CHEMOPREVENTATIVE AND CARDIOTOXIC EFFECTS FROM LONG-TERM EGFR PHARMACEUTICAL INHIBITION

A Dissertation

by

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ABSTRACT

The epidermal growth factor receptor *(EGFR)* is a gene expressed in epithelial cells and is involved in a variety of diseases, including preeclampsia, intrauterine growth restriction, embryonic lethality, diabetes, cardiac dysfunction, poxvirus propagation, and tumorigenesis. EGFR is a member of the ERBB family of receptor tyrosine kinases, consisting of EGFR/HER1, ERBB2/HER2, ERBB3/HER3, and ERBB4/HER4, and is a central point in an extensive network of growth-promoting intracellular signaling pathways, making it a prominent inducer of cellular proliferation and survival. Since EGFR is often co-opted by cancer cells through up-regulation of the receptor's expression and/or activity, there are several EGFR-targeted anti-cancer therapies used in the clinic and many more currently being explored in preclinical and clinical trials. Any potential cardiotoxicity rates for such therapies are unknown. Here we demonstrate that a subgroup in the population, those at risk of dilated cardiomyopathy, may experience severe adverse effects to EGFR inhibitors. BALB/cJ male mice treated with the EGFR tyrosine kinase inhibitor AG1478 had an exacerbated increase in left ventricular mass. Our findings support the utility of using genetically diverse mouse models to identify at risk groups. We also demonstrate genetic background-dependent differences in baseline cardiac structure and function, as well as differences in cardiac aging across four mouse strains (A/J, BALB/cJ, C57BL/6J, and FVB/NJ). We explored the utility of EGFR pharmacological inhibition in cancer prevention by exposing mice to AG1478 for 16 months. This timeframe allowed the mice to develop a variety of spontaneous neoplasia, with pulmonary adenomas being the most prominent neoplasm. Our findings demonstrate that EGFR-targeted treatment was ineffective for chemoprevention in a model of spontaneous somatic mutations in genetic backgrounds with familial risks of cancer.

Although our findings suggest that EGFR-targeted treatment is not beneficial for chemoprevention in the general population, high risk individuals with specific mutations or pre-existing lesions have not been excluded as groups that could potentially benefit from such treatment.

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TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION TO THE ROLE OF MISREGULATED EGFR SIGNALING IN CANCER AND CARDIOMYOPATHY

Introduction

The epidermal growth factor receptor *(EGFR)* is a gene expressed in epithelial cells and is critical for early development as well as tissue maintenance in adults. EGFR (also known as HER1) is the prototypical member of the ERBB family of tyrosine kinases and is a central point in an extensive network of growth-promoting intracellular signaling pathways, making it a strong inducer of cellular proliferation and survival. Disruption of these complex interactions at the level of extracellular signaling (i.e. EGFR mis-regulation) is implicated in a variety of diseases, including preeclampsia, intrauterine growth restriction, embryonic lethality, diabetes, cardiac dysfunction, poxvirus propagation, and tumorigenesis. Misregulation of EGFR activity is implicated in an array of cancers, with hyperactivity observed in about one-third of carcinomas (Mendelsohn, 2002). There are several EGFR-targeted antibodies and small molecule tyrosine kinase inhibitors (TKIs) currently approved by the Food and Drug Administration (FDA) for cancer treatment. Cancer patients are typically exposed to EGFR-targeted therapy for long periods of time that can range from months to several years. Extensive data has shown that chronic exposure to other chemotherapeutics, such as anthracyclines, can induce cardiotoxicity and heart failure at alarming rates. Such

findings have stressed the importance of monitoring for cardiotoxicity of chemotherapeutics in preclinical trials.

The exact function of EGFR in the adult heart remains to be determined. Mouse models that inhibit, knockout, or overexpress downstream effectors of EGFR have hinted at potential roles in cardiac hypertrophy, fibrosis, and response to stress. Given the long-term nature of EGFR-targeted therapy and the adverse cardiac effects associated with inhibiting signaling pathways downstream of EGFR, there is a strong need to understand EGFR's role in the heart for the purposes of cardiotoxicity avoidance. This information is especially critical if EGFR inhibition were to be used in long-term preventative medicine. Studies from our group and others have shown a strong benefit to reduced EGFR signaling in mouse models of colorectal cancer. Whether it be pharmacological inhibition or the use of the *EGFR*^{wa2} hypomorphic allele, studies have reported 50-90% reductions in tumor load (Rinella & Threadgill, 2012; Roberts et al., 2002). Such findings implicate EGFR inhibitors as possible chemopreventative agents. Given the wide range of cancers with hyperactive EGFR signaling, pharmacological inhibition of EGFR may be beneficial in other cancer prevention models.

Given that hyperactivity of growth-promoting pathways often has devastating consequences on the well-being of the organism, EGFR has evolved multiple mechanisms of regulation. EGFR can be categorized into three overarching domains: the extracellular ligand binding domain, the transmembrane domain, and the intracellular kinase domain. Interactions between the different components permit EGFR to be primed for activation while conversely it can be signficiantly inhibited during times

when activity is inappropriate. Here we briefly describe the activating and inhibitory functions of each domain and the ways that these functions are dysregulated in cancer cells. This review emphasizes that a detailed understanding of how cancer exploits EGFR is key to tailoring treatments and informative for the design of prevention studies. We examine why different EGFR-targeted treatments have had varying levels of success in treating cancers and the implications that such therapies might have on cardiovascular health as we integrate several cardiology studies involving the EGFR signaling network in an attempt to elucidate the potential functional roles of EGFR in the adult heart.

Availability of EGFR ligands

There are seven known growth factors that bind to EGFR's ligand binding domain: epidermal growth factor (EGF), heparin binding EGF-like growth factor (HB-EGF), transforming growth factor-α (TGFα), amphiregulin, betacellulin, epigen, and epiregulin (Singh et al., 2016). The growth factors all have a sequence of six cysteine residues with conserved spacing that allows them to bind to EGFR. The ligands must be cleaved from the cellular membrane by metalloproteinases. Once cleaved, the ligands are soluble and can activate EGFR in endocrine, paracrine, and autocrine fashions (Prenzel et al., 1999; Schneider & Wolf, 2009). There is growing data demonstrating/suggesting that ligands can also remain bound to the cell membrane and bind to EGFR in a juxtacrine manner (Schneider & Wolf, 2009).

Upon ligand binding to EGFR's extracellular domain, the receptor dimerizes with another EGFR molecule or another ERBB family member though a ligand-binding

conformational change that exposes the dimerization arm (Burgess et al., 2003; Endres et al., 2014). Dimerization is entirely receptor-mediated, with the ligands being on the outer ends of the dimer and therefore not making direct contact with the dimerization interface (Lemmon & Schlessinger, 2010). Receptor dimerization does not always require bound ligand, as studies have shown that that a significant portion of inactive EGFR receptors exist in the dimer conformation (Figure 1.1). Ligand binding triggers the kinase domains to change from a symmetrical orientation to an active asymmetric position (Purba et al., 2017). The EGFR inhibitor cetuximab preferentially binds untethered EGFR and sterically blocks the extension of the dimerization arm (Li et al., 2005). Conversely, this mechanism of action may contribute to resistance to cetuximab therapy in general as this inhibitor would have very limited effect in an environment where EGFR receptors are already dimerized. In normal cells, ligand availability tends to be short-lived and well controlled, ensuring that EGFR activation only occurs transiently and in response to specific stimuli.

In cancer cells, overexpression of EGFR ligands promotes EGFR activity and tumor growth, such as the overexpression of epiregulin and amphiregulin in cases of metastatic colorectal cancer (Khambata-Ford et al., 2007) and overexpression of TGFα found in several types of cancers (Chakraborty et al., 2014; Grandis et al., 1998; Sasaki et al., 2013; Umekita et al., 2000). Cancers that rely on ligand-dependent EGFR signaling tend to be responsive to treatment with EGFR tyrosine kinase inhibitors (TKIs) (Chakraborty et al., 2014; Khambata-Ford et al., 2007; Schiff et al., 2004). Elevated levels of both HB-EGF transcript and protein are a common finding in ovarian cancer,

with anti-HB-EGF antibodies showing promise in preclinical anti-cancer trials but demonstrating significant neurotoxicity in the initial clinical trial (Miyamoto et al., 2004; Sarantopoulos et al., 2016). Current therapies focus on antibodies against the ligands at the protein level as well as inhibition of the metalloproteinases that cleave ligands. Inhibition of ligands at the transcript level may be a possible route to explore for correcting situations where excess ligand is driven by high transcript levels and/or by gene amplification.

Non-EGFR-targeted treatments are also used to more directly target the source of excessive ligand release. G-protein coupled receptors (GPCRs) are known to promote metalloproteinase cleavage of ligands bound at the cellular membrane. Accordingly, misregulation of GPCRs is implicated in cancers, partly due to their roles in promoting excessive release of EGFR ligands (Almendro et al., 2010; Yu et al., 2018). The proto-oncogene, SRC, is a non-receptor tyrosine kinase that has functions both upstream and downstream of EGFR and is a conduit of GPCR signaling due to its role in activating metalloproteinases. For instance, GPR30 is a GPCR that promotes SRC activity and subsequent SRC-mediated cleavage of HB-EGF in an estrogen-mediated manner that is implicated in estrogen-receptor-negative breast cancer (Filardo, 2002; Filardo & Thomas, 2005). SRC may also be involved in hyperactivation of EGFR without GPCR involvement. Maretzky et al. (2011) showed that a constitutively active *Src* mutation promotes metalloproteinase-mediated shedding of TGFα and subsequent activation of EGFR signaling pathways, the effects of which were blocked with inhibitors of either SRC, the metalloproteinase (ADAM17) or EGFR. Taken together, both GPCRs and

SRC may be underappreciated yet potential targets for cancer mitigation. GPCRs, in particular, are popular targets for treatment of cardiovascular conditions but have largely been unexplored in cancer therapy (Belmonte & Blaxall, 2012; Yu et al., 2018). There are multiple other mechanisms not discussed here that promote ligand cleavage and are potential targets for therapy. For instance, hyperactivity of lysophosphatidic acid is associated with ovarian, breast, and prostate cancers, with the mechanism consisting of promotion of metalloproteinase activity, and subsequent excessive shedding of HB-EGF (David et al., 2014; Fishman et al., 2001; Willier et al., 2013).

