Although drugs like doxorubicin, tamoxifen and herceptin are widely employed in breast cancer therapy, they are known to cause side effects such as cardio-toxicity, endometrial cancer and congestive heart failure respectively. Treatment of triple negative breast cancer (TNBC) is still challenging for clinicians and researchers, as most of the patients do not respond to traditional chemotherapeutic approaches. Hence, identification of alternate less toxic forms of drugs is desperately needed to prevent and suppress the breast cancer. Emerging evidence from numerous studies shows encouraging results for the use of statins against many cancers including breast carcinoma. Our recent findings highlight that statins exhibit anti-proliferative and anti-invasive effects in breast cancer cells by altering intracellular iron, nitric oxide and H2O2 levels. Based on the recent advances made in understanding the mechanisms related to statin-mediated breast cancer cell death, more rigorous studies are warranted to employ statins at least as a neoadjuvant therapy to reduce the cancer-related morbidity and mortality.

**Keywords:** Statins; iron; NO; ROS; vimentin; proteomics; micro array; breast cancer

suggest the association between statin use and cancer incidence. Although epidemiological studies encourage the efficiency of statins as anti-cancer agents [6, 7], yet the clinical trials so far carried out indicated mixed results ranging from no association to both positive and negative associations [8, 9]. Perhaps, this divergence to some extent is attributed to the nature of statin that was employed in various clinical studies. Also, so far only few clinical studies were conducted with statins for their effect during breast cancer treatment protocols. Tumor cell proliferation rate was decreased in HMGCR-positive breast cancer patients who were on short-term, preoperative high dose of atorvastatin [10]. Furthermore, short term administration of fluvastatin as a neo adjuvant therapy (high dose compared to low dose) increased apoptosis and decreased proliferation in high grade breast cancer [11]. A long term clinical study from British population indicated that use of simvastatin after the diagnosis of breast cancer had reduced the mortality rate [12]. Statin users in a population-based cohort of breast cancer patients in Finland showed that both post-diagnostic and pre-diagnostic statin use were associated with a reduction in breast cancer related deaths [13]. Furthermore, it was also reported that lipophilic statins decrease the recurrence rate in early stage breast cancer survivors [14]. Interestingly, all these effects were pronounced only in the presence of hydrophobic statins but not with the hydrophilic statins. In tune with this, recently we and others have also reported that only hydrophobic statins but not hydrophilic statins like pravastatin induce breast cancer cell death [3, 15, 16]. These effects are in line with a meta analysis study conducted in Japan [17], suggesting that pravastatin had no role in the cancer incidence or suppression.

