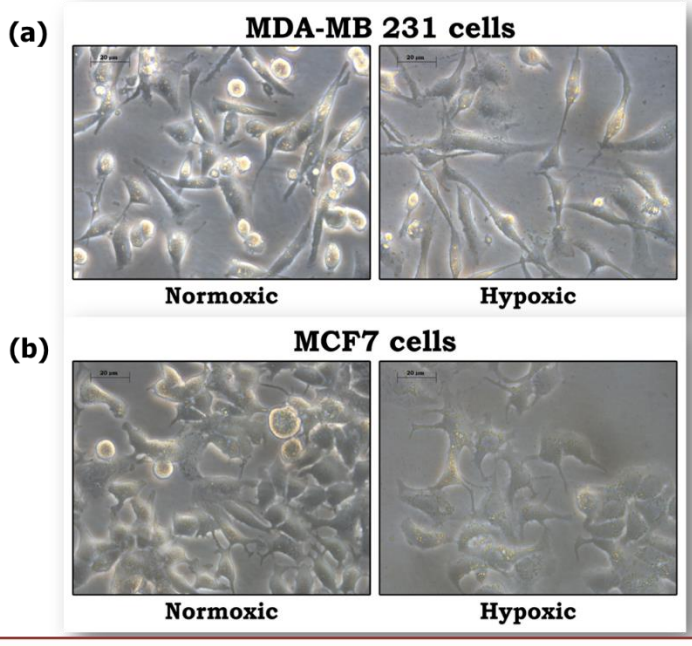


Fig.7 : Microscopic images for studies in morphological change of (a) MDA-MB 231 cells, (b) MCF7 cells under normoxic and hypoxic conditions (Scale – 20μm)

Hypoxia induced ROS, Autophagy and Lipid accumulation Hypoxia is reported to increase the intracellular ROS in a variety of cells. The MDA-MB-231 and MCF7 were exposed to hypoxia (2-5% oxygen) and ROS levels were measured using DCFDA stain by fluorescent microscope. The data showed significant increase in the ROS levels in both breast cancer cell lines

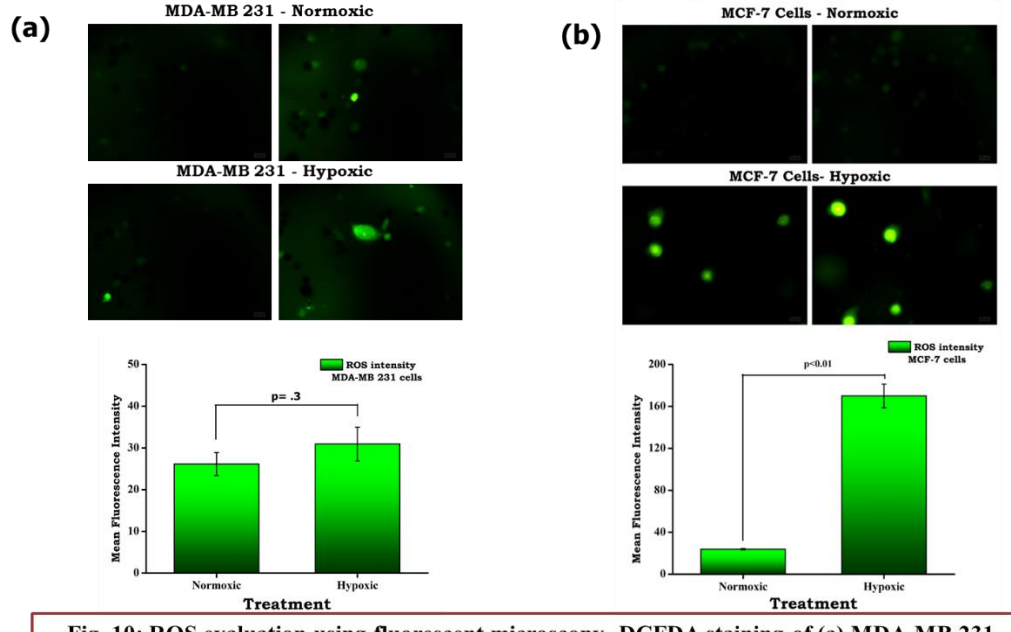


Fig. 10: ROS evaluation using fluorescent microscopy -DCFDA staining of (a) MDA-MB 231 cells, (b) MCF7 cells under normoxic and hypoxic conditions (Scale – 20μm). Images were processed by ImageJ and the MFI was evaluated indicating ROS intensity.

Kasper et al. hypothesized that autophagy, a lysosomal degradation pathway, may be involved in reducing ROS during periodic hypoxia through removal of ROS producing species. To study autophagy Acridine Orange (AO) was used and cells exposed to hypoxic and normoxic conditions were observed under fluorescent microscope (100x and 400x). The microscopic images were processed using ImageJ software and the number of acid vesicles (AVOs) and the degree of acidity (DOA) was analyzed. The data showed increase in the AVOs and DOA under hypoxia in both MDA-MB-231 and MCF7 cell lines (Figure 8 & 9). The work of He et al. (2012) also demonstrated hypoxia induced autophagy and the elevated autophagy activity is reported to be associated with increased radio-resistance of tumor cells. We also aimed at exploring the link between increased autophagy activities with drug resistance.

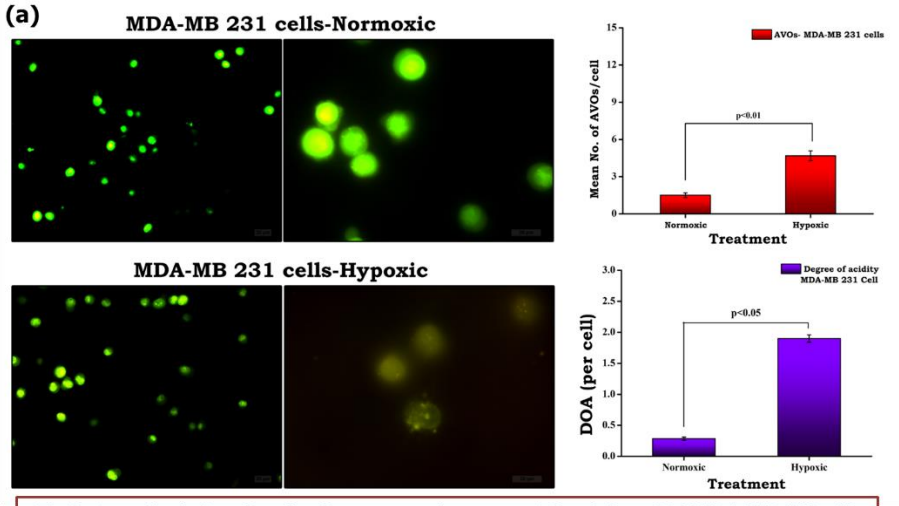


Fig.8 : Autophagical study using fluorescent microscopy -AO staining of (a) MDA-MB 231 cells under normoxic and hypoxic conditions. Images were processed and studied using ImageJ and the no. of acid vescicles were counted as shown in (b). (c) The degree of acidity for different conditioned cells were also calculated.

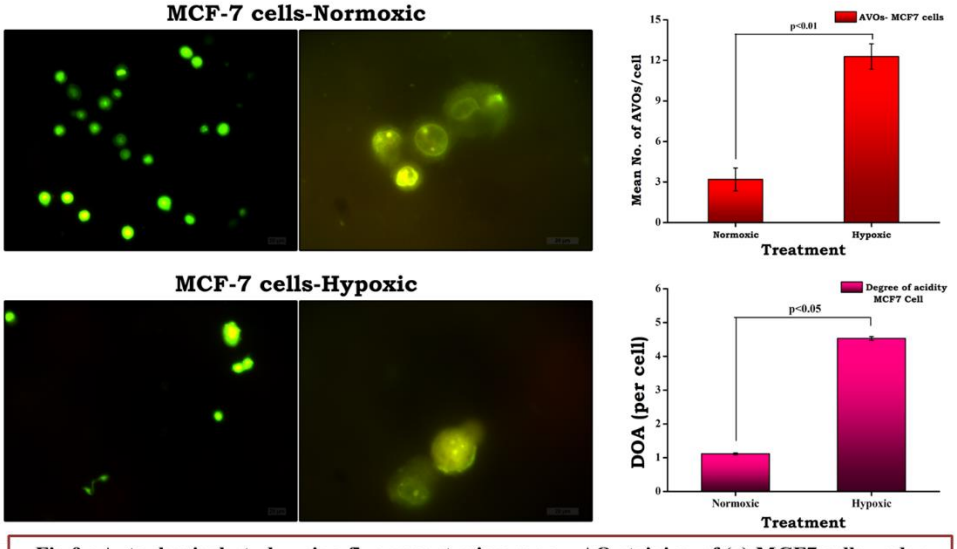


Fig.9 : Autophagical study using fluorescent microscopy -AO staining of (a) MCF7 cells under normoxic and hypoxic conditions. Images were processed and studied using ImageJ and the no. of acid vescicles were counted as shown in (b). (c) The degree of acidity for different conditioned cells were also calculated.

