RICE UNIVERSITY

NITRIC OXIDE: THE MISSING LINK IN OMENTUM-INDUCED METABOLIC REPROGRAMMING OF OVARIAN CANCERS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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ABSTRACT

Nitric oxide: the missing link in omentum-induced metabolic reprogramming of ovarian cancers

by

Bahar Salimian Rizi

A novel metabolic regulatory mechanism of ovarian cancer by omentum adipose-derived stroma cells (O-ASCs) has been discovered. O-ASCs induce survival, migration, and chemoresistance of ovarian cancer cells. However, the underpinning mechanism behind the metabolic modulation was not understood. Here, O-ASCs are shown to promote nitric oxide (NO) homeostasis in ovarian cancers by generating the pool of arginine. Ovarian cancer cells benefit from tumor microenvironment’s elements and expand their growth. In turn, cancer cells modify the elements’ fate to further take advantage of nutrients and resources. A unique combinatory drug treatment is proposed to target O-ASCs-induced chemoresistance of ovarian cancer cells.
Acknowledgments

First and foremost I wish to thank my PhD advisor, Dr. Deepak Nagrath for supporting me during these past five years. He has been supportive since the day I joined the group and has given me the high motivations to pursue various projects. I am also very grateful to Deepak for his scientific advice and knowledge and many insightful discussions and suggestions. He is my primary resource for getting my science questions answered. I appreciate all his contributions of time, ideas, and funding to make my Ph.D. experience productive and stimulating. He helped me come up with the thesis topic and guided me over years of development. And during writing this thesis, he gave me the moral support and the freedom I needed to move on.

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Special thanks to my husband’s family who all have been supportive and caring. My mother-in-law spoiled me by cooking the best dishes on earth whenever I was so busy with school.
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Bahar Saliamian Rizi
Rice University
October 2015
I dedicate this thesis to my family and my beloved husband, Masoud, for their constant support and unconditional love.

I love you all dearly.
Contents

Acknowledgments .................................................................................................................. iii

Contents ................................................................................................................................... vii

List of Figures .......................................................................................................................... x

List of Equations .................................................................................................................... xiii

Nomenclature ......................................................................................................................... 14

Introduction ............................................................................................................................. 18
  1.1. Ovarian Cancer ........................................................................................................... 18
    1.1.1. Ovarian tumor microenvironment ................................................................. 20
    1.1.2. Risk factors ....................................................................................................... 20
    1.1.3. Clinical treatment of ovarian cancer .............................................................. 21
    1.1.4. Biomarker (CA125) ......................................................................................... 21
  1.2. Cancer central energy metabolism ........................................................................ 22
    1.2.1. Glycolysis ......................................................................................................... 22
    1.2.2. TCA cycle ......................................................................................................... 26
    1.2.3. Electron transport chain .................................................................................. 28
    1.2.4. Warburg effect .................................................................................................. 31
    1.2.5. Glutaminolysis ................................................................................................. 34
    1.2.6. Fatty acid metabolism ...................................................................................... 34
      1.2.6.1. Fatty acid synthesis .................................................................................. 35
      1.2.6.2. Normoxia .................................................................................................. 35
      1.2.6.3. Fatty acid synthase .................................................................................. 38
      1.2.6.4. Hypoxia ...................................................................................................... 38
      1.2.6.5. Fatty acid oxidation .................................................................................. 39
      1.2.6.6. Lipogenesis ............................................................................................... 42
      1.2.6.7. Lipolysis .................................................................................................... 44
  1.3. Cancer-stroma interaction ....................................................................................... 46
    1.3.1. Cancer and wound healing .............................................................................. 46
    1.3.2. Stromal cells ...................................................................................................... 46
    1.3.3. Fibroblasts ......................................................................................................... 47
1.4. Adipose tissue ........................................................................................................ 48
1.5. Cellular Role of Nitric Oxide ................................................................................... 49
  1.5.1. S-nitrosation .................................................................................................... 52
  1.5.2. Nitric oxide in tumor progression .................................................................... 53
  1.5.3. Hypoxia-induced nitrite conversion to NO ...................................................... 54

A missing link between omentum and ovarian cancers’ metabolic coupling: nitric oxide homeostasis ............................................................................................................. 58
  2.1. O-ASCs induce nitric oxide synthesis of OVCAs and ECAs .............................. 60
  2.2. O-ASCs positively regulate OVCAs and ECAs growth through arginine ........ 63
  2.3. Inhibition of endogenous nitric oxide synthesis abrogates elevated viability of cancer cells induced by O-ASCs ............................................................. 67
  2.4. Citrulline induces adipogenesis of O-ASCs ...................................................... 69
  2.5. O-ASCs modulate cancer cells’ mitochondrial bioenergetics ......................... 74
  2.6. O-ASCs regulate OVCAs and ECAs metabolism via nitric oxide pathways .... 76
  2.7. O-ASCs induce chemo resistance of cancer cells ............................................ 79
  2.8. Conclusion ........................................................................................................ 82

Targeting NO-mediated pathways as a novel cancer treatment: Nitrite and S-nitrosylation ............................................................................................................. 92
  3.1. Nitrite is another source of NO in ovarian cancers ............................................. 95
  3.2. Inhibition of s-nitrosylation reduces ovarian cancer survival ......................... 102
    3.2.1. Nitrite-induced s-nitrosylation ...................................................................... 103
    3.2.2. NO-induced s-nitrosylation .......................................................................... 105
  3.3. Evolution of nitrogen metabolism during adipogenesis .................................. 107
  3.4. Conclusion ........................................................................................................ 109

Modulation of ovarian cancer metabolism by long chain fatty acids .......... 111
  4.1. Exogenous LCFA is consumed by ovarian cancer cells .................................... 111
  4.2. Lipid droplets are induced in ovarian cancers by LCFA ................................... 112
  4.3. O-ASCs mediates utilization of stored LCFA in ovarian cancers .................... 117
    4.3.1. Storage of LCFA in the coculture of ovarian cancer cells and O-ASCs ....... 118
    4.3.2. O-ASCs induce utilization of stored LCFA in ovarian cancers .................. 120
  4.4. Stimulation of lipase induces utilization of LD ................................................. 123
List of Figures

Figure 1-1. Percent of case and 5-year relative survival rate by stage at diagnosis .............................................................................................................................................................................. 20

Figure 1-2. Glycolysis pathway within the mammalian cells. ......................................................... 25

Figure 1-3. TCA cycle converts pyruvate and acetyl CoA to reducing agents .................. 28

Figure 1-4. Electron transport chain located in the mitochondrial matrix .......... 30

Figure 1-5. Aerobic and anaerobic glycolysis comparison ................................................. 33

Figure 1-6. Fatty acid synthesis pathway in normoxia and hypoxia ......................... 37

Figure 1-7. Long chain fatty acid oxidation within the human mitochondria.... 41

Figure 1-8. Triacylglyceride synthesis ........................................................................ 43

Figure 1-9. Lipolysis pathway in human cells. ........................................................................ 45

Figure 1-10. Cellular nitric oxide generation pathway ....................................................... 50

Figure 1-11. Two-step conversion of L-arginine to L-citrulline. ...................................... 50

Figure 1-12. Depiction of thiol and s-nitrosothiol functional groups ....................... 53

Figure 1-13. Basic mechanism of s-nitrosation. Nitric oxide experience a redox reaction generating s-nitrosothiols ......................................................................................................................... 53

Figure 1-14. Cellular nitric oxide (NO) synthesis. .................................................................. 57

Figure 2-1. Nitric oxide in tumor microenvironment. ................................................... 59

Figure 2-2. O-ASCs induce NO synthesis of OVCAs and ECAs. ................................. 63

Figure 2-3. O-ASCs positively regulate OVCAs and ECAs growth through arginine .................................................................................................................................................................................. 66

Figure 2-4. Inhibition of nitric oxide synthesis abrogates elevated viability of cancer cells induced by O-ASCs. ........................................................................................................................................ 68

Figure 2-5. Citrulline induces adipogenesis of O-ASCs. ................................................. 73
Figure 2-6. O-ASCs modulate cancer cells’ mitochondrial bioenergetics. .......... 75

Figure 2-7. O-ASCs regulate OVCA and ECA cells metabolism via nitric oxide pathways. ........................................................................................................................................................................ 79

Figure 2-8. O-ASCs induce chemo resistance in cancer cells. ...................... 82

Figure 2-9. O-ASCs’ conditioned-medium rescue reduced viability of cancer cells deprived of arginine. ........................................................................................................................................................................ 86

Figure 2-10. L-argininase-induced depletion of arginine. .............................. 87

Figure 2-11. Cancer cells induce glucose uptake and lactate secretion of O-ASCs and vice versa. ........................................................................................................................................................................ 88

Figure 2-12. Nitric oxide regulates chemo sensitivity of cancer cells............. 90

Figure 3-1. Cellular nitrogen metabolism................................................................. 93

Figure 3-2. Nitric oxide and tumor therapy. ......................................................... 94

Figure 3-3. Normoxic titration of sodium nitrite on ovarian cancer cells......... 98

Figure 3-4. Hypoxic titration of sodium nitrite on ovarian cancer cells......... 99

Figure 3-5. Ovarian cancers convert nitrite to nitric oxide in both normoxia dn hypoxia. ........................................................................................................................................................................ 100

Figure 3-6. Inhibition of NOS and mitochondrial complexes targets the conversion of nitrite to NO. ........................................................................................................................................................................ 101

Figure 3-7. Pathways of nitrite reduction to NO .................................................... 102

Figure 3-8. S-nitrosylation inhibition targets nitrite-induced viability of ovarian cancers........................................................................................................................................................................ 104

Figure 3-9. S-nitrosylation inhibition targets NO-induced viability of ovarian cancers ........................................................................................................................................................................ 106

Figure 3-10. NO modulation in SGBS adipogenesis........................................ 107

Figure 3-11. NO modulation in O-ASCs adipogenesis. .................................... 108

Figure 3-12. Inhibition of NO-mediated pathway at different stages blocks the increased growth of endometrial cancer cells caused by O-ASCs............. 110
Figure 4-1. LCFAs uptake in normoxia and hypoxia ........................................ 112
Figure 4-2. Ovarian cancer cells store LCFAs as lipid droplets ....................... 114
Figure 4-3. LCFAs induce lipid droplet formation in ovarian cancer cells ...... 115
Figure 4-4. Characterization of lipid droplets in ovarian cancer cells .......... 116
Figure 4-5. Induced neutral lipid and phospholipid formation in ovarian cancers treated with LCFAs ................................................................. 117
Figure 4-6. LD formation in direct coculture of ovarian cancers and O-ASCs .. 119
Figure 4-7. Colocalization of GFP-labeled ovarian cancer cells with AdipoRed Staining ................................................................. 120
Figure 4-8. O-ASCs induce utilization of stored LCFAs in ovarian cancer cells ........................................................................................................ 122
Figure 4-9. Lipase stimulation induces utilization of exogenous LCFAs .......... 124
Figure 4-10. Induced Chemiresistance of cancer cells caused by O-ASCs through stimulation of lipolsysis ......................................................... 126
Figure 4-11. FAS inhibition induces utilization of stored LCFAs ................. 128
Figure 5-1. LCFAs-reduced cellular NO levels ................................................ 132
Figure 5-2. O-ASCs stimulate ovarian cancers progression .......................... 136
List of Equations

Equation 1-1. Glucose conversion to pyruvate .......................................................... 24
## Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACLY</td>
<td>ATP citrate lyase</td>
</tr>
<tr>
<td>AGPAT</td>
<td>Acylglycerolphosphate acyltransferase</td>
</tr>
<tr>
<td>AP2</td>
<td>Adipocyte protein 2</td>
</tr>
<tr>
<td>ARG1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose stromal cells</td>
</tr>
<tr>
<td>ASL</td>
<td>Argininosuccinate lyase</td>
</tr>
<tr>
<td>ASS</td>
<td>Argininosuccinate synthase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned media</td>
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<tr>
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<td>Central nervous system</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
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<td>ER</td>
<td>Estrogen receptor</td>
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<td>FCCP</td>
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<td>FDG-PET</td>
<td>Fluorodeoxyglucose positron emission tomography</td>
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<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
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</tr>
<tr>
<td>---------</td>
<td>-----------</td>
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<tr>
<td>FGF2</td>
<td>Fibroblast growth factor 2</td>
</tr>
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<td>Forskolin</td>
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<tr>
<td>G3PDH</td>
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<td>Hepatocyte growth factor</td>
</tr>
<tr>
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<td>Hypoxia-inducible factor</td>
</tr>
<tr>
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<td>Hexokinase</td>
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<tr>
<td>HSL</td>
<td>Hormone sensitive lipase</td>
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<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
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</tr>
<tr>
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<td>Long chain fatty acids</td>
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<td>Monoacylglycerol lipase</td>
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<td>Matrix metalloproteinase</td>
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<td>NADH</td>
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<td>nNOS</td>
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<tr>
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</tr>
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<td>-----------</td>
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<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>O-ASC</td>
<td>Omentum adipose-derived stroma cells</td>
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<td>Phosphatidic acid</td>
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<td>Paclitaxel</td>
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<td>Phosphoinositide 3 kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
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<tr>
<td>PTIO</td>
<td>2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
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<td>Reactive oxygen species</td>
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<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
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<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
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<td>Transforming growth factor</td>
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<td>Description</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
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<tr>
<td>UPLC</td>
<td>Ultra-performance liquid chromatography</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Chapter 1

Introduction

Ovarian tumors are often detected at advance stages with dissemination of cancer cells into the peritoneum. Ovarian cancer tumors are frequently diagnosed to be resistant to chemo eventually. In this study, the intrinsic metabolic properties of ovarian tumors as well as particularities of the environment will be explained.

1.1. Ovarian Cancer

Ovarian cancer accounts for approximately three percent of cancers in women. While the 11th most common cancer among women, ovarian cancer is the fifth leading cause of cancer-related death among women, and is the deadliest of gynecologic cancers. Mortality rates are slightly higher for Caucasian women than for African-American women. The original causes of ovarian cancer are not fully understood although there are some speculations to explain the origins of ovarian cancer including the genetic malfunctions which may be the result of ovulation and the incorporated fluctuations in hormone levels [1]. Epigenetic changes also are believed to cause ovarian cancers [2].

High rates of mortality of ovarian cancers are because of the common late diagnosis of the disease since it shares the symptoms with other abdominal abnormalities. 75% of ovarian cancer patients are detected when the cancer has disseminated to peritoneum, more specifically into the omentum. The surgical
processes are principally successful and efficient; nevertheless, the metastasis of ovarian cancers into peritoneal cavity transforms them persistent and resistant to chemotherapy [3]. Ovarian carcinoma is a deadly disease, with a cure rate of only 30%.

Ovarian cancers were believed to initiate from the coelomic ovarian epithelium; however, ovarian carcinoma could originate from any of three potential sites: the surfaces of the ovary, the fallopian tube, or the mesothelium-lined peritoneal cavity.

Type I ovarian cancers proceed with their progression throughout a stepwise mutation process and acquire slow proliferation. They are categorized as borderline tumors which are usually resistant to conventional chemotherapies although they may respond to hormonal treatment. Type I ovarian cancers are frequently associated with KRAS, BRAF, phosphatase, and tensin homolog (PTEN) or phosphatidylinositide 3-kinase (PI3K) genes mutation [4]. On the other hand, type II ovarian cancers are frequently diagnosed at late stage with peritoneum metastasis. They grow fast and are responsive to conventional chemotherapies. The common mutations found in type II ovarian cancers are BRAC1/2 and p53 mutations.

As mentioned before, ovarian carcinoma dominantly metastasizes along the peritoneum throughout the pelvic and abdominal cavity although other metastatic sites are noticed including lung, skin, pleura, mediastina, and lymph nodes [5]. Isolated incidents of metastases in bone, brain, or gastrointestinal track have been detected [6].
Figure 1-1. Percent of case and 5-year relative survival rate by stage at diagnosis

1.1.1. Ovarian tumor microenvironment

Ovarian tumors are constituted of malignant cells and many other non-malignant cell types such as adipocytes, fibroblasts, endothelial cells, migratory hematopoietic cells, and stem cells [7]. The combination of these cells provides a unique stroma that can modulate the intrinsic properties of tumor cells. For example, there is a population of stem cell-like cells residing in the omentum called omentum-derived stroma cells (O-ASCs) that have been shown to regulate the extracellular matrix (ECM) composition and produce cytokines leading to attract ovarian carcinoma cells to the omentum [8].

1.1.2. Risk factors

Age, early menarche, late menopause radiation, geographic area [9] (North America and Northern Europe at highest risk), use of fertility drug, number of ovulatory cycles, number of pregnancies [9] (nullipara are at the highest risk and pregnancy is protective), caffeine consumption, and positive family history of ovarian, breast, uterine, or colon cancer are known as the main contributors to ovarian cancers. Race has been also shown to be an influential factor (lower survival rates for Black women compared to Whites) [10]. The impact of lifestyle
such as smoking and physical activity on ovarian cancer was investigated but the data were not reproducible by other researchers [11-13].

Breast feeding, first birth at elder ages, oral contraceptive, number of incomplete pregnancies, tubal ligation, and hysterectomy have been determined as protective in several studies but not consistent in all of them [9].

1.1.3. Clinical treatment of ovarian cancer

There has been an extension of 37% to 45% to five-year survival of ovarian cancer patients over the last thirty years mainly because of cytoreductive surgery and combination of chemotherapy with platinum compounds and taxanes [14].

Cytoreductive surgeries are often suggested by surgeons for ovarian cancer patients and six cycles of carboplatin and paclitaxel chemotherapy are considered standard adjuvant treatment for newly diagnosed cancers after the operation [15]. Carboplatin is alkylating compounds which binds to DNA and creates adducts that cast intra chain and inter chain cross-links. Paclitaxel binds to microtubules and also stabilizes them which results in their interference with mitotic spindle formation. Both these chemo drugs elevate the chance of apoptosis within cancer cells. Although recurrent ovarian cancers are commonly resistant to chemotherapy the drugs that are available can prolong survival. Apart from chemotherapy, recent research has shown that knockdown of various kinases induces the sensitivity of chemo drugs.

1.1.4. Biomarker (CA125)

The main biomarker of ovarian cancer is glycosylated transmembrane mucin, CA125 (MUC16), which has a high molecular mass (1MDa) and it is expressed by 80% of ovarian cancers [16, 17]. CA125 is vital for ovarian cancer adherence, motility, and their invasion and it is secreted by tumors and dispersed
in serum. The level of CA125 is measured as an indicator of chemotherapy drugs affectivity and also to monitor the recurrence of ovarian tumors.

1.2. Cancer central energy metabolism

Cancer cells exhibit significantly altered metabolism from normal differentiated-cells. Exploring their particular metabolism is crucial in order to understand the effect of fatty acids on malignant tumors. The revised metabolism of cancer cells is mainly characterized by high rate of glycolysis, fatty acid anabolism and glutaminolysis.

Normal and cancer cells implement similar mechanisms to generate energy in the form of adenosine triphosphate (ATP). These mechanisms include glycolysis, the Tricarboxylic Acid cycle (TCA cycle), and the Electron Transport Chain (ETC). In normal cells the majority of energy is provided within the mitochondria of cells and cells without proper mitochondrial activity are dependent on anaerobic glycolysis for the required ATP.

1.2.1. Glycolysis

Glucose is a vital substrate for all human cells [18] and Figure 1-2 shows the most possible pathways for this metabolite. Glycolysis is originated from Greek words glukus, “sweet”, and luis, “rupture”. Glycolysis generates ATP without the involvement of oxygen molecules. Glycolysis occurs in cytosol of most cells. During glycolysis, a glucose molecule with six carbon atoms is converted into two molecules of pyruvate, both of which contain three carbon atoms.

Glycolysis pathway involves a sequence of ten separate reactions, each producing a different sugar intermediate and each step is catalyzed by a different enzyme. The final product is pyruvate which is oxidized in CO$_2$ in TCA cycle in
order to generate large amounts of ATP through the process of oxidative phosphorylation.

Although no oxygen molecule is used in glycolysis oxidation occurs in that electrons are removed by NAD\(^+\) from some of the carbons derived from glucose molecule. The stepwise nature of the process releases the energy oxidation in small packets, so that much of it can be stored in activated carrier molecules rather than all of it being released as heat. Thus, some of the energy released by oxidation derives the direct synthesis of ATP molecules from ADP and Pi, and some remains with the electrons in the high-energy electron carrier NADH.

- First, glucose is phosphorylated to glucose-6-phosphate by hexokinase (HK). The phosphate group is borrowed from ATP which is consumed at this step.
- The enzyme phosphoglucone isomerase rearranges glucose 6-phosphate into its isomer fructose 6-phosphate.
- Another ATP molecule is utilized to transfer a phosphate group from fructose 6-phosphate to fructose 1, 6-bisphosphat by the enzyme phosphofructokinase.
- The enzyme aldolase then breaks fructose 1, 6-bisphosphate into two sugars which are isomers of each other. The products of this step are dihydroxyacetone phosphate and glyceraldehyde phosphate.
- The enzyme triosephosphate isomerase interconverts the molecules dihydroxyacetone phosphate and glyceraldehyde phosphate. Glyceraldehyde phosphate is removed as soon as it is formed to be used in the next step of glycolysis.
- The enzyme triosephosphate dehydrogenase plays two roles in this step. First hydrogen from glyceraldehyde phosphate is transferred to the oxidizing agent nicotinamide adenine dinucleotide (NAD\(^+\)) to form NADH. Next triosephosphate dehydrogenase adds a phosphate from the cytosol to the oxidized glyceraldehyde
phosphate to form 1, 3-bisphosphoglycerate. This occurs for both molecules of glyceraldehyde phosphate produced in previous step.

- The enzyme phosphoglycerokinase transfers a phosphate from 1, 3-bisphosphoglycerate to a molecule of ADP to form ATP. The process yields two 3-phosphoglycerate molecules and two ATP molecules because omission of phosphate group occurs for each molecule of 1, 3-bisphosphoglycerate.

