Chapter One:
Introduction

1.1 The global epidemiology of cancer:

The incidence of cancer continues to rise world wide, especially in low-income and middle income countries. An estimated 14 million patients were diagnosed globally in 2012 with a 50% projected increase to 21.6 million annually by 2030 (Allemani et al., 2018). Therfore to reduce cancer mortality, reduction of cancer incidence and improvement of cancer survival are both necessary. Among female population, breast cancer is one of the most frequent malignancies diagnosed among women worldwide and is the second leading cause of cancer-related deaths in women (Hutchinson, 2010). The high rate of recurrence due to drug resistance and metastatic phenotype alone accounts for more than 90% of deaths (Harbeck and Gnant, 2017). This continues to pose a major clinical challenge in the successful treatment of this disease. In light of these grim statistics, extensive efforts are being made to delineate the molecular mechanisms involved in the tumorigenesis of breast cancer. However, the physiological conditions leading to the origin, metastasis and relapse of human breast carcinoma is still not well understood.

1.2 Tumor-associated inflammation: Outcome of tumor-immune crosstalk

Inflammation is an integral feature of innate immune response which is classically viewed as self-limiting, with active resolution through apoptosis and clearance of debris and immune cells. Inflammation and tissue damage/stress, if persistent, leads to a chronic, dysregulated and unresolved immune response by a feed-forward loop mechanism which actively promotes tumorigenesis (Grivennikov et al., 2010). About 20% of human cancers are related to chronic, unresolved inflammation caused by bacterial or viral infections, exposure to irritants (such as asbestos or tobacco smoke) or autoimmune diseases (Aggarwal et al., 2009). Thus, it has become evident that all tumor promoting inflammation, whether it precedes or follows tumor development, is a part of cell-autonomous response to injury and infection that is utilized by cancer cells to their own advantage. For instance, an acute tumor-directed immune responses involving cytolytic T lymphocytes appear to protect against tumor development. whereas immune responses involving chronic activation of humoral immunity, infiltration by Th2 cells, and protumor-polarized innate inflammatory cells results in the promotion of premalignant breast tissue including

early ductal carcinoma in situ (DCIS) to invasive breast carcinomas (DeNardo and Coussens, 2007). However, the underlying mechanisms of the tumor-promoting immunosuppressive actions of the innate immune system in development of breast carcinoma remains under investigated.

Clinical and experimental studies suggest that solid tumors utilize a local network of inflammatory cytokines within its microenvironment to prevent activation of immunological effector function and sustain proliferation. An increased level of several pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-10, IL-18, transforming growth factor (TGF)-β, and macrophage migration inhibitory factor (MIF) have been reported with both experimental and clinical forms invasive breast carcinomas (Lippitz, 2013). An elevated concentration of intratumoral TNF-α is associated with a higher tumor grade and lymph node metastasis (Esquivel-Velazquez et al., 2015). However, the contribution of TNF-α regulated innate immune response to the aggressiveness of solid tumors is not well understood.

1.3 Immuno-metabolic interactions in tumor microenvironment

In addition to tumor-promoting inflammation, cancer cells overcome the cell-extrinsic regulation of nutrient acquisition- a fundamental barrier for cellular transformation. A reprogramming of cellular metabolism is a direct/indirect consequence of oncogenic mutation which enable tumor cells to acquire necessary nutrients from a nutrient-poor tumor microenvironment and utilize them for biosynthesis and unchecked proliferation (Palm and Thompson, 2017). Additionally, cancer cells use the catabolic function of autophagy to tolerate nutrient stress (Kimmelman and White, 2017). Autophagy is a cellular self-degradation mechanism to produce building blocks for macromolecular synthesis and maintain energy homeostasis through intracellular recycling. An upregulated basal autophagy is observed in established tumors. Autophagy supports tumor development by providing energy substrates during periods of nutrient limitation and preserving organelle function required for cell growth. Autophagy mitigates both cellular damage and maintains energy homeostasis to promote mammary tumorigenesis (White et al., 2015).

Interestingly, the metabolic competition between tumor and immune cells for key nutrients such as glucose, represses T-cell activation and causes T-cell dysfunction (Lyssiotis and Kimmelman, 2017). Indeed, recent studies within the past decade have demonstrated that metabolism has an essential role in controlling the fate of immune cells. For instance, metabolic reprograming by rapid induction of glycolysis and intermediates to support fatty acid biosynthesis is essential for the activation and proliferation of innate and adaptive immune cells (Biswas, 2015). ***Thus, an emerging concept is that metabolism could be a direct input to the immune system and act as a signal to control the immune function of a cell. This immuno-metabolic crosstalk is emerging as an important regulator of immune cell phenotype and function in tumor microenvironment.*** Tumor cells alter this immune-metabolic interaction to promote cancer-associated inflammation and immunosuppression, firstly, by reprogramming its own cellular metabolism to sustain inflammation and secondly, by outcompeting immune cells for key nutrients of tumor microenvironment. Importantly, TNF-α has emerged as a key cytokine that influences intermediary cellular metabolism by regulating mitochondrial function and its turnover through selective autophagy (mitophagy) (Bell et al., 2013). Chronic levels of TNF-α lead to increased ROS generation and alteration in mitochondrial structure and function (Kim et al., 2010; Yuan et al., 2017). However, the role of TNF-α-regulated innate immune response in controlling mitochondrial metabolism and the extent of this crosstalk in regulating tumorigenicity of cancer cells is not well understood.