The hypoxia-dependent regulation of lipid metabolism is not well found in literature. In our study we evaluated the presence of lipid droplet (LD) accumulation within MDA-MB-231 and MCF-7 cells (under normoxic and hypoxic conditions) using Oil Red O staining. The LDs could be seen interspersed between the clear vacuoles as depicted in the microscopic images. We observed increase in lipid accumulation expressed as absorbance (OD) per one lakh cells 1 lakh cells (Figure 11)

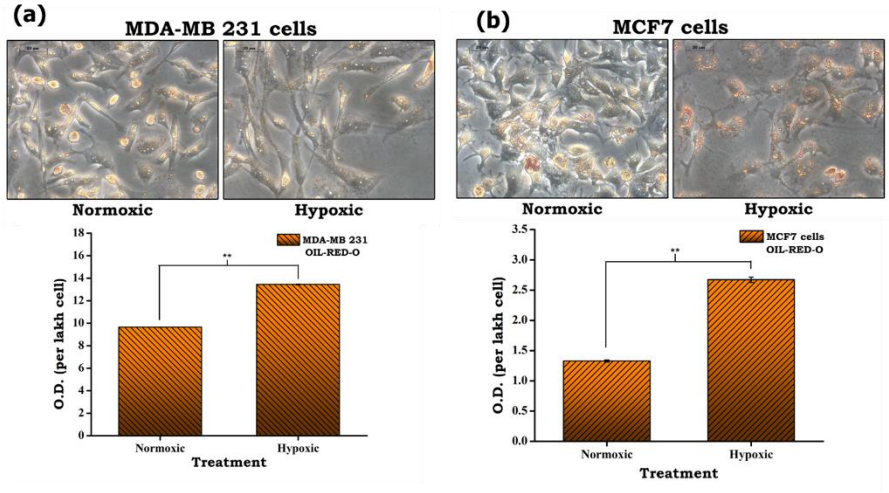


Fig.11 : OIL –RED O method results alongwith microscopic images for lipogenetic studies on (a) MDA-MB 231 cells, (b) MCF7 cells under normoxic and hypoxic conditions (Scale – 20µm)

Isolation and characterization of exosomes

The exosomes were isolated from MDA-MB 231 and MCF-7 cell lines cultured under hyoxic condition for 72 h. The size distribution of the exosomes were evaluated by Dynaic Light Scattering (DLS) method. The DLS analysis showed the exosomes size range from 50-1000 nm (Figure 12). The aggregation of exosome might have happened showing larger size of the particles.

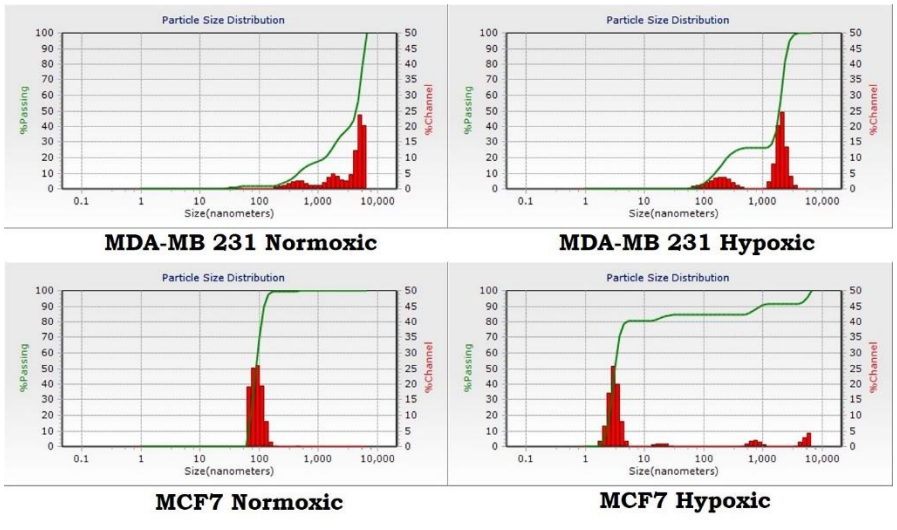


Figure 12 DLS study: Intensity vs size plot of exosomes isolated from the MDA-MB231 and MCF 7 breast cancer cell lines

Role of Exosomes in cell migration and drug resistance

Salnikov *et al.* (2012), suggested that hypoxia induces migratory potential in the cells. We have earlier reported that hypoxic exosomes increases migration in prostate cancer cells. Here we have examined the migration of MDA-MB 231 and MCF-7 cell lines treated with hypoxic and normoxic exosomes. The cells were seeded in 6-well plates and grown to confluence and then wound were scratched within the confluent cell layer using the fine end of a 10 ml pipette tip (time 0) and cells were treated with exosomes. The images of migrating cells collected from the wounded region for different time-points. The rate of wound closure increased in hypoxic exosome treated cells in both the breast cancer cells at 12h but for later time points decreased (Figure 13).

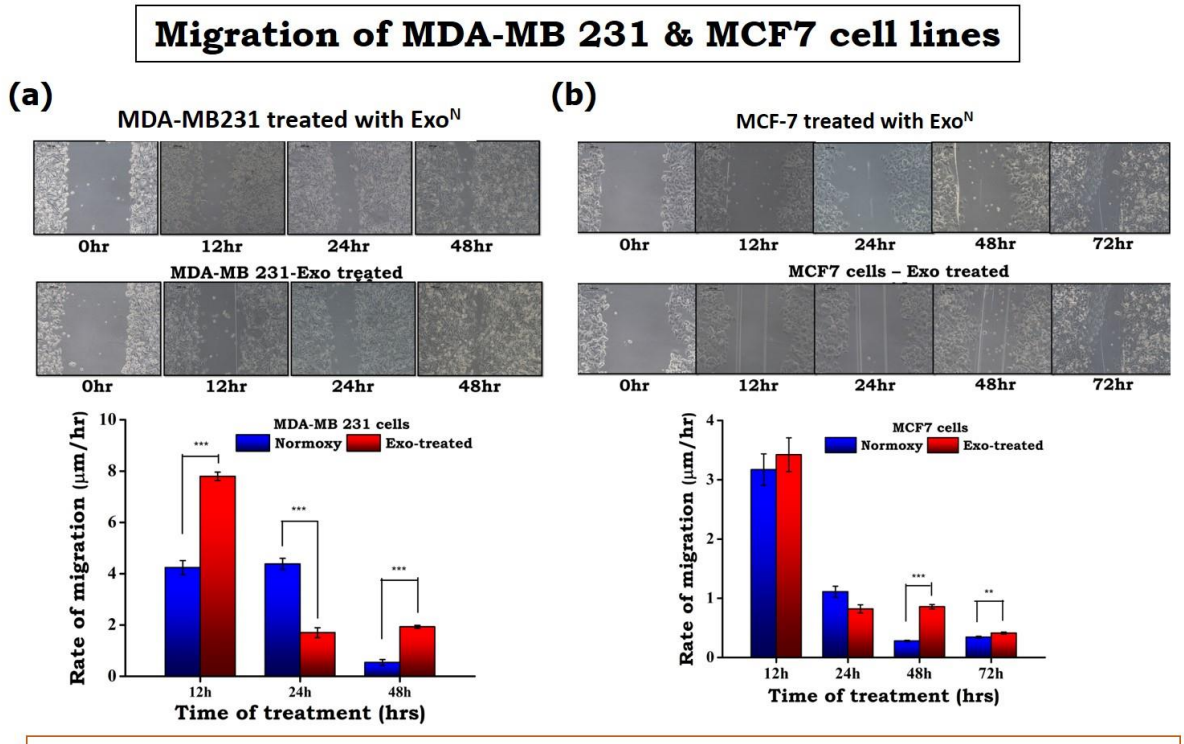


Fig. 13: Rate of migration of (a) MDA-MB 231 cells, (b) MCF7 cells treated with exosomes (Scale – 1000mm) (*=p<0.001, **=0.001<p<0.01, *=0.01<p<0.05)**

To study the role of exosomes in mediating drug resistance in cancer cells, we have exposed the MDA-MB 231 breast cancer cells with hypoxic exosomes at interval 6 and 12 h, followed by IC_{50} dose (0.84 µmole) of Epirubicin. Interestingly we observed significant restoration of cell viability were observed in the cells exposed to exosomes and IC_{50} dose of epirubicin (Figure 14). This indicates that exosomes may influence the efficacy of chemotherapeutic agents either directly or indirectly interacting with the drug. This needs to be further investigated.

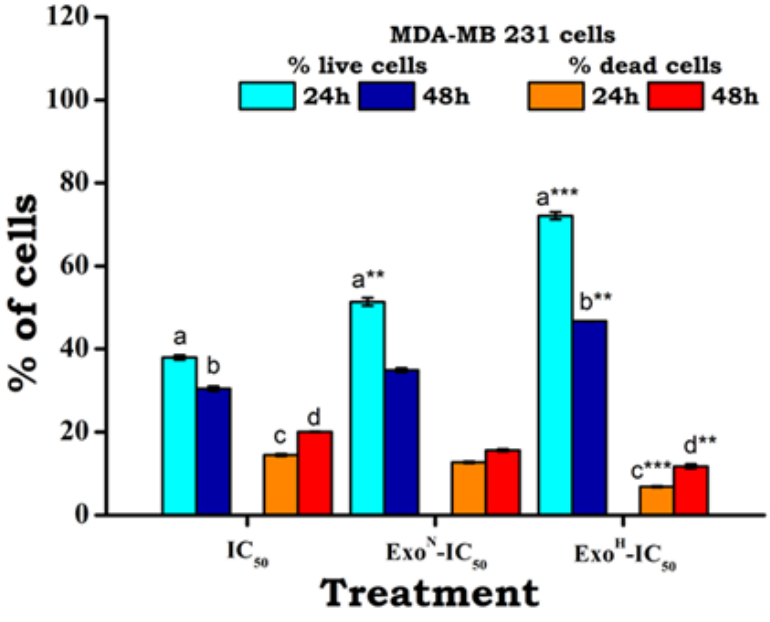


Figure 14: MDA-MB 231 cells treated with hypoxic exosomes for 6h and 12h followed by IC50 dose (0.84 µmole) of Epirubicin for 24h and 48 h and cell viability was checked with Trypan Blue exclusion method. (the annotations a,b,c & d are the ref. for significance test. ***=p<0.001, **=0.001<p<0.01, *=0.01<p<0.05

Exosomal microRNA and Proteome Studies:

We have collected the exosomes from the culture media of MCF-7 and MD-MB231 cells cultured under normoxic and hypoxic conditions and the exosomes were send to M/S Sandor Life Sciences Pvt. Ltd for extraction of microRNA and proteins for RNA sequencing and proteome analysis. The company experts were not able to extract the microRNA as result the RNA sequencing could not be performed. The proteins isolated were subjected to MS/MS analysis as per the methodology discussed above.

Here we have analyzed the proteome of exosomes isolated from MCF-7 and MD-MB231 cells exposed to hypoxia (3-5%) using LC-MS. The chromatogram of the exosome proteome profile of MD-MB231 and MCF-7 cells where we have identified 815 normoxic and 839 hypoxic proteins in MD-MB231 whereas in MCF-7 cells we have identified 919 and 921 in normoxic and hypoxic proteins respectively shown (Figure 15 & 16).