- The enzyme phosphoglyceromutase relocates the phosphate from 3-phosphoglycerate from the third carbon to the second carbon to form 2-phosphoglycerate.

- One molecule of water is removed from 2-phosphoglycerate to form phosphoenol pyruvate (PEP) in presence of the enzyme enolase.

- A phosphate is added to an ADP molecule from phosphoenol pyruvate and one molecule of ATP and pyruvate is produced. Since two molecules of PEP are produced from last steps this reaction yields 2 molecules of pyruvate and 2 ATP molecules.

- Subsequently, two molecules of pyruvate, two molecules of ATP, two molecules of NADH and 2 molecules of water are produced from a single glucose molecule in glycolysis. The overall reaction is

$$\text{Glucose} + 2 \text{NAD}^+ + 2 \text{P}_i + 2 \text{ADP} \rightarrow 2 \text{Pyruvate} + 2 \text{NADH} + 2 \text{ATP} + 2 \text{H}^+ + 2 \text{H}_2\text{O}$$

Equation 1-1. Glucose conversion to pyruvate
Figure 1-2. Glycolysis pathway within the mammalian cells.
1.2.2. TCA cycle

In the presence of oxygen, the pyruvate formed by glycolysis is rapidly transported into mitochondria. In contrast, for anaerobic conditions, the pyruvate and NADH electrons stay in cytosol and convert to lactate.

ATP, the energy currency within the cells, can be produced partially through sets of reactions in cytosol but the major source of generated ATP is mitochondria. Mitochondria are located within cells cytoplasm and occupy considerable volume of cytoplasmic region. Pyruvate produced from glycolytic pathway and the metabolism of sugars is accomplished in mitochondria. Pyruvate is transferred into mitochondria and oxidized to CO$_2$ and H$_2$O. This process produces 15 times more ATP compared to glycolysis.

Mitochondria are absolutely mobile and dynamic organelles. In some cells they are moving in the cytoplasm while they are constant and generate ATP directly to a site of high ATP consumer. They are large enough to be detected via light microscopes. They were first identified during nineteenth century. Mitochondria are energy converting organelles and their internal membrane supports the framework for a collection of complex electron-transport processes which lead to ATP synthesis. Each mitochondrion is surrounded by two membranes with totally different functions. The membranes together provide two compartments: the internal matrix and much narrower intermembrane space. Each of the membranes is composed of unique proteins.

The outer membrane consists of groups of proteins that form large aqueous channels through the lipid bilayer and it is permeable to molecules with molecular mass of 5000 daltons or less. These small molecules can enter intermembrane space but the rest are unable to pass the inner membrane. Thus the chemical composition of intermembrane is almost same as cytosol while the matrix contains highly selected molecules.
The inner membrane has a lipid bilayer which mostly consists of double phospholipids, cardiolipins, which have four fatty acids rather than two in order to equip the inner membrane to become impermeable to ions. Moreover, mitochondrial inner membrane has a variety of transport proteins which improve the permeability of vital metabolites such as pyruvate and fatty acids. There are specific enzymes in the matrix that convert fatty acids and pyruvate to acetyl CoA for further oxidation. The final products are CO₂ and H₂O which are expelled from cells as waste and NADH which is the main source of electron for transport along the respiratory chain. The enzymes for respiratory chain are embedded within the inner membrane and are necessary for oxidative phosphorylation.

Pyruvate and fatty acids can be metabolized within the mitochondria. Both of these molecules are transported across the inner membrane and converted to acetyl Coenzyme A (CoA) by matrix enzymes. The acetyl groups of acetyl CoA are then oxidized in citric acid cycle, tricarboxylic acid cycle or Krebs cycle. The citric acid cycle accounts for almost two-thirds of the total oxidation of carbon compound in most cells. Acetyl group is not oxidized directly. Instead, this group is transferred from acetyl CoA to a larger, four–carbon molecule, oxaloacetate, to from the six-carbon tricarboxylic acid, citric acid, for which the subsequent cycle of reactions is named. The chain of eight reactions forms a cycle because at the end the oxaloacetate is regenerated and enters a new round of a cycle.
1.2.3. Electron transport chain

Pyruvate and fatty acid oxidation generates high-energy electrons which are transferred to the inner membrane and drive the electron-transport chain [19]. Although the citric acid cycle itself does not use O$_2$, it requires oxygen in order to proceed because there is no other efficient way for the NADH to get rid of its electrons and thus regenerate the NAD$^+$ that is needed to keep the cycle going. The loss of electrons from NADH and FADH$_2$ produces the NAD$^+$ and FAD which is required for further oxidative metabolism.

NADH switches its electrons to oxygen through three respiratory enzyme complexes. The main reaction is $\text{H}_2 + \frac{1}{2}\text{O}_2 \rightarrow \text{H}_2\text{O}$ which is energetically favorable. This reaction occurs in different steps in order to prevent any energy loss. Hydrogen molecules are broken to protons at the first step. High energy electrons and generated protons are recombined at several stages. But only at the final step of electron-transport chain the protons are permanently returned.
i.e., when they are utilized to neutralize the negative charges created by the addition of electrons to the oxygen molecules.

The process of electron transport launches when hydride ion is removed from NADH and is transformed into a proton and two electrons. Then the electrons flow to different electron carriers in the respiratory chain. The electrons lose their energy gradually as they are transferred along the chain.

Electrons pass from one metal ion to another, each of which has a different electron affinity. Most of the proteins involved are classified into three large respiratory enzyme complexes. Each complex has transmembrane proteins that hold the complex firmly in the inner membrane. Each of complexes in the chain has greater affinity for electrons than its predecessor, such that electrons pass sequentially from one complex to another until they are finally transferred to oxygen, which has the greatest affinity of all for electrons.

The first stage is composed of high-energy electrons, derived from oxidation of carbohydrates such as pyruvate and fatty acids. The electrons make a journey through a series of electron carriers found within the membrane. Electron transfer releases energy to pump protons across the membrane which has two major consequences. It provides a pH gradient across the inner mitochondrial membrane. Also it produces a voltage gradient across the inner mitochondrial membrane, with the inside negative and the outside positive. The ion gradient across the membrane is a form of stored energy which could be utilized later. Ions generate favorable work when they are allowed to flow back across the membrane to their electrochemical gradient.

In the second stage, the protons shift back to their electrochemical gradient utilizing a protein machine called ATP synthase, which catalyzes energy requiring conversion of ATP from ADP and inorganic phosphate (P$_i$). ATP synthase supports hydrophilic pathways across the inner mitochondrial membrane that allows protons to flow down their electrochemical gradient. As these ions go through this cascade through the ATP synthase, they are utilized to
drive the energetically unfavorable reaction between ADP and P_i that makes ATP.

The electrochemical proton gradient also serves other membrane protein machines such as transportation of vital metabolites. All the proteins embedded in the membrane together are called electron-transport chain. The mechanism of electron transport is similar to an electric cell with a current but with a slight modification. The difference is that in biological systems, the transfer of electrons across the membrane is assisted with diffusion of small molecule and not by conducting wires. NAD^+ is the first electron carrier which absorbs two electrons (plus an H^+) and converts to NADH. NADH is water soluble and carries electron across the mitochondrial membrane. Therefore, for each glucose molecule that enters into the TCA cycle the net production of products are:

- 8 NADH
- 2 FADH_2
- 2 ATP
- 6 CO_2

Figure 1-4. Electron transport chain located in the mitochondrial matrix
1.2.4. Warburg effect

Warburg made a striking discovery in the 1920s. The Warburg effect has since been elucidated in different origins of tumors and the concomitant increase in glucose uptake has been exploited clinically for the detection of tumors by fluorodeoxyglucose positron emission tomography (FDG-PET). He found that cancer cells preferentially metabolize glucose through glycolysis even in the presence of abundant oxygen which seems a paradox as glycolysis, when compared to oxidative phosphorylation, is a less efficient pathway for producing ATP [20]. He hypothesized that a fundamental impairment of cellular respiratory capacity was the main cause of all cancer, a bold and controversial claim that was ultimately rejected despite his continued writing and lecturing on the subject for some 40 years [21].

Unique tumor microenvironment is one of the reasons that explain the altered metabolism of cancer cells for their survival and proliferation. During tumorigenesis cancer cells outgrow the diffusion limits of their local blood supply that leads to hypoxia and stabilization of the hypoxia-inducible transcription factor (HIF). HIF mediates a transcriptional program in order to support the cells with hypoxic stress [22]. HIF manipulates cell metabolism and forces a shift from mitochondrial metabolism toward glycolysis by inhibiting mitochondrial enzymes and stimulation of glycolytic enzymes and glucose transporter. Simultaneously angiogenesis is fastened because of vascular endothelial growth factor (VEGF) stimulation by HIF.

Moreover angiogenesis does not abate the expression of HIF mainly because blood vessel formation is not well organized within tumor microenvironments and defects in oxygen delivery are involved [23]. Oxygen accessibility is not uniform spatially and temporally which leads to glycolysis upregulation. Since HIF is coupled with oxygen in most cancer microenvironment [24] therefore it could not be the main cause of glycolysis induction in cancers. Other oncogenic activation or turmeric suppression needs to be involved
because recent studies have shown that the key components of the Warburg effect including increased glucose consumption, decreased oxidative phosphorylation, and accompanying lactate production are also characteristic properties of oncogene activation [25].

The signaling molecule Ras [26], Akt kinase [27], and Myc [28] are key oncogenic nodes that execute a reprogramming in metabolism of cancers. Furthermore, regulation of metabolism is not exclusive to oncogenes. Loss of the tumor suppressor such as p53 has shown to affect glycolysis rate within the cancer cells [29].

Targeting glycolysis leads to inhibition of cancer growth which shows that glycolysis is essential for cancer progression [30]. Apart from energy (in the form of ATP) proliferating cells demand also nucleotides, fatty acids, membrane lipids, and proteins, and this reprogrammed metabolism (glycolysis over oxidative phosphorylation) may support synthesis of macromolecules [25].
Figure 1-5. Aerobic and anaerobic glycolysis comparison.
This figure is adapted from [31]. In the presence of oxygen, most differentiated cells primarily metabolize glucose to carbon dioxide by oxidation of glycolytic pyruvate in the mitochondrial tricarboxylic acid (TCA) cycle. Normal cells generate NADH (via these processes) which in turn feeds oxidative phosphorylation to maximize ATP yield, with minimal production of lactate. It is only under anaerobic conditions that normal cells produce considerable amounts of lactate. In contrast, most cancer cells preferably produce enormous quantities of lactate regardless of oxygen presence which in known as aerobic glycolysis.
1.2.5. Glutaminolysis

Glutamine is produced from glutamate (generated by glucose) and ammonia so it is considered the non-essential amino acid for humans. However, in normal tissues glutamine synthetase is not expressed significantly and this makes cells dependent on exogenous glutamine. Glutaminolysis is the catabolic metabolism of glutamine, which produces substrates that support the TCA cycle, produce GSH and supply building blocks for amino acid and nucleotide synthesis. Upon glutamine entrance into cell, glutaminase enzymes convert it to glutamate. Glutamate is transformed directly into α-ketoglutarate, an intermediate in the TCA cycle [32]. Further conversion of α-ketoglutarate into citrate induces fatty acid synthesis under oxygen deprivation. It has been shown that c-MYC oncogene directly regulate genes involved in glutamine metabolism and to induce the expression of the mitochondrial glutaminase protein, which converts glutamine to glutamate [33, 34].

Since amino acids are generated from this cycle, as well as reducing equivalents for ATP synthesis via the electron transport chain, glutamine stimulates the synthesis of non-essential amino acids and provides the cell with energy. Glutamine is used for protein synthesis, synthesis of glucosamine or nucleotide biosynthesis because glutamine is a nitrogen donor [35] in purine and pyrimidine synthesis and thus improves cell growth.

1.2.6. Fatty acid metabolism

Normal and well-differentiated cells derive their free fatty acid supply from blood stream provided by diet. Dietary fatty acids and excessive carbohydrate are stored in liver, adipocytes or as fat droplets within the cytoplasm of cells. The majority of de novo fatty acids are synthesized in the liver, adipose tissue and the lactating breast.

However, because of nutritional deprivation due to limited vascular supply cancer cells rely on de novo fatty acid synthesis for generating substrates for
cellular biosynthesis and β-oxidation in a manner similar to embryonic tissues [36]. Although biological contributions of fatty acid into carcinogenesis are diverse, but their impacts could be classified into growth, energy and redox homeostasis, as well as supporting cancer cells for their distant metastases [37].

De novo fatty acid synthesis when they could be derived from bloodstream shows the fact that lipid synthesis itself is vital for tumorigenic phenotype. High rates of glycolysis converts glucose into lactate and cells need to regulate pH. It could be hypothesized that fatty acid synthesis is another carbon sink that prevents the conversion of more pyruvate into lactate and this pathway equilibrate cellular acidification to some extent [38]. Redox homeostasis is another assumption about the reason of high levels of fatty acid synthesis in cancers. Lipid synthesis produces enough NAD$^+$ (electron acceptor) to support highly active glycolysis in oxygen-deprived condition [39].

1.2.6.1. Fatty acid synthesis

High level of fatty acid synthesis is another hallmark of the altered phenotype of turmeric cells and may result in fat storage or membrane building blocks within the cancer cells. In 1992, high expression of fatty acid synthesis was observed for the first time in breast cancer [40].

1.2.6.2. Normoxia

There are 25 enzymes involved in the process of glucose conversion to fats [41]. Fatty acid synthesis initiates with mitochondrial export of citrate which is produced by glutaminolysis. Mitochondrial glutaminase converts glutamine to glutamate which is then altered into mitochondrial α-ketoglutarate to provide citrate through TCA cycle. A proportion of citrate is transferred into the cytoplasm by the SLC25A1 mitochondrial citrate uniporter [42]. Cytoplasmic citrate is then metabolized into acetyl-CoA and oxaloacetate by ATP-citrate lyase (ACLY), the expression of which is over expressed in cancer cells compared to normal cells and it has been shown that it is an important determinant for tumor growth [43].
Acetyl-CoA carboxylase (ACC) transforms cytoplasmic acetyl-CoA and yields malonyl-CoA, the precursor of saturated long chain fatty acid (LCFA) biosynthesis. Malonyl-CoA is next converted to LCFAs, such as palmitic acid, the most abundant LCFA in cancer cells. Palmitic acid is produced by the enzyme which plays the main role in synthesis of endogenous fatty acids, FASN; FASN is 250-270kDa multifunctional, homodimeric enzyme and it is NADPH dependent [44, 45].

There are two evidences that FASN contributes to the development and maintenance and promotion of malignant phenotype. Inhibition of FASN induces an intensive reduction in cell proliferation and ultimately apoptosis which suggests a role of FASN in metabolism. Moreover, high rate of de novo lipogenesis is linked to the ability of FASN in regulating signaling networks.

In other words the process for fatty acid synthesis from glucose is mainly comprised of four major reactions:

- Synthesis of acetyl-CoA from citrate while ATP citrate lyase is the involved enzyme
- Conversion of malonyl-CoA from acetyl-CoA using acetyl-CoA carboxylase, the primary rate-limiting enzyme.
- All the reactions involved in pentose phosphate pathway to produce nicotinamide adenine dinucleotide phosphate (NADPH), reducing equivalent, for fatty acid synthesis
- The rate limiting step which is the production of 16-carbon saturated free fatty acid palmitate from NADPH-dependent condensation of malonyl-CoA and acetyl-CoA with FASN as an enzyme.

In normal cells, FASN has a minimal role due to adequate levels of dietary fatty acids. Most of well-nourished cells develop newly-structured lipids from synthesized fatty acids. In non-cancerous conditions, FASN upregulation occurs
in embryogenesis and in fetal lung to produce lung surfactant. In highly lipogenic cells such as liver, and adipose tissue, fatty acid synthesis has two major rules:

- Converting excessive nutrition into triacylglycerols
- Storage of excess energy intake

Figure 1-6. Fatty acid synthesis pathway in normoxia and hypoxia.

There are almost twenty five enzymes involved in the metabolism of glucose to fatty acids (FAs). They key elements of the main synthetic pathways and their connections are shown here. Following cellular uptake by glucose transporters, glucose in phosphorylated and converted into pyruvate through glycolysis. Pyruvate is transformed into acetyl-CoA which enters TCA cycle in the mitochondria. Citrate is generated within the TCA cycle and can be driven to cytoplasm where it is converted back to acetyl-CoA (the building blocks for fatty acid synthesis) by ATP-citrate lyase. A portion of acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACC). This step is the rate limiting step and it could be manipulated in order to regulate this pathway. Fatty acid synthase (FASN) mediates the condensation of acetyl-CoA and malonyl-CoA to synthesize 16-carbon saturated fatty acids (long chain fatty acids, LCFAs). NADPH is
essential for fatty acid synthesis and can be acquired by pentose phosphate pathway. Saturated fatty acids can be further modified by elongases and desaturases to form more complex fatty acids (Appendix 1).

1.2.6.3. Fatty acid synthase

FASN is also up-regulated in hormone-sensitive cells. The expression of FASN is linked with estrogen receptor (ER) and progesterone receptor (PR) during menstrual cycle which represents a relation between FASN and E$_2$-ER-dependent signaling in the normal control of endometrial cell proliferation. FASN also provides fatty acid components of milk during lactation. Induction of FASN within the normal mammary glands during pregnancy and lactation involves several hormones such as prolactin, cortisol, insulin and progestin [46].

But in tumors, FASN expression is high irrespective of extracellular lipids. In 1950s, $^{14}$C-glucose labeling was utilized to show that in contrast to normal cells for cancers, most of esterifies fatty acids were originated from de novo and 93% of triacylglycerols were synthesized from endogenous fatty acids. It was inferred that endogenous fatty acids are an important source of fatty acids for growth and energy supply. The mechanism which causes the high expression of FASN within the cancer cells has not been depicted. Two possible mechanisms are discussed in Appendix 2. FASN inhibition has been widely used to impair cancer growth (appendix 3).

1.2.6.4. Hypoxia

The actual tumor microenvironment that tumors experience is hypoxia as a consequence of an increase in tumor volume and lack of vascularization. Low oxygen availability leads to the activation of the hypoxia-inducible factors (HIFs). HIF blocks entrance of pyruvate into TCA cycle by inducing the expression of pyruvate dehydrogenase kinase 1 (PDK1) thereby preventing glucose-derived
lipid synthesis [53]. However, FASN expression in breast cancer has been shown to be elevated in HIF-1 presence [54].

Since HIF-1 constricts the conversion of glucose into fatty acids, other carbon source should compensate the need of cells for fatty acid synthesis. Three papers have been published recently and their focus was to show synthesis of fats from glutamine in hypoxic condition. They also found that isocitrinate dehydrogenase-1 converts cytoplasmic glutamine derived α-ketoglutarate into citrate [53, 55, 56].

1.2.6.5. Fatty acid oxidation

Oxidation of fatty acids is an important source of energy in response to cellular energy depletion. Fatty acid oxidation occurs in both mitochondria and peroxisomes but oxidation in the latter does not produce energy and it is only responsible for breaking very long chain fatty acids for later oxidation in mitochondria. Entry of long chain fatty acids into mitochondria is generally considered to be the rate limiting step in their oxidation while medium- and short-chain fatty acids (12 carbons or less) enter mitochondria freely and they do not require any shuttle. Long chain fatty acids are transported into the mitochondrial matrix via a specific shuttle system named carnitine palmitoyl-transferase 1. The procedure includes four steps.

First, non-esterified LCFAs are activated into LCFA-CoA by long chain acyl-CoA synthase (ACS) which is located in the outer mitochondrial membrane. There are 26 genes encoding ACSs that have discriminatory affinities for their ability to activate short, medium, long, and very long chain fatty acids [57].

Secondly, carnitine palmitoyl transferase-I (CPT-I) transforms LCFA-CoA into long chain fatty acyl-carnitine in the outer mitochondrial membrane. Carnitine (β-hydroxy γ-trimethylaminobutyric acid) plays a vital role in LCFAs transportation [58]. Next, long chain fatty acyl-carnitine is then transported across mitochondrial
inner membrane and translocates to mitochondrial matrix via carnitine: acylcarnitine translocase (CACT).

In normal differentiated cells when ATP level is high enough, elevated levels of malonyl-CoA inhibit CPT-I activity and therefore β-oxidation is halted. While, during fasting when ATP level is low and AMPK is activated, ACC-dependent malonyl-CoA level drops and CPT-I is activated to promote β-oxidation of fatty acids. Later, carnitine palmitoyl transferase-II mediates regeneration of LCFA-CoA from long chain fatty acyl-carnitine. Among the previous steps that mentioned, the first step which is catalyzed by CPT-I is the rate limiting step of fatty acids entry into mitochondria.

Finally, LCFA-CoA is processed to be oxidized via β-oxidation pathway. The β-oxidation pathway composes of series of chain-length-specific enzymes, which consecutively cleave a molecule of Acetyl-CoA in each cycle from the initial fatty acid. The first β-oxidation enzyme, acyl-CoA dehydrogenous (ACD), has three basic isoforms: very long chain ACD, medium chain ACD, and short chain ACD [59, 60]. It has not been proven if the expression of each isoforms relies on the histological type of a cancer or other parameters are involved. Electrons from these reactions are conveyed to the oxidative phosphorylation pathway.

The next sets of reaction are catalyzed by long chain enoyl-CoA hydratase (LHYD) which hydrates long chain fatty acids. The enzymes are included in membrane-associated trifunctional enzyme complex called the mitochondrial trifunctional protein (TFP) [61].

In the last step of mitochondrial fatty acid oxidation reduces NAD⁺ to NADH. Long chain L-3-hyroxyacyl-CoA dehydrogenase (LCHAD) is an integral part of the TFP and is responsible for reduction when medium- and short-chain fatty acids are reduced by medium- and short-chain L-3-hyroxyacyl-CoA dehydrogenase (M, SCHAD). These reactions produces reducing equivalents
such as NADH and FADH$_2$ which can relegate their electrons ($e^{-}$) to respiratory chain and supplies NAD$^{+}$ and FAD required for further oxidation cycle [62].

