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Epidermal growth factor receptor mediated proliferation depends on increased Lipid Droplet density regulated via a negative regulatory loop with FOXO3/Sirtuin6

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Abstract

The proliferation of colon cancer cells is mediated in part by epidermal growth factor receptor (EGFR) signaling and requires sustained levels of cellular energy to meet its high metabolic needs. Intracellular lipid droplets (LDs) are a source of energy used for various cellular functions and they are elevated in density in human cancer, yet their regulation and function are not well understood. Here, in human colon cancer cells, EGF stimulates increases in LD density, which depends on EGFR expression and activation as well as the individual cellular capacity for lipid synthesis. Increases in LDs are blocked by inhibition of PI3K/mTOR and PGE2 synthesis, supporting their dependency on select upstream pathways. In colon cancer cells, silencing of the FOXO3 transcription factor leads to down regulation of SIRT6, a negative regulator of lipid synthesis, and consequent increases in the LD coat protein PLIN2, revealing that increases in LDs depend on loss of FOXO3/SIRT6. Moreover, EGF stimulates loss of FOXO3/SIRT6, which is blocked by the inhibition of upstream pathways as well as lipid synthesis, revealing existence of a negative regulatory loop between LDs and FOXO3/SIRT6. Elevated LDs are utilized by EGF treatment and their depletion through the inhibition of lipid synthesis or silencing of PLIN2 significantly attenuates proliferation. This novel mechanism of proliferative EGFR signaling leading to elevated LD density in colon cancer cells could potentially be therapeutically targeted for the treatment of tumor progression.

Keywords

Colon cancer; EGFR; Lipid Droplet; FOXO3; SIRT6

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Introduction

Colon cancer growth is characterized by enhanced proliferation of transformed cells mediated by various growth factor receptors including EGFR [1]. The proliferation of cancer cells requires cellular energy to meet their high metabolic needs, yet the source and regulation of this energy is unclear. Emerging findings show that intracellular LDs provide energy for diverse cellular functions, are increased in density in tumor tissue, and might be utilized for proliferation [2,3,4]. However, in colon cancer cells, regulation of LD density by EGFR signaling and the role LDs may have in providing metabolic energy necessary for tumor growth remain unexplored.

Epidermal growth factor receptor (EGFR) is a member of the receptor tyrosine kinase family and its activation in cancer cells stimulates cell cycle progression, cell survival, proliferation, and angiogenesis [5]. Binding of epidermal growth factor (EGF) ligand to the receptor's extracellular domain leads to a conformational change of the intracellular phosphorylation components, enabling downstream signaling that includes the phosphatidylinositol 3-kinase (PI3K) pathways, leading to stimulated proliferation [6]. Limited studies have shown that in lipid storing and secreting cells EGFR signaling can stimulate lipid metabolism [7,8]. However, in cancer cells, whether EGFR signaling stimulates lipid metabolism and if elevated intracellular lipids are used as a source of metabolic energy for proliferation are still unclear.

Lipid Droplets (LDs) are organelles constitutively present in adipocytes and their increase in density has been recently associated with human and mouse cancer tissues [2,4,9]. They contain a mixture of lipids enclosed by a family of regulatory proteins known as PERILIPINS (PLIN1-5) [10], which are involved in the regulation of lipid metabolism and correlate with LD status [11]. Elevated LDs are formed at the endoplasmic reticulum following increases in lipid synthesis and maturation steps [12]. LD dynamics have been studied in adipocytes and the liver; however, their regulation and function in non-lipid storing cells are not well understood. Recently, we have demonstrated elevated LD density in colonic tissue of Foxo3 deficient mice [4], linking this regulator of cell cycle arrest in colon cancer cells [13] with lipid metabolism. Additionally, one study showed that in colon cancer cells, PGE2 synthesis, which is essential for colon cancer growth [14], is interdependent with LD density [3]. While these limited findings indirectly support that elevated LD density might be involved in colon cancer progression, their regulation and function by growth factor receptor signaling are not well understood.

Here, we demonstrate that in human colon cancer cells EGFR signaling leads to elevated LDs mediated by a negative feedback loop with FOXO3/SIRT6 activity. Their increase in density is critical for EGFR stimulated proliferation and thereby provides the opportunity for new therapeutic approaches for the treatment of colon cancer.