In the proteome analysis of MDA-MD231 cells normoxic vs hypoxic conditions, we observed 21 proteins downregulated and 19 proteins upregulated (Fold change <0.5 is considered as downregulated and fold change >2.0 is considered as upregulated) The top five downregulated proteins are Adenylate cyclase type 9 (Fold Change = 0.084), Collagen alpha-1(XI) chain (Fold Change = 0.115), CD109 antigen (Fold Change = 0.160) Latent-transforming growth factor beta-binding protein 1(Fold Change = 0.201), Mediator of RNA polymerase II transcription subunit 12 (Fold Change = 0.214) and upregulated proteins are Erythroid differentiation-related factor 1(Fold Change = 3.320), Latent-transforming growth factor beta-binding protein 2 (Fold Change = 3.669) , A disintegrin and metalloproteinase (Fold Change = 3.706), Melanoma inhibitory activity protein

2 (Fold Change = 3.819) and Enamelin (Fold Change = 4.014). The pathway analysis of upregulated and downregulated proteins in MDA-MB231 breast cancer cells exposed to hypoxia revealed involvement of several signaling pathways. The top ten pathways are Aldosterone synthesis and secretion (p value: 8.66E-06), Adrenergic signaling in cardiomyocytes (p value: 6.15E-05), cGMP-PKG signaling pathway ((p value: 0.000110668), Salivary secretion (p value: 0.000138779), Pancreatic secretion (p value: 0.000178153), Calcium signaling pathway(p value: 0.000180904), Extracellular matrix organization(p value: 0.000195732), Proton Pump Inhibitor Pathway(p value: 0.000332693), Endocrine and other factor-regulated calcium reabsorption (p value = 0.000354654) and Reduction of cytosolic Ca++ levels (p value= 0.000553061).

The number of different proteins involved various biological process are Biological regulation (33), metabolic processes (27), response to stimuli (25), cell communication (23), developmental process (21), cellular localization (20), cellular component reorganization (17). The number of proteins associated with important cellular structures such as membrane (24), nucleus (17), macro-molecular complex (16) membrane associated lumen (13), endomembrane system (9), vesicles (9), cytoskeleton (7) and extracellular matrix (7)

MCF 7 breast cancer cell is ER/PR positive and highly invasive and ductal carcinoma. We have also examined proteome of the MCF 7 cells exposed to hypoxia and observed that 15 proteins are downregulated and 28 proteins are upregulated (Fold change <0.5 is considered as downregulated and fold change >2.0 is considered as upregulated). The top five down regulated proteins are Protein unc-13 homolog B (Fold Change: 0.040), Calreticulin (Fold Change:0.050), Large neutral amino acids transporter small subunit 1 (Fold Change: 0.155), Transmembrane protein 88 (Fold Change: 0.186), Type II inositol 3,4-bisphosphate 4-phosphatase(Fold Change: 0.188), Suppressor of tumorigenicity 14 protein(Fold Change: 0.208) and top seven upregulated proteins are: Cilia- and flagella-associated protein 65(Fold Change: 5.206), Anion exchange protein 4(Fold Change: 7.463), Probable ATP-dependent RNA helicase DDX58(Fold Change: 7.845), Protein virilizer homolog(Fold Change: 8.331), Leucine-rich repeat transmembrane neuronal protein 2(Fold Change: 10.277), Zinc finger protein 197(Fold Change: 38.474), ATP-dependent RNA helicase DHX8(Fold Change: 1465.570). The proteins upregulated and downregulated in MCF 7 on exposure to hypoxia are associated with following pathways based on p value: Phase 0 - rapid depolarization (p value 5.75E-06), Cardiac conduction (p value 2.56E-05), Interaction between L1 and Ankyrins (p value 6.42E-05), Scavenging by Class F Receptors (p value 0.000107141), O-glycosylation of TSR domain-containing proteins (p value 0.000157489), Muscle contraction (p value 0.00016963), Negative regulators of DDX58/IFIH1 signaling (p value 0.001755439), Antigen Presentation: Folding, assembly and peptide loading of class I MHC (p value 0.001911764), L1CAM interactions (p value 0.002559707), O-linked glycosylation (p value 0.003786493)

The number of different proteins involved various biological process are Biological regulation (30), metabolic processes (26), response to stimuli (21), cell communication (23), developmental process (20), cellular localization (24), cellular component reorganization (18). The number of proteins associated with important cellular structures such as membrane (27), nucleus (15), macro-molecular complex (17) membrane associated lumen (10), endomembrane system (9), cell projections (9), vesicles (14), cytoskeleton (4) and extracellular matrix (4). (Figure 15 &16, Table 1, 2, 3 & 4).

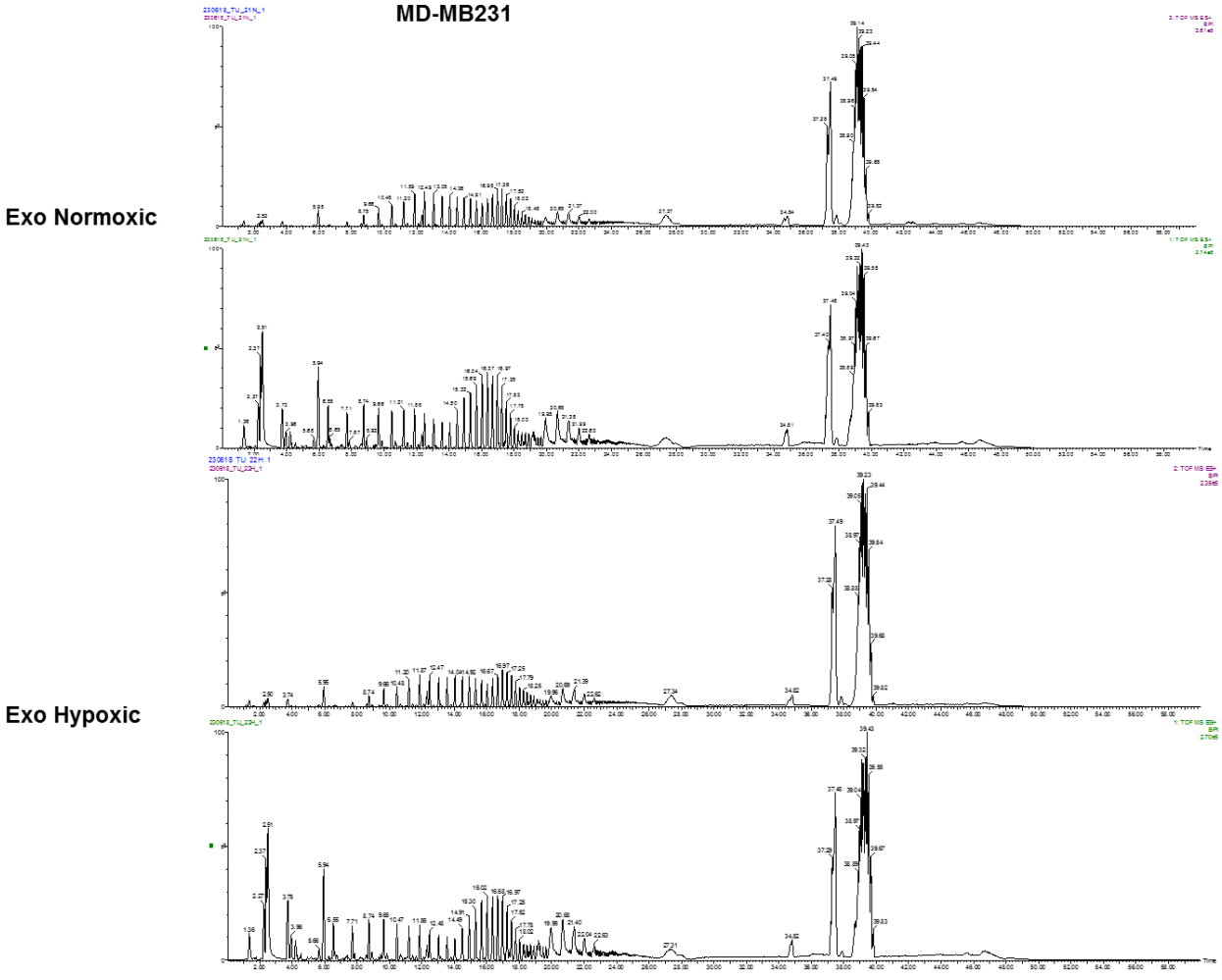


Figure 15 Chromatogram of Exosomal proteome profile of MD-MB231 exposed to normoxic and hypoxic conditions