Figure 1-7. Long chain fatty acid oxidation within the human mitochondria. Non-esterified LCFAs are activated into LCFA-CoA by long chain acyl-CoA synthase (ACS) which is located in the outer mitochondrial membrane. Next, carnitine palmitoyl transferase-I (CPT-I) transforms LCFA-CoA into long chain fatty acyl-carnitine in the outer mitochondrial membrane. Long chain fatty acyl-carnitine is then transported across mitochondrial inner membrane and translocates to mitochondrial matrix. Later, carnitine palmitoyl transferase-II mediates regeneration of LCFA-CoA from long chain fatty acyl-carnitine. Finally, LCFA-CoA is processed to be oxidized via β-oxidation pathway. The β-oxidation pathway composes of series of chain-length-specific enzymes, which consecutively cleave a molecule of Acetyl-CoA in each cycle from the initial fatty acid and feed TCA cycle.
1.2.6.6. Lipogenesis

Storage of excessive fatty acids as triacylglycerol (TAG) is necessary in order to preserve cells from probable cellular damage because of detergent-like effect of FAs [63]. Also it has been suggested TAGs synthesis and degradation generates lipid intermediates such as lysophosphatidic acid (LPA), phosphatidic acid (PA), and diacylglycerol (DAG) which regulates signaling pathways mediated by peroxisome proliferator-activated receptor-γ (PPARγ) [63].

Triacylglycerol synthesis requires fatty acyl-CoA (fatty acids which are activated by acyl-CoA) and glycerol-3-phosphate as substrates. Glycerol-3-phosphate is generated from glycolysis and glyceroneogenesis pathways. Next, triacylglycerol is produced as a consequence of a sequential esterification of the alcoholic functions of the glycerol-3-phosphate by different enzymes called glycerol-3-phosphate acyltransferases (GAPTs), 1-acylglycerol-3-phosphate acyltransferases (AGPATs) and diacylglycerol acyltransferases (DGATs). The entire enzymatic reactions occur within endoplasmic reticulum and mitochondria where all the enzymes are located [63, 64].
Figure 1-8. Triacylglyceride synthesis.
Excessive fatty acids are stored as triacylglycerols (TAGs). TAGs constitute molecules of glycerol to which three fatty acids have been esterified. Triacylglycerols synthesis requires fatty acyl-CoA (fatty acids which are activated by acyl-CoA) and glycerol-3-phosphate as substrates. Glycerol-3-phosphate is generated from glycolysis and glyceroneogenesis pathways. Glycerol-3-phosphate acyltransferase (GPAT) then esterifies a fatty acid to glycerol-3-phosphate generating the monoacylglycerol phosphate structure called lysophosphatidic acid (LPA). Two molecules of acyl-CoA are esterified to glycerol-3-phosphate to yield 1, 2-diacylglycerol phosphate (commonly identified as phosphatidic acid, PA) by 1-acylglycerol-3-phosphate acyltransferases (AGPATs). The phosphate is then removed, by phosphatidic acid phosphatases-1 (PAP1), to yield diacylglycerol (DAG), the substrate for addition of the third fatty
acid. Intestinal monoacylglycerol (MAG), derived from the hydrolysis of dietary fats, can also serve as substrates for the synthesis of diacylglycerols by activation on monoacylglycerol transferase (MAGT). The final step includes another addition of fatty acids to diacylglycerol to generate triacylglycerol through diacylglycerol transferase (DAGT). The entire enzymatic reactions occur within endoplasmic reticulum and mitochondria where all the enzymes are located.

1.2.6.7. Lipolysis

During fasting state endocrine-mediated utilization of stored fatty acids occurs. Stored fats are mostly triglycerols (TAGs) which are broken down in a stepwise reaction into diacylglycerol (DAG) and monoacylglycerol (MAG) in order to release three moles if free fatty acids and one mole of glycerol per mole of completely hydrolyzed TAG. Complete hydrolysis of TAG almost never happens in fat cells and always there is accumulation of the partial acyglycerol or lipolysis initiates from DAG or MAG [64]. More information about lipid classification can be found in Appendix 4.

The rate limiting enzyme in hydrolysis of TAG is hormone-sensitive lipase (HSL), and as comes from its name it is sensitive to hormones such as insulin and catecholamines [65]. Regulation of HSL occurs through its phosphorylation and also via lipolytic cascade.
Figure 1-9. Lipolysis pathway in human cells.

Stored fats are mostly triglycerols (TAGs) which are broken down during fasting state by catecholamine induction in a stepwise reaction into diacylglycerol (DAG) and monoacylglycerol (MAG) in order to release three moles if free fatty acids and one mole of glycerol per mole of completely hydrolyzed TAG. During fasting, catecholamines by binding to Gs-coupled β-adrenergic receptors (β-AR) activate adenylate cyclase (AC) to increase cAMP and thus activate protein kinase A (PKA). PKA phosphorylates HSL, resulting in translocation of HSL from the cytosol to the lipid droplet (LD) and increased lipolysis. The rate limiting enzyme in hydrolysis of TAG is hormone-sensitive lipase (HSL), and as comes from its name it is sensitive to hormones such as insulin and catecholamines. PKA also phosphorylates the lipid droplet associated protein perilipin. In the fed state, insulin binding to the insulin receptor (IR) results in decreased cAMP levels and decreased lipolysis.
1.3. Cancer-stroma interaction

1.3.1. Cancer and wound healing

Cancer-stroma interaction initiates with the recruitment of activated components of stromal cells such as myofibroblasts. Olumi et al. elucidated that stromal cells become activated after co-culture with prostate cancer cells *in vivo* and *in vitro* [72]. Chemokines, enzymes, and growth factors that are secreted by stromal cell types influence reprogramming and progression of cancer. This procedure resembles contribution of stromal cells in wound healing. Stromal cells defend the damaged organism by eliminating the offending agents. There are two notions about the stromal-cancer interaction: First, it is believed that stromal cells have defense mechanism toward the cancerous lesion and the second one which is more common is that cancer cells reprogram stromal cells to assist their abnormal growth.

The reality occurs between these two theories and probably in the beginning stromal cells attempt to defend against carcinogenesis but cancer cells modify them to improve their own survival. Prostate cancer-derived condition media affects the normal stromal cells [73]. The manipulated fibroblasts (after incubating with cancer derived-condition medium) shows higher keratinocyte growth factor (KGF), tumor necrosis factor-α (TNF-α), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) which in turn support cancer growth.

1.3.2. Stromal cells

Stromal cells or mesenchymal stem cells are adherent clonogenic cells that shape colonies. Stromal cells are mostly isolated from bone marrow but they are also involved in umbilical cord blood, adipose tissues and amniotic fluids. These cells are easily-cultured in vitro and are able to be differentiated to oesoblast, chondrocytes, or adipocytes if stimulated felicitously.
The stroma composes of fibroblasts or myofibroblasts, glial, epithelial, fat, immune, vascular, smooth muscle, and extracellular matrix (ECM). None of these cells types are malignant themselves but they acquire abnormal phenotype after interacting together and with cancer cells. Turmeric cells and stromal cells interact and evolve each other and it has not been shown which one in initiator of carcinogenesis. The mutual exchange of nutrients, secreted growth factors, and other proteins between stromal cells and cancer cells develops permanent alterations in cell function.

1.3.3. Fibroblasts

Growth factors and chemokines produced by fibroblasts and immune cells are manipulated by abnormal proliferator cancer cells although in normal cells fibroblasts are main producers of extracellular matrix (ECM). Fibroblasts provide the cells with the deposition of the fibrillar ECM such as type I, type III, and type V collagen and fibronectin and participate in formation of the basement membrane by secreting type IV collagen and laminin. Cellular environment ECM is dynamic and is reassembling through metalloproteinases (MMPs).

Fibroblasts supply a scaffold during wound healing and secrete levels of ECM proteins, growth factors, and chemotactic factors and crosstalk dynamically with the injured epithelium. They respond to factors secreted directly from the injured tissue such as fibroblast growth factor2 (FGF2), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor-β (TGF-β) [74]. Fibroblasts in tumor are similar to the kinds in wound healing and they are characterized by their α-smooth muscle actin (α-SMA) expression.

Ubaldo et al. found that loss of Cav-1(family of scaffolding proteins) in breast stromal cells is a single independent predictor of breast cancer patients [75]. Furthermore, Cav-1 loss in prostate stromal cells indicates patients in advanced stages with metastasis [76]. Ubaldo and his colleagues believed that loss of Cav-1 (inhibitor of TGF-β) upregulates the production of myofibroblast markers such as vimentin, calponin, and collagen. And also it stimulates
expression of glycolytic enzymes (LDHA and PKM2) and antioxidant markers (catalase and peroxiredoxin). Deficit of Cav-1 in stromal cells induces reactive oxygen species (ROS) and oxidative stress which leads to activation of certain key transcription factors (HIF-α and NF-κB) that elevates aerobic glycolysis in stromal cells. ROS transforms cancer cells more aggressive and apoptosis resistant; ROS upregulates the antiapoptotic protein TIGAR (TP53-induced glycolysis and apoptosis regulator) [77]. Other tumor microenvironment components are discussed in Appendix 5.

1.4. Adipose tissue

Adipose tissue has been widely studied for the past fifteen years because of their role in energy homeostasis in body. They secrete diverse metabolite and endocrines. Adipocytes are known to control the body mass after the discovery of leptin in 1994 [83]. Adipocytes are the main sites of imprecise-esterified fatty acids storage also their fast release makes them unique. Occurrence of global obesity is the other reason that adipose tissue caught numerous re-attentions recently. Adipocytes are classified into two main groups, white and brown fat.

Even among white adipocyte, cells from different locations are distinguished by their molecular and physiological properties. For instance, induced visceral adipose tissues are characterized with elevated risk of insulin resistance and cardiovascular disease while subcutaneous adipocytes are not. Visceral adipocytes are responsive to lipolytic stimuli though subcutaneous adipocytes are not. White fats compose the majority of fatty tissue in grownups while brown fats are present in newborn stages to provide body heat. White adipose tissue store fat in the form of esterified fatty acids, triglycerides, and release energy by free fatty acids back during starvation.

Adipocytes are derived from multipotent mesenchymal stem cells. Adipocytes are characterized and regulated by the sequential changes in the expression of special genes which define adipotic phenotypes. This is
determined by the expression of various early, intermediate, and late mRNA/protein markers and also triglyceride accumulation.

The first step in their differentiation is to arrest the growth by contact inhibition. And then treatment of the cells with appropriate combination of mitogenic and adipogenic signals results the conversion of cells into mature adipocytes that stored bulks of fats. During differentiation, distinct alterations occur in cell morphology, cytoskeletal components, and the amount and types of extracellular matrix. There are varieties of protocols for adipocyte differentiation. Dexamethasone is used to promote the glucocorticoide receptor pathway, and 3-isobutyl-1-methylxantine (IBMX) is implemented to stimulate the cAMP-dependent protein kinase pathway. Usually high concentrations of insulin are adapted in protocols. Adipogenic transcriptional cascade is more discussed in Appendix 6.

1.5. Cellular Role of Nitric Oxide

Before 1980s, Nitric oxide (NO) was merely an industrial pollutant and considered to be environmentally hazardous since NO reaction with atmospheric ozone depletes the protection of ozone layer. NO also reacts with atmospheric water and generates acidic precipitations. However, in 1992 nitric oxide became the molecule of the year and extensive research has been centering on the role of NO biological events. Even the Nobel Prize in Physiology and Medicine was awarded to three US scientists for the identification of NO as a signaling molecular in the cardiology[88]. There has been growing interests on investigating the role of NO in immune system, neurotransmission, vasodilation, cosmetics, cardiology and cancer progression. NO has been implicated in cancer biology and tumor progression for many years since then. Nitric oxide (NO) is a pleiotropic molecule and plays various physiological and pathophysiological roles in tumor progression. In the past decade, major advances in the investigation of NO biology have been witnessed. NO is an important physiological mediator in a
large number of biological actions including vascular tone, blood pressure, neurotransmission, immune response, and oxidation-sensitive mechanisms.

NO is a highly unstable molecule with one to five second half-life. NO is synthesized endogenously when one molecular of arginine and two molecules of oxygen are converted to one molecule of NO, one molecule of citrulline, and two molecules of water. The reaction occurs by the act of the enzyme nitric oxide synthase which is nicotinamide-adenine-dinucleotide phosphate (NADPH) dependent.

Figure 1-10. Cellular nitric oxide generation pathway

Synthesis of NO from arginine includes two separate steps [89]: arginine is hydroxylated to N-hydroxy-arginine (NHA), and then conversion of NHA to citrulline with a release of NO.

Figure 1-11. Two-step conversion of L-arginine to L-citrulline. L-arginine is first converted to a stable intermediate N-hydroxyarginine (NHA), which is subsequently converted to L-citrulline and NO. In both conversions, oxygen and the reducing power molecule NADPH are the co-substrates.

Currently, three isoforms of nitric oxide has been identified in mammals: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) that share roughly half of the sequence homology. Both eNOS and nNOS are known to be constitutive although iNOS can be inducible. eNOS role in cardiovascular system
has been extensively studied and has been shown to be essential for vasodilation and the maintenance of a healthy cardiomyocyte contractility. Chemical inhibition or ablation of eNOS induces vasoconstriction, hypertension, and chronical aneurism. eNOS is accountable for modulation of vascular-endothelial growth factor and angiogenesis as a result. nNOS is expressed not only in nervous system but also in some other cell types including muscles [90]. It is involved in many cellular functions such as synaptic plasticity in the central nervous system (CNS), central regulation of blood pressure, smooth muscle relaxation, and vasodilatation via peripheral nitrergic nerves. iNOS expression has been shown to be differentially regulated among different cell types, including endothelial and epithelial cells and variety of human cancer cells [91]. It can be expressed in many cell types in response to lipopolysaccharide, cytokines, or other agents for prolonged periods of time [92].

Deregulated NO production contributes to pathophysiological conditions including cancer. Expression of NOS has been differentially detected in obese and non-obese individuals [93, 94] as well as various cancers such cervical, breast, CNS, head, neck, pancreatic, and ovarian cancers. It has been shown that high levels of iNOS activity exist in malignant tissue from gynecologic cancers [95] and higher NOS expressions were correlated to the more advanced stages of breast and prostate cancers [96, 97]. Human colon adenocarcinoma showed to grow much more when injected to mice with iNOS transfection [98]. Similar results have been depicted with chemical inhibition of NOS [99]. The induction of tumor progression was detected to be because of 5-fold higher blood vessel volume compared to wild type mice. Lung tumorigenesis developed in iNOS knock out mice was significantly reduced compared to their wild type counterparts [100]. Chemical inhibition of NOS considerably decreases mammary cancer invasiveness and angiogenesis in mouse model [101, 102].

NO has to translocate from NOS site to apply cellular changes. It can either diffuse/transport or be stored as more stable NO-derived species for further actions since NO life time is too short to exert cellular modifications in
aerobic, biological milieu. S-nitrosothiols, metal-nitrosyl, nitrite [103], and nitrates are several NO reserves [104]. Irrespective of the source, NO utilize biomolecules to exert its effects; reactive NO reacts with a wide variety of redox-active species.

1.5.1. S-nitrosation

S-nitrosation is a covalent post-translational modification of cysteine residue that occurs in proteins in virtually all biological systems. Similar to other posttranslational modifications, such as phosphorylation, S-nitrosation modulates the biological activities of proteins and it is specifically involved in critical processes of the cellular lifecycle that include transcription regulation, DNA repair, and apoptosis. Deregulated s-nitrosation has been distinguished in many pathophysiological events. It has been reported to be involved both directly and indirectly in proliferation of cancer cells[105]. A number of studies have illustrated that abnormal s-nitrosation is implicated in cancer development and progression, as well as response to some therapeutics. Accumulating evidence suggests that deregulated s-nitrosation is a key event in cancer initiation that may considerably increase cancer risk [106-108].

Cysteine (Cys) residue plays a unique role in the function of proteins because of its thiol chain. This functional group is nucleophilic, acidic and redox active due to the existence of hybridized p- and d-orbitals. Numerous reactions are known to take place at thiol side of cysteine residue of the protein and as a result may alter the protein’s structure, activity, and function [109].

When a thiol group of cysteine binds with a nitric oxide an s-nitrosothiol is generated and the reaction is reversible. The abduction of a nitroso moiety and a cysteine thiolate occurs by several reactions:

- Acidic conditions (very low pH<3) enforce the formation of s-nitrosothiol from a cysteine thiol and nitrite (NO\textsuperscript{2-}) although it is not happening physiologically with the exception of stomach.
Another alternative pathway to generate s-nitrosothiol is the reaction between nitric oxide and a cysteine thiol when the redox requisites exist.

![Thio (-2) and S-nitrosothiol (0)](image)

Figure 1-12. Depiction of thiol and s-nitrosothiol functional groups

![Basic mechanism of s-nitrosation](image)

Figure 1-13. Basic mechanism of s-nitrosation. Nitric oxide experience a redox reaction generating s-nitrosothiols.

Protein s-nitrosation has been known as a principal mechanism by which nitric oxide apply biological changes in cells.

1.5.2. Nitric oxide in tumor progression

Since 1987, interest has grown in the investigation of the cellular role of NO. It has been shown that NO plays crucial roles in the immune system, neurotransmission, vasodilation, cosmetics, cardiology and cancer progression.

Deregulated NO production contributes to pathophysiological conditions including obesity and cancer [110, 111]. iNOS is the most common isoform expressed in tumors while nNOS and eNOS are detected in some patients [106].
Expression of NOS has been differentially detected in obese and non-obese individuals [93, 94] as well as various cancers such as cervical, breast, CNS, head, neck, pancreatic, and ovarian cancers. It has been shown that high levels of iNOS activity exist in malignant tissue from gynecologic cancers [95] and higher NOS expressions were correlated to the more advanced stages of breast and prostate cancers [96, 97]. Human colon adenocarcinoma is discovered to progress much more when injected to mice with iNOS transfection [98]. The induction of tumor progression was detected to be because of 5-fold increase in blood vessel volume compared to wild type mice. Lung tumorigenesis developed in iNOS knock out mice was notably reduced compared to their wild type counterparts [100]. Chemical inhibition of NOS considerably decreases mammary cancer invasiveness and angiogenesis in mouse model [101, 102].

NO is involved in tumorigenesis as well as tumor progression. NO provokes neoplastic transformation by inducing chronic inflammation. Moreover, NO and NO-derived reactive species elevate oxidative and nitrosative stress and cause further DNA damage. NO modulates s-nitrosylation of the caspase family, promotes resistant to cellular apoptotic reactions, and develops more mutations and clonal selection. iNOS has been suggested to initiate and progress tumors. Mice with silenced iNOS had reduced tumor formation compared to animals with adequate expression of iNOS [112]. Despite that, it has been reported that NO has antitumor effects and can be cytotoxic. The expression level and duration of NO exposure are major determinants of NO-regulated cellular outcome. High levels of NO (more than 300nM) induces apoptosis in tumors while low (less than 200nM) or mild, and chronic doses promote carcinogenesis [111]. As a result, the range of NO levels is an important factor in studying cellular role of NO and its therapeutic intervention [113, 114].

1.5.3. Hypoxia-induced nitrite conversion to NO

Tumors grow so rapidly that they do not allow proper vasculature to form and leaves portions of the tumor without the ample access to oxygen. Hypoxia-
inducible factor 1 (HIF-1) is the master transcriptional regulator of cellular and developmental response to hypoxia. Many genes are identified as HIF-1 target including the ones involved in angiogenesis, metabolic adaptation, apoptosis, invasion, and metastasis. Hypoxia is a characteristic feature of most solid tumors. Hypoxia induces tumor chemoresistance in proportion to distance from the vasculature. Tumors reprogram their metabolism to survive the harsh and challenging hypoxic environment [115]. HIF-1 inhibitors are being investigated as anticancer drugs while hypoxic cells are exposed to less chemo drugs. Shedding light on the regulation of HIF-1 is greatly demanded, since it affects the unfavorable outcome of cancer chemotherapy.

Exogenously added NO has been shown to increase the expression of HIF-1 in cancer cells [116, 117] by s-nitrosylation of a single cysteine residue of HIF-1 protein. Inhibition of iNOS with 1400W significantly sensitize cancer cells to radiotherapy because of the ablation of HIF-1. Identification of NO's role in HIF-1 upregulation proposes a promising combinatory drug therapy which implements NOS inhibitor simultaneously with conventional radiation or chemotherapy to reach the synergistic successful outcome [116].

NOS-independent generation of NO was first reported by two separate studies in 1994 [118]. Conversion of salivary-derived nitrite was the source of NO because of gastric acidity, and NO generation was greatly enhanced after intake of dietary nitrate. Later, NO generation from nitrite was shown in the ischemic heart, extending the occurrence of nitrite reduction outside the gastrointestinal tract [119]. Despite the traditional NO generation from arginine, the nitrate-nitrite-NO pathway is greatly enhanced by hypoxia and acidosis and may serve as a backup system to ensure NO generation during hypoxic conditions [104]. As a result, NO reserves, nitrite and nitrate, are involved in the regulation of blood flow, cell metabolism, and signaling, as well as tissue protection during hypoxia similar to that if NO. There are several proposed pathways by which nitrite and nitrate are converted to NO in hypoxia. Deoxygenated myoglobin (deoxy-Mb), xanthine oxidoreductase (XOR), mitochondrial complexes and even NOS are
known to donate electrons to nitrite and form NO [104, 120]. Nitrite- and nitrate-derived NO modifies the functions of proteins by s-nitrosylation of their cysteine residues. Numerous s-nitrosylated proteins have been identified to play a role in proliferation, apoptosis, angiogenesis, cell cycle modification, tissue invasion, metastasis, and the response of cancers to treatments [105].