Materials and Methods

Cell culture

Human colon cancer cell lines HT29, HCT116, SW480, and SW620 (ATCC) were propagated in appropriate media (Sigma) supplemented with 10% FBS (Fisher) and were serum starved 24 h prior to the treatment.

Treatment

EGF (100 ng/ml; Sigma) was used at the various time points indicated. The orange-red fluorescent fatty acid, BODIPY 558/569 C₁₂ (2 µM; Invitrogen), was employed to stimulate and visualize LDs. After 24 h of treatment with BODIPY C₁₂, one group was fixed (Con 0), while the others were washed (five times with PBS) and kept for another 24 h in serum free media (Con 24) or treated with EGF (EGF 24) before fixation. LD quantification was accomplished using Image J (NIH).

Inhibitors

The following inhibitors were employed: fatty acid synthase inhibitor, C75 (50 µg/ml; Sigma); EGFR inhibitor, PD153035 (1 µM; Calbiochem); PI3K inhibitor, wortmannin (200 nM; Calbiochem); mTOR inhibitor, rapamycin (2 nM; LC Laboratories); PGE2 inhibitor, sulindac (25 µM; Sigma).

BODIPY and Immunofluorescent staining

BODIPY and immunofluorescent stainings were performed as previously described [4]. Images were captured using fluorescent microscopy using an Olympus DP80 with camera software for CellSens Dimensions.

Protein extraction and Immunoblot

Total protein extraction and immunoblots were performed as previously described [15]. Antibodies specific to PLIN2 (Santa Cruz Biotechnology), FOXO3 (Cell Signaling), SIRT6 (Cell Signaling) and Tubulin (Cell Signaling) were used. Secondary antibody (Li-Cor Biosciences) signals were detected using an Odyssey infrared imaging system (Li-Cor Biosciencesgeneration).

Cell proliferation

Proliferation was quantified by MTS assay (Promega) as previously described [16].

FOXO3 and PLIN2 silencing

FOXO3 and PLIN2 siRNA (50 nM; Ambion) or equal amounts of negative control were added to 2×10^5 cells. Since lipids from transfection agents can interfere with LD status, cells were incubated with siRNA overnight in serum free media alone before being further utilized for experimentation.

Statistical analysis

ANOVA was used to determine significant differences between groups (means $\pm$ SEM). A p value of < 0.05 was considered to be statistically significant.

Results and Discussion

EGF mediated increases in LD density are dependent on receptor expression and activation as well as lipid synthesis

We examined if LD density depends on EGFR expression in human colon cancer cell lines recognized for high (HCT116), moderate (HT29, SW480), or very low (SW620) levels of the receptor [17]. Immunofluorescent staining of neutral lipids (BODIPY) shows that EGF (24 h) stimulates increases in LD density in all cell lines except SW620 cells, which have low EGFR expression (Fig 1A). Though EGFR expression in HT29 and SW480 cells is moderate compared to HCT116 cells [17], their increases in LDs are more prominent. HT29 cells appear to have the strongest capacity for EGF mediated LD accumulation, as the number of LD positive cells increases 2-fold and the number of LD aggregates per cell by 3-fold. Thus, while increases in LDs are dependent on EGFR expression, they also rely on each cell line's metabolic accumulation capacity. It is reasonable that the variation in capacity for LD accumulation might be due to different genetic mutations within each cell line. For example, in HT29 cells, mutations in the p53 tumor suppressor are found to drive lipid metabolism [18]. Also, mutations in PI3K in HCT116 cells could be accountable for their lower capacity for LD accumulation, as this pathway is critical for stimulating LDs [4]. Moreover, it is noticeable that within each cell line certain cells accumulate more LDs compared to surrounding cells, revealing a heterogenic response to EGF stimulation. Similar heterogeneity among cell populations is described by Herms et al., proposing that cells with high lipid levels accumulate more LDs in order to reduce overall lipotoxicity and provide fuel to surrounding cells [19]. These data reveal that in human colon cancer cell lines, EGF stimulates LD density to varying degrees that are dependent on receptor levels and each individual cell line's capacity for LD storage.