Currently, immunotherapy against EGF is being explored as an option for treating non-small-cell lung carcinoma. The anti-EGF vaccine CIMAvax-EGF has been shown to be efficacious in multiple clinical trials of advanced stage IIIB/IV non-small-cell lung carcinoma (NSCLC) (Rodriguez et al., 2016; Rodriguez et at., 2010; Saavedra & Crombet, 2017). The large response rates reported in these clinical trials (>50%) indicate the importance of tailoring treatments to individual biomarkers in order to improve treatment, as efficacy correlated strongly with serum EGF concentrations (Saavedra & Crombet, 2017).

Concentration of EGFR on the cellular membrane

The intracellular component of EGFR consists of a juxtamembrane segment, the catalytically active kinase domain, and the C-terminal tail containing multiple tyrosine residues. In the monomer and/or inactive states, the positively charged kinase domain docks to the plasma membrane by electrostatic interactions with the negative charge

from both the plasma membrane and the receptor's juxtamembrane segment (Arkhipov et al., 2013). This inactive configuration is key to sterically inhibiting dimerization between adjacent ERBB receptors during times when EGFR activity is inappropriate (Endres et al., 2013). In the active state, the transmembrane domains from the two ERBB receptors dimerize at the N-terminus, the juxtamembrane segment undocks from the cellular membrane and helps interlock the two kinase domains, and the kinase domain forms an asymmetric dimer that allows for the commencement of an autophosphorylation cascade (Arkhipov et al., 2013; Kovacs et al., 2015; Zhang et al., 2006). In the C-terminal tail, the receptors' tyrosine residues capture phosphate groups, creating phosphotyrosine binding docks at which other proteins and complexes can become phosphorylated and form complexes (Burgess et al., 2003; Endres et al., 2014). These interactions at the tyrosine residues result in a cascade of signaling pathways, among which the ERK, PI3K/AKT, STAT3, JNK and p38 MAPK signal transduction pathways are activated. There are many factors that coalesce to determine which pathways are activated, including but not exclusive to the type of ligand bound at the ligand binding domain and their relative availabilities, the mode of ligand binding (juxtacrine, paracrine, and so forth), and the dimerization partner of EGFR (each receptor has different combinations of binding docks) (Singh & Harris, 2005). Although EGFR activation is primed by the ability of dimers to readily become phosphorylated, 95% of EGFR receptors on the cell membrane are estimated to be in the inactive non-ligand bound state (Burgess et al., 2003; Johns et al., 2004). This demonstrates how

tightly EGFR activation is controlled by the cell, given how devastating hyperactivity can be to the overall organism.

The extracellular, transmembrane, and intracellular components of EGFR all cooperate to inhibit phosphorylation in the absence of a bound ligand. The transmembrane helix, often thought to be passive, has been shown to be crucial in maintaining the juxtamembrane segment docked to the plasma membrane (Endres et al., 2013). The inactive configuration of the extracellular component sterically blocks interaction between ERBB transmembrane domains by promoting dimerization at the C-terminal rather than the N-terminal configuration associated with activation. When the extracellular component is deleted or when there are flexible glycine-serine-threonine linkers, EGFR activity is extremely high despite there being no ligand bound (Endres et al., 2013). Both situations remove the steric block associated with the inactive/tethered extracellular domain, permitting receptor dimerization. Overexpression of EGFR can overcome inhibitory mechanisms by being a source of ligand-independent activity, given that high cell surface densities of EGFR stimulate phosphorylation even in the absence of ligands (Endres et al., 2013), indicating that crowding of receptors can overcome the inhibitory mechanisms intrinsic to the non-ligand-bound EGFR. High concentrations of EGFR enhance dimerization of the kinase domains and consequently trigger catalytic activity.

Although electrostatic interactions and ligand-dependent configurations help maintain EGFR in the inactive state, these mechanisms of inactivation are often disrupted in cancers via mutations in the *EGFR* gene. In glioblastoma, the most

prevalent type of brain cancer, more than 50% of tumors have amplification of the *EGFR* gene. Consequently, receptor overexpression and gene rearrangements are common occurrences (Chakraborty et al., 2014). Cancer cells further exploit receptor overcrowding by tampering with the extracellular domain's inhibitory functions. The EGFR version III mutation (*EGFRvIII*) is a gene rearrangement with deletions in the extracellular domain (exons 2-7). *EGFRvIII* is the most common *EGFR* mutation in glioblastoma, reported in 20-30% of cases, and has been found to a lesser extent in mammary carcinoma and non-small cell lung carcinoma (Gan et al., 2013). Despite the inability of EGFRvIII to bind ligands, these mutant receptors have low level activity. This activity may be caused by the absence of extracellular steric pressure, which under normal conditions blocks interactions between receptors, thereby inhibiting spontaneous kinase dimerization. The truncated extracellular domain in the EGFRvIII mutant receptor may be permissive to escaping steric inhibitory pressure. This mutation is particularly potent when accompanied by an environment of high density EGFR receptors on the cell membrane (i.e. overexpression) that is typical of gene amplification.

Several approaches have been tested in preclinical trials to target EGFRvIII mutant receptors, including immunotherapy, RNA-based therapies, and antibodies (Padfield et al., 2015). Immunotherapy (with dendritic cells carrying information on EGFRvIII specific peptides) has had success in eliminating *EGFRvIII* mutant-expressing cells *in vitro* but is largely lethal in *in vivo* mouse studies and undeliverable across the blood brain barrier with current methods (Padfield et al., 2015; Sampson et al., 2008). Rindopepimut, a vaccine against EGFRvIII, was reported to be ineffective and

potentially harmful in Phase III clinical trials, with the treatment group having slightly lower survival time than the control group (Weller et al., 2017). Other immunotherapies have consisted of using CAR T-cells to recognize antigens specific to EGFRvIII-expressing tumors, and is currently being evaluated in an initial clinical trial (Yang et al., 2017). Antibody therapy, in particular of ABT-414, is currently being tested in clinical trials, with the most recently completed trial reporting a 28.8% six-month progression free response rate (van den Bent et al., 2017; Yang et al., 2017).

Tumors highly expressing EGFR have a wide range of response to EGFR-targeted treatments. For instance, anaplastic thyroid cancer overexpressing EGFR is highly responsive to Gefinitib both in human cells *in vitro* and with an *in vivo* mouse model of thyroid carcinoma (Schiff et al., 2004). A Phase II clinical trial was recently completed (NCT00095836) but results are not yet published. In contrast, EGFR overexpression in breast cancer is generally resistant to treatment with EGFR inhibitors, likely due to functional redundancies shared with ERBB2, which can also promote MEK/ERK and PI3K/AKT signaling. Although EGFR TKIs may in part help with sequestering ERBB2 (Arteaga et al., 2005), signaling via ERBB2 could still persist by ERBB2-ERBB3 or ERBB2-ERBB4 heterodimers - a likely scenario given that ERBB2 is highly expressed in about 30% of breast cancers (Slamon et al., 1987). For these reasons, EGFR inhibitors are commonly used in conjunction with other chemotherapeutics, such ERBB2 inhibitors in treatment of breast cancer, a combination also explored in treating gastric, pancreatic and head and neck cancers (Gray et al., 2017; Larbouret et al., 2012; Wainberg et al., 2010). These experiments show that detailed

analyses of tumor biomarkers that consider signal transduction redundancies inherent to a network, such as the ERBB network, may yield more successful combinatorial therapies.

Activating mutations

The vast majority of *EGFR* mutations are somatic, with 93% of mutations occurring in the first four exons (exons 18-21) of the kinase domain (Kobayashi & Mitsudomi, 2016; Siegelin & Borczuk, 2014). Mutations in the tyrosine kinase domain of the *EGFR* gene are particularly common in NSCLC, with about 10-12% incidence in the United States and over 50% incidence in East Asian populations. The *del746-750,* which is an exon 19 inframe deletion of 4 amino acids, accounts for about 45% of the mutations in NSCLC, whereas the L858R missense mutation in exon 21 accounts for 40% (Kobayashi & Mitsudomi, 2016). Both of these mutations have been reported to encourage dimerization at the kinase domain, allowing for tyrosine activity without upstream ligand binding (Okabe et al., 2007). *EGFR* amplification is not a requirement for mutant-induced activation (Okabe et al., 2007), meaning that overexpression may not be the mechanism permitting dimerization. The likely mechanism is by conformational changes induced by the mutations that facilitate dimerization in the absence of ligand (Arteaga et al., 2005; Okabe et al., 2007). In fact, even bound EGFR inhibitors, such as AG1478, trigger conformational changes that facilitate receptor dimerization and formation of inactive dimers (Arteaga et al., 2005). The exon 19 deletion and L858R mutations have a 60% response rate to EGFR TKIs (Kobayashi & Mitsudomi, 2016). In

fact, non-smoking East Asian females with lung adenocarcinoma, a group with a particularly high incidence of activating *EGFR* mutations (Shi et al., 2014; Shigematsu et al., 2005), tend to respond favorably to EGFR-targeted therapy (Yang, 2008; Yang et al., 2014).

Cancers often develop resistance to EGFR TKI treatment, with the T790M EGFR missense mutation at exon 20 accounting for over 60% of NSCLC TKI-resistant cases (Yu et al., 2013). The T790M mutation has been shown to have higher ATP affinity, thus making it a potent competitor against small molecular inhibitors and a common mechanism of tumor resistance to therapy. Sharma et al. (2007) postulate that the T790M mutation may have a two-fold advantage to cancer cells, occurring early in tumorigenesis to enhance tumor outgrowth and later providing a selective advantage against EGFR TKI treatment. Ultimately, EGFR-targeted treatments are thought to fail due to tumor heterogeneity, in which selective pressure by TKIs such as gefinitib inadvertently promotes persistence of a subgroup of cells harboring the T790M mutation. More recently, the third generation inhibitors osimertinib and olmutinib were designed to target T790M mutant receptors by forming covalent bonds at the tyrosine residues. However, the problem of selective pressure persists, with the C797S mutation (*in trans* to T790M) conferring resistance to these inhibitors by disrupting the covalent bond with the TKI. Fortunately, the C797S mutation is sensitive to first generation TKIs (such as gefinitib and erlotinib) and combinatorial therapy of first and third generation TKIs were shown to be beneficial at treating both mutations simultaneously in multiple preclinical trials (Kobayashi & Mitsudomi, 2016; Li et al., 2017). These findings

highlight the significance of sampling the cell population and targeting multiple mutations in order to minimize a selective advantage, although even this technique has limits. Combinatorial therapy with first and third generation TKIs was applied clinically to a patient with advanced lung adenocarcinoma and was reported to be initially effective in stopping tumor progression. However, the tumor cell population transitioned to an *in cis* preference for the C797S mutation, re-enabling resistance to therapy (Wang et al., 2017).