Tumor protein p53 is a tumor suppressor and impacts the cancer responses to DNA damage and abnormalities. Inactivation or mutation of p53 leads to uncontrolled cell proliferation and persistent DNA damage, being correlated with various tumors. S-nitrosylation of p53 has shown to impair the normal response of cells to DNA damages [121].

Fas ligand (FasL or CD95L) is a type-II transmembrane protein which belongs to the tumor necrosis factor (TNF) family. FasL is involved in the induction of cell apoptosis when it binds with its receptor. S-nitrosylation of FasL induces its capacity to target unwanted cancer cell growth by promoting FasL-mediated apoptosis [122].

B-cell lymphoma-2 (Bcl-2) downregulates mitochondria-mediated apoptosis pathway (reduction of Cytochrome C) and regulates cell survival. In most cancer cells, Bcl-2 has shown to be upregulated and to cause chemo resistance. S-nitrosylation of Bcl-2 enhances the protein stabilization and increases the survival of tumor cells. Inhibition of s-nitrosylation has been indicated to increase the chemo sensitivity of melanoma cells by targeting Bcl-2 activity [123].

Phosphatase and tensin homolog (PTEN) mutation is commonly detected in tumors from different origins. It acts a tumor suppressor and is fed primarily by phosphoinositide 3-kinase (PI3K) which has been shown to play a role in the angiogenesis of tumors. Enhancement of PI3K leads to significant elevation of tumor vasculature and results tumor growth. PTEN inactivates PI3K and has been utilized in cancer treatment mostly as an angiogenesis inhibitor [124]. S-nitrosylated PTEN does not inhibit PI3K and the involved angiogenic activities.
Figure 1-14. Cellular nitric oxide (NO) synthesis.
In normoxia, nitric oxide is synthesized by the action of enzyme NOS (nitric oxide synthase). NOS transforms L-arginine to L-citrulline and NO. L-citrulline can be recovered to L-arginine through the argininosuccinate synthetase (ASS) and Argininosuccinate lyase (ASL). Generated NO with the presence of ample oxygen is later oxidized to nitrite and nitrate, respectively. They are reserved sources of NO. In hypoxia, since there is not enough oxygen for NOS activity, cellular nitrite and nitrate are reduced to form NO. The reactions occur at various sites such as mitochondrial electron transport chain complexes, Deoxygenated myoglobin (deoxy-Mb), xanthine oxidoreductase (XOR), and even NOS.
A missing link between omentum and ovarian cancers’ metabolic coupling: nitric oxide homeostasis

Omental adipose stromal cells (O-ASCs) are multipotent population of mesenchymal stem cells contained in the omentum tissue which promote endometrial and ovarian tumor proliferation, migration and drug resistance [126, 127]. The mechanistic underpinnings of O-ASCs role in tumor progression and growth are unclear. Here, we propose a novel nitric oxide (NO) mediated metabolic coupling between O-ASCs and gynecological cancer cells in which O-ASCs support NO homeostasis in malignant cells. NO is synthesized endogenously by the conversion of L-arginine into citrulline through nitric oxide synthase (NOS). Through arginine depletion in the media using L-arginase and NOS inhibition in cancer cells using L-NAME, we demonstrate that patient derived O-ASCs increase NO levels in ovarian and endometrial cancer cells and promote proliferation in these cells. O-ASCs and cancer cell cocultures revealed that cancer cells utilize O-ASCs-secreted arginine and in turn secrete citrulline in the microenvironment. Interestingly, citrulline increased adipogenesis potential of

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the O-ASCs. Furthermore, we found that O-ASCs increased NO synthesis in cancer cells which led to decrease in mitochondrial respiration in these cells. Our findings suggest that O-ASCs upregulate glycolysis and reduce oxidative stress in cancer cells by increasing NO levels through paracrine metabolite secretion. Significantly, we found that O-ASC mediated chemoresistance in cancer cells can be deregulated by altering NO homeostasis. A combined approach of targeting secreted arginine through L-arginase, along with targeting microenvironment secreted factors induced increased NO synthesis in cancer cells using L-NAME, may be a viable therapeutic approach for targeting ovarian and endometrial cancers.

Figure 2-1. Nitric oxide in tumor microenvironment.
Nitric oxide synthase (NOS) expression increases when cells become cancerous. Overall cellular microenvironment changes and activated macrophages generate higher nitric oxide (NO) which in turn induces angiogenesis and chemoresistance. Tumor microenvironment promotes NO synthesis within cancer cells which modifies cell cycle as well as cellular metabolism.
2.1. O-ASCs induce nitric oxide synthesis of OVCA and ECAs

We hypothesized that “NO homeostasis” is a key player in regulating reciprocal communication between O-ASCs and cancer cells. We tested NO levels in coculture media supernatants of OASCs and OVCA cells (Figure 2-2 A) as compared to tumor cells cultured alone. Significantly higher NO was detected in cocultures. To further confirm the increased NO levels in cancer cells we measured NO in cancer cell homogenates. Indeed, NO levels were higher in cell homogenates of cancer cells which were in cocultures compared to cancer cells cultured alone (Figure 2-2 A). Interestingly, control cell line (IMR-90, human fibroblasts) was unable to increase NO synthesis in cocultures (Figure 2-2 B). These results suggest that O-ASCs selectively increase NO synthesis in cancer cells. Further, O-ASCs increased expression of iNOS in HEC-1-A cells in transwell cocultures (Figure 2-2 C). Cancer cells in turn increased NO synthesis in O-ASCs; O-ASCs synthesized higher NO when transwell-cocultured with OVCA and ECA cells (Figure 2-2 D).

To confirm that NO synthesis in these cells is by conversion of arginine into citrulline through NOS, we measured NO levels in presence of NOS inhibitor N^G^-nitro-L-arginine methyl ester (L-NAME) (Figure 2-2 E). As seen in Figure 2-2 E, L-NAME reduced the NO production in a dose-dependent manner in cancer cells. The hydrolysis of L-NAME results in L-NNA, a fully functional inhibitor of i-NOS. L-NAME action on i-NOS was further confirmed using L-NNA, an active form of the i-NOS inhibitor. Further, D-NAME, an inactive enantiomer of L-NAME which served as negative control was ineffective in reducing the NO production in cancer cells. To ascertain if arginine is a major source for NO synthesis in these cells, we measured NO synthesis under arginine depletion conditions with and without L-NAME. As see in Figure 2-2 F, under arginine deprived conditions NO synthesis is drastically reduced, thus indicating that other nutrients contribution towards NO synthesis is negligible. Moreover, when L-NAME is added to inhibit
NO production through endogenously synthesized arginine, the decrease of NO levels were not significant. To confirm whether arginine levels in cancer cells were regulated through arginase, an enzyme which converts arginine into ornithine and urea, we measured urea secretion in these cells. Our results show that cancer cells have negligible arginase activity as measured through urea secreted in the medium (Figure 2-9 A). Further, gene expression analysis using the Oncomine database [128] showed that arginase-1 (ARG1) expression in ovarian and endometrial cancers were similar to normal ovarian epithelium (Figure 2-9 B). The urea secretion data were in line with TCGA data which didn’t show any upregulation of arginase gene expression (ARG1) in cancer cells.

We next sought to determine if NO regulates cancer proliferation. We used S-nitroso-N-acetyl-DL-penicillamine (SNAP), a NO donor, with varying concentrations to investigate NO’s effect on cancer cell growth under complete media conditions. Our results illustrate that SNAP plays a bimodal role in the growth of OVCAs and ECAs. At low concentrations of SNAP (less than 0.1μM) increased growth of cancer cells (Figure 2-2 G), whereas, higher concentrations of SNAP (greater than 1μM) have cytotoxic effects. To confirm NO’s role in increasing proliferation, we used low to high concentrations of L-NAME. As seen in Figure 2-2 H, L-NAME and L-NNA had similar behavior at low concentrations (< 1mM) where they marginally reduced viability. On the contrary, both L-NNA and L-NAME significantly reduced viability at 10mM concentration compared to D-NAME control.
Figure 2-2. O-ASCs induce NO synthesis of OVCAs and ECAs.
(A) OVCAR429 cells were transwell cocultured with O-ASC 35 for three days. The media was replaced with fresh RPMI media, 3 hours before sample collection. NO content was assessed in the samples (cell homogenate and cell supernatant) using Sievers NO analyzer. Protein content of samples was utilized for normalization of data. (B) Cancer cells were transwell cocultured with either O-ASC 10 or IMR-90 before NO content was measured in cancer cell homogenate samples. (C) iNOS mRNA expression of HEC-1-A was measured after 4 days of transwell coculture with O-ASC 34. (D) NO content in O-ASC 10 were measured upon its transwell coculture with OVCAR429 and HEC-1-A for three days. O-ASC 10 cell homogenate was used for NO analysis. (E) Cancer cells were treated with NOS inhibitors for 48 hours prior to NO measurements in cell homogenates. (F) OVCAR429 and HEC-1-A were cultured with either complete RPMI or arginine free media for 48 hours. L-NAME (10mM) was added to inhibit endogenous NO synthesis. (G) Cancer cells were treated with varying concentrations of SNAP and viability of cells was measured. (H) Cells were treated with NO inhibitors for 48 hours before their viability was measured. Data are expressed as means ± SE; n>6. *P<0.05, §P<0.01, and #P<0.001. T-test was used for single comparisons. Multiple comparisons versus a control group were analyzed by Dunnett’s method. All pairwise multiple comparisons were analyzed by Bonferroni test to compare lean and overweight patients’ samples.

2.2. O-ASCs positively regulate OVCAs and ECAs growth through arginine

To understand OASCs’ role in regulating cancer cells growth, we first cultured OVCAs and ECAs cells with and without O-ASCs cocultures for 5 days (Figure 2-3 A). O-ASCs increased the proliferation of both endometrial (HEC-1-A) and ovarian (OV429) cancer cell lines. Next, to elucidate a precise role for the NO pathway in O-ASCs induced tumor pathogenesis, we cocultured OVCA and ECA cells under complete media and arginine deprived conditions (Figure 2-3 B). Cells cultured under arginine deprived conditions will have reduced NO
synthesis. We found that OVCA and ECA cancer cells are arginine dependent and hence had reduced proliferation. Significantly, transwell cocultures of both overweight and lean O-ASCs with HEC-1-A and OV429 cells rescued the proliferation rate in these cells under arginine deprivation conditions. Interestingly, the rescue of proliferation was higher when cancer cells were cocultured with overweight O-ASCs compared to their lean counterparts. Similar results were obtained when cancer cells were cultured in conditioned media obtained from lean and overweight O-ASCs. As seen in the figure, the rescue effect with conditioned media for both lean and overweight O-ASCs is less pronounced compared to rescue of proliferation obtained with transwell cocultures. We further confirmed the effect of O-ASCs in rescuing proliferation under arginine deprivation conditions under direct contact cocultures (Figure 2-3 C). Interestingly, control cell line was ineffective in rescuing the reduced proliferation in cancer cells under arginine deprivation conditions. In line with these findings, IMR-90 was found to be arginine dependent for proliferation. This is in contrast to complete media conditions where IMR-90 cells did increase proliferation of cancer cells.

To establish if the O-ASCs mediated rescue under arginine deprivation is through the secreted arginine, we measured arginine using ultra performance liquid chromatography (UPLC) in spent media of transwell cocultures of O-ASCs and cancer cells under arginine deprivation conditions (Figure 2-3 D). As seen in the figure, both lean and overweight O-ASCs had significant arginine secretion in coculture with both ovarian and endometrial cancer cells, while tumor cells alone did not secrete arginine. Interestingly, cancer cells exhibited reciprocal effect by increasing arginine synthesis from O-ASCs (Figure 2-3 E). As seen in the figure, arginine secretion from O-ASCs is higher when they were cocultured with cancer cells.
Figure 2-3. O-ASCs positively regulate OVCAs and ECAs growth through arginine.

(A) O-ASCs and stably luciferase transfected OVCA and ECA cells were in contact cocultures in 96-well plate. Luciferin (150μg/mL) was added and luminescence was assessed to quantify viable cells for 5 days. The media was changed to RPMI after each measurement for further viability assessment. (B) OVCAR429 and HEC-1-A cells were seeded in 6-well plates, while O-ASCs were seeded on top of transwell inserts. The media was changed to RPMI without arginine during three days of indirect coculture. Cancer cells viability was measured and was reported in million units. Cancer cells without cocultures are shown in white (medium with arginine) and black (medium without arginine) bars. Cocultured cancer cells (both OVCAR42 and HEC-1-A) are labeled according to the O-ASCs they were cocultured. Cancer cells without coculture and without arginine were used as the control. (C) Cancer cells were cocultured in direct contact either with O-ASCs or IMR-90 for three days before their viability was measured. The coculture media was complete RPMI or RPMI without arginine. Cancer cells without coculture were used as the control. (D) illustrate arginine contents of cancer cells and O-ASCs in mono and coculture, respectively. OVCAR429 and HEC-1-A were transwell cocultured with O-ASCs for three days in arginine free media. Fresh media was replaced 24 hours before sample collection. Arginine contents of collected samples were analyzed by ultra-performance liquid chromatography (UPLC). (E) O-ASCs from transwell coculture were separated and incubated with arginine free media for 24 hours prior to the sample collection. Arginine contents of the spent media were measured by UPLC. Data are expressed as means ± SE; n>6. *P<0.05, §P<0.01, and #P<0.001. T-test was used for single comparisons. Multiple comparisons versus a control group were analyzed by Dunnett's method. All pairwise multiple comparisons were analyzed by Bonferroni test to compare lean and overweight patients’ samples.
2.3. Inhibition of endogenous nitric oxide synthesis abrogates elevated viability of cancer cells induced by O-ASCs

The above experiments demonstrated that O-ASC coculture rescued the growth inhibitory effect of arginine depletion. We further investigated whether O-ASCs secrete arginine, an essential metabolite for NO synthesis, or they secrete factors which upregulate NO synthesis in cancer cells. To assess if O-ASCs secrete arginine under arginine deprivation conditions, we added L-arginase, an enzyme which converts arginine to ornithine and urea, in direct cocultures of O-ASCs and cancer cells seeded in a ratio of 3:1. We first evaluated the efficacy of arginase in depleting arginine in the medium (Figure 2-10). As seen in the figure, arginine levels decreased with increasing arginase concentration. The L-arginase treatment depletes secreted arginine and thereby disrupts the rescue effect of O-ASCs. Indeed, adding L-arginase (10U/mL) disrupted the rescue potential of O-ASCs, thus suggesting that O-ASCs secreted arginine is the possible cause behind the rescue potential of O-ASCs (Figure 2-4 A-B).

We added L-NAME in contact cocultures and monocultures under arginine deprivation conditions to determine if NO signaling is required for the O-ASC effect on cancer cell proliferation (Figure 2-4 C-D). Consistent with L-arginase results, the addition of L-NAME decreased the rescue effect of O-ASCs on cancer cells’ proliferation in cocultures, thereby suggesting that O-ASCs effects on cancer cell proliferation are mediated by NO signaling. Interestingly, overweight O-ASCs had stronger rescue effect in cocultures for both ovarian and endometrial cancer cells. To determine if the O-ASCs effect on cancer cell proliferation is mediated by secreted factors, we added L-NAME to transwell cocultures (Figure 2-4 E-F). In agreement with L-arginase results, L-NAME addition significantly reduced proliferation in cocultures. To further demonstrate, the direct involvement of NO pathway in rescue of cancer cells growth, we added S-nitroso-N-acetyl-DL-penicillamine (SNAP, 100nM), a NO donor, under arginine deprivation and NOS inhibition conditions (using L-NAME) (Figure 2-4 G).
Remarkably, SNAP rescued the reduced proliferation of cancer cells under both conditions and the rescue effect was similar to O-ASCs induced rescue of cancer cell growth under L-NAME and arginine deprivation conditions.

Figure 2-4. Inhibition of nitric oxide synthesis abrogates elevated viability of cancer cells induced by O-ASCs.

(A and B) Stably luciferase transfected OVCA and ECA cells (OVCAR429 and HEC-1-A) were cocultured in direct contact with O-ASCs for three days (seeding ratio of 1:3). L-arginase (10U/ml) was added to deplete arginine from media. Luciferin (150μg/mL) was added to cells and luminescence was assessed to
determine viability of cancer cells. Cancer cells without coculture and without L-arginase treatment were used as a control. (C and D) L-NAME (20mM) was added to inhibit nitric oxide synthesis by blocking NOS. Cancer cells and O-ASCs were cocultured in direct contact for 72 hours with L-NAME. All the conditions were compared to cancer cells without coculture and without L-NAME treatment. (E and F) Cancer cells were seeded in 6-well plates while O-ASCs were on top of transwell inserts (The ratio was 1:2, respectively). The media was changed to RPMI without arginine, with and without L-NAME (20mM), during three days of indirect coculture. OVCAR429 and HEC-1-A without coculture and without L-NAME treatment were used as control. (G) SNAP (100nM) and L-NAME (10mM) were simultaneously added to cancer cells for 48 hours, and the results were compared to the viability of cells treated with L-NAME alone. Moreover, arginine was excluded from the cancer media, and the results were compared to arginine free media containing SNAP (100nM). Data are expressed as means ± SE; n>9. *P<0.05, §P<0.01, and #P<0.001. Dunnett’s method was implemented to compare multiple groups versus a control group. Comparisons of lean and obese results were analyzed by Bonferroni test.

2.4. Citrulline induces adipogenesis of O-ASCs

Cells with high NOS activity convert arginine into citrulline and release NO. In our previous study, we found that OVCA cells secreted citrulline, suggesting significant levels of NOS activity. Thus, we measured citrulline in spent media from transwell cocultures. Interestingly, we found high levels of citrulline in cocultures compared to tumor cells alone (Figure 2-5 A). These results suggest that cancer cells utilize arginine from the tumor microenvironment and in turn secrete citrulline to alter the tumor microenvironment. To reveal the reciprocity of cancer cells in modulating O-ASCs, we hypothesized that cancer cells secrete citrulline which could induce adipogenesis. To confirm the hypothesis, we cultured O-ASCs in presence of citrulline for 48 hours and
measured the citrulline content in the spent media using UPLC. Both lean and overweight O-ASCs consume exogenous citrulline and our results show that there is an uptake when cultured in the media supplemented with citrulline (Figure 2-5 B).

We monitored the growth rates of O-ASCs in the presence of citrulline and no alterations were observed (data not shown). To confirm our hypothesis, we cultured O-ASCs into adipogenic differentiation media with and without citrulline during the differentiation period. As seen in Figure 2-5 C, citrulline markedly increased the adipogenic differentiation in the three tested primary patient derived O-ASC cell lines. The extent of differentiation was similar in lean (O-ASC1, O-ASC 35) and overweight (O-ASC 21) O-ASCs. Noticeably, citrulline increased the size of fat droplets in O-ASCs (Figure 2-5 B). We further measured the activity of glyceraldehyde 3-phosphate dehydrogenase (G3PDH), an enzymatic marker for measuring adipogenesis. G3PDH activity is elevated during adipogenesis to support the production of key metabolites such as glycerol. Our results show increased G3PDH activity with citrulline at day 14 of O-ASC differentiation (Figure 2-5 D). We further measured the mRNA expression of AP2 (adipocyte protein 2) and LPL (lipoprotein lipase) in O-ASCs when adipogenesis was induced with and without citrulline. As seen in Figure 2-5 E, citrulline increased the expression of these adipogenesis markers.

To confirm, if the cancer cells indeed could increase adipogenesis of O-ASCs, we cultured O-ASCs in conditioned media obtained from cancer cells (Figure 2-5 F). As seen in the figure, conditioned media from cancer cells significantly increased adipogenesis in patient derived O-ASC cells. G3PDH activity of O-ASCs was higher when differentiation media was conditioned by cancer cells in the presence of arginine. Because cancer cells converts the arginine content of media to citrulline which induces the adipogenic capacity of O-ASCs. These results confirmed our hypothesis, that cancer cells increase adipogenesis in O-ASCs and this increase is mediated through secreted citrulline.
To prove that cancer cells induce adipogenesis via citrulline, we measured adipogenesis using cancer cells’ conditioned media with and without arginine. Cancer cells cultured under arginine deprived conditions will have significantly reduced citrulline in the media. Thus, decreased adipogenesis will be consequent of reduced citrulline in the conditioned media. As seen in Figure 2-5 G, G3PDH activity of O-ASCs differentiated with was significantly higher for conditioned media with arginine than the conditioned media without arginine. These results confirm our hypothesis, that cancer cells increased adipogenesis in O-ASCs and this increase is mediated through secreted citrulline.
Figure 2-5. Citrulline induces adipogenesis of O-ASCs.
(A) OVCAR429 and HEC-1-A cells were transwell cocultured in arginine-free media for three days. Fresh media was replaced 24 hours before sample collection. Citrulline contents of collected samples were analyzed by ultra-performance liquid chromatography (UPLC). Cancer cell viability was measured and reported in million units to normalize the results. Cancer cells without coculture were used as a control. (B) O-ASCs were incubated with MEM-α along with citrulline (0.5mM) for 48 hours. O-ASCs’ supernatant were collected and citrulline content of samples was assessed with UPLC and compared to fresh media at Day 0. (C) O-ASCs were seeded on a 12-well plate and cultured until confluency. The media was replaced with adipogenic media containing insulin (10μg/ml), rosiglitazone (2μM), indomethacin (200 μM), cortisol (100nM), IBMX (500 μM), and human transferrin (10μg/ml) with or without citrulline (0.5mM) for four days. Next, the media was exchanged to adipogenic media including only rosiglitazone (2μM) and insulin (10μg/ml) with or without citrulline (0.5mM) for another ten days. Lipid droplet formation was evaluated with Oil red O staining. The bright field images were taken with 4X and 20X magnifications by EVOS® XL Core Cell Imaging System. (D) G3PDH activity was measured for adipogenic induction with and without citrulline conditions as described in B. (E) AP2 and LPL mRNA expressions were measured at Day 14 of adipogenic induction in the presence or the absence of citrulline (0.5mM). (F) Cancer cells were conditioned 24 hours with MEM-α and the conditioned-media was used to induce adipogenesis in O-ASCs. O-ASCs induced by adipogenic media were utilized as a control. (G) Cancer cells were conditioned with either arginine-free or arginine containing media. Cancer conditioned media without arginine was then replenished with 0.2g/L arginine before adipogenic induction. G3PDH activity was measured at Day 14 and results from cancer complete conditioned media were compared to arginine free conditioned media. Data are expressed as means ± SE; n>6. *P<0.05, §P<0.01, and #P<0.001. T-test was used for single comparisons. Dunnett's method was implemented to compare multiple groups versus a control group.
2.5. O-ASCs modulate cancer cells’ mitochondrial bioenergetics

Recently, cancer cells have been found to have altered metabolism and this metabolic rewiring promotes tumor growth and increases malignancy. Previous studies have shown that NO regulates mitochondrial respiration in hepatocytes and cardiomyocytes [129]. To elucidate the precise role of O-ASCs in regulating cancer cells’ metabolic pathways by altering NO homeostasis, we performed mitochondrial bioenergetics analysis. Our results show that NO reduced respiration of both OVCA and ECA cells. Oxygen consumption rate (OCR) of cancer cells under arginine deprived conditions was significantly higher than arginine replete conditions (Figure 2-6 A). Inhibiting NO synthesis using L-NAME under complete media conditions similarly increased OCR. Further, when SNAP was added OCR drastically decreased. The addition of L-arginine in arginine-free media also decreased OCR, since arginine is a substrate for NO synthesis. These results are consistent with the hypothesis that NO decreases mitochondrial respiration in cancer cells. To determine the effect of O-ASCs on mitochondrial respiration of cancer cells through NO, we measured OCR of cancer cells cocultured with O-ASCs in arginine deprived condition for 72 hours. We found that cancer cells which were cocultured with both lean and overweight O-ASCs had significantly lower respiration when compared with cancer cells without cocultures (Figure 2-6 B-C). Further, cancer cells in cocultures with O-ASCs had lower maximal respiratory capacity (measured using FCCP, a protonophoric uncoupler) (Figure 2-6 B). We next measured OCR under NO inhibition conditions. Consistent with previous results, adding L-NAME increased OCR (Figure 2-6 D). Further, cocultures of both lean and overweight O-ASCs decreased OCR. Thus, from this data we can conclude that O-ASCs increase NO synthesis in cancer cells, resulting in suppression of mitochondrial respiration in these cells.
Figure 2-6. O-ASCs modulate cancer cells’ mitochondrial bioenergetics.