Next, we assessed in colon cancer cells if EGF mediated increases in LD density depend on EGFR activation and de novo lipid synthesis. Pharmacological inhibition of EGFR or lipid synthesis blockades EGF (6 h) stimulated LDs as shown by BODIPY staining (Fig 1B). Moreover, EGF increases expression of the LD coat protein PLIN2 by $86.0 \pm 8.4\%$ relative to control, which is blockaded by inhibition of EGFR or lipid synthesis (Fig 1C). These data suggest that in colon cancer cells EGF stimulated increases in LD density occur via receptor activation leading to de novo lipid synthesis. Limited studies have shown in liver and mammary epithelial cells that EGF could also increase LDs used either for intracellular lipid storage or secretion [7,8]. However, here we demonstrate that in colon cancer cells select EGF receptor signaling tightly controls increases in LDs that may be utilized for proliferative needs.

EGFR mediated increases in LD density are mediated by PI3K/mTOR activation and PGE2 synthesis

As EGFR signaling leads to downstream activation of PI3K/mTOR signaling, known to be critical in colon cancer progression [20], we assessed if this pathway is involved in LD increases. Following treatment with PI3K (wortmannin) and mTOR (rapamycin) inhibitors, EGF (6 h) mediated increases in LD density are blocked as shown by BODIPY staining and immunoblotting (Fig 2A,B). These findings reveal that in colon cancer cells, EGFR mediated increases in LD density depend on activation of the PI3K/mTOR pathway. Similarly, in colon cancer cells, increased LD density mediated by oleic acid treatment depends on PI3K activation [4] and because HCT116 cells with mutated PI3K [21] have a lower capacity for LD accumulation, this pathway might play a critical role in controlling LD density. Moreover, in lipid storing adipocytes, mTOR is found to be an essential mediator in the accumulation of intracellular lipids [22]. Hence, it appears that PI3K/mTOR is a ubiquitous regulator of LD density and is utilized by metabolically active colon cancer cells to produce intracellular lipids needed for tumor growth.

Additionally, blockade of PGE2 synthesis with the selective inhibitor sulindac effectively lowers EGF (6 h) mediated increases in LDs as shown by BODIPY staining and PLIN2 immunoblotting (Fig 2A,B). These data support that in colon cancer cells EGFR dependent increases in LD density rely on PGE2 synthesis, further linking LDs with regulators of tumor growth. While it is unclear whether PI3K/mTOR and PGE2 independently stimulate LD density, it is plausible that colon cancer cells utilize different mechanisms to generate lipids needed for their growth. Although pharmacological inhibition of PI3K/mTOR and PGE2 is well established, these inhibitors might exhibit non-specific effects on other pathways that could potentially be involved in the regulation of LD density. Our findings suggest that in colon cancer cells, EGFR mediated increases in LD density occur, in part, via two signaling mechanisms: the PI3K/mTOR pathway and PGE2 synthesis.

EGFR signaling controls loss of FOXO3 and Sirtuin6 via a negative feedback loop with lipid synthesis

Our group has demonstrated that deficiency in Foxo3 in mice leads to elevated LD density in colonic tissue via decreases in Sirtuin 6 (SIRT6) [4], a negative regulator of lipid metabolism [23]. Also, though EGF stimulates loss of FOXO3 function via PI3K [16], whether EGFR signaling utilizes this mechanism in colon cancer cells to promote increases in LDs is unclear. Silencing of FOXO3 in HT29 cells leads to a significant decrease in SIRT6 and elevation of the LD coat protein PLIN2 by $73.3 \pm 5.4\%$ (Fig 3A), supporting that in colon cancer cells loss of FOXO3/SIRT6 leads to LD elevation. In both HT29 and HCT116 cell lines, EGF stimulates loss of FOXO3 (translocation from the nucleus to the cytosol) and decreases in SIRT6 (Fig 3B,C). Moreover, EGF mediates inactivation of FOXO3/SIRT6 to a lesser degree in HCT116 cells relative to HT29 cells (SIRT6 decrease $37.7 \pm 8.2\%$, $57.9 \pm 4.3\%$, respectively), supporting that in cells with a lower capacity for LD accumulation this signaling mechanism is also utilized, but is less efficient. Furthermore, the EGF mediated loss of FOXO3/SIRT6 is prevented by blockade of mTOR and PGE2 synthesis (Fig 3B,C), indicating that their loss of function depends on activation of select upstream pathways. Moreover, we assessed whether inhibition of lipid synthesis restores FOXO3/