There is an abundance of rare mutations in pulmonary adenocarcinomas that trigger ligand-independent EGFR activity not covered here but well documented in reviews such as Siegelin & Borczuk (2014). An understanding of the gain-of-function properties of each mutation would aid in designing optimally-targeted therapies and improve response to treatment. For instance, first generation TKIs are generally more effective on the exon 19 deletion than on the L858R mutation. An understanding of the functional difference between the two types of mutations and their interactions with the TKIs would aid in the design of future therapies tailored to specific mutations. For example, a functional study investigated why treatment with cetuximab (an EGFR monoclonal antibody that binds to the extracellular domain) is not effective with certain EGFR mutations, such as the dual L858R/T790M mutation (Cho et al., 2013). The study demonstrated the functional role of cetuximab in preventing dimer formation, explaining why cetuximab is effective with certain mutations (i.e. that still depend on receptor dimerization for kinase activity) but not with the mutations associated with resistance to therapy (i.e. the mutant receptors that can be catalytically active as monomers). By

understanding the functional role of cetuximab, Jia et al. (2016) were able to predict and test a novel therapeutic use of cetuximab when combined with an allosteric inhibitor of EGFR, EAI045. Jia et al. (2016) observed that despite its high specificity to L858R/T790M mutant receptors, EAI045 was much less effective in cells than what was predicted in biochemical modeling. cetuximab/EAI045 combinatorial treatment effectively treated cellular and mouse models of lung cancer with either the L859R/T790M or L859R/T790M/C797S mutations that are typically resistant to cetuximab (Jia et al., 2016). Combinatorial therapy was successful because when a kinase pair is in the dimerized conformation, only the activator's allosteric site is exposed, whereas the allosteric site of the other kinase is oriented toward the interior of the dimer. Cetuximab prevented dimer formation, which exposed the allosteric sites in all receptors, thereby enhancing the efficacy of EAI045 (Jia et al., 2016).

Cross-talk among kinases and activation of alternative pathways

Redundancies in the activation of EGFR-mediated signal transduction pathways are a major source of resistance against EGFR-targeted therapies. There are several receptor families that can influence EGFR activity. G-protein coupled receptors (GPCRs) indirectly activate EGFR by encouraging release of EGFR ligands from their membrane bound states with the aid of metalloproteinases (Prenzel et al., 1999; Schneider & Wolf, 2009). The A disintegrin and metalloproteinase 17 (ADAM17) is often upregulated in human cases of colon cancer (Mustafi et al., 2017). Consequently, inhibition of ADAM17 suppresses EGFR activation and colon tumor formation in mouse

models (Mustafi et al., 2017). ADAM17 also activates ERBB2 and ERBB3 and its activity is linked to resistance to EGFR-TKIs. The estrogen receptor (ER), hepatocyte growth factor receptor (MET), and insulin growth factor receptor 1 (IGFR-1) have been shown to cross-talk with EGFR (Adams et al., 2004; Karamouzis et al., 2009; Skandalis et al., 2014), in which activation of one receptor promotes activity in the others. Colon cancer cells have been shown to utilize IGF-1-mediated cleavage of TGFα and subsequent upregulation of EGFR activity (Adams et al., 2004; Wang et al., 2002). Furthermore, IGFR-1, MET, and GPCRs (such as Angiotensin-II receptor type 1) can activate some of the same signal transduction pathways as EGFR by alternative means independent of EGFR phosphorylation. For instance, lung adenocarcinomas resistant to gefinitib often correlate with IGF-1 overexpression (Hurbin et al., 2011). Cross-talk also likely contributes to hyperactivity of effectors downstream of EGFR in tumorigenesis (such as ERK1/2, mTOR, AKT and STAT3), especially when there are no activating mutations in the *EGFR* gene. Cross-talk among downstream effectors (reviewed in Avraham & Yarden, 2011) further delineate outcomes.

Receptors with the EGFRvIII mutation can dimerize with wildtype EGFR when ligand is available. In the absence of ligand, EGFRvIII triggers recycling of the wildtype EGFR and switches to forming a complex with the MET receptor, allowing MET to become active (Li et al., 2015). Activated MET triggers pathways canonical to EGFR, such as PI3K/AKT and MEK/ERK. MET is also expressed in cells of epithelial origin, and its activity is a source of resistance to EGFR TKIs (Karamouzis et al., 2009). Given the tumorigenic properties of EGFRvIII-MET heterodimers, combinatorial therapy

targeting both EGFRvIII and MET has proven immensely beneficial in treatment of lung cancer and glioma in preclinical trials (Greenall et al., 2015; Greenall & Johns, 2016; Nakagawa et al., 2012). In large-scale clinical trials, combinatorial therapy for EGFRvIII and MET has had only modest effects or has been efficacious in a few properly designed clinical trials, but often with adverse toxicities such as diarrhea and skin rash (Solomon, 2017). MET is rarely amplified in non-treated adenocarcinomas, meaning that it is through TKI selective pressure that its prominence arises (Siegelin & Borczuk, 2014).

Improved efficacy in combinatorial therapies can also be observed within the ERBB family. Takezawa et al. (2012) reported that ERBB2 amplification was found in 12% of lung adenocarcinomas that were resistant to EGFR-targeted therapy. The occurrence of ERBB2 amplification was mutually exclusive to the T790M mutation (Takezawa et al., 2012). Combinatorial therapy with the EGFR inhibitor cetuximab and afatinib (which targets EGFR, ERBB2, and ERBB4) inhibited both receptors much more effectively than mono therapy, with a 40% response rate (Takezawa et al., 2012).

Downstream effectors of EGFR are often mutated in cancers, such as deleterious mutations in *PTEN* and upregulation of STAT3 activity in lung adenocarcinoma (Takata et al., 2012). Likewise, resistance to TKIs is often due to aberrant activity of downstream effectors. The PI3K/AKT pathway is particularly key to survival of cancer cells, due to its role in phosphorylating an abundance of downstream effectors and transcription factors that promote survival and proliferation. *PTEN* deleterious mutations are often found in cancers due to the role of PTEN in preventing phosphorylation of AKT. Given that hyperactive EGFR activity and loss of PTEN both serve similar roles in promoting

signal transduction, mutations in *EGFR* and *PTEN* tend to be mutually exclusive. When both mutations are found in the same tumor, such as in glioma EGFRvIII xenografts transduced with a *PTEN* loss-of-function mutation, a combination treatment with both EGFR and PI3K inhibitors was shown to suppress tumor growth (Fan & Weiss, 2010). KRAS is constitutively active in about 50% of colorectal cancer cases and is largely responsible for resistance to EGFR-targeted treatment due to its role in promoting AKT and ERK signaling. Mutations in *KRAS* are also a source of resistance against EGFR-targeted therapies in NSCLC, accounting for 10% of resistant cases. In a worldwide study with 617 cases of NSCLC, *KRAS* mutations were not present in samples with EGFR activating mutations (Shigematsu et al., 2005). It is generally reported that *EGFR* and *KRAS* mutations are mutually exclusive, only rarely occurring within the same tumor. *KRAS* mutations occur most frequently among smokers, whereas *EGFR* mutations are more prevalent among nonsmokers, suggesting that different mutations are selected in different tissues, microenvironments, and in response to different environmental toxicants. Genetic mutations tend to be mutually exclusive when they share redundant physiological roles. *EGFR*, *KRAS*, and *PTEN* share the role of activating AKT and mutations in all three genes have been reported to be mutually exclusive to each other (Hosgood et al., 2013; Ikeda et al., 2000; Shigematsu et al., 2005). This suggests that generally there is little selective advantage to redundant mutations within the same pathway, at least in tumors that have not undergone selective pressure by targeted therapy. RAC is another downstream effector of EGFR that can become deregulated. In a heterogeneous breast cancer population consisting of both EGFR-

sensitive and resistant cells, resistance was attributed to RAC hyperactivity. Combinatorial therapy with EGFR and RAC inhibitors reduced cell viability by 80% (Borrero-Garcia et al., 2017).

In vitro experiments with lung adenocarcinoma cells showed that even with EGFR inhibition, AKT pathway activity persists. Several other tyrosine kinase receptors, such as MET and IGFR-1, were shown to be active and involved in promoting AKT signaling (Shimamura et al., 2008). Interestingly, inhibition of heat shock protein 90 (HSP90) effectively reduced phosphorylation of EGFR and other receptor tyrosine kinases, also suppressing downstream AKT activation and enhancing regression of adenocarcinomas in mice. This mode of treatment was effective even with mouse and cell models harboring the L858R mutation in conjunction with the T790M mutation (Shimamura et al., 2008). HSP90 may be involved in upstream activation of several tyrosine kinase receptors and has been shown to form a complex with EGFR, including the double mutant EGFR T790M/L858R. HSP90-targeted therapy might have two-fold benefits in inhibiting EGFR, by preventing HSP90 trans-activation of EGFR and by inadvertently targeting EGFR for degradation when the HSP90-EGFR complex is targeted (Ahsan et al., 2012; Sharma et al., 2007).

The best modes of treatment may be complex and multi-targeted, given the redundancies in the EGFR network that allow cells to adapt to specific treatments. For instance, compensatory PI3K upregulation has been observed when mTOR is inhibited. Currently, both are targeted to more effectively suppress the PI3K/AKT/mTOR pathway (Sharma et al., 2007). Several preclinical studies with breast cancer and glioma patients

have shown that combinatorial targeting of EGFR, PI3K, and mTOR are more effective at halting tumor progression than monotherapies (Fan & Weiss, 2010; Deng et al., 2015). However, apoptosis is not triggered and more effective outcomes are seen when the combination of inhibitors are used concurrently with other treatments that promote cytotoxicity (Fan & Weiss, 2010).

Misregulated endocytosis of EGFR

Inhibiting EGFR signaling is crucial to downstream signaling network activity. Mechanisms of EGFR signaling inhibition include dephosphorylation by tyrosine-specific phosphatases, degradation, and recycling of the receptor. Endocytosis of dimerized ERBBs is triggered by a variety of stimuli and is mediated by several adaptor proteins. Internalized EGFR is either returned to the cell membrane or degraded by a ubiquitin-dependent mechansism, depending on an array of conditions. EGFR is able to remain active during this process (reviewed in Avraham & Yarden, 2011).