(A) Cancer cells were seeded in XF Seahorse multi-well plates and were incubated overnight until cells were attached to the surface. Cancer cells oxygen consumption rate (OCR) levels were measured after treating cancer cells with SNAP (100nM), exogenous L-arginine (15mM), and L-NAME (10mM) for three
hours before assay execution. (B) and (C) Cancer cells were reseeded in XF-seahorse multi well plates after three days of transwell coculture with O-ASCs. The media of coculture did not include arginine and cells were reseeded with diluted (1:1) coculture media. Oligomycin (2µg/mL), FCCP (1µM), and rotenone (1µM) were injected through the cartridge ports. Cells were lysed and quantified for their protein contents and used for normalization of data. (D) Cancer cells from transwell cocultures were injected with L-NAME (20mM) in the cartridge. Data are expressed as means ± SE; n>6. *P<0.05, §P<0.01, and #P<0.001. All pairwise multiple comparisons were analyzed by Bonferroni test. Dunnett's method was implemented to compare multiple groups versus a control group.

2.6. O-ASCs regulate OVCAs and ECAs metabolism via nitric oxide pathways

To expand our findings on O-ASCs regulation of cancer cells' metabolism, we examined O-ASCs effect on the glycolysis. NO increased glucose uptake and lactate secretion in cancer cells (Figure 2-7 A). Both lean and overweight O-ASCs increased glycolysis in cancer cells under coculture conditions in the absence of arginine. These results confirm that O-ASCs induced NO induced changes consistent with the Warburg effect in these cells. Interestingly, pyruvate uptake was increased in cancer cells cocultured with O-ASCs (Figure 2-11 A). To investigate if there was reciprocal communication between cancer cells and O-ASCs in regulating metabolism, we measured metabolic activity of O-ASCs with and without cocultures of cancer cells. Interestingly, O-ASCs from cancer cells coculture had higher glucose uptake and lactate secretion (Figure 2-11 B). However, pyruvate uptake was reduced in O-ASCs which were cocultured with cancer cells (Figure 2-11 C). In line with results previously reported, our results suggest that cancer cells transform the microenvironment cells by increasing their glucose metabolism [130]. To further confirm NO's regulation of cancer cells' metabolism in cocultures, we measured glycolytic and mitochondrial ATP
contribution using oligomycin (inhibits electron transport chain) and 2-DG (inhibits glycolysis), respectively. Consistent with the above results, we found that O-ASCs increased the glycolytic ATP generation but decreased the mitochondrial ATP generation in both OVCA and ECA cells (Figure 2-7 B). Since cancer cells may divert increased glucose to the pentose phosphate pathway for NADPH generation to decrease ROS, we next measured nicotinamide adenine dinucleotide phosphate (NADPH). We found that NO increased NADPH synthesis (Figure 2-7 C) and decreased ROS (Figure 2-7 D). Notably, O-ASCs increased NADPH and reduced ROS in cocultures under arginine deprivation conditions. These results substantiate the role of O-ASCs secreted factors which modulate NO homeostasis in cancer cells and thereby upregulate glycolysis and reduce oxidative stress [131]. Schematic illustrates reciprocal interaction between cancer cells and O-ASCs before and after coculture (Figure 2-7 E).
Figure 2-7. O-ASCs regulate OVCA and ECA cells metabolism via nitric oxide pathways. OVCAR429 and HEC-1-A were indirectly cocultured with O-ASCs for three days. The control cells from monoculture were seeded at the same time as cells with transwell coculture. Similar culture methods were used for cells in coculture and monocultures. The culture media was RPMI 1640 without arginine during three days of indirect coculture. Cocultured media was collected on the third day and diluted (1:1) with fresh RPMI (without arginine). Cancer cells were incubated with diluted media for 24 hours prior to supernatant collection. Collected samples were analyzed for their extracellular metabolites content. (A) Glucose uptake and lactate secretion of cancer cells. (B) Cancer cells from transwell cocultures of O-ASCs were reseeded with diluted media in 96-well plates. The cells were incubated at 37°C overnight until cells are attached to the surface. Oligomycin (2μg/μL) or 2-DG (100mM) was added three hours before assay execution. Glycolysis and mitochondrial ATP contribution were assessed with these inhibitors. Cancer cells without coculture and without arginine were utilized as control. Cancer cells from transwell cocultures of O-ASCs were reseeded and assessed for (C) NADPH and (D) reactive oxygen species (ROS) levels. (E) Schematic illustrates reciprocal interaction between cancer cells and O-ASCs before and after coculture. Data are expressed for each cell type as the mean ± SE; n>3. *P<0.05, §P<0.01, and #P<0.001. Dunnett's method was used to compare multiple groups versus a control group.

2.7. O-ASCs induce chemo resistance of cancer cells

The above results show that O-ASCs secrete factors which modulate NO homeostasis and increases cancer cells' proliferation and alters cancer metabolism. We recently showed that O-ASCs induce chemo-resistance in cancer cells [8]. Here, we asked if this O-ASC mediated chemoresistance can be deregulated by disrupting NO homeostasis. We added L-arginase in direct-
contact cocultures of O-ASCs and cancer cells. The L-arginase depletes any secreted arginine by O-ASCs and blocks NO synthesis in cancer cells. Remarkably, we found that addition of L-arginase to direct-contact cocultures increased chemosensitivity of paclitaxel in cancer cells (Figure 2-8 A). Furthermore, addition of L-NAME in direct-contact cocultures also had similar effect and increased sensitivity of paclitaxel in cancer cells (Figure 2-8 B). Similar results were obtained with additional O-ASCs patient samples treated with L-arginase at 10U/mL (Figure 2-8 C). To confirm the involvement of NO in increasing resistance of cancer cells to paclitaxel we added SNAP in cancer cells cultured with either L-arginase or L-NAME in presence and absence of paclitaxel (Figure 2-12). We found that SNAP decreased sensitivity of cancer cells to paclitaxel thus corroborating our previous results. We further evaluated if combinatorial addition of L-NAME and L-arginase will have synergistic effect in disrupting the NO mediated communication between O-ASCs and cancer. Indeed, adding both L-NAME and L-arginase significantly reduced the cell viability in cancer cells in cocultures with O-ASCs (Figure 2-8 D). These results suggest that combined approach of targeting secreted arginine through L-arginase, along with targeting microenvironment secreted factors induced increased NO synthesis in cancer cells using L-NAME, may be a viable therapeutic approach for targeting OVCAs and ECAs.
Figure 2-8. O-ASCs induce chemo resistance in cancer cells. Stably luciferase transfected cancer cells and O-ASCs were seeded in 96-well plate and cocultured in direct contact for 48 hours before their exposure to different concentrations of paclitaxel along with (A) L-arginase (10U/ml) or (B) L-NAME (20mM). Viability of cancer cells were assessed by addition of luciferin (150μg/mL) for 1 hour and the corresponding luminescence was measured. (C) More patient samples were incorporated to illustrate the increased chemo resistance of cancer cells when directly cocultured with O-ASCs and L-arginase (10U/ml). (D) Cancer cells and O-ASCs (ratio 1:3) were directly cocultured in 96-well plates and treated with L-arginase, L-NAME, or their combination for three days. Data are expressed for each cell type as the mean ± SE; n>6. *P<0.05, §P<0.01, and #P<0.001. The curves are compared at each point using Bonferroni test. Dunnett’s method was used to compare multiple groups versus a control group (C).

2.8. Conclusion

Here, our results revealed mechanism behind the interaction between O-ASCs and cancer cells. We found that O-ASCs promoted the growth of both OVCA and ECA cells through NO. Interestingly, O-ASCs secrete arginine under arginine deprivation conditions and this secreted arginine was uptaken by cancer cells, thereby increasing NO synthesis and cancer cells’ growth rate. Arginine depletion is currently used as a therapy for melanoma and hepatocellular carcinoma [132, 133]. We showed that when O-ASCs secreted arginine is depleted (using L-arginase) or when O-ASCs secreted factors induced increased NO synthesis in cancer cells is inhibited (using L-NAME), there is a decline in the growth rate of both OVCA and ECA cells. It was previously reported by our group that OVCA cells secrete considerable amount of citrulline, thereby indicating high NOS activity and arginine utilization [134]. Here, our data showed that OVCA and ECA cells utilize arginine produced by O-ASCs and generated citrulline. Remarkably, citrulline secreted by cancer cells increased the adipogenesis of O-
ASCs. Thus, our findings propose a previously unexplored metabolic coupling between cancer cells and O-ASCs.

Recent studies proposed metabolic-symbiosis as a reciprocal coupling between cancer cells and its microenvironment [135]. In these studies, tumor microenvironment cells, mainly cancer associated fibroblasts, were shown to be in catabolic state, thus generating energy rich metabolites (such as lactate, glutamine, fatty acids, and other amino acids) which are then used by cancer cells’ mitochondria for OXPHOS [136]. However, our findings show that O-ASCs rescue cancer cells loss of growth under arginine deprivation conditions, by secreting arginine for cell growth/biosynthesis but not for energetic needs. Arginine utilization for NO as a signaling molecule dominated other roles of arginine in cellular functions. As seen in Figure 2-4 G, growth of cancer cells decreased significantly when arginine was excluded from media and SNAP compensated this decrease induced by arginine deprivation. These results emphasize the crucial role of arginine in NO synthesis.

In contrast with Warburg effect, recent data suggest that cancer cells have healthy mitochondria; however, they have upregulated glycolysis [137, 138]. Interestingly, our data suggests that O-ASCs promoted glycolysis in cancer cells by elevating NO synthesis, which has been shown to have inhibitory effects on enzymes involved in mitochondrial respiration. Previous studies showed that NO affects glycolysis through s-nitrosylation of hexokinase [139]. Hexokinase converts glucose to glucose-6-phosphate in the first step of glycolysis and is highly expressed in cancer cells[140]. Low concentrations of NO (below 100nM) induces hypoxia inducible factor 1-α (HIF-1α) expression and mimics low oxygen conditions [117]. HIF-1α, a key regulator of hypoxia, switches energy metabolism from oxidative phosphorylation to glycolysis by regulating glucose transporter-1 (GLUT-1), lactate dehydrogenase (LDH), and pyruvate dehydrogenase (PDH) expression [141]. We here showed that O-ASCs positively regulates the Warburg effect by modulating the NO homeostasis. O-ASCs secreted arginine increased NO synthesis in cancer cells which reprogrammed cancer cells by
increasing glycolysis and reducing mitochondrial ATP generation. Treating cancer cells with arginine depleted media showed that reducing NO levels reduced glucose and pyruvate consumption of cells as well as their lactate secretion levels. Remarkably, O-ASCs interaction with cancer cells compensated the reduced levels of metabolites. Consistent with our hypothesis, we found that O-ASCs increased glucose uptake and lactate secretion of cancer cells under arginine deprivation conditions. O-ASCs modulated ovarian and endometrial cancer cell metabolism via arginine secretion which when uptaken by cancer cells increased NO in these cells. Figure 2-7 E summarizes our results obtained on metabolic coupling between O-ASCs and cancer cells. Based on our results, “NO homeostasis” could be a key player in regulating reciprocal communication between O-ASCs and gynecological cancer cells. The reciprocal communication was observed between cancer cells and O-ASCs, where cancer cells were found to increase glucose metabolism and adipogenesis in O-ASCs.

NO has been known to influence respiration rates of cancer cells by targeting mitochondrial complexes, Complex I and IV [142, 143]. Previous studies carried out primarily in liver cells showed that NO regulates mitochondrial respiration by targeting terminal enzyme of electron transport chain, cytochrome c oxidase by competing with oxygen [144]. Inhibition of complex IV is rapid (milliseconds), reversible, and occurs at low NO concentrations (nM), whereas inhibition of complex I occurs after a constant exposure of higher NO concentrations [145, 146]. NO’s inhibition of mitochondrial respiration in cancer cells shifts them from oxidative phosphorylation to glycolysis. Here, we showed that arginine deprivation decreases NO; thereby increases OCR of OVCAR429 and HEC-1-A cells (Figure 2-6 A). O-ASCs decreased OCR of cancer cells by secreting arginine, a substrate for NO synthesis under arginine deprivation conditions. We demonstrated that NO can shift source of ATP generation in cancer cells cocultured with O-ASCs by increasing glycolytic ATP production and concomitantly decrease mitochondrial contribution towards ATP production (Figure 2-7 B).
Recent studies have shown that O-ASCs induce chemoresistance in cancer cells [147]. Multiple lines of evidence support the link between NO and chemoresistance [123, 148-150]. Herein, we show that O-ASCs regulate cancer cells’ response to chemo-drugs through NO pathway. Inhibition of NO synthesis, sensitized cancer cells cocultured with O-ASCs to paclitaxel (Figure 2-8 A-C). Further, our studies suggest that combinatorial therapy of depleting arginine using L-arginase, along with inhibiting NO using L-NAME, could disrupt the communication between O-ASCs and cancer cells. Our data present mechanistic insights into O-ASC mediated metabolic reprogramming in cancer cells and also reciprocal modulation of O-ASCs adipogenesis by cancer cells. Future studies are needed to investigate the therapeutic strategies targeting the impact of O-ASC on cancer initiation and progression. The detailed analysis of altered NO metabolism of cancer cells in presence of O-ASCs will shed light on the molecular pathways regulated by O-ASCs and thus allow development of targeted therapies linking signaling, transcriptional changes with metabolic signatures linking obesity with cancer.
Figure 2-9. O-ASCs’ conditioned-medium rescue reduced viability of cancer cells deprived of arginine.

Cancer media (RPMI 1640 without arginine) was conditioned with O-ASCs for 24 hours. Cancer cells were cultured in conditioned media for three days while the media was changed every other day. Cancer cells cultured without arginine was used as control condition. Data are expressed as means ± SE; n>6. *P<0.05, §P<0.01, and #P<0.001. Multiple comparisons versus a control group were analyzed by Dunnett's method. All pairwise multiple comparisons were analyzed by Bonferroni test to compare lean and obese patients’ samples.
Figure 2-10. L-arginase-induced depletion of arginine.
Figure 2-11. Cancer cells induce glucose uptake and lactate secretion of O-ASCs and vice versa.

OVCAR429 and HEC-1-A were transwell cocultured with O-ASCs for three days. The media was RPMI 1640 without arginine during three days of indirect coculture. Cocultured media was collected on the third day and diluted (1:1) with
fresh RPMI (without arginine). Transwell inserts containing O-ASCs from coculture were separated from cancer cells and were placed in 6-well plate. Cancer cells and O-ASCs were separately cultured with diluted media for 24 hours prior to supernatant collection. Collected samples were analyzed for their extracellular metabolites content. (A) Results show pyruvate uptake of HEC-1-A, OVCAR429, and O-ASCs, respectively. (B) Glucose uptake and lactate secretion are shown for O-ASCs with and without coculture of O-ASCs. Cancer cells and O-ASCs without coculture were used as controls. Data are expressed for each cell type as the mean ± SE; n>3. *P<0.05, §P<0.01, and #P<0.001. Dunnett’s method was used to compare multiple groups versus a control group.
Figure 2-12. Nitric oxide regulates chemo sensitivity of cancer cells.
HEC-1-A and OVCAR429 were seeded in 96-well plate and treated with paclitaxel (500nM), L-NAME (20mM), SNAP (100nM), and L-arginase (10U/ml) or combinations thereof. W/O ARG labeling denotes cancer media, RPMI 1640,
without arginine. Viable cancer cells were determined by measuring luminesces of luciferin (150μg/mL) conversion after 72 hours. Data are expressed as means ± SE; n>6. *P<0.05, **P<0.01, and ***P<0.001. All pairwise multiple comparisons were analyzed by Bonferroni test.
Targeting NO-mediated pathways as a novel cancer treatment: Nitrite and S-nitrosylation

Our results in the previous chapter shows that nitric oxide synthesis plays an important role in the progression of gynecologic cancers as well as their resistance to chemo drugs. O-ASCs-induced NO generation in ovarian cancer cells were targeted either by removing the feeder metabolite, L-arginine, or by inhibiting nitric oxide synthase. Inhibition of NO synthesis results in more response to the most common ovarian cancer chemo drug, paclitaxel.

One of the challenges in the practice of cancer treatment is the relapse of the disease. It occurs when cancer cells become resistant to the former therapy. For example, depletion of arginine has been taken under clinical trial as a cancer treatment strategy for the patients lacking argininosuccinate synthetase (ASS) and Argininosuccinate lyase (ASL) gene expression [151]. ASS and ASL convert L-citrulline to L-arginine in two separate steps (as shown in Figure 3-1). The patients without ASS and ASL expression are dependent on dietary arginine and if exogenous arginine is depleted there is no other source for arginine-dependent

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2 This chapter is the Rice version of a preparing manuscript to be submitted to Cell Death and Disease
cancer cells. In vivo models of mice with silenced ASL and ASS have demonstrated extensive tumor shrinkage upon arginine depletion; however, reoccurring of cancer does exist.

![Cellular nitrogen metabolism](image)

Figure 3-1. Cellular nitrogen metabolism.

The role of NO has been contradictory because of the dose-dependency of the tumor response to NO. NO is a tumor advocator at low concentrations while it is known to be cytotoxic at high concentrations. Interestingly, tumor cells are detected with elevated levels of constitutive NO compared to non-malignant tissues. On the one hand, targeting endogenous NO pathways such as inhibition of NOS has been consistently shown to arrest tumor growth and been proposed as an enhancement for drug efficacy.

On the other hand, overexpression of NO generation by several folds has been depicted to initiate cell apoptosis mechanisms and cause cell death. The cytotoxic effect of NO is though the induction of nitrosative stress. Inhibition of NO by blocking NOS with N\textsuperscript{G}-monomethyl-L-arginine (L-NMA) targets human colorectal adenocarcinoma, and for the first time the results showed the supportive role of NO in tumor progression [152]. Low levels of NO donors such as s-nitro-n-acetyl-penicillamine (SNAP) increase the vascular tubular formation and improve tumor supply of nutrients as well as oxygen. Released NO significantly modulates tumor progression by inducing angiogenesis up to 46%
In 1990’s several studies depicted that oral administration of \(N^\text{G}\)-nitro-L-arginine methyl ester (L-NAME), which is the non-specific NOS inhibitor, diminished tumor vascularization and caused tumor shrinkage [154-156].

More recently, NO role in cancer metastasis was discovered, and inhibition of iNOS with aminoguanidine drastically abrogated tumorigenic and metastatic characteristics of cancer cells [157]. Ablation of NO was efficient to overcome the chronic inflammation of tumors and arrest the cancer metastasis [131].

Moreover, NO makes the lung adenocarcinoma resistant to cisplatin which is the most common chemo drug for solid tumors. NO impairs the cellular apoptotic mechanisms and increases the resistance to cisplatin-induced cell death in human lung and melanoma carcinomas [123, 158]. Concurrent utilization of NO scavengers and cisplatin may increase the sensitivity of lung cancers to cisplatin-induced apoptosis [158, 159].

Figure 3-2. Nitric oxide and tumor therapy.
Nitric oxide plays a double edged sword role in cancer progression. It induces tumor growth at low concentrations while it causes DNA damage and apoptosis when produced at high levels. When NO a tumor promoter, inhibition of NOS is proposed as a therapy. L-NAME has been shown to target gynecologic cancer growth because of deregulation of tumor and microenvironment crosstalk [123,
125, 160, 161]. Overexpression of NOS and NO generation is utilized when NO is assumed to damage tumor progression and causes cell death. NO donors release constant dose of NO in the range of activated macrophages which induces cancer cell apoptosis [162, 163]. iNOS gene therapy results in the inhibition of murine thyroid cancer advancement [164]. Induction of NOS with the use of chemokines and drugs results in the shrinkage of tumors [165]. Development of vasculature diminishes cancer growth by delivery of prominent oxygen as well as chemodrugs. eNOS is known to promote angiogenesis and its expression is enhanced to progress tumor vasculature [166, 167].