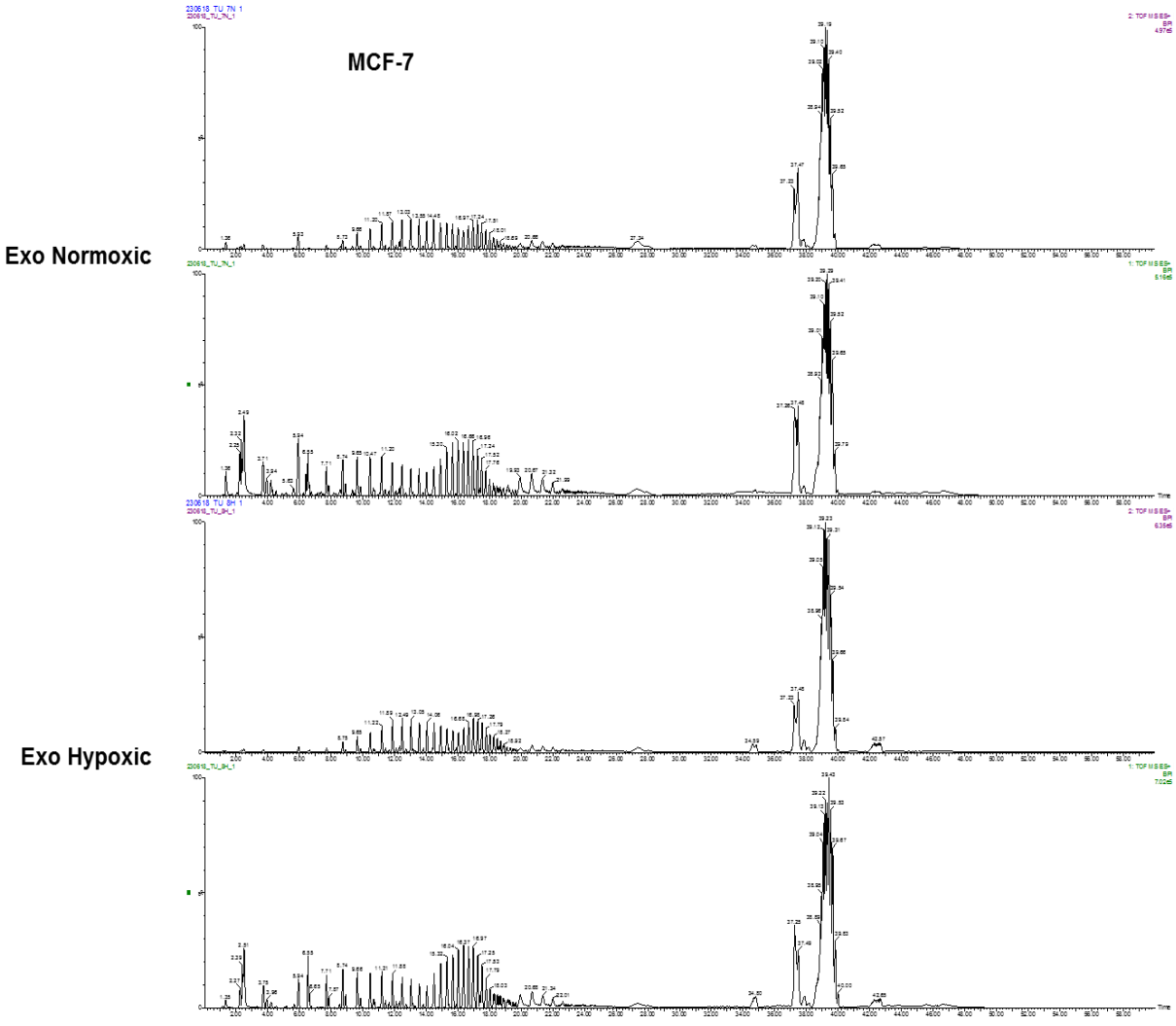


Figure 16 Chromatogram of Exosomal proteome profile of MCF-7 exposed to normoxic and hypoxic conditions

Table 1: Exosomal proteins upregulated/down regulated in MD-MB231 breast cancer cell line exposed to 3-5% hypoxic condition. Fold change <0.5 is considered as downregulated and fold change >2.0 is considered as upregulated

S.N.	Accession	Description	Score	Fold Change
1	O60503	Adenylate cyclase type 9 OS=Homo sapiens OX=9606 GN=ADCY9 PE=1 SV=4	54.01	0.084
2	P12107	Collagen alpha-1(XI) chain OS=Homo sapiens OX=9606 GN=COL11A1 PE=1 SV=4	72.12	0.115
3	Q6YHK3	CD109 antigen OS=Homo sapiens OX=9606 GN=CD109 PE=1 SV=2	87.05	0.160
4	Q14766	Latent-transforming growth factor beta-binding protein 1 OS=Homo sapiens OX=9606 GN=LTBP1 PE=1 SV=4	127.44	0.201
5	Q93074	Mediator of RNA polymerase II transcription subunit 12 OS=Homo sapiens OX=9606 GN=MED12 PE=1 SV=4	29.43	0.214
6	P27816	Microtubule-associated protein 4 OS=Homo sapiens OX=9606 GN=MAP4 PE=1 SV=3	89.24	0.220
7	Q06418	Tyrosine-protein kinase receptor TYRO3 OS=Homo sapiens OX=9606 GN=TYRO3 PE=1 SV=1	69.97	0.239
8	Q92824	Proprotein convertase subtilisin/kexin type 5 OS=Homo sapiens OX=9606 GN=PCSK5 PE=1 SV=4	56.47	0.244
9	O14529	Homeobox protein cut-like 2 OS=Homo sapiens OX=9606 GN=CUX2 PE=1 SV=4	60.43	0.280
10	Q96SB3	Neurabin-2 OS=Homo sapiens OX=9606 GN=PPP1R9B PE=1 SV=2	138.29	0.280
11	P20020	Plasma membrane calcium-transporting ATPase 1 OS=Homo sapiens OX=9606 GN=ATP2B1 PE=1 SV=3	65.69	0.289
12	Q14152	Eukaryotic translation initiation factor 3 subunit A OS=Homo sapiens OX=9606 GN=EIF3A PE=1 SV=1	81.29	0.313
13	P78504	Protein jagged-1 OS=Homo sapiens OX=9606 GN=JAG1 PE=1 SV=3	44.4	0.332
14	O94911	ATP-binding cassette sub-family A member 8 OS=Homo sapiens OX=9606 GN=ABCA8 PE=1 SV=3	118.92	0.353
15	Q6IE81	Protein Jade-1 OS=Homo sapiens OX=9606 GN=JADE1 PE=1 SV=1	104.06	0.353
16	Q00722	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-2 OS=Homo sapiens OX=9606 GN=PLCB2 PE=1 SV=2	100.49	0.375
17	O14497	AT-rich interactive domain-containing protein 1A OS=Homo sapiens OX=9606 GN=ARID1A PE=1 SV=3	42.27	0.379
18	Q8IZF0	Sodium leak channel non-selective protein OS=Homo sapiens OX=9606 GN=NALCN PE=1 SV=1	110.89	0.379
19	Q13438	Protein OS-9 OS=Homo sapiens OX=9606 GN=OS9 PE=1 SV=1	118.37	0.390
20	Q5JV73	FERM and PDZ domain-containing protein 3 OS=Homo sapiens OX=9606 GN=FRMPD3 PE=2 SV=2	72.08	0.449

21	O14513	Nck-associated protein 5 OS=Homo sapiens OX=9606 GN=NCKAP5 PE=1 SV=2	20.23	0.472
22	P82987	ADAMTS-like protein 3 OS=Homo sapiens OX=9606 GN=ADAMTSL3 PE=1 SV=4	30	2.095
23	P53355	Death-associated protein kinase 1 OS=Homo sapiens OX=9606 GN=DAPK1 PE=1 SV=6	111.8	2.181
24	A6NKB5	Pecanex-like protein 2 OS=Homo sapiens OX=9606 GN=PCNX2 PE=2 SV=3	49.34	2.534
25	Q9P260	LisH domain and HEAT repeat-containing protein KIAA1468 OS=Homo sapiens OX=9606 GN=KIAA1468 PE=1 SV=2	55.69	2.664
26	Q9NQC3	Reticulon-4 OS=Homo sapiens OX=9606 GN=RTN4 PE=1 SV=2	56.46	2.691
27	Q5JU85	IQ motif and SEC7 domain-containing protein 2 OS=Homo sapiens OX=9606 GN=IQSEC2 PE=1 SV=2	316.3	2.857
28	Q9BXW9	Fanconi anemia group D2 protein OS=Homo sapiens OX=9606 GN=FANCD2 PE=1 SV=2	30.22	2.915
29	A3KMH1	von Willebrand factor A domain-containing protein 8 OS=Homo sapiens OX=9606 GN=VWA8 PE=1 SV=2	71.58	2.915
30	O60346	PH domain leucine-rich repeat-containing protein phosphatase 1 OS=Homo sapiens OX=9606 GN=PHLPP1 PE=1 SV=3	76.79	2.915
31	P49756	RNA-binding protein 25 OS=Homo sapiens OX=9606 GN=RBM25 PE=1 SV=3	152.81	3.034
32	P23634	Plasma membrane calcium-transporting ATPase 4 OS=Homo sapiens OX=9606 GN=ATP2B4 PE=1 SV=2	12.01	3.126
33	P01023	Alpha-2-macroglobulin OS=Homo sapiens OX=9606 GN=A2M PE=1 SV=3	136.83	3.158
34	Q13698	Voltage-dependent L-type calcium channel subunit alpha-1S OS=Homo sapiens OX=9606 GN=CACNA1S PE=1 SV=4	102.12	3.189
35	Q8N108	Mesoderm induction early response protein 1 OS=Homo sapiens OX=9606 GN=MIER1 PE=1 SV=2	116.39	3.254
36	Q9UN74	Protocadherin alpha-4 OS=Homo sapiens OX=9606 GN=PCDHA4 PE=1 SV=1	76.02	3.254
37	Q3B7T1	Erythroid differentiation-related factor 1 OS=Homo sapiens OX=9606 GN=EDRF1 PE=1 SV=1	37.69	3.320
38	Q14767	Latent-transforming growth factor beta-binding protein 2 OS=Homo sapiens OX=9606 GN=LTBP2 PE=1 SV=3	45.78	3.669
39	Q9P2N4	A disintegrin and metalloproteinase with thrombospondin motifs 9 OS=Homo sapiens OX=9606 GN=ADAMTS9 PE=1 SV=4	51.44	3.706
40	Q96PC5	Melanoma inhibitory activity protein 2 OS=Homo sapiens OX=9606 GN=MIA2 PE=1 SV=4	135.35	3.819
41	Q9NRM1	Enamelin OS=Homo sapiens OX=9606 GN=ENAM PE=1 SV=3	54.88	4.014

Table 2: Pathway Analysis Exosomal proteins upregulated/down regulated MD-MB231breast cancer cell line exposed to 3-5% hypoxic condition.