Cancer cells can maintain EGFR signaling by preventing dephosphorylation and degradation of the receptor. Avraham & Yarden (2011) noted that the tyrosine-specific phosphatase *PTPRJ* gene tends to have compromised function/mutations in carcinomas, likely due to its role in dephosphorylating EGFR. The Casitas B-lineage lymphoma proto-oncogene (CBL) is a commonly recruited ligase protein that is often misregulation in EGFR-dependent tumors due to its role in targeting EGFR for ubiquitination. For instance, mitogen-inducible gene 6 (*MIG6)* can acquire deleterious mutations in cancer and consequently interfere with CBL recruitment to EGFR, essentially preventing

ubiquitination of EGFR (Boopathy et al., 2018). In fact, MIG6-deficiency has been implicated in initiation and progression of various cancers, including incomplete degradation of mutant receptors harboring the exon 19 deletion or the L858R missense mutation (Ferby et al., 2006; Maity et al., 2015).

There are multiple ways that EGFR internalization can be disrupted. For instance, the poxvirus has very low affinity to EGFR, but once bound, the virus keeps the receptor anchored to the cell membrane and prevents endocytosis of EGFR. The EGFR TKI Gefinitib has been shown to be effective in stopping the spread of poxvirus infection (Langhammer et al., 2011). It is interesting to note that poxvirus-based cancer therapies have had excellent clinical outcomes, due to preferential lysing of cancer cells and targeting them to elicit immune system responses (Sharp & Lattime, 2016). Cancer cells can also evade endocytosis of EGFR, such as in the case of the EGFRvIII mutation. EGFRvIII mutant receptors have low-level, prolonged signaling that evades internalization. The mutant receptor also evades ubiquitination by hypophosphorylation at the tyrosine 1045 residue which destabilizes binding to CBL, (Grandal et al., 2007; Zeineldin et al., 2010).

Persistent EGFR signaling and crowding of receptors at the plasma membrane may also be due to endocytotic deregulation. Menard et al. (2018) showed that the balance between EGFR recycling and degradation is deregulated in cells with *EGFR* mutations, in favor of excessive receptor recycling and persistent signaling. Shifting the balance toward receptor degradation could be a potential route for future therapies. The clathrin-mediated EGFR recycling pathway is usually most active when EGF

concentrations are low (Sigismund et al., 2018), a likely mechanism to preserve a baseline of EGFR receptors at the plasma membrane. Inhibition of clathrin-mediated EGFR recycling induced EGFR degradation and apoptosis in cells with mutant EGFR, including TKI-resistant mutations such as T790M and C797S (Menard et al., 2018).

Ali & Wendt (2017) postulated that transformation of tumors from primary to metastatic involves a switch from EGFR signaling dependency to suppression of EGFR transcription, non-canonical EGFR internalization signaling, and activation of other pathways that fulfill EGFR's growth-promoting functions, making these tumors inappropriate candidates for EGFR TKI treatment at the metastatic stages. This process is termed the EGFR paradox, in which EGFR agonist treatment at the metastatic stage triggers apoptosis of cancer cells, rather than promote growth (Ali & Wendt, 2017).

Cardiac hypertrophy

The heart is abundantly studied because heart disease is the leading cause of death worldwide and is also the most common cause of death among cancer survivors due to chemotherapeutic-induced cardiotoxicities. The heart is a dynamic organ that can adapt to the body's physiological demands. Increased work demands can come from a variety of sources, some of which include exercise, hypertension, viral infections, and aortic valve abnormalities. Since cardiomyocytes are terminally differentiated cells that do not divide, the common mode of adaptation is through hypertrophy, in which cardiomyocytes increase in size by creating and organizing new sarcomeres, a process termed remodeling (Figure 1.2). The orientation of the sarcomeres depends on the type

of hypertrophy (concentric vs eccentric) and its underlying cause. For instance, aortic valve stenosis (such as in the case of calcification) can lead to the situation termed pressure overload, which is increased pressure that the heart must overcome when ejecting blood during systole. The heart can overcome this pressure overload by increasing the force of contraction, accomplished by thickening of the walls. In concentric hypertrophy, the sarcomeres are organized in parallel, expanding the thickness of the walls while not affecting the chamber diameter (Fernandes et al., 2011). Functionally, this can be detected by 2D echocardiography as increased diameters of walls along with increased ejection fraction (percent of blood ejected out of the left ventricular chamber during systole). In contrast, eccentric hypertrophy consists of sarcomeres created in series, so that the endocardium elongates in adaptation to an enlarged left ventricular (LV) chamber. In this situation, echocardiography would detect no change in wall thickness, but an enlargement of the LV chamber diameter and maintained ejection fraction (EF). Eccentric hypertrophy occurs as a consequence of volume overload, as is often the case when the aortic valve does not close properly after systole and there is regurgitation of blood back into the LV. This has a balloon-like effect in which the LV chamber expands to accommodate the extra volume. Eccentric hypertrophy can be a physiological response seen in endurance athletes whose hearts have to meet higher exercise demands, whereas concentric hypertrophy occurs with strength training (Fernandes et al., 2011). Eccentric hypertrophy, hypertrophic remodeling, and concentric hypertrophy can all occur in pathological cases. In addition, the heart can switch between the different types of hypertrophy as a response to changes

in work demand. With time, the initial mode of adaptation can further diminish the capabilities of the heart to maintain cardiac output, leading to continuous changes in sarcomere structure.

Eccentric hypertrophy has the propensity to become dilated cardiomyopathy, in which contractility is compromised due to the large LV volume that needs to be ejected at systole. At this stage, echocardiography can detect reduced contractility (decrease in EF). This is the most widely used diagnostic parameter in detecting cardiomyopathy and heart failure. While wall thickening and LV dilation are adaptive responses to pressure or volume overload that may accumulate with age, continually increased cardiac mass is linked to mortality and morbidity and leads to compromised contractile function. As the heart increases in mass due to hypertrophy, there is greater energy demand placed on cardiomyocytes that cannot be met, placing cardiomyocytes at risk of apoptosis. Since cardiomyocytes cannot divide, apoptotic cells are replaced by fibrotic or necrotic tissue. Hearts with fibrosis/collagen replacement of cells, have compromised function and are a hallmark of cardiomyopathies and heart failure.

EGFR signaling pathways involved in cardiomyopathies

EGFR's role in heart physiology is not yet well-defined, but several studies have begun revealing cardiovascular consequences of misregulated EGFR signaling. Abundant data has shown that the ERBB family and their ligands are required for cardiac development (reviewed in Makki et al., 2013). EGFR-deficient mice in particular develop aortic valve defects as well as genetic background-dependent

hypertrophic remodeling (Barrick et al., 2009). Studies on ERBB signaling in the heart have historically focused on ERBB2 and ERBB4. This is likely due to the fact that ERBB2 and ERBB4 are more abundantly expressed in cardiomyocytes than EGFR. However, function is often more relevant than expression level in revealing the relevance of a protein, since many enzymes have been shown to be highly effective at low expression levels. It is also possible for EGFR expression levels to fluctuate in a stimulus-dependent manner - a topic, that to our knowledge, has not been studied.

Cardiotoxicity studies have focused more on ERBB2 because pharmacological inhibition of ERBB2 is associated with severe cardiotoxicity and heart failure in 10-28% of breast cancer patients, often associated with pre-existing heart conditions (Martin et al., 2009; Wadhwa et al., 2009; Zeglinski et al., 2011). Trastuzumab, an ERBB2 inhibitor, has been shown to suppress activity of intracellular signaling pathways essential for cardiomyocyte survival such as MAPK/ERK and PI3K/AKT, pathways which are downstream of the ERBB receptors (De Keulenaer et al., 2010). Even though these signal transduction pathways are also regulated by EGFR, much less is known about cardiotoxicity rates of EGFR inhibitors and more generally, of EGFR's role in the cardiovascular system. EGFR-specific TKIs are generally considered to be of low cardiotoxicity risk, with few reports in the literature showing adverse effects (Grisanti et al., 2015). In one such study, Barrick et al. (2008) demonstrated that after 3 months of EGFR TKI treatment, C57BL/6J mice had a higher incidence of apoptotic cells in the left ventricle, thinning of the left ventricular walls and dilation of the chamber, and exacerbated calcification of the aortic valve.

The Angiotensin II type 1 receptor (AT1R) is a key GPCR in the cardiovascular system that has been well-studied (Belmonte & Blaxall, 2012). Deregulation of AT1R signaling, especially by high levels of Angiotensin-II (Ang-II), is implicated in several heart diseases including cardiomyopathy. EGFR is trans-activated via two Ang-II-mediated mechanisms. One method is via Ang-II-mediated activation of SRC, which then phosphorylates the cytoplasmic domain of EGFR. A second method is Ang-II-mediated cleavage of HB-EGF via the metalloproteinase ADAM17, reported in vascular smooth muscle cells and cardiac fibroblasts (Chen, 2006; Makki et al., 2013). Blocking of this trans-activation mechanism with an ADAM17 inhibitor was shown to attenuate left ventricular hypertrophy even in the presence of Ang-II infusion, demonstrating that EGFR is the conduit for Ang-II-mediated hypertrophy (Asakura et al., 2002). This conclusion has been supported by several experiments (Paradis et al., 2000; Peng et al., 2016; Thomas et al., 2002), including the use of the EGFR TKIs for reducing incidence of Ang-II induced hypertrophy (Peng et al., 2016). Studies have shown that mice exposed to *EGFR* antisense oligonucleotides have lower incidence of developing Angiotensin II-induced hypertrophy (Kagiyama et al., 2002). These experiments demonstrate that EGFR stimulation is sufficient to induce cardiac hypertrophy, suggesting a functional role for EGFR in the heart's adaptation to stimuli. Kagiyama et al. (2002) demonstrated that Ang-II-mediated trans-activation of EGFR leads to activation of the MEK/ERK pathway, which several studies have shown directly impacts the occurrence of cardiac hypertrophy (De Pasquale et al., 2018). For instance, Neurofibromin (which deactivates RAS) knock-out mice have hyperactivity of the

RAS/RAF/MEK/ERK pathway with increased incidence of hypertrophy and cardiomyopathy (Xu et al., 2009).