In this section, other sources of NO which may lead to better pinpointing the NO-mediated cancer treatment will be explored. As mentioned in the former chapter we proposed a combinatory drug treatment including inhibition of NO and depletion of arginine. Here, it is suggested that other sources of NO production as well as the downstream impacts of NO need to be addressed.

### 3.1. Nitrite is another source of NO in ovarian cancers

Tumors grows so rapidly and do not allow proper vasculature to form and leave portions of the tumor without ample access to oxygen. Hypoxia-inducible factor 1 (HIF-1) is the master transcriptional regulator of cellular and developmental response to hypoxia. Many genes are identified as HIF-1 target including ones involved in angiogenesis, metabolic adaptation, apoptosis, invasion, and metastasis. Hypoxia is a characteristic feature of most solid tumors. Hypoxia induces tumor chemoresistance in proportion to distance from the vasculature. Tumors reprogram their metabolism to survive the harsh and challenging hypoxic environment [115]. HIF-1 inhibitors are being investigated as anticancer drugs since hypoxic cells are exposed to less chemo drugs because of lack of defined vasculature.
NOS-independent NO generation was first reported by two separate studies in 1994 [118]. Conversion of salivary-derived nitrite was the source of NO because of gastric acidity. And NO generation was greatly enhanced after intake of dietary nitrate. Later, NO generation from nitrite was shown in the ischemic heart thus extending the occurrence of nitrite reduction outside the gastrointestinal tract [119]. Despite the traditional NO generation from arginine, the nitrate-nitrite-NO pathway is greatly enhanced by hypoxia and acidosis and may serve as a backup system to ensure NO generation during hypoxic conditions [104]. As a result NO reserves, nitrite and nitrate, are involved in the regulation of blood flow, cell metabolism, and signaling, as well as tissue protection during hypoxia. There are several proposed pathways by which nitrite and nitrate are converted to NO in hypoxia. Deoxygenated myoglobin (deoxy-Mb), xanthine oxidoreductase (XOR), mitochondrial complexes and even NOS are the known to donate electrons to nitrite and to form NO [104, 120].

To confirm the impact of nitrite on the ovarian cancer cells’ growth, I measured viability of four different cancer cell lines in the presence of various concentrations of sodium nitrite (SN) in normoxia as well as in hypoxia (low oxygen). As shown in Figure 3-3 and Figure 3-4, SN did increase the viability of cancer cells both in normoxia and hypoxia and more interestingly that no toxicity was detected for higher concentrations of SN (up to 1mM). It is important to note that SN not only affects the cancer’s growth in the absence of oxygen it increases the proliferation when ample oxygen exists.

My next step was to determine whether increased viability of cancer cells by SN was caused by the conversion of SN to NO. Thus, I measured the levels of NO synthesis with various concentration of SN treatment. The results show that NO synthesis of ovarian cancers is directly correlated to SN treatment (Figure 3-5). This proves that SN-induced viability of ovarian cancers is through the conversion of SN to NO.
As mentioned, the reduction of nitrite to NO occurs at different sites including nitric oxide synthase (NOS) and mitochondrial complexes (Figure 3-7). We utilized NOS inhibitor, N\textsuperscript{\textgreek{g}}-nitro-L-arginine methyl ester (L-NAME), and mitochondrial complex I and III inhibitors rotenone and antimycin C, respectively. Our aim was to illustrate how the inhibitors target the induced growth of ovarian cancer cells caused by nitrite. Two different cell culture medium for cancers were implemented: RPMI 1640 with and without arginine to explore whether presence of other source of NO affects the conversion of nitrite to NO. As shown in Figure 3-6, SN increases the levels of viable cells which were targeted by the cocktail treatments. The cocktails included pair combination of L-NAME and one of the mitochondrial inhibitors or the combination of all the drugs. Interestingly, the results show that blocking the pathways of nitrite transformation to NO inhibits the induced-proliferation of ovarian cancer cells caused by nitrite.
Figure 3-3. Normoxic titration of sodium nitrite on ovarian cancer cells. Cancer cells were seeded (10,000 cells per well) in 96-well plates and incubated until they attached to surface. Next, the media was changed to RPMI 1640 including different concentrations of sodium nitrite (SN). The horizontal axis demonstrates the SN concentrations in nM. Viability of cells were monitored at day 2, 4, and 6 by measuring luminescence of luciferase-labeled cancer cells.
Figure 3-4. Hypoxic titration of sodium nitrite on ovarian cancer cells.
Cancer cells were seeded (10,000 cells per well) in 96-well plates and incubated in normoxic chamber until they attached to surface. Next, the media was changed to RPMI 1640 including different concentrations of sodium nitrite (SN) and cells were transferred to hypoxic incubator for the rest of the experiment. The horizontal axis demonstrates the SN concentrations in nM. Viability of cells were monitored at day 2, 4, and 6 by measuring luminescence of luciferase-labled cancer cells.
Figure 3-5. Ovarian cancers convert nitrite to nitric oxide in both normoxia and hypoxia.

The cancer cells were seeded in 6-well plates and incubated in normoxic chamber until the cells attached to the surface. Then the medium was changed to RPMI 1640 or/and different concentrations of sodium nitrite. The cells for hypoxia were kept in hypoxic incubator thereafter. NO levels were monitored using Sievers and the data was normalized to the protein content of samples.
Figure 3-6. Inhibition of NOS and mitochondrial complexes targets the conversion of nitrite to NO.

Cancer cells were seeded in 96-well plates (10,000 cells per well) and incubated until the cells attached to the surface. Next, the media was changed to RPMI 1640 with and without arginine. SN, L-NAME, rotenone, antimycin C or their combination was added to the cells depending on the labeling of the horizontal axis. The cells were incubated wither in normoxic or hypoxic chambers for three days before the viability of cells were assessed.
3.2. Inhibition of s-nitrosylation reduces ovarian cancer survival

S-nitrosylation is the primary posttranslational protein modification caused by NO. S-nitrosylation is a rapidly reversible and precisely targeted ubiquitous posttranslational modification. NO is covalently attached to a thiol group of a protein cysteine reside (S-NO). More than 1000 proteins have been shown to be modified by s-nitrosylation [168]. S-nitrosylation of proteins exerts profound impacts on cellular signaling and overall response to microenvironment [169]. The S-NO bond is formed by NO-derived species such as N₂O₃, low molecular-weight nitrosothiols (s-nitroso-glutathione) [170], and by a reaction between NO and cysteine thiol where the redox requirement is met by loss of one electron. Decomposition of S-NO bonds occurs by multiple mechanisms including ascorbate, thioredoxin, GSNO reductase, and trans-nitrosylation. S-nitrosylated proteins acquire altered enzymatic activity, function, localization, and stability [171]. Aberrant s-nitrosylation has been reported being responsible in carcinogenesis and the poor response to therapies [172-174].
3.2.1. Nitrite-induced s-nitrosylation

Nitrite- and nitrate-derived NO modifies the functions of proteins by s-nitrosylation of their cysteine residues. Numerous s-nitrosylated proteins have been identified to play role in proliferation, apoptosis, angiogenesis, cell cycle modification, tissue invasion, metastasis, and the response of cancers to treatments [105].

Tumor protein p53 is a tumor suppressor and impacts the cancer responses to DNA damage and abnormalities. Inactivation or mutation of p53 leads to uncontrolled cell proliferation and persistent DNA damage and it has been correlated with various tumors. S-nitrosylation of p53 has shown to impair the normal response of cells to DNA damages [121]. Fas ligand (FasL or CD95L) is a type-II transmembrane protein which belongs to the tumor necrosis factor (TNF) family. FasL is involved in the induction of cell apoptosis when it binds with its receptor. S-nitrosylation of FasL induces its capacity to target unwanted cancer cell growth by promoting FasL-mediated apoptosis [122].

B-cell lymphoma-2 (Bcl-2) downregulates mitochondria-mediated apoptosis pathway (reduction of Cytochrome C) and regulates cell survival. In most cancer cells, Bcl-2 has shown to be upregulated and it causes chemo resistance. S-nitrosylation of Bcl-2 enhances the protein stabilization and increases the survival of tumor cells. Inhibition of s-nitrosylation has been indicated to increase the chemo sensitivity of melanoma cells by targeting Bcl-2 activity [123].

Phosphatase and tensin homolog (PTEN) mutation is commonly detected in tumors from different origins. It acts a tumor suppressor and is fed primarily by phosphoinositide 3-kinase (PI3K) which has been extensively shown to play role in angiogenesis of tumors. Enhancement of PI3K leads to significant elevation of tumor vasculature and resulted tumor growth. PTEN inactivates PI3K and has been utilized in cancer treatment mostly as an angiogenesis inhibitor [124]. S-nitrosylated PTEN does not inhibit PI3K and the involved angiogenic activities.
Here I utilized s-nitrosylation inhibitors to verify the nitrite-derived NO induces ovarian cancers’ growth through s-nitrosylation (Figure 3-8). Interestingly, the results show that the increased viability levels caused by SN is targeted with s-nitrosylation inhibitors such as 2-Phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), sodium azide (SA), and sodium cyanide (SCN). It is important to note than only SA showed very high toxicity on the cells although other inhibitors did affect viability of cancer cells in the range that was used in the experiments.

Figure 3-8. S-nitrosylation inhibition targets nitrite-induced viability of ovarian cancers.

Cancer cells were seeded in 96-well plates (10,000 cells per well) and incubated until the cells attached to the surface. Next, the media was changed to RPMI 1640 with and without arginine. SN, SA, SCN, PTIO or their paired combination was added to the cells depending on the labeling of the horizontal axis. The cells were
incubated wither in normoxic or hypoxic chambers for three days before the viability of cells were assessed.

3.2.2. NO-induced s-nitrosylation

Cellular NO induces s-nitrosylation and leads to higher proliferation rates of ovarian cancer cells. Here we studied the effect of NO-donor SNAP on s-nitrosylation of ovarian cancer cells. As it is shown in Figure 3-9 deprivation of arginine significantly targets cancer cells’ viability in normoxia and hypoxia. PTIO was implemented to inhibit s-nitrosylation and added at concentrations which did not affect the viability of cells. Addition of SNAP increases viable cells which emphasizes the crucial role of NO on progression of cancers. The results depict that PTIO targets induced viability of ovarian cancer cell lines caused by SNAP. It is inferred that SNAP-elevated viability of ovarian cancers is because of an induction of s-nitrosylation. It is important for a successful treatment not only to inhibit NO synthesis by arginine depletion but also targeting downstream NO-related pathways such as s-nitrosylation.
Figure 3-9. S-nitrosylation inhibition targets NO-induced viability of ovarian cancers.

Cancer cells were seeded (10K) in 96-well plates and were incubated at 37°C until they attached to surface. Next, the media was changed to arginine-free RPMI and RPMI as a control. DNBC and PTIO was added to the cells alone and also simultaneously with SNAP to study the role of s-nitrosation in the regulation of cancer cells by nitric oxide. The cells were incubated in normoxia and hypoxia before viability assessment.
3.3. Evolution of nitrogen metabolism during adipogenesis

As mentioned before in section 2.4 that cancer secreted citrulline induces adipogenesis of O-ASCs. Addition of exogenous citrulline increases fat droplet formation considerably as is shown in Figure 2-5. However, the mechanism by which citrulline affects O-ASCs’ adipogenesis capacity was not clear. Here, it is proposed that consumed citrulline is converted to arginine which is a source of NO generation in cells. Synthesized NO modulates cellular proteins in O-ASCs and promote fat storage. So we measured the NO generation of SGBS cells over the period of adipogenic differentiation. SGBS cells are a unique tool for studies of human fat cell biology and are used as a control in our fat differentiation experiments [175]. Our results (Figure 3-10 A) show that higher NO levels are correlated with the period of induced adipogenic differentiation. We also measured the impact of s-nitrosylation on adipogenic capacity of SGBS cells and interesting PTIO treatment significantly reduces the differentiation of SGBS cells into fat cells. It is concluded that NO-related pathways affect the transformation of primary SGBS cells to adipose cells.

Figure 3-10. NO modulation in SGBS adipogenesis.
SGBS cells were seeded in 6-well plate and incubated until the cells attached to the surface. Next, the media was changed to DMEM F12 including the adipogenic differentiation cocktail. (A) Cell pellets were collected at Day 0, 1, and 14. Cell samples were measured for their NO content and the data was normalized to their protein content. (B) Cell pellets were collected at Day 10 upon complete adipogenic differentiation. Cell samples were utilized for monitoring G3PDH activity.

We next tested the same hypothesis in O-ASCs which have been shown to have high capacity to differentiate to adipose cells [8]. O-ASCs also produce more NO while are being transformed to adipose cells (Figure 3-11 A and B). The expression of nNOS and arginase 1 (ARG1) mRNA expression was measured at different days of induced differentiation. Omental adipose tissue samples were utilized as a control. Interesting we found that nNOS expression was elevated when cells become more adipocyte-like while ARG1 expression decreased (Figure 3-11). It is inferred that NO-related pathways are upregulated and other side pathways which borrow arginine are downregulated. The cells focus the arginine to NO generation.

Figure 3-11. NO modulation in O-ASCs adipogenesis.

O-ASCs were seeded in 6-well plate and were incubated until the cells attached to the surface. Next, the media was changed to DMEM F12 including the adipogenic differentiation cocktail. (A and B) Cell pellets were collected at Day 0, 4, 7 and 14. Cell samples were measured for their NO content and the data was normalized to
their protein content. (C and D) Cell pellets were collected at Day 0, 4, 14 upon complete adipogenic differentiation. Omental adipose tissue was utilized as a control. nNOS and ARG mRNA expressions were monitored by RT-PCR. The results are normalized to mRNA expressions at Day 0.

3.4. Conclusion

NO and its derivatives affect ovarian cancer progression through protein modification called s-nitrosylation. Arginine depletion has been studied extensively for targeting cancer growth since it is the main source of NO synthesis. However, the relapse of cancer is known to occur due to intricate-metabolic reprogramming pathways that enable the tumors to adapt to the new condition. In chapter, we showed that NO and nitrite exert their cancer promotive effect through s-nitrosylation. Targeting these molecules at synthesis levels is not adequate and here we proposed that inhibition of NO-related pathways at different stages is necessary. Our preliminary results show simultaneous targeting of NOS (L-NAME) and s-nitrosylation (sodium azide, SA, sodium thiocyanate, SCN, and PTIO) lead to the inhibitory results in more pronounced reduction of viable endometrial cancer cells even in the presence of O-ASCs (Figure 3-12). It is illustrated that L-NAME, PTIO, and SA synergistically inhibit the effect of O-ASCs on endometrial cancer growth.
Figure 3-12. Inhibition of NO-mediated pathway at different stages blocks the increased growth of endometrial cancer cells caused by O-ASCs.

Cancer cells were seeded in 96-well plates and incubated until the cells attached to plate’s surface. Next, O-ASCs were seeded on top of cancer cells in the same wells to mimic direct contact of cells in tumor microenvironment. When O-ASCs attached to the surface the media was changed to RPMI 1640 or/ and L-NAME, PTIO, SCN, SA, or their paired combination depending on the labeling of the horizontal axis. The cells were incubated in normoxia for 72 hours before cell viability assessment. Relative viability of endometrial cancer cells in mono and coculture was used to normalize the data to remove the effect of secreted factors during the coculture.
Chapter 4

Modulation of ovarian cancer metabolism by long chain fatty acids

Cancer cells are characterized by their altered metabolism. Tumor cells are known to acquire higher expression of FASN compared to their normal counterparts. FASN is responsible for synthesis of long chain fatty acids (LCFAs). Upregulated FASN aids cancer cells’ growth by synthesizing more fats which incorporate into cell membrane and nuclei. High expression of FASN is independent of the excessive pool of exogenous fats accessible for tumors. This chapter focuses on the role of exogenous long chain fatty acids on ovarian cancer metabolism. Also, the crucial role of tumor microenvironment in fatty acid metabolism is uncovered.

4.1. Exogenous LACFs are consumed by ovarian cancer cells

Overexpression of endogenous fatty acid synthase is associated with significantly enhanced proliferation of tumor cells. Pharmacological inhibition of FASN has been explored to arrest tumor growth. However, the direct effect of exogenous fatty acids have not been investigated. Here, my aim is to study the role of LCFAs in ovarian cancer metabolism. My first step was to measure whether LCFAs are consumed by cancer cells [176].
The cells were incubated with 100µM of LCFAs, palmitic acid (PA), oleic acid (OA), and linoleic acid (LA) for 24 hours. Free fatty acids (FFAs) uptake was measured by comparing the FFAs in fresh media and spend media. The data illustrates that cancer cells consume LCFAs and the extent is much higher in hypoxia (Figure 4-1) [177].

Most cancers experience chronic hypoxic environment during tumor progression since the rapid cell growth does not allow the proper vasculature to form. As shown in Figure 4-1 cancer cells depend on exogenous pool of LCFAs when they are deprived of ample oxygen so studying this class of fats is crucial in ovarian cancer investigation.

![Fatty acid uptake in Normoxia](image1)
![Fatty acid uptake in Hypoxia](image2)

Figure 4-1. LCFAs uptake in normoxia and hypoxia.
Fatty acid contents of fresh and spent media was measured upon treating ovarian cancers with 100µM of LCFAs for 24 hours. The cells were incubated in either complete RPMI or RPMI without glucose.

4.2. Lipid droplets are induced in ovarian cancers by LCFAs

Lipid droplets consist of a large core of neutral lipid, primarily TAG, surrounded by a monolayer of phospholipids [178]. Perilipin, ADPR, TIP47, the PAT family, are the most abundant lipid droplet-associated proteins constitute. PAT proteins are generally believed to operate as shells on lipid droplets, with packaging properties that protect the neutral lipid core from hydrolysis by
cytoplasmic lipases. Lipid droplet formation has been proposed to initiate from lipid esters which are accumulated between the two leaflets of the endoplasmic reticulum (ER) membrane (or other membranes), gradually grow into a globular shape, and are finally separated from the ER to become independent.

Lipid droplets are usually included in cytoplasm of eukaryotic cells which stores excess fatty acids and cholesterol as neutral lipids, predominantly triglycerides (TAG). Knowledge about their detailed composition and role in cellular functions are limited but it is believed that they are not static and only sites for storage of excessive lipids, but they are dynamic [179]. Lipid droplets are involved in membrane trafficking, cell signaling and administering specific lipids and proteins [180]. Moreover, Lipid droplets are sites for cytokine storage in inflammatory leukocytes which means that lipid droplets induce regulation of signaling that leads to inflammatory mediator secretion during inflammation. Under hypoxia or when excessive amounts of free fatty acids are present (exogenously in the medium or serum and when oxidation of fatty acids are blocked), accumulation of lipid droplets are elevated. Impaired lipid homeostasis because of elevated in lipid accumulation introduces membrane disruption and activation of lipoapoptosis [181].

The presence of lipid droplets in non-adipocytes has not been paid much attention although lipid droplets are normal organelles in any cell type. Although given the same name, those in non-adipocytes are much smaller than the adipocyte lipid droplet (generally less than a few μm in diameter).

Ovarian cancer cell lines (OVCAR3 and SKOV3ip1) were supplemented with exogenous saturated and unsaturated long chain fatty acids in order to study the capacity of cancer cells for lipid formation. Interestingly, ovarian cancer cells generate considerable amount of lipid droplets (Figure 4-2). The quantified AdipoRed™ staining is shown in Figure 4-3. It is concluded from the results that LCFAs are stored as lipid droplets in ovarian cancer cells. We further characterized the lipid droplets by implementing the phospholipid staining. The
results (Figure 4-4) depict that the droplets are constituted of a core of non-polar lipids (in green) which are surrounded by phospholipids (in red). Quantification of the results proved that LCFAs induce the content of both neutral lipids and phospholipids in ovarian cancer cells (Figure 4-5).

Figure 4-2. Ovarian cancer cells store LCFAs as lipid droplets. Cells were seeded in 12-well plates and incubated over night for adherence. Next, the cells were treated with fatty acids (palmitic acid, oleic acid, and linoleic acid all with the concentration of 100µM) for 24 hours. On the day of assay execution, cells were washed with PBS two times and stained with AdipoRed™ according to
the manufacturer’s protocol. The cells in normal medium, RPMI, were used as a control and no staining of AdipoRed™ was observed.

![AdipoRed Staining of lipid droplets](image)

Figure 4-3. LCFAs induce lipid droplet formation in ovarian cancer cells.

LCFAs were extensively studied for their role in central energy metabolism; however, no significant change was observed. So it is concluded that there is a missing element in tumor microenvironment that prevents cancer cells from utilizing LCFAs. Chemokines and hormones secreted by tumor microenvironment are potential candidates and their presence substantially regulate fatty acid trafficking and utilization. As a result, a new model was developed and direct coculture of ovarian cancer cells with O-ASCs were implemented for studying fatty acid metabolism.
Figure 4-4. Characterization of lipid droplets in ovarian cancer cells. OVCAR3 and SKOV3ip1 cells were seeded in 96-well plates and were incubated until the cells were attached to the surface. Next, the media was changed to RPMI and RPMI containing 100µM of LCFAs. A, B, C, and D represent RPMI, LA, PA, and OA, respectively. Neutral lipids are shown in green while phospholipids are stained with red. The cells' nuclei were stained with Hoechst 33343 and are shown in blue.
Figure 4-5. Induced neutral lipid and phospholipid formation in ovarian cancers treated with LCFAs.
Neutral lipid and phospholipid staining of OVCAR3 and SKOV3ip1 were quantified and is shown in A and B, respectively.