SIRT6 function. In colon cancer cells, inhibition of lipid synthesis blockades the EGF mediated loss of FOXO3/SIRT6 (Fig 3B,C), revealing a negative regulatory loop between lipid levels and the activity of these transcription factors. Together, these data show that in colon cancer cells EGFR utilizes select signaling and lipid synthesis to control a negative regulatory loop between LDs and FOXO3/SIRT6. Limited studies have shown in muscle cells that loss of FOXO3 is critical in providing cellular energy [24,25], supporting the critical role of this molecule in regulation of cellular metabolism. Since cancer cells require high metabolic energy for proliferation it is reasonable that EGFR signaling would utilize this mechanism needed to sustain tumor growth. While this emerging novel metabolic characteristic of cancer cells needs further understanding, it provides the opportunity to develop novel treatment options for cancer.

EGFR signaling utilizes elevated LDs for proliferative needs

Next, we assessed whether EGFR mediated increases in LDs are used as an energy source during proliferation. In HT29 cells, LD density is pre-stimulated with fluorescently labeled fatty acid (BODIPY C₁₂) and then treated with EGF for 24 h. During EGF stimulation (EGF 24), pre-stimulated C₁₂ labeled LDs are significantly lowered, relative not only to cells with initial accumulation (Con 0), but also in cells kept in serum free media for 24 h (Con 24) (Fig 4A). Compared to Con 24, EGF treatment lowers the number of pre-stimulated LD positive cells and the number of LDs per cell by $31.5 \pm 5.2\%$ and $50.2 \pm 3.5\%$, respectively (Fig 4B). Since these data indicate that in colon cancer cells pre-stimulated LDs are utilized during EGF treatment, next we assessed if LDs are required for EGFR mediated proliferation. In HT29 cells, EGF stimulated proliferation is significantly attenuated following inhibition of lipid synthesis (from $130.2 \pm 3.3\%$ to $89.4 \pm 3.6\%$ relative to the control) (Fig 4C). Moreover, following knockdown of PLIN2 in HT29 cells, EGFR mediated proliferation is lowered from $134.2 \pm 9.7\%$ (EGF) to $87.9 \pm 7.9\%$ (EGF, siRNA) relative to the control (Fig 4D). These data support that in colon cancer cells, EGF signaling utilizes elevated LDs to support proliferation. Since FOXO3/SIRT6 regulate LDs, and since both act as tumor suppressors in cancer cells [26,27], it is plausible that EGFR signaling utilizes their loss of function to synchronize the energy stored within LDs with cellular proliferation. Thus, we suggest that this novel mechanism of colon cancer cell proliferation mediated by EGFR signaling is employed to control and coordinate the increases in intracellular, metabolic energy with cancer growth.

Emerging studies demonstrate the importance of LDs as cellular energy stores [28] and that abnormal LD dynamics, which are not currently understood, are associated with the pathobiology of many metabolic diseases, including: obesity, diabetes, atherosclerosis, fatty liver, and cancer [29]. Our current study highlights that in colon cancer cells a) EGFR signaling stimulates increases in LD density b) via a negative regulatory loop with FOXO3/SIRT6, and c) that elevated LDs are utilized for proliferative needs. This regulatory network could serve as a new target for therapeutic intervention in colon cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
LD	lipid droplet
PI3K	phosphatidyl inositol 3-kinase
PGE2	prostaglandin E2

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Highlights

- In colon cancer cells, EGFR activation leads to increases in LD density.
- EGFR signaling includes PI3K/mTOR and PGE2 leading to lipid synthesis.
- Increases in LDs are controlled by a negative regulatory loop with FOXO3/SIRT6.
- EGFR mediated colon cancer cell proliferation depends on increased LD density.

Fig 1.