p-value	q-value	pathway	source
8.66E-06	0.001108739	Aldosterone synthesis and secretion - Homo sapiens (human)	KEGG
6.15E-05	0.003579099	Adrenergic signaling in cardiomyocytes - Homo sapiens (human)	KEGG
0.000110668	0.003579099	cGMP-PKG signaling pathway - Homo sapiens (human)	KEGG
0.000138779	0.003579099	Salivary secretion - Homo sapiens (human)	KEGG
0.000178153	0.003579099	Pancreatic secretion - Homo sapiens (human)	KEGG
0.000180904	0.003579099	Calcium signaling pathway - Homo sapiens (human)	KEGG
0.000195732	0.003579099	Extracellular matrix organization	Reactome
0.000332693	0.005043961	Proton Pump Inhibitor Pathway, Pharmacodynamics	PharmGKB
0.000354654	0.005043961	Endocrine and other factor-regulated calcium reabsorption - Homo sapiens (human)	KEGG
0.000553061	0.007023631	Reduction of cytosolic Ca++ levels	Reactome
0.000603593	0.007023631	Transport of small molecules	Reactome
0.000840716	0.008967641	Cortisol synthesis and secretion - Homo sapiens (human)	KEGG
0.000920988	0.009068189	Beta-agonist/Beta-blocker Pathway, Pharmacodynamics	PharmGKB
0.000998087	0.00912537	chromatin remodeling by hswi/snf atp-dependent complexes	BioCarta
0.001999578	0.017063068	Insulin secretion - Homo sapiens (human)	KEGG
0.002280478	0.017823695	Dilated cardiomyopathy (DCM) - Homo sapiens (human)	KEGG
0.002453704	0.017823695	Hypothesized Pathways in Pathogenesis of Cardiovascular Disease	Wikipathways
0.002506457	0.017823695	GnRH signaling pathway - Homo sapiens (human)	KEGG
0.002686623	0.018099358	cAMP signaling pathway - Homo sapiens (human)	KEGG
0.003074444	0.019676442	Platelet calcium homeostasis	Reactome
0.003296039	0.020090145	the information processing pathway at the ifn beta enhancer	BioCarta
0.003524904	0.020508534	Molecules associated with elastic fibres	Reactome
0.004368205	0.024310011	Cholinergic synapse - Homo sapiens (human)	KEGG
0.004776785	0.024670597	Elastic fibre formation	Reactome
0.004818476	0.024670597	Thyroid hormone signaling pathway - Homo sapiens (human)	KEGG
0.005419558	0.0266809	Vascular smooth muscle contraction - Homo sapiens (human)	KEGG
0.00590484	0.027993316	O-glycosylation of TSR domain-containing proteins	Reactome
0.006510139	0.029111307	Inositol phosphate metabolism	INOH
0.006752472	0.029111307	Cardiac conduction	Reactome
0.006822962	0.029111307	Splicing factor NOVA regulated synaptic proteins	Wikipathways

0.007468757	0.029370903	PLC beta mediated events	Reactome
0.00763801	0.029370903	Apelin signaling pathway - Homo sapiens (human)	KEGG
0.00763801	0.029370903	Estrogen signaling pathway - Homo sapiens (human)	KEGG
0.007801646	0.029370903	G-protein mediated events	Reactome
0.00848721	0.03103894	Transcriptional regulation of white adipocyte differentiation	Reactome
0.009433256	0.033237305	Retrograde endocannabinoid signaling - Homo sapiens (human)	KEGG
0.009607659	0.033237305	Calcium Regulation in the Cardiac Cell	Wikipathways

Figure 17: GoSlim summary exosomal proteins showing canonical pathways based on biological processes, cellular components and molecular functions for MD-MB231 cells exposed to 3-5% hypoxia

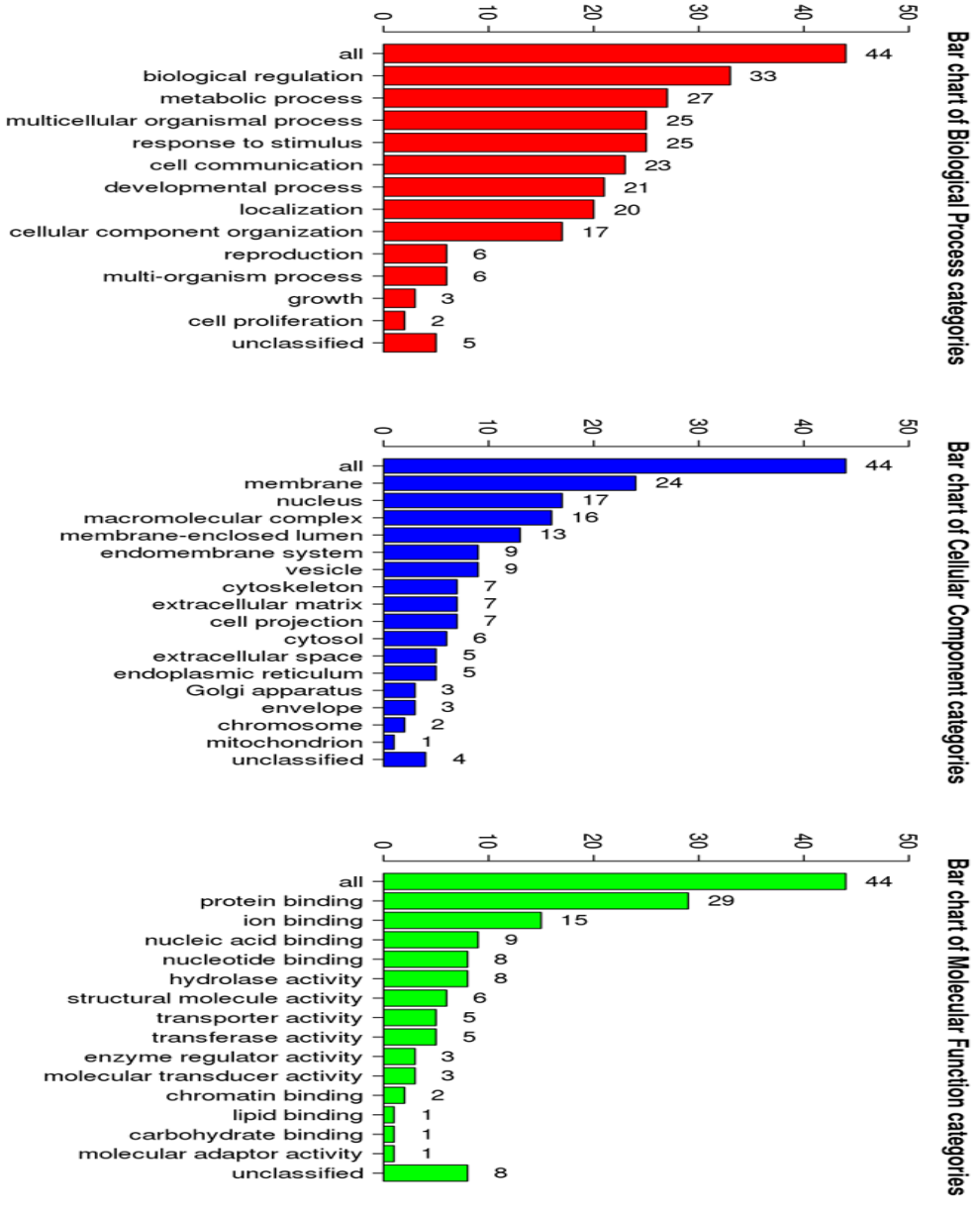


Table 3: Exosomal proteins upregulated/down regulated in MCF-7 breast cancer cell line exposed to 3-5% hypoxic condition. Fold change <0.5 is considered as downregulated and fold change >2.0 is considered as upregulated

S.N.	Accession	Description	Score	Fold Change
1	O14795	Protein unc-13 homolog B OS=Homo sapiens OX=9606 GN=UNC13B PE=1 SV=2	102.12	0.040
2	P27797	Calreticulin OS=Homo sapiens OX=9606 GN=CALR PE=1 SV=1	171.5	0.050
3	Q01650	Large neutral amino acids transporter small subunit 1 OS=Homo sapiens OX=9606 GN=SLC7A5 PE=1 SV=2	117.43	0.155
4	Q6PEY1	Transmembrane protein 88 OS=Homo sapiens OX=9606 GN=TMEM88 PE=1 SV=1	513.14	0.186
5	O15327	Type II inositol 3,4-bisphosphate 4-phosphatase OS=Homo sapiens OX=9606 GN=INPP4B PE=2 SV=4	287.25	0.188
6	Q9Y5Y6	Suppressor of tumorigenicity 14 protein OS=Homo sapiens OX=9606 GN=ST14 PE=1 SV=2	45.85	0.208
7	P07510	Acetylcholine receptor subunit gamma OS=Homo sapiens OX=9606 GN=CHRNG PE=1 SV=2	8.61	0.275
8	P07996	Thrombospondin-1 OS=Homo sapiens OX=9606 GN=THBS1 PE=1 SV=2	153.89	0.329
9	Q8NEY1	Neuron navigator 1 OS=Homo sapiens OX=9606 GN=NAV1 PE=1 SV=2	25.87	0.349
10	P35498	Sodium channel protein type 1 subunit alpha OS=Homo sapiens OX=9606 GN=SCN1A PE=1 SV=2	72.35	0.364
11	Q8N6G6	ADAMTS-like protein 1 OS=Homo sapiens OX=9606 GN=ADAMTSL1 PE=1 SV=4	32.79	0.402
12	P48634	Protein PRRC2A OS=Homo sapiens OX=9606 GN=PRRC2A PE=1 SV=3	58.34	0.444
13	Q96N67	Dedicator of cytokinesis protein 7 OS=Homo sapiens OX=9606 GN=DOCK7 PE=1 SV=4	64.21	0.449
14	O60315	Zinc finger E-box-binding homeobox 2 OS=Homo sapiens OX=9606 GN=ZEB2 PE=1 SV=1	102.57	0.453
15	Q5TH69	Brefeldin A-inhibited guanine nucleotide-exchange protein 3 OS=Homo sapiens OX=9606 GN=ARFGEF3 PE=1 SV=3	65.69	0.463
16	Q86YW9	Mediator of RNA polymerase II transcription subunit 12-like protein OS=Homo sapiens OX=9606 GN=MED12L PE=1 SV=2	29.62	2.013
17	Q8TEM1	Nuclear pore membrane glycoprotein 210 OS=Homo sapiens OX=9606 GN=NUP210 PE=1 SV=3	67.84	2.054
18	Q9NZV1	Cysteine-rich motor neuron 1 protein OS=Homo sapiens OX=9606 GN=CRIM1 PE=1 SV=1	71.59	2.075
19	Q92824	Proprotein convertase subtilisin/kexin type 5 OS=Homo sapiens OX=9606 GN=PCSK5 PE=1 SV=4	29.49	2.138
20	Q95604	HLA class I histocompatibility antigen, Cw-17 alpha chain OS=Homo sapiens OX=9606 GN=HLA-C PE=1 SV=1	36.24	2.159
21	Q01668	Voltage-dependent L-type calcium channel subunit alpha-1D OS=Homo sapiens OX=9606 GN=CACNA1D PE=1 SV=2	135.06	2.181
22	P20020	Plasma membrane calcium-transporting ATPase 1 OS=Homo sapiens OX=9606 GN=ATP2B1 PE=1 SV=3	132.43	2.203