4.3. O-ASCs mediates utilization of stored LCFAs in ovarian cancers

Our extensive investigations revealed that LCFAs are not utilized by ovarian cancer cells. The results were implausible since most classes of fats have been shown to affect cancers one way or another. However, our results showed that LCFAs neither did increase the respiration nor ATP generation in ovarian cancer cells. Instead they are stored within the cells and increase the growth rates only when cells are cultured with LCFAs for long time (more than 7 days). We even studied utilization of LCFAs under various nutrient deprivation
conditions including glucose, glutamine, and sodium pyruvate depletion from the media. They cells became apoptotic but did not utilize the stored LCFAs. So we pondered that there is a missing link in the utilization of stored fat. After further exploration we hypothesized that soluble factors such as chemokines and hormones secreted by the tumor microenvironment are required for the lipolysis of stored fat in ovarian cancer cells.

4.3.1. Storage of LCFAs in the coculture of ovarian cancer cells and O-ASCs

We designed a coculture experiment in which cancer cells were in direct contact with O-ASCs before they are exposed to LCFAs. Next, the cells were treated with LCFAs for 48 hours. Our aim was to determine whether LCFAs are stored in cells in the new culture. Our results have illustrated that LCFAs are dominantly stored in ovarian cancer cells and interestingly O-ASCs do not have the same capacity to store LCFAs (Figure 4-6). The magnified images are designated with arrows for LD staining which are localized majorly in ovarian cancers. Elongated cells in the picture are O-ASCs while the smaller and more compact cell colonies are cancer cells surrounded by O-ASCs.

We utilized labeled ovarian cancer cells lines to better tell apart the exact LD locations in direct coculture. The cells were double stained with AdipoRed and Hoechst 33343 when HEYA8, ovarian cancer cell line, are constitutively labeled with GFP. Green regions represent GFP-labeled cancer cells while blue and red stained spaces are cells' nuclei and LDs, respectively. As it is shown in Figure 4-7 AdipoRed staining is only co-localized with blue Hoechst labeling where the green GFP labeling exists. It is concluded that LCFAs are only stored in cancer cells even if they are cocultured directly with O-ASCs. This proves that addition of LCFAs into culture media does directly impact cancer cells even if they are cocultured with O-ASCs.
Figure 4-6. LD formation in direct coculture of ovarian cancers and O-ASCs. Cancer cells were seeded in 6-well plated and incubated until cell were attached to the surface. O-ASCs were seeded on top. When the direct contact was developed the cells were treated with LCFAs including linoleic acid (LA), oleic acid (OA), stearic acid (SA), myristic acid (MA), and palmitic acid (PA) for 4 consecutive days. The media was changed every other day. On the day of assay
execution, the cells were fixed with 1% glutaraldehyde for 20 minutes before staining with OilRedO according to the manufacturer’s protocol. EVOS microscope was implemented to take transmitted-light images.

Figure 4-7. Colocalization of GFP-labeled ovarian cancer cells with AdipoRed Staining.
Cancer cells were prepared in direct coculture in 96-well plates and treated with LCFAs for 48 hours before they were fixed and double stained with AdipoRed and Hoechst 33343.

4.3.2. O-ASCs induce utilization of stored LCFAs in ovarian cancers

LCFAs have been shown to be stored in cancer cells although they did not alter proliferation or respiration rates of cancer cells. We speculated that the presence of tumor microenvironment’s elements such as stromal cells (O-ASCs) assist utilization of stored LCFAs. So, we designed the experiment in which ovarian cancer cell lines directly interact with O-ASCs while treating with LCFAs. Our data (Figure 4-8 A) depicts that O-ASCs induce growth rates of ovarian cancer cells. The elevated growth rates were assumed to be because of the releasing of the previously stored fatty acids. Thus DEUP (diethylumbelliferyl phosphate) was utilized to inhibit lipolysis in cancer cells which showed to inhibit elevated viability of cancer cells induced by O-ASCs (Figure 4-8 A).

O-ASCs-conditioned media (CM) also has shown to increase the respiration rates of ovarian cancer cells treated with LCFAs. As shown in
Figure 4-8 B, O-ASCs shift the central energy metabolism of ovarian cancer cells from glycolysis to more OXPHOS by inducing β-oxidation of the released LCFAs. Triggered cancer respiration rates caused by O-ASCs’ CM was inhibited with DEUP treatment (Figure 4-8 C). DEUP targets O-ASCs-induced lipolysis in ovarian cancers and decreases OCR levels (Figure 4-8 C). 2-Deoxy-D-glucose (2DG), the glucose homologue that blocks glycolysis, was utilized to inhibit glycolysis. The cells had higher OCR levels upon 2-DG treatment to compensate the loss of energy production by glycolysis pathway. The increase is more pronounced in cancer cells exposed to O-ASCs’ CM without DEUP since they acquire higher β-oxidation. Furthermore, O-ASCs induce the ovarian cancer cells mRNA expression of CPT-1 which is the key enzyme in the transportation of LCFAs into mitochondria as shown in Figure 4-8 D. O-ASCs modulate the growth rates of LCFAs-pre-treated cancer cells by promoting the lipolysis pathway. ERLIN2 (endoplasmic reticulum lipid raft-associated 2) expression has been shown to be correlated with the amount of cellular stored lipid droplets [182]. And it is interesting our results show that O-ASCs’ CM decrease mRNA expression of ERLIN2 in ovarian cancer cells by inducing LD depletion (Figure 4-8 D).
Figure 4-8. O-ASCs induce utilization of stored LCFAs in ovarian cancer cells. (A) Viability of ovarian cancer cells were measured upon their treatment with LCFAs and DEUP for 24 hours. (B and C) Respiration of LCFAs-treated ovarian cancer cells were monitored with and without O-ASCs' CM. DEUP was added to
inhibit lipopase for 24 hours before OCR was measured. (D) CPT-1 and ERLIN2 mRNA expression was measured in the presence of RPMI or RPMI conditioned with O-ASCs.

4.4. Stimulation of lipase induces utilization of LDs

As mentioned in section 1.2.6.7, activation of adenylate cyclase (AC) increases cAMP and thus activate protein kinase A (PKA). PKA phosphorylates HSL, resulting in translocation of HSL from the cytosol to the lipid droplet (LD) and increased lipolysis. The rate limiting enzyme in lipolysis is hormone-sensitive lipase (HSL). Forskolin (FSK) has been shown to induce lipolysis by stimulating cAMP [183]. It has been extensively utilized to activate lipolysis cascade and to induce the release of cellular stored fat [184].

Cancer cells were pre-treated with LCFAs and exposed to FSK or DEUP to study the effect of stimulation and inhibition of lipolysis on the utilization of exogenous LCFAs. Cancer cells acquired higher viability levels when lipolysis was induced by FSK while DEUP reduced the amount of viable cells (Figure 4-9 A). Etomoxir (ETO, CPT-1 inhibitor) has been shown to significantly inhibit FSK-induced viability of cancer cells (Figure 4-9 B) [185]. ETO targets β-oxidation of the released LCFAs and causes a drop in levels of viable cancer cells.

The effect of FSK on the release of LCFAs were studied. The cells were pre-incubated with LCFAs for 48 hours to accumulate adequate LDs and then were treated with FSK. Our results illustrate that storage of LCFAs is a reversible phenomenon and the cellular LD content diminishes if the cells are deprived of exogenous LCFAs. The depletion of LD happened significantly faster with FSK treatment (Figure 4-9 C). FSK also induces the oxygen consumption rates of ovarian cancer cells which are pre-treated with LCFAs as it is shown in the Figure 4-9 D. Liberated LCFAs induce β-oxidation in ovarian cancer cells and stimulate the respiration rates.
Figure 4-9. Lipase stimulation induces utilization of exogenous LCFAs.

(A) Ovarian cancer cells treated with LCFAs were exposed to either DEUP or Forskolin (FSK) for 48 hours before measuring cells’ viability. (B) Cancer cells were treated with LCFAs, FSK, Etomoxir (ETO), or their combinations for 48 hours before viability measurement. (C) Cancer cells were seeded in 96-well plate and incubated until they attached to surface. The media was changed to LCFAs, DEUP, FSK, or their combinations for 24 hours. On the day of assay execution, the cells were washed with PBS two times and fixed with 1% gluteraldehyde for 20
minutes. Next, the cells were stained with AdipoRed® according to the manufacturer’s protocol. The stained area was quantified with ImageJ and Hoechst 33343 staining was utilized to normalize the results. (D) Ovarian cancer cells were incubated with LCFAs for 48 hours and FSK was injected through Seahose cartridge injection ports before OCR levels were monitored.

4.5. O-ASCs makes ovarian cancers chemoresistant via lipolysis stimulation

Our results in the previous section illustrate that inhibition of lipase targets induced viability of ovarian cancer cells caused by LCFAs. Our aim in this section is to study whether combinatory drug treatment arrest the LCFAs-induced growth rates of ovarian cancer cells. Inhibition of lipolysis of stored LCFAs induces chemo resistance of ovarian cancer cells pre-treated with LCFAs.

We utilized paclitaxel which is the most common chemo drug for ovarian cancers. We tested the effect of lipolysis on ovarian cancers’ response to chemo by incorporating two drugs: DEUP for the inhibition of lipolysis pathway and Forskolin for the induction of LD release. The single black bar represents the cells with RPMI in Figure 4-10. The first grouped white bars next to RPMI, are showing the effect of the drugs alone. We utilized DEUP and FSK in the range that does not affect the viability of ovarian cancers although paclitaxel was implemented at 100nM which cause slight decrease in viable cells. The effect of DEUP and FSK was studied when cancer cells were pretreated with LCFAs and as a result lipid droplets were accumulated. As previously shown, FSK induced viability of pretreated cancer cells while DEUP blocks the lipolysis in pretreated cancer cells and brings the levels of viable cells to RPMI.

It is noteworthy to mention that pretreated cancer cells acquire higher viability when treated with PAC compare to their counterparts without pretreatment with LCFAs. In other words, lipolysis of accumulated lipid droplets induce resistance of cancers to chemo drugs. This phenomena is emphasized
when FSK was used. However, the levels are much higher than the control RPMI which is counterintuitive. The last two grouped bars from the left is depicting the effect of FSK and DEUP on the response of cancer cells to PAC. DEUP and PAC together reduces viable cells when compared to only PAC treatment because of lack of utilization of accumulated lipid droplets. DEUP did not recover the levels of viable cells to those in PAC only (without pretreatment of LCFAs). FSK reduces chemo response of ovarian cancers to PAC since it liberates more of stored lipids.

Figure 4-10. Induced Chemiresistance of cancer cells caused by O-ASCs through stimulation of lipolsysis
4.6. FAS inhibition induces utilization of stored LCFAs

Fats are known to be tumor promotive because they can generate energy and supply building blocks for rapid proliferation of cancer cells. It is imperative that extra fats can supply cancer cells which are deprived of ample nutrients. Fast growth of tumors does not allow uniform angiogenesis and as a result shortage of nutrients exists in the tumor microenvironment. Cancerous cells alter their metabolism to survive in such harsh environment [186]. They are known to have higher endogenous synthesis of fatty acids, FAS has been shown to be overexpressed in many tumors. So it is logical to test the effect of exogenous LCFAs on ovarian cancer cells when they are deprived of endogenous pool.

In our experiments, we supplemented the cells with LCFAs and complete culture media (RPMI) for 48 hours before inhibiting FAS with Orlistat. We used the cells in complete media as a control. Interestingly our results show that after two days, pretreated cancer cells acquire higher viabilities compared to the cells without any LCFAs exposure when they are treated with Orlistat (Figure 4-11). Orlistat inhibits endogenous synthesis of LCFAs and ovarian cancers are prompted to utilize their exogenous fat resource which is stored as LDs. And as a result the cells without LD contents have reduced viability levels. The cells in complete RPMI also benefit from exogenous LCFAs source but only after day 3 (Figure 4-11). It can be inferred that cancer cells store LCFAs so they can be utilized when the cells do not access to enough LCFAs.
Conclusion

Altered metabolism is a hallmark of cancer cells which is believed to assist the cells to better adapt to their harsh environment [187]. Elevated fatty acid synthesis is one of the metabolic reprogramming that occurs during tumorigenesis. Inhibition of the key lipogenic enzymes targets the growth of tumor cells and impairs their survival. However, the contributions of extracellular fats in cancer progression are not well-studied. In this chapter we shed light on the metabolic regulation of ovarian cancers induced by exogenous LCFAs. Our results show that ovarian cancer cells store dietary LCFAs as LDs (Figure 4-3).

We characterized the LDs with neutral lipid staining as well as phospholipid probes. LDs are composed of a neutral lipid core and a layer of phospholipids [188]. Our findings proved that tumor microenvironment-secreted factors are necessary for utilization of stored fats in ovarian cancer cells (Figure 4-8). It is concluded from section 4.3 that adopting the right model is necessary to study cancer lipid metabolism. We also developed a model in which cancer cells are directly contacting O-ASCs and treated with LCFAs. O-ASCs induce lipolysis of stored fat and Inhibition of lipase with DEUP targets the induced viability of cancer cells caused by LCFAs (Figure 4-9). Our results also suggest a combinatory drug for ovarian cancer treatment. Concurrent treatment
of ovarian cancer cells with DEUP and paclitaxel increases sensitivity of cells to
the chemo drug when cancer cells have access to extracellular fats. Inhibition of
lipase does not allow the cells to convert the stored neutral lipids to usable forms
and limits the cells from using extra nutrients.

Interestingly we found that cancer cells utilize their stored source of
LCFAs when they are deprived of endogenous fat synthesis. LCFAs are directed
to biosynthesis of membrane and signaling molecules and promote tumor
progression [189]. To further explore this phenomenon, DEUP can be added to
the culture medium to inhibit not only the endogenous synthesis but also the
release of stored fat. The results may lead to the development of therapeutics for
ovarian cancer cells which are shown to have high metastasis in omentum which
is a fatty pad [190]. Ovarian cancers are known to be highly regulated by lipids
and study of their altered fat metabolism is crucial.
Chapter 5

Future missions

Ovarian cancer is among the most lethal gynecological malignancy worldwide, with most of the disease detected at later stages. The disease has been studied through proteins- and genes-involved pathways and extensive effort has been spent to explain the development and progression of ovarian cancer based on upregulation aforementioned pathways. However, understanding the metabolic regulation and changes in metabolism involved in ovarian cancer is lacking, and this understanding may lead to development of next generation of therapeutics.

5.1. The crosstalk of NO and lipid metabolism

During the past years of my research, I focused extensively on studying the effect of fatty acids on ovarian cancers’ metabolism. Ovarian cancers are drastically impacted by obesity-derived factors such as abundant source of lipids. Also, our recent results have shown the importance of NO in the modulation of ovarian cancer metabolism. We have shown that NO stimulates glucose uptake and lactate secretion of cancer cells. Upregulated glycolysis may be the direct result of diminished OXPHOS capacity of tumor cells caused by constitutive NO. There is similar evidence that NO triggers glucose uptake in skeletal muscle by stimulating the translocation of GLUT4 which is the key enzyme in cellular
glucose transport [191]. However, the effect of NO on lipid metabolism has to be further studied.

It is a controversial debate in the body of literature regarding the effect of NO on fatty acid metabolism. For example, it has been shown that NO affects the release of free fatty acids (FFAs) and more uptake of FFAs was observed during the inhibition of NOS. The hypothesis was further supported when higher respiration rates were observed after NOS inhibition which was believed to be due to the higher fatty acid oxidation rates. These findings suggested that NO downregulates fatty acid metabolism in skeletal muscle [191].

In another study, it has been shown that NO can potentially mediate fatty acid oxidation [192]. NO specifically modulates LCFAs oxidation by depleting them; NO reacts with lipid-derived radicals and causes a termination in fatty acid peroxidation cascade of reactions which is a common effect of LCFAs [193]. Homeostasis of NO and LCFAs are two crucial factors in regulations of inflammatory responses [194]. The biological half-life of NO is less than a minute; however, it is long enough to penetrate hydrophobic milieu of cell membrane and bilayers of lipoproteins because of its small size and lipophilic nature.

I have measured the cellular NO levels upon LCFAs treatment and the results have interestingly shown that LCFAs attenuate NO levels in ovarian cancers (Figure 5-1). It was hypothesized that exogenous LCFAs absorbed cellular NO when being stored as LD in cytoplasm of ovarian cancer cells. As a result, LDs are not only the reserved source of neutral and phospholipids but also nitrogen. When LDs are released, the NO content can exert its metabolic modulation [195]. Nitrated lipids are also a class of cell signaling molecules and understanding the role of these molecules in inflammation and metabolic regulation needs deep characterization of the fatty acids modified by nitro group. This area of research has not been explored in detail and requires much attention. It may be a key factor in explaining the regulation of fat tissues on
ovarian cancers and in the bigger picture the effect of obesity on hormonal sensitive cancers.

Figure 5-1. LCFAs-reduced cellular NO levels. Cancer cells were seeded in 6-well plate and incubated until they attached to surface. Next, the media was changed to RPMI or RPMI containing 100µM LCFAs for 24 hours. On the day of assay execution, the cells were washed with ice cold PBS and cell layer was collected as treated for NO measurement with Sievers NO Analyzer.

NO also alters the synthesis of endogenous fatty acids and has been characterized to change the fate of FAPs which are the population of mesenchymal fibro-adipogenic progenitors residing in the muscle [196]. NO affects adipogenesis of FAPs by modulating cGMP-independent inhibition of PPARγ [197] which is discussed extensively in the section 1.2.6.6. By inhibiting PPARγ, NO targets the formation of adipocytes and fibrous tissues and prevents muscular dystrophy, and can thus be potentially targeted therapeutically. Moreover, NO-donors have been shown to reduce the adipocyte markers such as perilipin, adiponectin, and FABP4 (fatty acid binding protein 4) [198].

In conclusion, there is a missing link between lipid metabolism and NO. It is necessary to investigate the reciprocal effect of constitutive NO and altered lipid metabolism of ovarian cancers which are usually diagnosed when infiltrated into fat pad, omentum with high expressions of NOS.
5.2. The link of obesity and ovarian cancer

It is estimated that 97 million adults in the United States are overweight or obese, a condition that substantially raises their risk of morbidity from various diseases such as hormonal cancers including ovarian, endometrial, breast, and prostate [199].

Overweight is described as a body mass index (BMI) of 25 to 29.9 kg/m$^2$ and obesity as a BMI of 30 kg/m$^2$. Body mass index (BMI, also called Quetelet’s index) is defined as weight (in kilograms) divided by height (in meters squared) [200].

Obesity is a complex multifactorial chronic disease that evolves from an interaction of genotype and the environment. More than half of adults in the United States [201] and growing percentage of the population worldwide [202] are overweight or obese. Increasing rates of obesity is not limited to the US and other wealthy industrialized countries, developing countries are suffering from the growth in their obese populations. In 2003 obesity was accounted for 14% of all cancer deaths in men and 20% of those in women in the United States [203]. Fat storage expands as the intake of calories exceeds their expenditure. There are several factors involved such as urbanization, calorie-intense food, palatable foods, lack of adequate physical activity which leads to energy balance disruptions and increase adiposity in the population.

The impact of obesity on cancer development and progression has been underestimated when compared to its cardiovascular impacts. The risk of endometrial cancer and breast cancer after menopause is much higher for overweight women because of the elevation in estrogen levels. Several studies suggest that there is a correlation between the increased adiposity and the death incidence from various cancers such as ovary, prostate, pancreas, colon, rectum, esophagus, kidney, gallbladder, cervix, liver, and certain hematopoietic cancers [204].
Single gene mutations rarely account for obesity and obesity is the result of the interaction of multiple genes and further interactions with environmental factors such as energy availability, and physical activity. A survey of literature indicates the crucial roles of about 15 genes on obesity including common polymorphisms in the β-adrenergic receptors (β-AR2, β-AR3), the peroxisome proliferator-activated receptor gamma (PPARγ), uncoupling protein genes (UCP-1, UCP-2, and UCP-3), and tumor necrosis factor alpha (TNFα) [205]. Leptin is a hormone secreted by adipocytes that acts in the hypothalamus to inhibit food intake and is known to affect obesity in humans. However, not enough evidence is available to prove the singular role of leptin pathways in human energy homeostasis. Body weight has significant impacts on metabolic symbiosis and may subsequently induce cancer risk; in particular, on circulating levels of peptide and steroid hormones and their binding factors. Some factors could depend on gender and menopausal status in women.

There is a linear correlation between the blood insulin levels and increased BMI in both men and women. Insulin command the uptake and usage of glucose in peripheral tissues. Calorie-intense food and weight gain reasons in tissues with insulin resistance. Insulin-like growth factors (IGFs) are mitogens that modulate energy deponent cell growth [206]. IGF-I stimulates cell proliferation and inhibits IGF-I stimulates cell proliferation and arrests apoptosis and has been depicted to significantly affect various cancer cell lines. Moreover, insulin and IGF-I modulates synthesis and bioavailability of sex steroids which are the key player in the initiation and progression of hormonal cancers.

Persistent high blood insulin level inhibits hepatic synthesis of sex hormone-binding globulin (SHBG) which then increases bioavailable androgens and estrogens unbound to SHBG. The unbound portion of hormones determines the actual biological activity of androgens and estrogens which affect the growth, differentiation and function of many tissues in both men and women [207]. Adipose tissue alone also can increase the production of sex hormones through the aromatization of androstenedione to estrone [208].
Furthermore, emerging evidence indicates that adipose tissue is a functional endocrine source which generates various adipocytokines or adipokines [209]. The imbalance of cellular levels of adipocytokines may cause the development of obesity related metabolic disorders such as cancer. Adiponectin and omentin are adipokines which are secreted by visceral adipose tissues and has been shown to affect the growth of tumors [209, 210].

We revealed that O-ASCs-secreted arginine could rescue reduced proliferation rates of ovarian cancers caused by arginine deprivation [125]. Notably, overweight O-ASCs were able to rescue the reduced proliferation of cancer cells more than their lean counterparts. However, more studies are required to shed light on the role of obesity in ovarian cancer development and progression. The impact of obesity in ovarian cancer initiation and progression has been studied and further studies are needed to explore the role of NO metabolism in this modulation [211].