Although different mechanisms have been proposed to implicate statin-induced anti-proliferative effects in various cancer cell types, we have recently shown a novel mechanism by which statins affect intracellular iron, nitric oxide (NO) and reactive oxygen species (ROS) levels, in turn leading to TNBC cell death. Recent evidences suggest that iron, NO, and ROS play a dual role (progression or regression) in cancer depending on their intra cellular levels. Either a beneficial or deleterious effect of iron in cancer has been debated for decades. Iron is essential for the maintenance of various cellular functions including proliferation, differentiation, activities of respiratory complexes, DNA synthesis, cell cycle, detoxification (catalase and other peroxidises) [18-21] and so on. Transferrin (Tf) is the major iron transporter in the blood that binds to transferrin receptor (TfR1) on the cell surface. TfR1 in turn assists the uptake of Fe-Tf complex through receptor mediated endocytosis [22]. TfR1 protein in general is expressed at elevated levels in a variety of cancers conceivably to support the increased demands of iron by these cells including rapid proliferation. TfR1 serves as a validated predictive biomarker in cancer patients including mammary cancer [23-26]. Therefore, depletion of intracellular iron levels by inhibiting TfR1 is one of the prospective approaches to regress tumor growth. Iron chelators, antibodies and small molecules against TfR1 have been employed in the suppression of different cancers [27-29]. TfR1 expression in the cell is regulated based on the intracellular iron levels by Iron Regulatory Protein (IRP). Over expression of IRP2, but not IRP1, reduced ferritin H and increased TfR1 expression in MDA-MB-231 breast cancer cells [30]. In tune with this, silencing of IRP2 in MDA-MB-231 cells increased ferritin H and decreased TfR1 protein levels, resulting in diminished labile iron pool along with a reduction in the growth of MDA-MB-231 cells in mouse mammary fat pad [30]. The results of our recent study indicated that statins deplete intracellular iron pools via iNOS-dependent increased NO generation along with an inhibition of ROS levels because of the induction of catalase activity and GSH levels in TNBC cells. This ‘cross talk’ between iron, NO and ROS was in-part responsible for statin-mediated anti-proliferative and anti-invasive effects in TNBC cells [16]. This study also showed that fluvastatin or geranylgeranyl transferase inhibitor but not farnesyl transferase inhibitor dys-regulated iron homeostasis via inhibiting TfR1 and intracellular iron levels in TNBC cells. All these effects of fluvastatin were hampered in the presence of mevalonate, an immediate downstream product of HMG-CoA. These results clearly suggest that fluvastatin-altered intracellular iron pool may have a role in cytostasis/cell death of TNBC cells. It is known that the activity of small GTPases (Rho and Rac) is regulated by statins. To this end, we recently showed that Rho-C is majorly involved in TfR1 regulation in breast cancer cells [16]. Thus suggesting that, intracellular iron homeostasis is involved in statin-induced pro-apoptotic or cytostatic effects and also geranylgeranylation of Rho GTPases is indeed necessary for TfR1 regulation in TNBC cells. Furthermore, our studies on statin-induced global transcriptome alterations in MDA-MB-231 cells revealed a transcriptional down regulation of ribonucleotide reductase subunit M2 (RRM2) upon fluvastatin treatment (Kanugula AK and Kotanraju S unpublished data). RRM2 is one of the two subunits of iron-dependent ribonucleotide reductase, which catalyzes the formation of deoxyribonucleotides from ribonucleotides. This observation further supports a role for iron in statin-mediated inhibition of nucleotide synthesis, which in turn reduces cell proliferation. However in a related study and to our surprise, in contrast to estrogen negative (ER-) breast cancer cells, fluvastatin treatment increased TfR1 protein expression in estrogen positive (ER+) MCF-7 breast cancer cells. Thus indicating that, iron regulation may differ in ER+ and ER- breast cancer cells by statins. Endogenous
levels of TfR1 are low in malignant MCF-7 compared to metastatic MDA-MB-231 cells (Kanugula AK and Kotamraju S. Unpublished observations). We are currently investigating the mechanisms responsible for the differential regulation of TfR1 expression by statins in ER+ and ER- breast cancer cells, although both these breast cancer cell types are susceptible to statin mediated cell death.

The cardio protective effects of statins are due to an increase in NO levels through increased expression of endothelial nitric oxide synthase (eNOS). The results of previous studies have implicated a dual role for NO in cancer. Based on the intracellular concentrations, NO can either promote tumorogenesis or tumoricidal activity [31, 32]. In an earlier study, we showed that iNOS-induced NO was in part responsible for the anti-proliferative effects of statins in MCF-7, MDA-MB-231 and BT-549 breast cancer cells because, on one hand, ADMA (non-specific NOS inhibitor) and 1400w (specific iNOS inhibitor) marginally, albeit significantly, reversed the fluvastatin-induced cytotoxicity; and on the other hand, sepiapterin (BH4 analogue, which facilitates increased generation of NO) exacerbated statin-induced breast cancer cell death [15,16]. High concentrations of nitric oxide levels generated by either over expression of iNOS or by supplementation of NO donors caused cytotoxic, cytostatic and decrease of metastatic process in breast cancer cells [33-35]. DETA/NO-mediated release of NO decreased cyclin-D1 levels and hypophosphorylation of retinoblastoma protein was responsible for its cytostatic effects in MDA-MB-231 cells [30]. Recent reports suggest that NO alters iron levels and furthermore, NO donors hamper iron uptake mediated through Tf-TfR1 process [37, 38]. Along these lines, we have shown that, NOS inhibitors (ADMA and 1400w) restored fluvastatin-mediated inhibition of TfR1 expression and iron levels in TNBC cells and on the contrary, sepiapterin by increasing NO generation, downregulated TfR1 and depleted iron levels. All these results propose a ‘cross-talk’ between nitric oxide and intracellular iron pools in TNBC cells.