The literature is undecided on the exact function of ERK in cardiac hypertrophy, with some studies reporting that ERK activity promotes hypertrophy and others demonstrating that inhibition of ERK also contributes to hypertrophy. The explanation for such contrasting results may be that ERK functions as a switch between two types of hypertrophy (Kehat et al., 2011). ERK has been shown to be involved in promoting a concentric hypertrophic response, especially to pressure overload, although it is not the only pathway necessary for this (Bernardo et al., 2010; Mutlak & Kehat, 2015). In addition to promoting an adaptive concentric hypertrophic response, ERK is critical for protection against eccentric hypertrophy, as demonstrated with ERK1/2-deficient mice (Kehat et al., 2011; Mutlak & Kehat, 2015). EGFR is a likely upstream mediator of the ERK hypertrophic response, given that EGFR knockout mice have been shown to have lower blood pressure and increased incidence of severe eccentric hypertrophy and that rats treated with the EGFR inhibitor AG1478 had impaired cardiac function and early indications of eccentric hypertrophy (Schreier et al., 2013).

Both ERK and EGFR are often highly expressed in cases of dilated cardiomyopathy, as well as in heart failure (Haq et al., 2001). The necessity of ERK to prevent eccentric hypertrophy and the abundance of ERK expression in dilated cardiomyopathy seem to be opposing observations. There are several possible explanations for the emergence of ERK overexpression in dilated cardiomyopathy (DCM). The first hypothesis is that ERK may be upregulated to suppress existing

eccentric hypertrophy, but this may be a futile effort once progression to dilated cardiomyopathy has already commenced. The second potential explanation is that the function of ERK may switch in a sort of paradox, in which ERK activity initially suppresses eccentric hypertrophy but after dilated cardiomyopathy is established, ERK activation may serve a different function. Experiments that inhibit ERK within a dilated cardiomyopathy mouse model would help in determining which is the most likely explanation of why ERK is upregulated in cases of DCM. For instance, if ERK inhibition has no effect on or worsens DCM, then upregulation of ERK may function as a minimally effective mechanism to suppress DCM. If instead ERK inhibition reduces severity of DCM, then the results would support the paradox explanation. Results from our group demonstrate that BALB/cJ mice, a strain prone to developing DCM, treated with the EGFR TKI AG1478 developed more severe hypertrophy than untreated mice (Howe et al., unpublished), supporting the first explanation and suggesting that EGFR-mediated ERK activity may play a role in suppressing DCM.

Many studies have correlated upregulated AKT activity with cardiac hypertrophy and there is a general consensus that short-term AKT phosphorylation is involved in physiological cardiac hypertrophy (associated with improved cardiac function) whereas long-term AKT activity is associated with pathological cardiac hypertrophy (Kemi et al., 2008; Matsui et al., Nagoshi, 2003; Shiojima & Walsh, 2006; Shiojima et al., 2002). There are numerous combinations of initiating stimuli (including exercise, hypertension, aortic valve stenosis), membrane receptors, and downstream feedback loops that contribute to the large range of outcomes reported in the AKT signaling pathway

(Adams et al., 1998; Arkun, 2016; Bernardo et al., 2010; Condorelli et al., 2002; DeBosch et al., 2006; DeBosch et al., 2006; Dent, 2014; Dorn & Force, 2005; Ikeda et al., 2015; Pillai et al., 2014; Sakata et al., 1998; Sundaresan et al., 2012). EGFR can trigger activation of the PI3K/AKT/mTOR pathway, thus promoting cardiomyocyte survival and preventing fibrosis. For example, Gαq expression was shown to protect cardiomyocytes against apoptosis while also promoting pressure overload-induced hypertrophy (Adams et al., 1998; Howes et al., 2006; Sakata et al., 1998). Howes et al. (2006) demonstrated that the anti-apoptotic properties of Gαq were due to EGFR-mediated AKT activity, which was protective against apoptosis in cardiomyocytes cultured with 2-deoxyglucose. Treatment with inhibitors that targeted either EGFR or the upstream mediator, SRC, removed the protective effects (Howes et al., 2006). These experiments, taken together with *in vivo* findings by Barrick et al. (2008) showing that EGFR TKI treatment promoted apoptosis within the left ventricle, signal that there are potential adverse consequences to cardiomyocyte survival when inhibiting EGFR.

While EGFR and AKT were necessary for cell survival, inhibition of either kinase had no impact on cardiac hypertrophy (Howes et al., 2006), indicating that Gαq-mediated hypertrophy occurred in an EGFR and AKT-independent manner. Although EGFR is not required to induce hypertrophy in these scenarios, the EGFR/Ras/Raf/MEK/ERK signaling pathway may still play a key role in regulating AKT activity. For instance, ERK was shown to activate mTOR in rat cardiomyocytes in an AKT-independent manner (Martin et al., 2001; Sato et al., 2014; Wang et al., 2001). Carriere et al. (2010) demonstrated that ERK was able to directly phosphorylate mTOR-

bound raptor. Other experiments have shown that ERK can also phosphorylate GAB1 and thus prevent GAB1-mediated recruitment of PI3K to EGFR (Yu et al., 2002), essentially inhibiting the AKT pathway. ERK-mediated inhibition of AKT is also reported in insulin resistant diabetes, which can be relieved by treatment with ERK inhibitors (Ozaki et al., 2016). There is the caveat that EGFR may not always be necessary in mediating ERK, as IGFR-1 can also activate RAS and initiate the RAS/RAF/MEK/ERK cascade. Taken together, AKT activity is associated with cardiac hypertrophy (both concentric and eccentric depending on the stimuli and agonists involved), and ERK plays a role in cross-inhibiting the AKT pathway. We postulate that when ERK is inhibited, as in the case of ERK1/2 knockout mice or more indirectly with mice exposed to pharmacological inhibitors of EGFR (Kehat et al., 2011; Howe et al., unpublished), ERK-mediated inhibition of AKT is removed, which effectively activates the AKT pathway and permits subsequent eccentric hypertrophy. In a study with EGFR knockout mice, Schreier et al. (2013) reported increased incidence of eccentric hypertrophy as well as NADPH-oxidase 4 (Nox4) levels. NADPH-oxidase is an upstream regulator of Ang-II-mediated AKT activation and is important to Ang-II induced hypertrophy (Hingtgen et al., 2006). AKT/mTOR has been shown to be the conduit for Nox4-mediated hypertrophy (Zhao et al., 2015) and many studies have implicated EGFR in Ang-II-mediated hypertrophy as well (Asakura et al., 2002; Paradis et al., 2000; Peng et al., 2016; Thomas et al., 2002). Studies have shown that not only does Nox4 activate AKT, but it also triggers SRC-mediated activation of internalized EGFR as well as subsequent ERK activation (Gorin & Block, 2013; Kim et al., 2017;

Truong & Carroll, 2012; Zhang et al., 2014). It is possible that loss of EGFR would disrupt ERK-AKT crosstalk, resulting in hyperactivity of the AKT pathway, increased hypertrophy, and increased levels of NOX4 in compensation for the absence of EGFR. Increased oxidative stress in response to EGFR inhibition was also reported in studies that treated rats with AG1478 (Mak et al., 2015; Weglicki et al., 2012). Reduced systolic and diastolic functions as well as early stages of dilated cardiomyopathy were observed, and the authors concluded that the cardiac changes were a consequence of oxidative stress induced by hypomagnesemia, given that EGFR activity promotes the magnesium channel transient receptor potential melastatin 6 (TRPM6).

EGFR genetic modifiers

Modifier genes are key to the prediction of individual disease risk and response to treatment. The existence of genetic modifiers is most evident when individuals carrying the same mutation have different phenotypic and disease outcomes. For example, EGFR-null mouse embryos have been shown to have a range of outcomes, from lethality during gestation to robust survival to term. Their survival depended largely on genetic background-mediated modification of EGFR's role in the developing placentae (Dackor et al., 2007). In human oncology, SNPs associated with expression of EGFR, as well as a nearby gene encoding a GPCR, were found to modify progression free survival among adenocarcinoma patients treated with EGFR TKIs (Chang et al., 2016). Another study identified 18 genes (including some from the *SRC* family) involved in determining the degree of dependency that NSCLC cells may have on the

EGFR activating mutations that they harbor, i.e. predict how effective EGFR TKI treatment would be on these cells based on how dependent they are on their EGFR mutation (Sharifnia et al., 2014). There is currently an ongoing Genome-Wide Association Study that aims to identify genetic modifiers that determine EGFR TKI treatment outcome (NCT01838577). The high incidence of EGFR mutations found among NSCLC patients of Southeast Asian ethnicity may indicate the presence of genetic modifier polymorphisms that are more permissive to the development of adenocarcinomas carrying EGFR mutations (Sellers & Meyerson, 2005), although the influence of polymorphisms within *EGFR* must be considered when determining influences from genetic modifiers. Overall, modifiers genes determine the availability and roles of target genes and gene products, thereby contributing to genetic interactions within a network and ultimately influencing cellular decisions. Utilizing a single mouse strain when studying complex diseases, such as cardiomyopathies and cancers, restricts interpretations of experimental findings, as a single inbred mouse strain only represents one possible combination of modifier alleles.

Specificity of EGFR inhibitors

Although EGFR inhibitors are designed to have high specificity for EGFR, the possibility remains that these inhibitors also bind to other tyrosine kinases and consequently contribute to off-target side effects. Potential cardiotoxicity effects, for example, may be due to a generalized tyrosine kinase inhibition rather than EGFR inhibition alone. It is generally accepted that compounds that have high affinity to their

target are intrinsically of high specificity as well. AG1478 for example, has an affinity for EGFR of IC_{50} = 3 nM, where 100 uM is the minimum concentration required for non-specific binding to ERBB2 and PDGF (Osherov & Levitski, 1994). However, the *in vivo* outcomes of physiologically-relevant doses of AG1478 likely yield different specificity results than what is typically tested in a cell culture system. The use of a genetic model of EGFR inhibition, such as the combination of the hypomorphic $EGFR^{wa2}$ variant with an inducible EGFR knockout allele, would help clarify whether side effects of EGFR inhibitors are partly caused by non-specific binding of the compound.

Conclusions

The EGFR signaling network is essential for cell survival and metabolism. Hence, there is much redundancy in activation of EGFR and its downstream pathways, allowing the network to adapt in response to perturbations. More advanced stages of cancer require combinatorial therapies that inhibit growth-promoting networks through targeting of multiple factors. This is due to the inherent heterogeneity observed in late stage tumors. In this review, we hope to have conveyed that there is a large amount of redundancy in signaling networks and therefore numerous ways for a heterogenous cell population to escape targeted therapies.