5.3. Omentum the hospitable site for other hormonal cancers

A large fat pad that extends from the stomach and covers the intestines supplies nutrients and induces the spread and growth of ovarian and prostate cancer [190, 212]. However, the reason of omentum being metastatic site for neoplasms is unknown. Omentum contain fat cells which can be the extra energy source for the lethal spread of tumors. Recent studies have shown that omentum contains a population of stem cell-like cells such as adipose stromal cells (ASCs) that engraft in tumors and encourage cancer progression [8]. Omentum-derived ASCs (O-ASCs) have been demonstrated to contribute to the formation of a hospitable environment for the development of ovarian cancer metastasis [8, 213].
Figure 5-2. O-ASCs stimulate ovarian cancers progression. O-ASCs supply cancer cells with the pool of L-arginine for NO synthesis. The conversion of L-arginine into citrulline is through nitric oxide synthase (NOS). Moreover, secreted citrulline by cancer cells is consumed by O-ASCs and enhances adipogenicity of O-ASCs. The generated NO may induce s-nitrosylation of metabolic enzymes and may alter ovarian cancer’s metabolism.

Previous studies have shown that O-ASCs enhance proliferation and migration of ovarian cancer cells while reducing their response to chemotherapy and radiation [8]. However, the mechanistic underpinnings of O-ASCs’ role in tumor progression and growth are still unclear. Recently we demonstrated that nitric oxide (NO)-mediated metabolic coupling between O-ASCs and ovarian cancer cells contributes to ovarian cancers’ growth and increased chemo sensitivity [125]. The future plan is to investigate the link between omentum and other hormonal tumors such breast and prostate cancers to shed light on the exclusive impact of omentum on ovarian cancers. Recent studies demonstrated
that extra depots of nutrients such as arginine and fatty acids from omentum is the principal incitement of ovarian cancer metastasis. However, other types of hormonal cancers marginally metastasize in the omentum and the incidents are isolated. For instance, risk of prostate cancer metastasis in the omentum is not as high as ovarian cancers [212]. Investigating the discrepancies in metabolic characteristics of ovarian and prostate cancers may result in pinpointing the metastatic motivation of ovarian cancers [214]. Male and female-originated omentum tissues may assist to characterize undiscovered factors impacting ovarian cancer metastasis but no prostate cancers. Omentum-derived hormones and growth factors such as omentin may be the key metastatic factor. The metabolic regulation caused by omentin which as chemokine secreted by the oemtum fats needs to be further explored. Even as new mechanisms implicating omentum for ovarian cancer metastasis keep emerging, many aspects of its biology still remain to be unveiled [215].
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Elongation and desaturation of fatty acids

Further elongation and desaturation of newly synthesized fatty acids occurs within the endoplasmic reticulum membrane. For instance longer chain fatty acids compared to palmitate are made through the addition of carbons to the end of the chain in each cycle of reactions. The reaction is catalyzed by “elongation of very long chain fatty acid protein”. The protein family includes seven members responsible for different chain length (ELOVL 1 to 7). ELOV7 has been shown to be over expressed in prostate cancer cells maybe because of synthesis of steroids such as androgen.

Desaturation steps are activated by fatty acid-CoA desaturases (SCDs). SCD1 adds a double bond in the Δ9 position if palmitate and stearate to produce monounsaturated fatty acids. Fatty acyl-CoA desaturases are responsible for synthesis of highly unsaturated fatty acids from essential polyunsaturated fatty acids. Physical properties of unsaturated fatty acids influence synthesis of membrane phosphoglycerides which identify cell membrane fluidity.
Appendix 2

Tumor-associated fatty acid synthase

In the first proposed mechanism, growth factors (GFs) and growth factor receptors (GFRs) are the primary modulators of FASN at transcriptional level. EGF receptors (EGFRs) also stimulate FASN expression. The cascade of this mechanism is shown in figure 9. Tumor FASN over expression also occurs at post transcriptional level for instance, there is a lack of correlation between FASN amplification or transcription and FASN protein expression. In prostate cancer cells, it has been shown that USP2a, a preproteasomal ubiquitin-specific protease strongly stabilizes FASN. The figure 9 illustrates the mechanism thoroughly.

General alteration in the genetic program controlling lipogenesis is the most likely reason for FASN over expression in cancer cells. This deregulation in FASN expression is just an indication of major alteration in upstream regulatory circuits [47, 48].
Figure A. 1. Regulation of tumor-associated FASN expression.
This figure is adapted from [49]. (a) On the surface of tumor cells, growth factor (GF)-dependent or independent auto phosphorylation of receptor tyrosine kinases (RTKs) such as the GF receptors (GFRs) epidermal growth factor receptor (EGFR, also known as ERBB1) and ERBB2 (also known as HER2) leads to downstream activation of phosphatidylinositol-3 kinase (PI3K)–Akt and extracellular signal-regulated kinases (ERK1 and ERK2) signal transduction cascades. Steroid hormones (SH) including oestradiol (E2), progestins (P) and androgens (A) bound to their corresponding SH receptors (SHRs; ER, PR and AR, respectively) can also trigger identical transduction mechanisms. These ultimately stimulate fatty acid synthase (FASN) expression through the modulation of the expression and/or nuclear maturation of the transcription factor sterol regulatory element-binding protein 1c (SREBP1c), which binds to and activates sterol regulatory elements in the promoter region of FASN. Cross-talk
between GFs–GFRs and SHs–SHRs converging on PI3K–Akt and mitogen-activated ERK kinase (MEK)–ERK cascades amplify the responses of FASN expression in hormone-responsive cancer cells. (b) Tumor-associated FASN overexpression can also be achieved at the post-translational level through interaction with USP2a, a preproteasomal ubiquitin-specific protease that, by removing ubiquitin from FASN, strongly stabilizes the enzyme. It should be noted that these two pathways regulating FASN might concurrently take place in tumor cells.
FASN inhibition as a cancer therapy

The difference between the expression and activity of FASN in normal and cancer cells makes this enzyme as an appropriate target for cancer therapy. Pharmacological inhibition of FASN preferentially impairs cancer cells and halts the growth of tumors. There are several mechanism proposed to explain anti-tumeric effect of FASN inhibition.

FASN supports the synthesis of phospholipids by providing adequate primary substrate. FASN inhibition impairs the ability of cells to generate enough phosphatidycholine which are membrane building blocks for highly-proliferative cells such as cancers which results an apoptosis. FASN depletion interferes with phospholipids production and alters lipids rafts and localization of tyrosine kinase receptors (such as epidermal growth factor receptor, EGFR). FASN deletion arrest cell cycle mainly through activation of p53. FASN inhibition results an accumulation of malonyl-CoA which then assists carnitine palmitoyl-transferase-1 (CPT-1) inhibition and diminish β-oxidation thus pro-apoptotic genes are induced [50].

FASN is a complex multifunctional enzyme consisting of two identical multifunctional monomers [44]. Each of FASN peptides has seven catalytic domains: β-ketoacyl synthase (KS), malonyl/acetyltransferase (MAT), dehydrase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP) and thioesterase (TE). FASN forms an x-shape structure and all of his active sites are present to bind to designed drugs. FASN monomers should be bind as a dimer for palmitate synthesis.
There are several FASN blockers that are designed to be specific. Cerulenin was one of the first FASN inhibitors that have been used for breast cancer. Cerulenin isolated from *Cephalosporium caerulens* and it includes an epoxy group that binds to β-ketoacyl synthase domain (KS) of FASN and has demonstrated promising antitumor activity. Its cytotoxicity effect depends on FASN activity. Cerulenin has a reactive epoxy group which makes it chemically unstable and it is its drawback [51].

C75 was synthesized based on cerulenin structure just without epoxy group in order to resolve the instability issue. C75 reacts with FASN irreversibly by binding to β-ketoacyl synthase (KS), enoyl reductase (ER) and thioesterase (TE) domains. Like cerulenin, C75 also have been proven to cause significant implications on food intake and body weight in mice. Upregulation of palmitoyl transferase-1 (CPT1) in mitochondria by FASN inhibition seems to be responsible for stimulation of fatty acid oxidation and weight loss [51].

Orlistat has been used as a rather selective to inhibit FASN. Orlistat binds to thioesterase (TE) function of the enzyme, interferes with cellular fatty acid synthesis and subsequently halts tumor cell proliferation and induces tumor cell apoptosis [52].
Figure A. 2. Fatty acid synthase and its inhibitors.
FASN is a complex multifunctional enzyme consisting of two identical multifunctional monomers. Each of FASN peptides has seven catalytic domains: β-ketoacyl synthase (KS), malonyl/acetyltransferase (MAT), dehydrase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP) and thioesterase (TE). FASN forms an x-shape structure and all of his active sites are present to bind to designed drugs. Cerulenin has an epoxy group that binds to β-ketoacyl synthase domain (KS) of FASN and demonstrates promising antitumor activity. C75 was synthesized based on cerulenin structure just without epoxy group in order to resolve the instability issue. C75 reacts with FASN irreversibly by binding to β-ketoacyl synthase (KS), enoyl reductase (ER) and thioesterase (TE) domains. Orlistat binds to thioesterase (TE) function of the enzyme, interferes with cellular fatty acid synthesis and subsequently halts tumor cell proliferation and induces tumor cell apoptosis. Triclosan blocks FASN activity by specifically inhibiting the ER domain. Epigallocatechin-3-gallate (EGCG) is the main component of green tea acts similarly to cerulenin and C75 by blocking FASN KS domain.
Lipid classification

Lipids are organic compounds that contain hydrocarbons which are the foundation for the structure and function of living cells. Lipids are non-polar so they are soluble in non-polar environments thus not being water soluble because water is polar. Most naturally occurring fatty acids contain an even number of carbon atoms and are un-branched. Lipids are classified into eight main categories according: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol Lipids, prenol Lipids, saccharolipids, and polyketides.

Fatty acids

Fatty acids are carboxylic acids (or organic acid), often with a long aliphatic tails (long chains), either saturated or unsaturated. Fatty acids are building blocks of phospholipids and glycolipids. These amphiphilic molecules are important components of biological membranes. Many proteins are modified by the covalent attachment of fatty acids, which targets them to membrane locations. Fatty acids are fuel molecules. They are stored as triacylglycerols (also called neutral fats or triglycerides), which are uncharged esters of fatty acids with glycerol. Fatty acids mobilized from triacylglycerols are oxidized to meet the energy needs of a cell or organism. Fatty acid derivatives serve as hormones and intracellular messengers. Fatty acids are classified based on the number of double bond in their chain.

Long chain fatty acids are critically vital for cellular homeostasis as they are involved in a wide variety of processes including phospholipid synthesis,
protein post-translational modifications, cell signaling, membrane permeability, and transcriptional modifications.

**Saturated fatty acids**

Saturated fatty acids have no carbon-carbon double bonds but they have at least one carbon-carbon double bond. Saturated fatty acids have higher melting points than fatty unsaturated acids of corresponding size.

Palmitic acid or hexadecanoic acid (18 carbons) is the first fatty acid which is synthesized endogenously by cells. Palmitic acid inherited it name from palm where it is found mainly. Figure 13 shows the structure of palmitic acid.

![Figure A. 3. Palmitic acid structure](image)

**Unsaturated fatty acids**

Unsaturated fatty acids have more than one double bond. Monounsaturated fatty acids are those with only one double bond in their chain.

Oleic acid (also known as oleate) is the most common monounsaturated fatty acids in diets and it is odorless, colorless oil at room temperature. Oleic acid 18:1(n-9) has one "cis" double bonds; the double bond is located at the ninth carbon from the omega carbon (ω-9). Oleic acid is produced naturally in various animals and plants. *De novo* synthesis of oleic acid involves the enzymatic reaction of stearoyl-CoA via stearoyl-CoA 9-desaturase. In fact, one mole of stearic acid is dehydrogenated and produces oleic acid. Figure 14 illustrates oleic acid structure and biosynthesis.
Fatty acids with more than two double bonds in their backbone are named polyunsaturated (PUFA). PUFA are identified by position of the double bond nearest the methyl end (CH$_3$) of the carbon chain; this is described as omega number. Polyunsaturated fatty acids such as linoleic acid (LA; 18:2 n-6) and α-linoleic acid (ALA; 18:3 n-3) are essential in mammalian diet mainly because endogenously synthesized fatty acids can be desaturated by cells up to the Δ9 position due to the lack of specific enzymes [66]. They are vital for maintaining normal structure of tissues and membranes. Although studies have been shown that they are not required to be supplemented for cultured cells and no impaired membrane has been observed by using fat free medium [67]. The most common polyunsaturated fatty acids are linoleic and linolenic acid.

Polyunsaturated fatty acids (PUFAs) such as linoleic acid influences carcinogenesis directly or indirectly through [68]:

- Induction of oxidative stress and generation of reactive lipid peroxidation which increases DNA damage because of peroxidation of double bonds in PUFAs
- Production of short-lived hormone-like lipids which are produced from conversion of essential fatty acids into eicosanoids
- Interaction with signal transduction which introduces alterations in gene expression
Linoleic acid (also known as linoleate) is the most common polyunsaturated fatty acids which is a colorless liquid at room temperature. The word "linoleic" comes from the Greek word linon (flax) and oleic means "of, relating to, or derived from oil or olive" or "of or relating to oleic acid" because saturating the n-6 double bond of LA produces oleic acid. Linoleic acid 18:2(n-6) has two "cis" double bonds; the first double bond is located at the sixth carbon from the omega carbon (ω-6). Linoleic acid (LA) is a precursor of biosynthesis of arachidonic acid (AA) and thus some prostaglandins.

Linoleic acid is a ubiquitous component of plant lipids, and of all the seed oils such as corn, sunflower and soybean. Linoleic acid must be dietary supplemented in animal tissues. Figure 15 shows the structure of LA and its metabolism.

Endogenously synthesized long chain fatty acids (LCFAs) are then desaturated to monosaturated fatty acids (MUFAs) by stearoyl-CoA desaturase (SCD)-1 [69-71]. MUFAs are important in study of tumorigenesis because they are the major constitutes of phospholipid cell membrane and they are thought to influence cells biomechanical integrity.
Figure A. 5. Linoleic acid is a polyunsaturated fatty acid and its metabolism. The first step in the metabolism of Linoleic Acid (LA) is performed by Δ-6-desaturase, which converts LA into gamma-Linolenic acid (GLA). Next, GLA is converted to Dihomo-gamma-linolenic acid (DGLA), which in turn is converted to Arachidonic acid (AA). One of the possible fates of AA is to be transformed into a group of metabolites called eicosanoids, a class of paracrine hormones.

**Glycerols**

Triacylglycerols are non-polar complex lipids that act as energy reserves. These lipids are triesters formed from three fatty acids esterified to the three hydroxyl groups of glycerol. Natural fatty acids found in plants and animals
are typically composed of even numbers of carbon atoms, reflecting the pathway for their biosynthesis of their fatty acids from the two-carbon building-block acetyl CoA. One of the very important functions of triglycerides is their conversion into phospholipids which contribute to the structure of membranes by the formation of a lipid bilayer. They also provide energy for cellular activity.
Immune cells

Monocytes /macrophages, neutrophils, and lymphocytes are part of the tumor stroma but macrophages are abundantly included in tumor microenvironment. Monocytes differentiate into tumor-associated macrophages (TAMs) upon recruitment of precursors (CCL2 chemokine induced by cancer cells is a key factor). TAMs are included in necrotic and hypoxic conditions where they upregulate hypoxia-induced transcription factors (HIFs). Macrophages are the main secretors of promoting vascularization factors including VEGF, HGF, and IL-8 in ovarian, breast, and leukocytes cells. Furthermore; TAMs remodel the ECM through expression of MMPs.

Vascular cells

VEGF is the major stimulator of vessel formation in tumors and it is released by cancer cells, fibroblasts, and inflammatory cells in the stroma. Not-organized blood vessels introduce higher levels of VEGF, hypoxia, acidosis, and increased interstitial fluid pressures to malignant tissues.

Cytokines and growth factors along with inflammatory species

Cytokines and growth factors are important factors that influence cancer proliferation directly or promote carcinogenesis by modulating the phenotype and function of cells and their microenvironment. They are released into stroma by stromal cells as well as cancer cells [78]. The main factors are
• Transforming growth factor-β (TGF-β) is known the most immunosuppressive factor that affects proliferation, invasion of cancer cells. It also induces the migratory capacity of cancer and makes them more metastatic [79].

• Tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) which are stimulated by hypoxia (the main feature of cancer cells). Both cytokines promote each other and also themselves, making an established pro-inflammatory loop. IL-1 family increases the levels of cancer-promoting genes including, cyclooxygenase-2 (COX-2), the inducible nitrogen oxide synthase (NOS) and also cytokines like MMPs. IL-2 family also increases angiogenesis and invasiveness [80].

• Stromal cell derived factor-1 (SDF-1)
• Secreted protein acidic rich in cysteine (SPARC)
• Matrix metalloproteinases (MMP)
• Vascular endothelial growth factor (VEGF)
• Hypoxia-induced transcription factors-1α (HIF-1α)
• Reactive oxygen species (ROS), reactive nitrogen species (RNS) that are produced by activated immune cells in tumor microenvironment. ROS and RNS promote inflammation and introduce DNA damage that leads to more errors replication and genomic instability [81]. Reactive nitrogen species condemn the expression of p53 (protein 53, tumor suppressor) and nitrogen oxides improve migration, invasion and metastasis of breast cancer cells by promoting MAPK pathway [82].
Adipogenic transcriptional cascade

There are several transcription factors which have been identified to regulate adipocyte differentiation. Nuclear peroxisome proliferator-activated receptor γ (PPARγ) and members of CCAAT/enhancer binding protein (C/EBP) family are the most influential transcription factors in adipogenesis. They are shown to be stimulators of adipocyte-specific genes and improver of growth arrest.

PPARγ is known as the master regulator of adipogenesis and sufficient to stimulate adipocyte differentiation in fibroblasts [84]. No other factors have been found to induce differentiation of adipocyte in the absence of PPARγ. Despite all the effort no endogenous ligand has been investigated for PPARγ. In mouse model (3T3-L1) cells, cyclic AMP-dependent ligand improves differentiation in the first two days although it is temporary and does not maintain. Transcription factors sterol response element-binding protein-1c (SREBP1c) has been demonstrated to stimulate synthesis of PPARγ ligand [85, 86]. PPARγ is necessary not only for initiation of differentiation but also for maintenance of differentiation in adipocytes.

Several C/EBPs are involved in adipogenesis including C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and CHOP (transcription factor similar to CCAAT-enhancer binding protein). Activation of C/EBPβ and C/EBPδ results the induction of C/EBPα which in turn stimulates many adipocyte genes directly although the affectivity is negligible in the absence of PPARγ.

KLFs are substantial regulators of adipogenesis as well. The KLFs are from C2H2 zinc-finger protein families that regulate apoptosis, proliferation and
differentiation. KLF15 was the prominent family member which induces
differentiation of adipocytes through stimulation of insulin-dependent glucose
transporter-4 (GLUT4). KLF5 is also regulated by C/EBPβ and C/EBPδ during
adipogenesis. KLF5 stimulation then increases the activity of PPARγ2 promoter.
On the other hand KLF2 and KLF7 have contrary effect and they repress
differentiation. So it could be concluded that a replacement of KLFs takes place
during differentiation. KLF2 and KLF7 are displaced with KLF5 and KLF15 [87].

Adipocyte determination- and differentiation-dependent factor-1/ sterol
regulatory element-binding protein-1 (ADD-1/SREBP-1) is stimulated in early
differentiation which induces many regulatory genes such as C/EBPβ and
C/EBPδ and PPARγ.

GATA-binding and forkhead families are repressor transcription factors.
They do not bind to DNA directly but their effect is through protein-protein
interactions.

Figure A. 6. The adipogenic transcriptional cascade.
Nuclear peroxisome proliferator-activated receptor γ (PPARγ) and members of
CCAAT/enhancer binding protein (C/EBP) family are the most influential
transcription factors in adipogenesis. They are shown to be stimulators of
adipocyte-specific genes and improver of growth arrest. Transcription factors sterol response element-binding protein-1c (SREBP1c) has been demonstrated to stimulate synthesis of PPARγ ligand. Several C/EBPs are involved in adipogenesis including C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and CHOP (transcription factor similar to CCAAT-enhancer binding protein). Activation of C/EBPβ and C/EBPδ results the induction of C/EBPα which in turn stimulates many adipocyte genes directly although the affectivity is negligible in the absence of PPARγ. KLFs are substantial regulators of adipogenesis as well. KLF15 was the prominent family member which induces differentiation of adipocytes through stimulation of insulin-dependent glucose transporter-4 (GLUT4). KLF5 is also regulated by C/EBPβ and C/EBPδ during adipogenesis. KLF5 stimulation then increases the activity of PPARγ2 promoter. On the other hand KLF2 and KLF7 have contrary effect and they repress differentiation. Adipocyte determination- and differentiation-dependent factor-1/ sterol regulatory element-binding protein-1 (ADD-1/SREBP-1) is stimulated in early differentiation which induces many regulatory genes such as C/EBPβ and C/EBPδ and PPARγ. GATA-binding and forkhead families are repressor transcription factors. They do not bind to DNA directly but their effect is through protein-protein interactions.

Hormones and signal transduction pathways regulating adipogenesis

Hormones and growth factors impact adipogenesis through mediation of external growth and differentiation signals. Insulin-like growth factor-1 (IGF-1) has been demonstrated to be a promoter of adipocyte maturation.

Glucocorticoids are regulators of differentiation of adipocytes. Dexamethasone hormonally induces the activation of glucocorticoid receptor which is the homologous receptor family as PPARγ. IBMX increases the expression of C/EBPδ through cAMP dependent pathway. Adipogenesis is highly controlled by estrogen which is regulated by ovarian status.