Similar to iron and NO, ROS also exhibits a dual role in either tumor suppression or promotion in many cancers. This bi-functional role of ROS has been credited due to an imbalance in the net radical scavenging ability by the cells [39]. Elevated H2O2 levels in breast cancer cells induce metastatic nature via suppressing the protein phosphatase activity, in turn, guiding increased cell proliferation signaling cascades [40, 41]. By further exploring this phenomenon, in a recent study, we showed that fluvastatin treatment induced MnSOD (by repressing DDB2, a known transcriptional epigenetic negative regulator of MnSOD) and catalase activities in TNBC cells. This increased catalase activity up on fluvastatin treatment resulted in the decomposition of H2O2 which in turn lead to the inhibition of both MMP-2 and MMP-9 levels along with a significant reduction in the invasive potential of TNBC cells. All these effects were blocked by either 1400w (iNOS inhibitor) or aminotriazole (catalase inhibitor). Thereby suggesting that, fluvastatin-dependent increase in NO generation and inhibition of ROS plays a key role in statin-mediated suppression of invasion in TNBC cells. Also, either silencing of MnSOD by SiRNA or inhibition of catalase by aminotriazole (AT) in TNBC cells reversed statin-induced depletion of TfR1 expression, thereby indicating a role for ROS in TfR1 regulation. Previously, we reported an inverse relation between oxidants and iron uptake in endothelial cells [42-44]. Apart from increasing NO generation, fluvastatin treatment to TNBC cells also greatly elevated intracellular GSH levels which in turn may lead to the increased formation of nitrosothiols via nitrosonium species (NO+) that inactivate iron-responsive element-binding protein 2 (IRP2) and thereby decreasing TfR1 transcription [45]. Nevertheless, at present we are not sure of the redox nature of NO (whether NO+ or NO-), which is generated in TNBC cells upon fluvastatin treatment. Overall, statin mediated anti-proliferative and anti-invasive effects in TNBC cells are ascribed due to the depletion of intracellular iron pools through the up regulation of nitric oxide and antioxidant levels.

Although several molecular and signaling mechanisms have been proposed for the anti-proliferative and anti-cancer actions of statins, information is inadequate on statin-induced global proteome or gene expression changes to identify possible molecular signatures responsible for their anti-cancer properties. To understand the statin-mediated proteome alteration in breast cancer cells, we have recently reported a 2D-gel-based fluvastatin-altered global proteome changes in MDA-MB-231 breast cancer cells. The results of this study showed that statins target various cytoskeletal proteins [46]. In the proteome analysis, we have identified about 800 spots of which 39 spots were found to be differentially regulated upon fluvastatin treatment. Differentially regulated proteins were identified using LTQ-fourier transform-ion cyclotron resonance (LTQ-FT-ICR) and these 39 differentially regulated proteins corresponded to 35 different proteins. Interestingly, vimentin (VIM), translationally controlled tumor protein (TPT1) and eukaryotic elongation factor 1-D (EEF1D) proteins were identified as multiple protein spots in 2D-gel. All the differentially regulated proteins were mapped together using Ingenuity Pathway Analysis (IPA) and found that vimentin, a known epithelial mesenchymal transition (EMT) marker, was centrally focused by connecting with all other nodes. It is known that vimentin is elevated in epithelial cancers including breast cancer and recently it was also highlighted...
as a prospective therapeutic target because of its role in proliferation, migration and invasion \cite{47,48}. To further emphasize that statins target vimentin and induces proteolysis, we found that vimentin was identified at three different regions and the fragmented spots corresponded to different molecular weights (~ 48, 28, 18 kDa) in the fluvastatin treated proteome map. Fluvastatin mediated vimentin degradation was further confirmed by downregulation of its protein levels by Western blot analysis. Under these conditions, however, vimentin mRNA levels were not changed upon fluvastatin treatment. The decrease in vimentin protein levels was reversed in the presence of mevalonate thereby, signifying a role for vimentin in statin-induced breast cancer cell death. Earlier we have shown that statins increased caspase 3 and 8 like activities in breast cancer cells and this increased caspase 3 like activity was responsible for vimentin degradation, as vimentin levels were restored in the presence of a caspase 3 inhibitor \cite{15,16}. However, interestingly, fluvastatin treatment failed to affect vimentin levels in MCF-10A (normal mammary epithelial) cells. Apart from regulating vimentin levels, fluvastatin treatment to MDA-MB-231 cells resulted in the alterations of proteins involved in various cellular and molecular functions including cell-to-cell signaling, binding, cell cycle, cellular movement and cell morphology.