Current methods of anti-cancer targeted treatment can be of limited scope, often generating selective pressure for specific mutations in a population and/or triggering adaptation by the EGFR network, ultimately conferring resistance to treatment (Figure

1.3). Understanding the interactions and limitations of EGFR's signaling network is crucial to correcting signal imbalances common to human diseases. Treatments that target mechanisms that are more generally utilized by cancer cells, such as favoring EGFR degradation by inhibiting receptor recycling (Menard et al., 2018), may become more popular as more is understood about how EGFR is misregulated in cancer. Cancer can be viewed as an evolutionary process that is sensitive to selective pressures within its microenvironment, including pressures from targeted treatments, requiring re-evaluation of patients throughout the treatment process. Interestingly, selective pressures sometimes work in favor of treatment, such as when TKI-treated NSCLC transforms into the much more treatable small cell carcinoma. This begs the question of whether artificial selection, such as manipulation of the microenvironment, can be used to drive the cell population toward a more treatable outcome.

Early stage cancers are much more homogenous and responsive to treatment, with fewer rogue cells able to acquire a selective advantage after exposure to treatment. Taken together, early detection and preventative treatment are of extreme importance in avoiding tumor heterogeneity. Tang et al. (2005) examined samples of lung adenocarcinomas with *EGFR* mutations and found that 43% of the patients also had *EGFR* mutations in normal lung epithelium. The authors concluded that *EGFR* mutations are present at the early stage of tumorigenesis and that EGFR might be a good target for chemoprevention. Overall, early detection and prevention could be immensely beneficial since cancer cells would have a smaller chance to acquire new mutations and undergo selection by bottleneck or selective pressures.

With EGFR-targeted therapies becoming more common there is an increased need to understand how genetic background confers resistance to treatment and susceptibility to toxicities. Cardiotoxicity resulting from ERBB2-targeted treatment is much more widely observed than with EGFR-targeted therapies. A possible explanation is that ERBB2 may be essential for maintenance of cardiomyocytes, whereas EGFR may be more important for response to stress. Therefore, inhibition of EGFR may have adverse consequences on the heart during times when the heart is undergoing compensatory changes. Individuals at risk for therapy resistance and toxicity are more likely to be identified through the use of comprehensive preclinical models encompassing both genetic diversity and sensitive assays that detect cardiotoxicity. The fact that inhibition of either EGFR or ERK promotes eccentric hypertrophy highlights the significance of targeting cancer cells specifically while avoiding off-target cells such as cardiomyocytes. Many advancements have been made since the discovery of EGFR and early trials with targeted treatment, yet there is much still to learn on how genetic networks cross-talk, how cancer cell populations evolve, and how allelic differences between individuals determine disease risk and treatment outcomes.

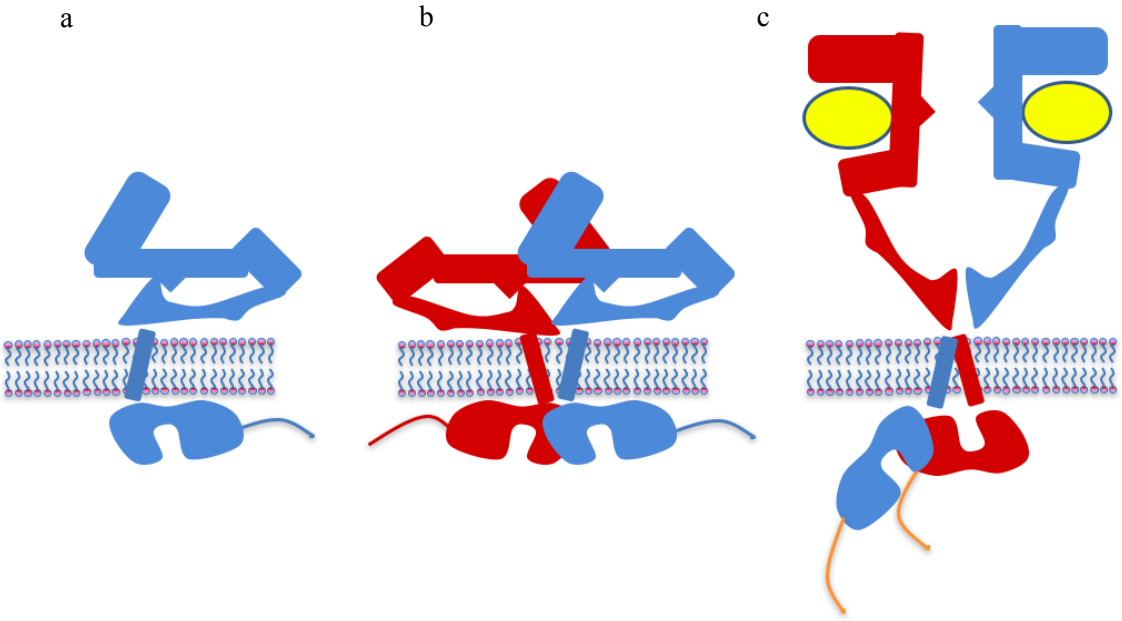

Figure 1.1. Diagram of EGFR protein. EGFR in the following configurations: (a) inactive, tethered monomer, (b) inactive, tethered dimer, and (c) active, ligand-bound dimer.

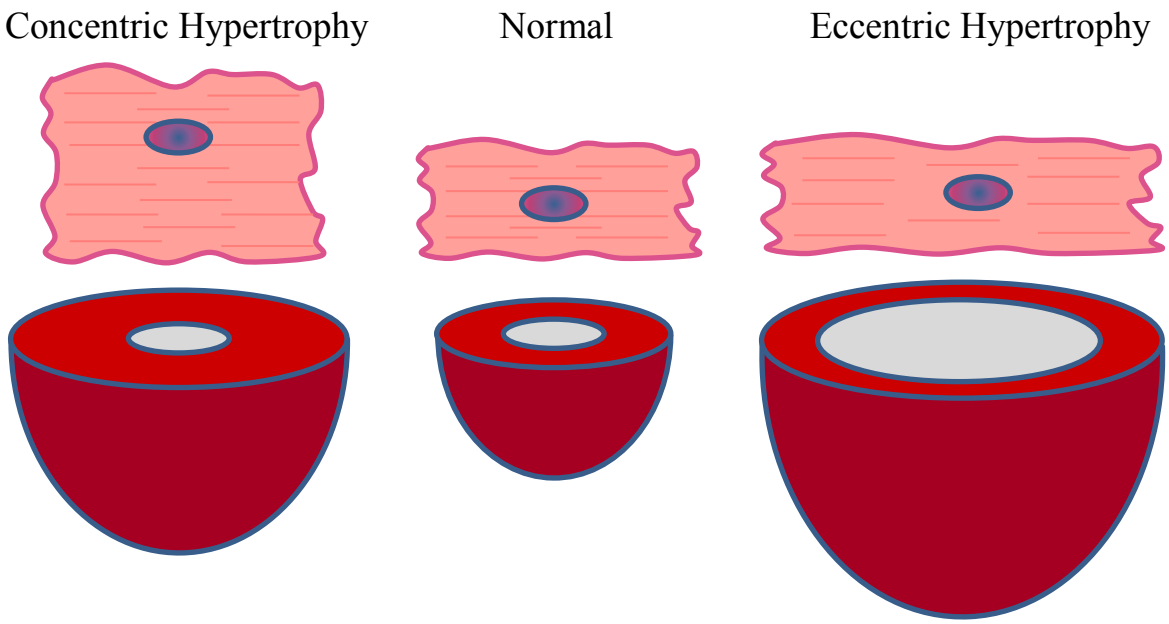

Figure 1.2. Diagram of cardiac concentric and eccentric hypertrophies. Concentric hypertrophy is characterized by the orientation of sarcomeres in parallel, resulting in thickened left ventricular walls. In eccentric hypertrophy, the sarcomeres are arranged in series, permitting stretching of the left ventricular walls and enlargement of the left ventricular chamber.

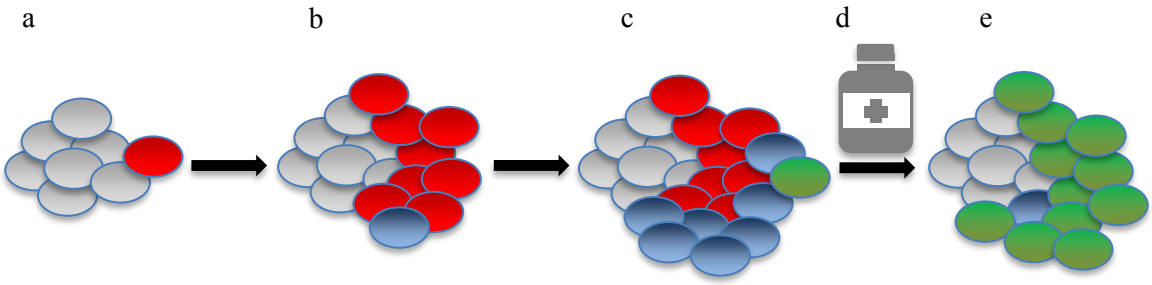

Figure 1.3. Adaptation of a cancer cell population to targeted treatment. (a) A mutation arises, which (b) promotes proliferation of mutant cells. (b-c) One cell acquires an additional mutation that further enhances proliferation. (d) Targeted treatment commences to target the rapidly expanding red and blue cells in an increasingly heterozygous population. (e) Treatment eliminates the target cells, but cells resistant to treatment, in green, survive and expand.

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CHAPTER II

INHIBITION OF EGFR BY AG1478 AUGMENTS HYPERTROPHY IN AGED BALB/CJ MICE WITH DILATED CARDIOMYOPATHY

Introduction

The epidermal growth factor receptor (EGFR) is a member of the ERBB family of receptor tyrosine kinases, consisting of EGFR/HER1, ERBB2/HER2, ERBB3/HER3, and ERBB4/HER4. The ERBB receptors are broadly expressed during embryonic development, normal adult physiology, and cancer progression due to their pivotal roles in promoting cell proliferation, differentiation, survival, and migration. EGFR is often co-opted by cancer cells through up-regulation of the receptor's expression and/or activity, even in the absence of activating mutations in the *EGFR* gene (Chakraborty et al., 2014; Gan et al., 2013; Khambata-Ford et al., 2007; Kobayashi & Mitsudomi, 2016; Maretzky et al., 2011; Schiff et al., 2004; Siegelin & Borczuk, 2014). The prevalence of aberrant EGFR activity in human cancers has made EGFR-targeted mono or combinatorial therapies common strategies for inhibiting tumor growth (Wang et al., 2018). EGFR antibodies that bind EGFR's ligand-binding domain (for example, panitumumab and cetuximab) as well as small molecule inhibitors (such as gefinitib and erlotinib), which compete with ATP for binding sites on EGFR's kinase domain, are used clinically to treat a variety of cancers including breast, pancreatic, non-small cell lung carcinoma, colorectal and head and neck cancers (Hervent & De Keulenaer, 2012; Kobayashi & Mitsudomi, 2016).