23	Q14162	Scavenger receptor class F member 1 OS=Homo sapiens OX=9606 GN=SCARF1 PE=1 SV=3	70.17	2.316
24	O94986	Centrosomal protein of 152 kDa OS=Homo sapiens OX=9606 GN=CEP152 PE=1 SV=4	34.42	2.363
25	P10586	Receptor-type tyrosine-protein phosphatase F OS=Homo sapiens OX=9606 GN=PTPRF PE=1 SV=2	11.88	2.509
26	Q8TE56	A disintegrin and metalloproteinase with thrombospondin motifs 17 OS=Homo sapiens OX=9606 GN=ADAMTS17 PE=2 SV=2	67.27	2.691
27	P35499	Sodium channel protein type 4 subunit alpha OS=Homo sapiens OX=9606 GN=SCN4A PE=1 SV=4	64.7	2.745
28	Q9UJ55	MAGE-like protein 2 OS=Homo sapiens OX=9606 GN=MAGEL2 PE=1 SV=2	163.15	2.944
29	Q9NXF1	Testis-expressed protein 10 OS=Homo sapiens OX=9606 GN=TEX10 PE=1 SV=2	44.9	3.095
30	O75095	Multiple epidermal growth factor-like domains protein 6 OS=Homo sapiens OX=9606 GN=MEGF6 PE=1 SV=4	93.97	3.126
31	P56715	Oxygen-regulated protein 1 OS=Homo sapiens OX=9606 GN=RP1 PE=1 SV=1	29.75	3.353
32	Q6UB98	Ankyrin repeat domain-containing protein 12 OS=Homo sapiens OX=9606 GN=ANKRD12 PE=1 SV=3	20.23	3.632
33	O15031	Plexin-B2 OS=Homo sapiens OX=9606 GN=PLXNB2 PE=1 SV=3	56.45	3.743
34	Q9UI33	Sodium channel protein type 11 subunit alpha OS=Homo sapiens OX=9606 GN=SCN11A PE=1 SV=2	102.21	3.974
35	Q6BDS2	UHRF1-binding protein 1 OS=Homo sapiens OX=9606 GN=UHRF1BP1 PE=1 SV=1	119.97	4.0146
36	Q86WI3	Protein NLRC5 OS=Homo sapiens OX=9606 GN=NLRC5 PE=1 SV=3	104.85	4.437
37	O75096	Low-density lipoprotein receptor-related protein 4 OS=Homo sapiens OX=9606 GN=LRP4 PE=1 SV=4	94.83	4.854
38	Q6ZU64	Cilia- and flagella-associated protein 65 OS=Homo sapiens OX=9606 GN=CFAP65 PE=1 SV=2	39.33	5.206
39	Q96Q91	Anion exchange protein 4 OS=Homo sapiens OX=9606 GN=SLC4A9 PE=2 SV=2	91.45	7.463
40	O95786	Probable ATP-dependent RNA helicase DDX58 OS=Homo sapiens OX=9606 GN=DDX58 PE=1 SV=2	59.77	7.845
41	Q69YN4	Protein virilizer homolog OS=Homo sapiens OX=9606 GN=VIRMA PE=1 SV=2	51.77	8.331
42	O43300	Leucine-rich repeat transmembrane neuronal protein 2 OS=Homo sapiens OX=9606 GN=LRRTM2 PE=2 SV=3	46.73	10.277
43	O14709	Zinc finger protein 197 OS=Homo sapiens OX=9606 GN=ZNF197 PE=2 SV=1	82.47	38.474
44	Q14562	ATP-dependent RNA helicase DHX8 OS=Homo sapiens OX=9606 GN=DHX8 PE=1 SV=1	27.29	1465.570

Table 4: Pathway Analysis Exosomal proteins upregulated/down regulated in MCF-7 breast cancer cell line exposed to 3-5% hypoxic condition.

p-value	q-value	pathway	source
5.75E-06	0.000396739	Phase 0 - rapid depolarisation	Reactome
2.56E-05	0.00088379	Cardiac conduction	Reactome
6.42E-05	0.001475454	Interaction between L1 and Ankyrins	Reactome
0.000107141	0.00184818	Scavenging by Class F Receptors	Reactome
0.000157489	0.00195074	O-glycosylation of TSR domain-containing proteins	Reactome
0.00016963	0.00195074	Muscle contraction	Reactome
0.001755439	0.016488962	Negative regulators of DDX58/IFIH1 signaling	Reactome
0.001911764	0.016488962	Antigen Presentation: Folding, assembly and peptide loading of class I MHC	Reactome
0.002559707	0.019624422	L1CAM interactions	Reactome
0.003786493	0.026126805	O-linked glycosylation	Reactome
0.005517925	0.034612441	Binding and Uptake of Ligands by Scavenger Receptors	Reactome
0.007593835	0.036233081	Calcium Regulation in the Cardiac Cell	Wikipathways
0.007734524	0.036233081	MicroRNAs in cancer - Homo sapiens (human)	KEGG
0.007806727	0.036233081	Antigen processing-Cross presentation	Reactome
0.007876757	0.036233081	Phagosome - Homo sapiens (human)	KEGG
0.009085808	0.038231172	Influenza Life Cycle	Reactome
0.009419274	0.038231172	TGF-beta Receptor Signaling	Wikipathways

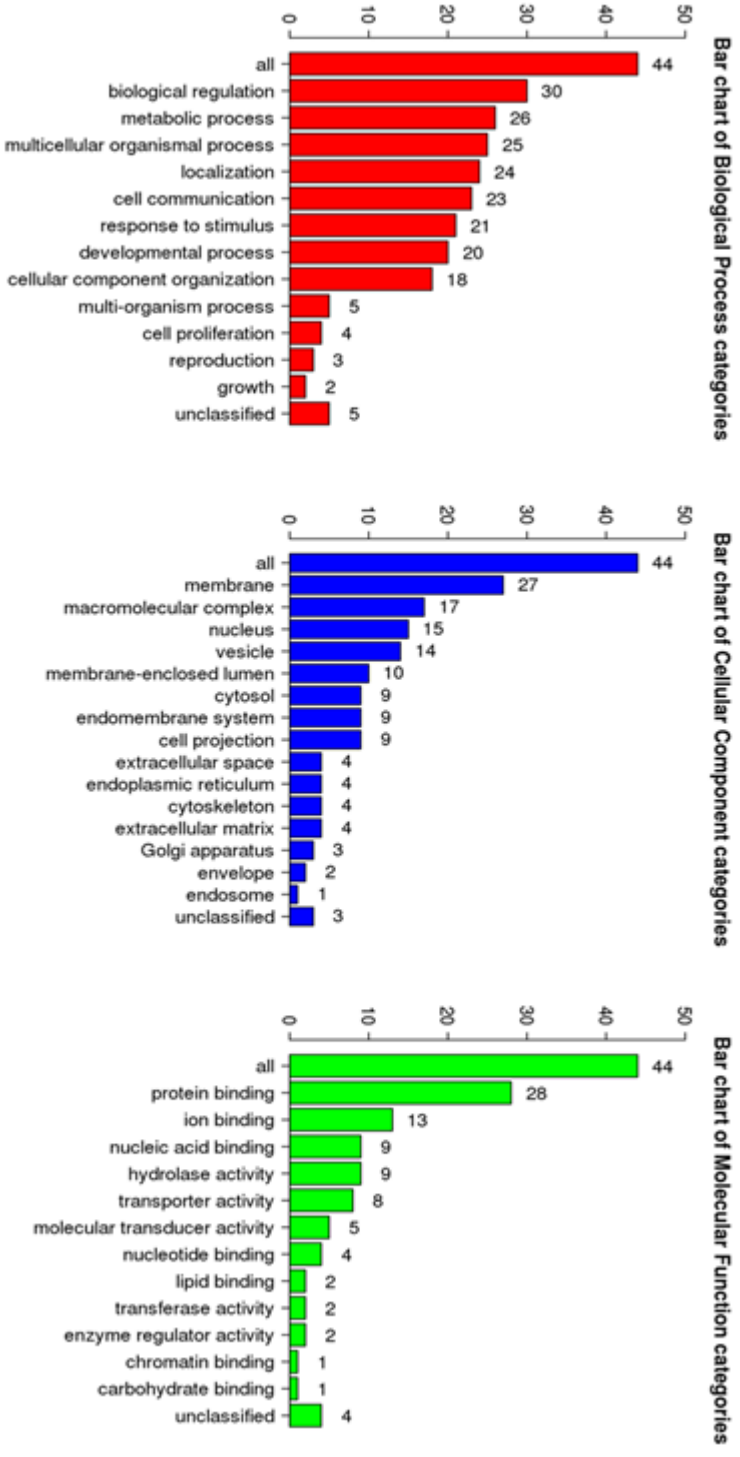


Figure 18: GoSlim summary exosomal proteins showing canonical pathways based on biological processes, cellular components and molecular functions for MCF-7 cells exposed to 3-5% hypoxia

10. Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject:

Despite improved means of diagnosis and treatment, multiple drug resistance is the main cause of poor clinical outcome in breast cancer. Studies have shown that hypoxia in the tumor microenvironment is an independent prognostic indicator of poor outcome in breast cancer and several other malignancies. Hypoxia-driven genes and protein changes enhances epithelial-to-mesenchymal transition, remodels the extracellular matrix, drives drug resistance, supports cancer stem cells and aids evasion from immune cells. However, the precise mechanisms through which hypoxic condition promotes disease aggressiveness and drug resistance are not well understood. The evidences have also linked hypoxia with tumor progression and chronic inflammatory processes, leading to dysregulated activity of various immune cells.