In colon cancer cells, EGF mediated increases in LD density are dependent on receptor expression and activation as well as lipid synthesis. A) Human colon cancer cells HT29, HCT116, SW480, and SW620 were treated with EGF (24 h). LDs were stained with BODIPY and visualized using immunofluorescent microscopy (objective 60X, representative images from three independent experiments). While EGF stimulated differential increases in LD density in HT29, HCT116, and SW480 cells, SW620 cells, which express less EGFR, did not show increases in LDs. B) BODIPY stains of HT29 cells showed that EGF treatment (6 h) increased LD density, while pharmacological blockade of EGFR (PD153035) and lipid synthesis (C75) effectively attenuated this increase (objective 60X, representative images from three independent experiments). C) Similarly, immunoblotting and densitometric analysis revealed that EGF (6 h) increased levels of PLIN2, and this increase was lowered in the presence of PD153035 and C75 (* $p < 0.05$ compared to untreated Con, # $p < 0.05$ compared to EGF treated cells, $n = 3$).

Fig 2.

EGFR mediated increases in LD density depend on PI3K/mTOR signaling and PGE2 synthesis. A) The effect of the respective PI3K and mTOR inhibitors wortmannin and rapamycin, and the PGE2 inhibitor sulindac, on EGF stimulated LD density in HT29 cells was assessed by BODIPY staining (objective 60X, representative images from three independent experiments). All three inhibitors diminished EGF stimulated LDs in HT29 cells. B) Protein from HT29 cells treated with EGF (6 h) with or without PI3K/mTOR or PGE2 inhibitors were immunoblotted for PLIN2. Immunoblots and corresponding densitometric analysis indicated that in the presence of these inhibitors, the EGF mediated increase in PLIN2 was attenuated (* $p < 0.05$ compared to untreated Con, # $p < 0.05$ compared to EGF treated cells, $n = 3$).

Fig 3.

EGFR signaling controls loss of FOXO3/SIRT6 via negative feedback with lipid synthesis.

A) Protein from control HT29 cells, or those transfected with either scrambled ((-)siRNA) or FOXO3 siRNA (FOXO3 siRNA) were immunoblotted for FOXO3, SIRT6 and PLIN2. Immunoblots and corresponding densitometric analysis revealed that silencing FOXO3 lowered SIRT6 and significantly elevated PLIN2 (*p<0.05 compared to Con and (-)siRNA, n=3). B) In HT29 and HCT116 cells treated with EGF (2 h) with or without rapamycin, sulindac, or C75, the localization of FOXO3 was assessed by immunofluorescent staining (objective 60X, representative images from three independent experiments). EGF dependent translocation of FOXO3 from the nucleus to the cytosol (inactivation) as indicated by arrows was prevented in the presence of rapamycin, sulindac, and C75. C) Immunoblots showed that in HT29 and HCT116 cells, treatment with EGF led to decreases in levels of SIRT6. In HT29 cells, the EGF dependent reduction of SIRT6 levels was prevented by rapamycin, sulindac, and C75 (*p<0.05 compared to untreated Con, n=3).

Fig 4.

EGF stimulated proliferation depends on increases in LD density. A, B) Following 24 h incubation of HT29 cells with fluorescently labeled fatty acid (BODIPY C₁₂), monolayers were washed and one group was fixed (Con 0), while the other cells were kept for another 24 h in serum free media (Con 24) or treated with EGF (EGF 24) before fixation. Monolayers were visualized using immunofluorescent microscopy and LDs were quantified (Image J). Images and corresponding graphs showed significant decreases in pre-stimulated LD positive cells and LDs per cell in those that were treated with EGF relative to Con 0 and Con 24 (*p<0.05 compared to Con 0, #p<0.05 compared to Con 24, n=250 cells). C) In HT29 cells EGF stimulated proliferation was attenuated by C75 (*p<0.05 compared to Con, n=8). D) In HT29 cells, silencing of PLIN2 through siRNA (PLIN2 siRNA) as shown in the immunoblot attenuated proliferation induced by EGF while nonspecific effects of scrambled siRNA ((-)siRNA) were not observed (*p<0.05 compared to control, n=8).