Heart disease is the leading cause of death worldwide and is also the most common cause of death among cancer survivors due to chemotherapeutic-induced cardiotoxicities. For instance, studies have shown that an alarming number of adults who survived cancer during childhood may still have long-lasting cardiotoxic effects years later (Lipshultz et al., 2015). The most common side effects of EGFR-targeted therapy are skin rashes, follicular damage to hair, hypomagnesemia, and diarrhea (Fakih et al., 2006; Li & Perez-Soler, 2009; Solomon, 2017; Weglicki et al., 2012). Interstitial lung disease is a less common (with about 1% incidence) but severe adverse effect of treatment (Inoue et al., 2003; Qi et al., 2015). To our knowledge, there are no reports in the literature showing cardiotoxicity effects caused directly by EGFR inhibition, apart from previous studies from our group (Barrick et al., 2009; Barrick et al., 2008). Barrick et al. (2008) demonstrated that C57BL/6J adult mice treated with either the EGFR small molecular inhibitor EKB-569 or AG1478, for three months had adverse cardiac changes, involving left ventricular apoptosis, wall thinning, left ventricular dilation, and an exacerbation of the mouse strain's tendency toward aortic valve calcification. These studies have shown serious implications of potential adverse consequences to EGFR-targeted therapies. Any potential cardiotoxicity rates for EGFR-targeted therapies are unknown and likely missed due to insufficient clinical modeling and understanding of EGFR's role in the heart. De Keulenaer (2012) and others have stressed that the functional role of EGFR in the cardiovascular system is of greater importance than EGFR's expression levels when trying to predict cardiotoxicity outcome.

The ERBB2 receptor, which is more abundantly expressed in cardiac tissue than EGFR, is upregulated in about one-third of breast cancer cases (Mendelsohn, 2002). There is a plethora of evidence from ERBB2-positive breast cancer studies showing adverse cardiac effects caused by treatment with the ERBB2 antibody trastuzumab (Martin et al., 2009; Wadhwa et al., 2009; Zeglinski et al., 2011). Trastuzumab has been shown to suppress activity of intracellular signaling pathways essential for cardiomyocyte survival such as MAPK/ERK and PI3K/AKT, pathways which are downstream of the ERBB receptors (De Keulenaer et al., 2010). An estimated 10-28% of ERBB2-positive breast cancer patients are at increased cardiotoxicity risk, partly due to pre-existing heart conditions. Despite the fact that the MAPK/ERK and PI3K/AKT signaling pathways are also regulated by EGFR, far less is known on the cardiac side effects of EGFR inhibitors or more generally of EGFR's role in the cardiovascular system. There may be unidentified groups at risk for adverse cardiac effects when undergoing EGFR-targeted therapy.

One hypothesis is that any cardiotoxic effects of EGFR inhibition may be due to impaired ERBB2 signaling, since ERBB2 is the preferred binding partner for activated EGFR. However, EGFR has specifically been shown to play a critical role in the development of cardiac hypertrophy. While ERBB2 is essential for cardiomyocyte maintenance and survival, accumulating evidence supports the hypothesis that EGFR is an important regulator of cardiac remodeling during times of adaptive change. Angtiotensin-II (Ang-II) receptor hyperactivity has long been linked to cardiac hypertrophy among other conditions. The likely mechanism for Ang-II-induced

hypertrophy is transactivation of EGFR (Kagiyama et al., 2002). Inhibition of EGFR by either blocking Ang-II-induced transactivation or by the small molecule inhibitor of EGFR, AG1478, was sufficient to prevent Ang-II induced hypertrophy (Paradis et al., 2000; Peng et al., 2016). Studies have implicated upregulation of ERK activity in the process of promoting adaptive concentric hypertrophy and in suppressing eccentric hypertrophy and dysfunction (Kehat et al., 2011; Mutlak & Kehat, 2015). Taken together, the upregulation of EGFR and ERK during adaptive cardiac remodeling suggests that EGFR has an adaptive mediator function. From this, we can hypothesize that inhibition of EGFR would impair the ability of the heart to cope with stress, particularly with stress that promotes eccentric hypertrophy.

There is a need to understand individual response to targeted therapy for the purpose of toxicity avoidance. In the present study, we evaluated the spectrum of cardiotoxicity responses to EGFR inhibition using four genetically distinct mouse strains (A/J, BALB/cJ, C57BL/6J, FVB/NJ) treated with varying sub-therapeutic to therapeutic doses (0, 5, 50 and 144 mg/kg) of AG1478 for 16 months. We chose subtherapeutic doses for the purposes of low dose cancer prevention based on previous research in which inhibition of EGFR reduced tumor load in mouse models of colorectal cancer (Rinella & Threadgill, 2012; Roberts et al., 2002; Torrance et al., 2000), whereas 144 mg/kg is a clinically-relevant therapeutic level dose. EGFR activity has been shown to be a requirement for Western diet-induced development of colon cancer in mouse models (Dougherty et al., 2008; Fichera et al., 2007; Mustafi et al., 2017). To more realistically model the Western diet lifestyle and health risks, AG1478 was incorporated

into a Western Diet mouse chow (high fat, high sugar), which has been demonstrated to be an effective mode of administering the drug (Barrick et al., 2008). Recently, EGFR's downstream effector, ERK, has been implicated in metastatic prostate cancer under a high fat diet influence, demonstrating the importance of metabolic changes induced by diet (Chen et al., 2018). We monitored cardiac changes *in vivo* throughout the 16 months of treatment by 2D echocardiography (M-mode, speckle tracking, and tissue doppler), with the goal of assessing whether long-term EGFR inhibition has adverse cardiovascular outcomes. Heart and spleen tissues were analyzed by histopathology to further evaluate cardiotoxicity-related changes and to investigate whether genetic background-dependent immune responses contributed to instances of decompensated eccentric hypertrophy.

Materials and Methods

Animals and Husbandry

We obtained mice that were 6-to-8-weeks-old (A/J, BALB/cJ, C57BL/6J, and FVB/NJ strains) from The Jackson Laboratory (Bar Harbor, ME). There were 160 mice per strain, 80 males and 80 females, to equal a total of 640 animals on study. Baseline measurements were taken after one week of acclimation to the facility and treatment commenced at ages 8-10 weeks. Animals were measured for all parameters at 4-month intervals, yielding a total of 5 time points (0, 4, 8, 12, and 16 months), with some time points omitted for particular parameters as noted in the corresponding sub-methods sections. The mice were housed 5 per cage, in Nextgen filtered cages, at 22° C under a

12-hour light/dark cycle. In all our experiments, we adhered to Animal Use Protocols approved by the Texas A&M University Institution Animal Care and Use Committee. Mice were euthanized by carbon dioxide asphyxiation and immediately necropsied. Tissues were either flash frozen in liquid nitrogen or fixed in formalin.

Diet and Delivery of AG1478

Tryptosin AG-1478 was purchased from LC Laboratories (Woburn, MA) and incorporated into Western Diet mouse chow (D12079B) by Research Diets, Inc. (New Brunswick, NJ). The therapeutic-level dose chosen was 144 mg of AG1478/kg of food, shown to be sufficient to inhibit EGFR phosphorylation (Barrick et al., 2008; Rinella & Threadgill, 2012). The 50 mg/kg sub-therapeutic dose was selected due to its efficacy at inhibiting cancer cell growth at an equivalent dose *in vitro* (Lee et al., 2005), with the lowest dose chosen to be $1/10^{th}$ of the 50 mg/kg dose (i.e. 5mg/kg). The control chow (0 mg/kg) consisted of the Western diet alone. Food dyes were added for ease of feeding purposes, and mice received food and water *ad libitum*.

Food consumption

Mice were singly-housed in Phenomaster Metabolic cages (TSE Systems, Chesterfield, MO) for 48 hours. Data collected during the first 24 hours were used as an acclimation period and were thus omitted from analysis. Food and water consumption were measured by the system's weighing sensors that attached to feeding and drinking

dispensers. Mice that drank less than 0.5 mL within the first 24-hour period were omitted from analysis.

Echocardiography

Animals were prepared for the procedure the previous day by removal of chest hair with a depilatory cream under 4% anesthesia. 2D echocardiography images were acquired with the Vevo 3100 Preclinical Imaging System (VisualSonics/FujiFilm, Toronto, ON). The mice were scanned un-anesthetized with a 40 MHz transducer (MX550D). 2-dimensional video loops were collected for M-mode, B-mode, and PW Tissue Doppler. Long axis and parasternal short axis images were acquired at the level of the papillary muscles under the B and M-mode settings. Long axis B-mode and short axis M-mode videos were recorded for all 5 time points (0, 4, 8, 12, and 16 months on diet). Long and short axis B-mode images were analyzed with the VevoStrain package, allowing for quantification of myocardial deformation, although the short axis B-mode videos were acquired only for the 8, 12 and 16-month time points. PW Tissue Doppler images were obtained only for the 12 and 16-month time points. Images were traced on the VevoLab software by two independent and blinded observers and the values from both observers were averaged for each animal. Among the parameters obtained were left ventricular anterior and posterior wall thicknesses, left ventricular internal diameter, stroke volume, fractional shortening, cardiac output, heart rate, mitral E'/A' ratio, longitudinal strain, circumferential strain, and radial strain. Given the complex interactions among variables and the non-normality nature of the data distribution, results were analyzed by multivariate analysis and the Wilcoxon statistical test.

Body composition

Values for fat and lean masses, measured in grams, were obtained using the EchoMRI-100H body composition analyzer (EchoMRI, Houston, TX).

Histopathology

At necropsy, hearts were cut at necropsy with a scalpel, with an initial transverse cut at the apex followed by a longitudinal cut toward the cranial end. The sections obtained for histology (the apex piece and half of the cranial end piece) were fixed in formalin for 48 hours, then stored in 70% ethanol until processed and paraffin embedded. Five μm sections were obtained at the plane of the papillary muscles and stained with hematoxylin and eosin. Spleens were fixed and processed as whole tissues and later cut lengthwise along a transverse plane to prepare for sectioning and H&E staining.