Hypoxia is considered as one of the hallmarks of tumor microenvironment resulting in poor oxygen and nutrient supply and is associated with decrease in effectiveness of the chemotherapy and poor prognosis. Here we observed in reduction in the cell proliferation rate in response to hypoxic condition in both MCF 7 and MDA-MB 231 cells. Lower proliferation rate in hypoxic condition has been accredited to hypoxia-associated deficiencies in other nutrients such as glucose which causes cells to stop or slow their rate of progression.

Reports have shown that solid tumors exhibits high level of tissue hypoxia which causes increase in cellular reactive oxygen species (ROS) and limits to supply of nutrients to the tumor cells. In the present study we observed significant increase in the ROS levels in the both MCF 7 and MDA-MB 231 cells when exposed to hypoxia. The increased levels of ROS causes endoplasmic reticulum (ER) stress leading to the disruption of the signalling pathway that controls cell proliferation and growth which might be responsible for the decreased rate of cell proliferation.

Emerging evidences suggests that various signaling pathways converge on autophagy in response to hypoxia. Recent reports have shown that autophagy plays important role of hypoxia in reprogramming tumors cells and confers resistance to chemotherapeutic agents. Autophagy was studied using Acridine Orange (AO) staining of the cells exposed to hypoxia. Here we observed increase in the number of acid vesicles (AVOs) and the degree of acidity (DOA) in both MDA-MB-231 and MCF7 cells in response to hypoxia. Kasper et al. hypothesized that autophagy, a lysosomal degradation pathway, may be involved in reducing ROS during exposure of hypoxia through removal of ROS producing species and protecting the cells from damage. He et al. (2012) also demonstrated hypoxia induced autophagy and the elevated autophagy activity is reported to be associated with increased radio-resistance of tumor cells. The induction of autophagy is suggested to be involved in chemo/radio resistance in the tumor cells suggests possibility of autophagy pathway as therapeutic targets for breast cancer.

There are also evidences suggesting elevated rate of lipid production is critical for cancer cell survival and expression of lipogenic enzyme, fatty acid synthase is strongly correlated with cancer progression. The hypoxia-dependent regulation of lipid metabolism is not well found in literatures. In our study we observed increased levels of lipid droplet (LD) accumulation within MDA-MB-231 and MCF-7 cells in response to hypoxia. Reports have exhibit significant metabolic alterations with respect to several critical nutrients and substrates including changes in metabolism of both glucose and glutamine. The tumor cells exhibits increase demand for fatty acids which are derived endogenously from citrate or taken up from exogenous source. As suggested in the literatures the

rate of cell proliferation is directly related to lipid synthesis in tumor cells the accumulation of lipid in response to hypoxia in both MDA-MB-231 and MCF7 cells might be due to reduction in the cell proliferation rate.

Recently it has been reported that exosomes released by the cancer cells modulate tumor micro-environment and drug response. These exosomes are nano-sized membrane bound vesicles of endocytic origin and have been implicated in proliferation, angiogenesis, immune-suppression and more lately in the preparation of pre-metastatic niches in the secondary organs. The factors and stimuli that regulate exosomes packaging and release have not been fully characterized.

Xiao et al (2014) reported that the exosomes released in the tumor microenvironment can be taken up by another cell which might affect the recipient cell signaling. Reports have also demonstrated that the decreased sensitivity of lung cancer A549 cells to cisplatin which might be mediated by miRNA and mRNA exchange by exosomes via cell to cell communication. Corcoran et al (2012) have also showed that the resistance to docetaxel and associated phenotypic changes are mediated through exosomes but the associated molecular mechanism on the role of hypoxic exosomes in drug resistance are not fully understood.

To study the role of exosomes in cell migration and drug response, we have isolated exosomes from MDA-MB 231 and MCF-7 cells cultured under hypoxic condition. The size distribution of the exosomes were evaluated by Dynaic Light Scattering (DLS) method, showed the exosomes size range from 50-1000 nm. The aggregation of exosome might have happened showing larger size of the particles.

Salnikov *et al.* (2012), suggested that hypoxia induces migratory potential in the cells. We have earlier reported that hypoxic exosomes increases migration in prostate cancer cells. Here in present study we also observed the rate of wound closure increased in hypoxic exosome treated cells in both the breast cancer cells at 12h but for later time points decreased This suggests that the exosomes might be loaded with factor or molecules that enhances the migratory potential of breast cancer cells.

Here we also tried to explore the role of exosomes in drug resistance of breast cancer cells. The breast cancer cell MDA-MB 231 was treated for 6h and 12h with exosomes isolated from cells exposed to hypoxia and then by IC_{50} dose (0.84 µmole) of Epirubicin. Interestingly significant restoration of cell viability were observed in cells exposed the exosomes isolated from hypoxic condition (Figure 14). This indicates that exosomes may influence the efficacy of chemotherapeutic agents either directly or indirectly interacting with the drug.

As we have observed that the hypoxic exosomes induces cell migration and is able restore cell viability in breast cancer cells treated IC_{50} dose of epirubicin, it is important to find out the cargo of exosomes responsible for change in the phenotype of breast cancer cells. So we have collected exosomes from breast cancer cell cultured under normal and hypoxic condition for microRNA and proteome analysis. The samples were sent to Sandor Life Sciences Pvt. Ltd but unfortunately the microRNA could not be extracted from the exosomes and only proteome data were obtained.

The proteome analysis of MDA-MD231 cells normoxic vs hypoxic conditions, revealed 21 proteins downregulated and 19 proteins upregulated and in MCF 7 cells we observed that 15

proteins are downregulated and 28 proteins are upregulated (Fold change <0.5 is considered as downregulated and fold change >2.0 is considered as upregulated). These proteins are involved in various regulatory pathways as mentioned in the data above. The data analysis suggested their role in various biological process such as biological regulation, metabolic processes, response to stimuli, cell communication, developmental process, cellular localization, cellular component reorganization. These proteins are suggested to be associated with important cellular structures such as membrane, nucleus, macro-molecular complex, membrane associated lumen, endomembrane system, vesicles, cytoskeleton and extracellular matrix. The functional studies if top five upregulated/downregulated proteins using knock down approach is needed to further establish their role in cell proliferation, migration and chemo-resistance in response to hypoxia.

11. Conclusions summarizing the achievements and indication of scope for future work:

1. Our finding showed increased levels of lipid droplet (LD) accumulation within MDA-MB-231 and MCF-7 cells in response to hypoxia. This alteration in the lipid metabolism could be used as a therapeutic intervention to target cancer cells using inhibitors lipid metabolism pathways that could also provide new strategies to sensitize the cancer cells.

2. Recent reports have shown that autophagy plays important role of hypoxia in reprogramming tumors cells and confers resistance to chemotherapeutic agents. Here we observed increase in the number of acid vesicles (AVOs) and the degree of acidity (DOA) in both MDA-MB-231 and MCF7 cells in response to hypoxia. The induction of autophagy is suggested to be involved in chemo/radio resistance in tumor cells suggests possibility of autophagy pathway as therapeutic targets for breast cancer.

3. Breast cancer cells co-cultured with hypoxic exosomes and then treated with IC50 dose (0.84 µmole) of Epirubicin resulted in restoration of cell viability. This indicates that exosomes may influence the efficacy of chemotherapeutic agents either directly or indirectly interacting with the drug.

4. The proteome analysis of MDA-MD231 cells normoxic vs hypoxic conditions, revealed 21 proteins downregulated and 19 proteins upregulated and in MCF 7 cells we observed that 15 proteins are downregulated and 28 proteins are upregulated. The functional studies if top five upregulated/downregulated proteins using knock down approach is needed to further establish their role in cell proliferation, migration and chemo-resistance in response to hypoxia.

12. S&T benefits accrued:

i. List of Research publications: in process of communication

ii. Manpower trained on the project

a) Research Scientists or Research Associates

b) No. of Ph.D. produced

c) Other Technical Personnel trained: JRF trained 02
PhD scholars 03

iii Patents taken, if any NIL

13. Financial Position

S.No.	Financial Position/ Budget Head	Funds Sanctioned (Rs)	Expenditure (Rs)	% of Total cost
I	Salaries/ Manpower costs	4200000.00	792000.00	18.857
II	Equipment		1800000.00	42.857
III	Supplies & Materials		1106985.00	26.356
IV	Contingencies		66633.00	1.586
V	Travel		38537.00	0.917
VI	Overhead Expenses		393265.00	9.364
	Total	4200000.00	4197420.00	99.938

14. Procurement/ Usage of Equipment

S.No.	Name of Equipment	Make/Model	Cost (FE/ Rs)	Date of Installation	Utilization Rate (%)	Remarks regarding maintenance
1	CO2 incubator	Eppendorf	900000.00	12/01/2017	100%	Project grant
2	UV-VIS spectrophotometer	Eppendorf			100%	Project grant
3	Refrigerated Centrifuge	Eppendorf			100%	Project grant
4	Laminar Air Flow Hood (Biosafety Cabinet)	Haier	308016.00	04/04/2017	100%	Project grant
5	Gas Blowing evaporator	N. Biotek	347053.00	06/10/2017	50%	Project grant
6	Inverted Microscope	Leica	346479.00	8/12/2016	100%	Project grant

b) Plans for utilizing the equipment facilities in future

Equipment facility will be utilized by the Research scholars and M.Sc. project studies for carrying out research in the department

Name and Signature with Date:
(Principal Investigator)

Project Completion Report

Title of the project: “Studies on exosomal lipidomics and micro RNAs and their clinical utility in the management of metastatic and multiple drug resistant breast cancer”

Sanction Order: SB/EMQ-215/2014 dated 23/01/2016

Submitted By

Principal Investigator
Professor Anand Ramteke
Dept. of Molecular Biology and Biotechnology
TEZPUR UNIVERSITY
PO Napaam, Tezpur, Assam 7840228

Annexure-II

REVISED ANNUAL INSTALMENT WITH UP-TO-DATE STATEMENT OF EXPENDITURE (2019-2020)