1.4 Mitochondrion: an emerging regulator of immunometabolism

Recently, mitochondria has emerged as key nutrient and immune sensor suggesting that it could participate in immuno-metabolic crosstalk. Indeed, in addition to its primary role in energy metabolism, outer mitochondrial membrane as well as MAMs (mitochondria-associated membranes) provide a molecular platform for the assembly of the signalosomes involved in innate immune pathways (Arnoult et al., 2011; Monlun et al., 2017). Mitochondria acts as a coordinating site for the innate immune regulators of several key host responses against microbial infection and sterile inflammation including apoptosis, type-I IFN and IL-1β signaling (Mills et al., 2017; West et al., 2011b). For instance, mi-

tochondrial antiviral signaling protein (MAVS) localizes at the outer membrane of mitochondria (OMM) and recruits downstream signaling proteins to activate NF-κB and type-I IFN pathways during an anti-viral response. Importantly, MAVS associates with MFN1 and MFN2 to regulate mitochondrial fusion, thereby facilitating its efficient oligomerization and downstream signaling (Mills et al., 2017). Further, COX5B, a complex IV subunit of mitochondrial respiratory chain, interacts with MAVS to control its aggregation as well as represses mitochondrial ROS generation to attenuate NF-κB activation and promote mitophagy (Zhao et al., 2012). Thus, a controlled activity of mitochondrial electron transport chain coordinated with autophagy pathway negatively regulates MAVS-mediated activation of innate immune responses.

Similarly, toll-like receptors (TLRs) engage TRAF6-adaptor protein which translocates to mitochondrial outer membrane during bacterial infection. Mitochondrial TRAF6 leads to enrichment of ECSIT, which alters the activity and assembly of complex-I to regulate the generation of ROS required for the bactericidal response (West et al., 2011a). ECSIT-induced generation of mitochondrial ROS (mtROS) is negatively regulated by antioxidant protein, PRDX6 which translocates to mitochondria and control excess ROS production (Min et al., 2017). Thus, a reorganization of complex-I structure and function is mediated through ECSIT to positively regulate mtROS generation during anti-bacterial innate immune response.

Stimulator of interferon genes (STING), also known as MITA, is a MAM-localized immune adaptor protein of anti-viral signaling (Ishikawa and Barber, 2008). STING associates with MAVS at OMM to facilitate RIG-I mediated activation of IRF3 and NF-κB, resulting in subsequent production of interferons and inflammatory cytokines such as TNF-α and IL-6 (Zevini et al., 2017). Similarly, activation of STING through cGAS (cyclic GMP-AMP synthase) also results in production of type-I IFN during DNA virus infection (Barber, 2015). STING plays an essential role in micro-managing the sterile inflammation resulting from the recognition of cytosolic DAMPs such as leaked mitochondrial DNA (mtDNA) and escape of nuclear DNA during stress conditions (Abe et al., 2013). Similarly, the engagement of NLRP3 inflammosome with mtDNA at OMM induces the activation of caspase-1 mediated autoinflammatory response via the cleavage

and secretion of IL-1β and IL-18 cytokines (Subramanian et al., 2013). ***These examples suggest that the role of mitochondria in sensing stress by the innate immune system has been co-opted as an indirect mechanism to regulate the inflammatory response and metabolism resulting from intrinsic cellular damage.*** The alteration of mitochondrial regulated innate immune responses in malignant cells to maintain the chronic inflammatory state is not well understood.

1.5 NLRX1: an innate immune receptor regulating mitochondrial metabolism

The nucleotide-binding domain and leucine-rich repeat containing (NLR) proteins are an emerging class of innate immune sensors of cytosolic PAMPs and DAMPs. A fundamental role of NLR family protein is to regulate pro-inflammatory cytokines and chemokines that drive host innate immune response to pathogens and cellular insults (Meunier and Broz, 2017). Recent studies have identified a functional group of NLRs that negatively regulates inflammation (Allen, 2014). NLRX1 is unique among these NLRs due to its mitochondrial localization and its functionally undefined C-terminal LRR domain (Xiao and Ting, 2012). Earlier reports suggested that NLRX1 function as a negative regulator of canonical NF-κB and type-I IFN signaling during acute viral infection (Moore et al., 2008; Xia et al., 2011). Subsequent studies also indicated that NLRX1 is a mitochondrial matrix protein and positively regulates ROS generation and autophagy during host antiviral innate immune response (Arnoult et al., 2009; Lei et al., 2012). Given the important role of these immuno-metabolic pathways in tumor-promoting immunosuppressive actions of the innate immune system and metabolic adaptation of cancer cell, we hypothesized that NLRX1 could be a potential regulator of immunometabolic functions which may integrate innate immune responses and mitochondrial function to regulate the tumorigenic potential of cancer cells. NLRX1 expression in different cancer type would significantly influence tumor progression and metastasis.

Here, we systematically investigated the role of NLRX1 in altering mitochondrial form/function and its turnover to regulate cell death and survival response in the presence of TNF-α in human breast cancer cells lines. We further characterized the mechanism of this regulation by NLRX1 to reveal its role in preserving the mitochondrial homeostasis of breast cancer cell lines during inflammatory stress conditions.