In continuation, to classify the statin sensitive breast cancer patients by identifying the gene specific signatures, we are currently making efforts to understand the global transcriptomic changes brought about by fluvastatin in ER-positive (MCF-7) and ER-negative (MDA-MB-231) breast cancer cells. cDNA-based micro array (illumina) analysis revealed that there were about 3000 genes differentially regulated between MCF-7 and MDA-MB-231 cells (GSE59006; GSE59007). Interestingly, fluvastatin altered six times more global transcriptome changes in MDA-MB-231 breast cancer cells compared to MCF-7 breast cancer cells (Kanugula AK and Kotamraju S et al unpublished observations). Furthermore, 48 genes were identified as common among the differentially regulated genes (DRG’s). To know the functional relevance of fluvastatin mediated DRG’s, we have mapped all these genes using Search Tool for the Retrieval of Interacting Genes (STRING) analysis. Surprisingly, all the down regulated genes of both the cell types were highly interconnected and the up regulated genes were scattered around, suggesting that there is a strong mutual co-regulation between the down regulated genes. The Gene Ontology (GO) analysis of these altered genes implied that they are involved in various molecular functions (calcium ion binding, antioxidant, catalytic, enzyme regulatory, nucleic acid binding transcription factor, receptor and transporter activities) and also in different biological processes such as apoptotic process, biological adhesion, cellular component organization, cellular process, localization, metabolic process and developmental process. Some of these DRG’s (ACOX2, HMGCS1, TIMP3, PCNA, RRM2, CD45R, MCM4, ALDH1B1, BCL2, PPP1CC, FTH1) of various pathways were selected and validated using real time-PCR. Interestingly, none of these DRGs were altered in statin treated normal human mammary epithelial cells (MCF10-A) indicating that these differentially regulated genes possibly regulate breast cancer cell proliferation. These results conclude that, fluvastatin altered DRG’s are high in ER+ cells compared to ER+ breast cancer cells and perhaps, this large number of altered genes may play a role in statin sensitivity toward basal (ER-) versus luminal (ER+) cell types. Also it was observed that, TNBC cells (MDA-MB-231 and BT-549) are relatively more sensitive to fluvastatin-induced cell death compared to ER+ breast cancer cells (MCF-7). This differential sensitivity could be due to the variation in HMGCR activity or wild type versus mutant p53 status or differences in the hormonal status between ER+ and ER- cell types. Currently, we are exploring these fluvastatin altered common gene’s role in breast cancer cell proliferation and metastasis in conjunction to the hormonal receptor status and p53 activity across various types of breast cancer cells.

Taken together, statins act differently in malignant and metastatic breast cancer cells by perturbing various intracellular key components like iron, NO and ROS. Furthermore, disruption of vimentin protein in metastatic breast cancer cells by fluvastatin treatment also plays a key role in statin-induced anti-proliferative and anti-invasive effects. Overall, the results of these studies provide value addition to the already existing literature on statin-mediated breast cancer cell death mechanisms and may eventually aid in the identification of statin-sensitive breast cancer patients for the effective treatment.

**Conflicting interests**

The authors have declared that no competing interests exist.

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