1. SERB Sanction Order No. and Date : SB/EMQ-215/2014 dated 23/01/2016
2. Name of the PI : Dr. Anand M Ramteke
3. Total Project Cost : Rs. 5403200/-
4. Revised Project Cost (if applicable) : NIL
5. Date of commencement : 23-01-2016
6. State of Expenditure : 01/04/2019 to 22/01/2020

(Month wise expenditure incurred during current financial year, April 2019 to March 2020)

Month & Year	Expenditure incurred/committed
25 July 2019	10597.00
14 November 2019	24877.00
17 December 2019	19867.00

7. Grant received in each year:
   a. 1st year : Rs. 2700000. (Non-Recurring- Rs 1800000/- and Recurring Rs. 900000/-)
   b. 2nd Year : Rs. 600000.00
   c. 3rd Year : Rs 900000.00
   d. Interest, if any : Rs 14990.00
   e. Total (a+b+c+d) : Rs. 4214990.00

Ramtek

Final Consolidated Statement of Expenditure (21/01/2016 to 20/01/2020)

(FOR FINAL SETTLEMENT OF ACCOUNTS)

1. Title of the Project: **Studies on exosomal lipidomics and micro RNAs and their clinical utility in the management of metastatic and multiple drug resistant breast cancer**

2. Sanctioned Project Cost : Rs. 5403200.00
3. Grant Released : Rs. 4200000.00
4. Duration of the Project : Four Years
5. Sanction Order : SB/EMQ-215/2014 dated 23/01/2016
6. Date of Commencement of Project : 21/01/2016
7. Date of Completion of Project : 20/01/2020

S.No (I)	Sanctioned Heads (II)	Total Funds Allocated (indicate sanctioned or revised) (III) (Rs)	Expenditure Incurred				Total Expenditure till 20/01/2020 (VII=IV+V+VI) (Rs)	Balance as on 20/01/2020 (Rs)	Remarks
			1st Year (DOS to 31st March 2017) (IV)	**2nd Year 1st April 2017 to 31st March 2018 (V)**	**3rd Year 1st April 2018 to March 2019 (VI)**	**4th Year 1st April 2019 to 20/01/2020 (VI)**			
1.	Equipment	1800000.00	1246479.00	553521.00	0.00	0.00	1800000.00	0.00	NIL
2.	Manpower Costs	2400000.00	300000.00	150000.00	300000.00	42000.00	792000.00	2580.00	
3.	Consumables		166635.00	572979.00	367371.00	0.00	1106985.00		
4.	Travels		3063.00	0.00	0.00	35474.00	38537.00		
5.	Contingencies		50962.00	15671.00	0.00	0.00	66633.00		
6	Overhead		76750.00	118350.00	178298.00	19867.00	393265.00		
7.	*Interest Earned if any	14915.00	0.00	0.00	0.00	0.00	0.00	14915.00	0.00
8.	Total	4214915.00	1843889.00	1410521.00	845669.00	97341.00	4197420.00	17495.00	

*Interest is for period DOS i.e. 21/01/2016 to 20/01/2020

Name and Signature of Principal Investigator
Date: 20/06/2022

Signature of Competent Financial authority:
(with Seal)
Date:

Finance Officer
Tezpur University

(Head of the Institution)
Registrar
Tezpur University

(16)

Final UC for Recurring Grant

FINAL UTILISATION CERTIFICATE (2 COPIES)
FOR THE FINANCIAL YEAR – April 2019 to 20/01/ 2020

UC pertains to √ appropriate box

First Release	Second Release	Third Release	Fourth Release	**Final √ settlement**

1. Title of the Project/ Scheme :**"Studies on exosomal lipidomics and micro RNAs and their clinical utility in the management of metastatic and multiple drug resistant breast cancer"**

2. Name of the Institution : Tezpur University

3. Principal Investigator : Dr Anand Ramteke

4. SERB Sanction order No and date : SB/EMQ-215/2014 dated 23/01/2016

5. Amount brought forward from the previous Financial year quoting DST letter no and date in which the authority to carry forward the said amount was given
   i. Rs 99921.00
   ii. Letter No
   iii. Date

6a. Amount received during the financial year (Please give DST letter/order no and date)
   i. NIL
   ii.
   iii.

6b. Interest earned (period DOS i.e. 21/01/2016 to 20/01/2020): Rs 14915.00

7. Total amount that was available for expenditure (excluding commitments) during the financial year (Sr. No. 5+6a+6b) : Rs 114836.00

9. Actual Expenditure (excluding commitments) Incurred during the financial year (upto 31st March 2019) : Rs 97341.00

10. Balance amount available at the end of the financial year: Rs 17495.00

11. Unspent balance refunded, if any (please give details of cheque no etc.):Rs17495.00

12. Amount to be carried forward to the next financial year (if applicable): NIL

Ramteke

UTILIZATION CERTIFICATE
(Recurring)

Certified that out of **Rs NIL** of grants-in-aid sanctioned during the year 2019-2020 in favour of **Dr Anand Ramteke** under SERB letter/ order **No** NIL and **Rs 114836.00** on account of unspent balance of the previous year, a sum of **Rs 97341.00** has been utilized for the purpose of **project work** for which it was sanctioned and that the balance of **Rs 17495.00** remaining unutilized at the end of the project and is refunded to **Fund for Science and Engineering Research** as Demand Draft No. ..847003.....dated ..28/06/2022

Certified that we have satisfied ourselves that the conditions on which the grants-in-aid was sanctioned have been fulfilled/are being fulfilled and that we have exercised the following checks to see that the money was actually utilized for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. Cash book
2. Ledge book

Signature of PI

Date

Signature of Registrar/ Signature of Head

Registrar
Tezpur University

Date

Accounts Officer of the Institute

Date

Finance Officer
Tezpur University

Guidelines for preparation of UC

1. U.C. should be only for the grants released by the SERB. Please do not account for Security deposits/other matching grants/account opening charges and miscellaneous itemsr
2. SERB Sanction No and Dt. should be accurately shown in the U.C.
3. Even if the grant is utilized in the financial year in which the grant was released by SERB a NIL U.C. needs to be forwarded to SERB along with a request for carrying forward the grant to the next financial year. Such grants which are carried forward must be shown in the subsequent UC as carried forward grant and not amount received in the subsequent year (ref SL No. 5 on pre-page)

Science and Engineering Research Board

UC has been accepted by

Signature _____

Name of the SERB officer_____

Designation _____

Final UC for Non-Recurring Grant

FINAL UTILISATION CERTIFICATE (2 COPIES)
FOR THE FINANCIAL YEAR – April 2019 to 20/01/2020

UC pertains to
√ appropriate box

First Release	Second Release	Third Release	Fourth Release	**Final √Settlement**

1. Title of the Project/ Scheme :**"Studies on exosomal lipidomics and micro RNAs and their clinical utility in the management of metastatic and multiple drug resistant breast cancer"**

2. Name of the Institution : Tezpur University

3. Principal Investigator : Dr Anand Ramteke

4. SERB Sanction order No and date : SB/EMQ-215/2014 dated 23/01/2016

5. Amount brought forward from the previous Financial year quoting DST letter no and date in which the authority to carry forward the said amount was given
   i. NIL
   ii. Letter No
   iii. Date

6a. Amount received during the financial year (Please give DST letter/order no and date)
   i. NIL
   ii.
   iii.

6b. Interest earned, if any :

7. Total amount that was available for expenditure (excluding commitments) during the financial year (Sr. No. 5+6a+6b) : NIL

9. Actual Expenditure (excluding commitments) Incurred during the financial year (upto 31st March 2019) : NIL

10. Balance amount available at the end of the financial year: NIL

11. Unspent balance refunded, if any (please give details of cheque no etc.): NIL

12. Amount to be carried forward to the next financial year (if applicable): NIL

Ramteke

UTILIZATION CERTIFICATE
(Non-Recurring)

Certified that out of **NIL** of grants-in-aid sanctioned during the year 2016-2017 in favour of **Dr Anand Ramteke** under SERB letter/ order dated SB/EMQ-215/2014 dated 23/01/2016 and Rs **NIL** on account of unspent balance of the previous year, a sum of Rs **NIL** remaining unutilized at the end of the year.

Certified that we have satisfied ourselves that the conditions on which the grants-in-aid was sanctioned have been fulfilled/are being fulfilled and that we have exercised the following checks to see that the money was actually utilized for the purpose for which it was sanctioned.

Kinds of checks excerised:

1. Cash book
2. Ledge book

Signature of PI

Date

Signature of Registrar/ Signature of Head

Date

Registrar
Tezpur University

Accounts Officer of the Institute

Date

Finance Officer
Tezpur University

Guidelines for preparation of UC

1. U.C. should be only for the grants released by the SERB. Please do not account for Security deposits/other matching grants/account opening charges and miscellaneous itemsr
2. SERB Sanction No and Dt. should be accurately shown in the U.C.
3. Even if the grant is utilized in the financial year in which the grant was released by SERB a NIL U.C. needs to be forwarded to SERB along with a request for carrying forward the grant to the next financial year. Such grants which are carried forward must be shown in the subsequent UC as carried forward grant and not amount received in the subsequent year (ref SL No. 5 on pre-page)

Science and Engineering Research Board

UC has been accepted by

Signature _____

Name of the SERB officer _____

Designation _____

Details of Assets acquired wholly or substantially out of Govt. grants Register to be maintained by Grantee Institution

DST-SERB project (SB/EMQ-215/2014 dated 23/01/2016)

"Studies on exosomal lipidomics and micro RNAs and their clinical utility in the management of metastatic and multiple drug resistant breast cancer"

S.No.	Name of Equipment/Make	Amount in Rupees
1	Eppendorff (a) CO2 incubator, (b) UV-VIS spectrophotometer (c) Refrigerated Centrifuge	900000.00
2	Leica Inverted Microscope Custom Clearance	298448.00 48031.00
3	N. Biotek Gas Blowing evaporator	347053.00
4	Haier Laminar Air Flow Hood (Biosafety Cabinet)	308016.00
	Total	1901548.00
	Excess amount paid from Overhead	101548.00

(PROJECT INVESTIGATOR)

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University