RT-qPCR

Heart tissues were homogenized at 4º C using the TissueLyser II (Qiagen, Hilden, Germany). For the RNA extraction, we utilized the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Norcross, GA) and added an incubation step with TRIzol (Invitrogen, Waltham, MA). The Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland) was used to set up the reverse transcription reaction. The

Timp1 and *Spp1* primers were obtained from (Sigma-Aldrich, St. Louis, MO). The sequence for the sense and anti-sense primers for *Timp1* were: CATCCTCTTGTTGCTATCAC and CATGAATTTAGCCCTTATGACC, respectively. The primers for *Spp1* were: GGATGAATCTGACGAATCTC and GCATCAGGATACTGTTCATC. The qPCR reaction was performed on the LightCycler 96 System with 480 SyBr Green 1 Master (Roche Diagnostics, Risch-Rotkreuz, Switzerland). cDNA was diluted at 1:5. Primers were used at a concentration of 10uM and had efficiencies close to 100% (with ΔC_T scores near 3.3). *Timp1* and *Spp1* expression were normalized to the average of *β-actin*, *Gapdh* and *Hprt*. Fold differences were calculated by the $2^{(\Delta\Delta C_T)}$ formula.

Results

Genetic background-dependent differences in baseline echocardiographic parameters.

During baseline echocardiographic measurements, we observed consistent differences between mouse strains (Figure 2.1; Table 2.1) that can be separated into two groups that had some major distinctions. The A and BALB strains have larger left ventricular internal diameters (LVID) than the B6 and FVB group. The A and BALB strains also had higher cardiac output (CO) and stroke volume (SV). In contrast, the B6 and FVB group had greater left ventricular mass (LVM) than the A and BALB mice. Correspondingly, the B6 and FVB mice also had thicker left ventricular anterior and posterior walls (LVAW and LVPW). A key difference between the B6 and FVB strains is that FVB mice have greater LVM, LVAW, and LVPW than the B6 mice. Likewise, a

difference between the A and BALB strains is that the A mice had a slightly thicker LVAW and the BALB had a larger LVID. These genetic background-dependent patterns were consistent for both male and female mice (Table 2.1).

Age-related cardiovascular changes vary by genetic background.

In order to evaluate the presence of cardiotoxicity, we first had to understand which changes occurred naturally due to aging (Figure 2.2). We compared trends over time among control mice and separated the trends by strain but not sex, since there were significant baseline differences in cardiac structure between mouse strains but no differences in overall trends between sexes within each strain (Tables 2.2-2.5). All genetic backgrounds had increased left ventricular mass (LVM) by the 16th month time point, compared to corresponding baseline LVM (Figure 2.2; Figures 2.3-2.6). The magnitude of the trends was different for each mouse strain, with the BALB and B6 mice having greater increases in LVM than the A and FVB mice. LVID followed similar trends, with one distinct difference in that LVID did not change for the FVB/NJ mice. Some consequences of these structural changes were that CO and SV increased the most for the mice with the greatest increases in LVM and LVID (BALB and B6). CO and SV increased only slightly for the A mouse strain, which had only minor increases in LVM and LVID in comparison to BALB and B6. Although the FVB mouse strain also had a minor increase in LVM, the lack of change in LVID corresponded to a slight decrease in CO and SV. All genetic backgrounds had notable increases in left ventricular anterior wall (LVAW) thickness, with A mice having a smaller change in magnitude than the

other mouse strains, which had a similar amount of change in LVAW relative to each other. There were genetic background-dependent trends in left ventricular posterior wall (LVPW) thickness. FVB and A mouse strains had an increase in LVPW thickness that was equivalent to the degree of change in LVAW, resulting in a uniform increase in overall LV wall thickness. The BALB and B6 mice had a non-uniform LV wall thickness, with little to no change in LVPW despite the increased LVAW thickness.

The BALB/cJ genetic background is predisposed toward developing dilated cardiomyopathy.

We measured heart mass with two methods: calculation of left ventricular mass by 2D echocardiography and organ weight at necropsy. For each animal, we normalized heart mass to the lean body mass. While the A, B6, and FVB mouse strains had similar cardiac mass by the 16th-month time point, the BALB mouse strain had considerably more massive hearts, as demonstrated by both heart weight and LVM (Figure 2.7a-d). We monitored changes in cardiac structure and function by 2D echocardiography and observed a progression toward dilated cardiomyopathy (DCM) among the BALB males (Figure 2.7e), defined by an extreme increase in LVID, reduced systolic function, gross spherical (globular) shape, and cardiomegaly. The results confirm previous reports that the BALB genetic background is genetically predisposed toward development of dilated cardiomyopathy. Interestingly, male mice clearly demonstrated the DCM phenotype, whereas the female mice did not (Figure 2.7). The BALB male hearts that displayed the DCM phenotype exhibited a 2-fold increase in *Timp1* gene expression and a 10-fold

increase in *Spp1* gene expression when compared to the control (Figure 2.7g). *Timp1* is a tissue inhibitor of matrix metalloproteinases (MMPs) which are involved in breaking down the LV myocardial collagen matrix.

Treatment with AG1478 exacerbates severity of dilated cardiomyopathy

A subgroup of the study population, the BALB/cJ males, demonstrated sensitivity to the therapeutic dose (144 mg/kg) of AG-1478 but not to the sub-therapeutic doses (data not shown). During age-related progression toward increased heart mass, there is a statistical difference between control and therapeutic-level treatment at the 8th-month time point, in which the treatment mice had a larger average LVM ($p < 0.05$; Figure 2.7f). This trend persisted from the 8th-month forward, although there was a large statistical variation within the treatment group in the 12 and 16th month time points. The variation was large enough to not yield statistical significance, but the upward trend was clearly maintained, showing that treatment BALB/cJ males had greater increases in LVM compared to control mice. Correlation analysis revealed a positive correlation between dose and two components of LVM, the LV anterior wall ($r = .20$, $p < 0.05$) and posterior wall ($r = .23$, $p < 0.05$; Supplementary Figure 2.2). At necropsy, the hearts from the therapeutic-level treatment group had significantly increased weight compared to the control group ($p < 0.01$; Figure 2.7b), supporting the trend that the treatment group accumulated more mass than the control, although the frequency of dilated cardiomyopathy is similar among both dose groups (Figure 2.7h). Hearts that displayed

the DCM phenotype also exhibited a 2-fold increase in *Timp1* and a 10-fold increase in *Spp1* gene expression (Figure 2.7g) when compared to normal hearts (without DCM).

Considerations about timing of treatment: impact on weight

We measured body weight and composition at 4-month intervals during the 16-month duration of the study and compared the 5, 50, and 144 mg/kg dose groups to the 0 mg/kg control per sex and strain. We found no significant statistical trends, using both ANOVA per time point and linear regression with time as the X variable. This is in contrast to findings by Barrick et al. (2008), who reported that wild-type C57BL/6J female mice (but not male mice) had reduced weight gain over the course of 90 days of treatment with 144 mg/kg of AG-1478. Since our experimental design consisted of gathering data in 4-month intervals (i.e. we did not record data in the time between 0 to approximately 120 days of treatment) and Barrick et al. (2008) showed that the difference between treatment and control female mice was significant between 20-60 days of treatment but non-significant at 90 days (where the weight-time points began to merge), we conclude that any mild effects on weight gain are temporary and normalize over time. The temporary nature of this effect may be due to initial effects of treatment followed by adaptation of the organism or by an effect that is dependent on whether the mice were at the young adulthood period in which they gained weight, presumably before they were 120 days old. The Barrick et al. (2008) study, as with the present study, commenced treatment when the mice were approximately 8 weeks of age. This means that the timelines are comparable and demonstrates that the 20 to 60-day window of significant reduction in weight gain was not captured in our current study. We

demonstrate that after 8 months, there is little change in weight (Figures 2.8-2.10) and that the most rapid increase in both weight and fat mass occurred within the first 4 months of treatment (approximately the first 130 days of age), indicating that the impact of EGFR inhibition on the physiology of weight gain may be dependent on the age of the mice, specifically on whether the mice are less than 130 days old and are in the period of their lives when they are gaining weight rapidly. A follow-up experiment that measures weight gain at regular intervals over the course of the first 130 days of age would aid in demonstrating that weight gain is reduced in the treatment group temporarily but normalizes with time. In addition, having a separate cohort of mice who are introduced to AG1478 treatment as older adults, at least 130 days of age, would aid in confirming whether the temporary effect is dependent on age or initial physiological response to treatment. This discrepancy would be important in identifying whether EGFR inhibitors are safe to administer to young adults (former hypothesis). If there is an initial weight loss response (latter hypothesis), there should be as assessment for whether a patient who is already underweight (as is common with cancer treatments) should be considered for treatment or given supplements to prevent the initial weight reduction. Weight loss is a commonly reported side effect of EGFR TKIs, and has recently been associated with reduced survival among patients with non-small cell lung carcinoma (Lin et al., 2018).

Treatment with AG1478 had no impact on survival

We saw no significant differences in survival (Kaplan-Meier curve) among treatment groups when separated by strain and sex (Figure 2.11). We aimed to maintain

a sample size of at least 10 of the original 20 mice per treatment group (per strain and sex) for the 16-month duration of the study and were able to meet this goal. However, the BALB and FVB males had markedly higher mortality rates than the other strain/sex groups.

Splenomegaly in BALB/cJ males with dilated cardiomyopathy

Differences in genetic-dependent immunology is a major determinant of heart disease outcome, with BALB/cJ being naturally at higher risk for DCM (Nishimura et al., 2001; Huber & Lodge, 1986; Li et al., 2012; Liao et al., 2005). Liao et al. (2005) linked the development of DCM in BALB/cJ male mice to a heightened B lymphocyte-mediated response when mice were exposed to human ADP/ATP. The membrane bound protein programmed cell death (PD1) plays a role in preventing autoimmunity by inhibiting T cell activity. PD1-deficient BALB/cJ mice also had higher rates of DCM, heart failure, and mortality compared to the PD1-deficient C57BL/6J mice (Nishimura, 2001). Overall, there is a convincing connection between inappropriate immune response and development of DCM. Since the BALB/cJ genetic background is much more sensitive to autoimmune disorders in terms of development of DCM, we examined mouse spleens for potential abnormalities that could correlate to DCM incidence. Spleen weights and length were significantly high for C57BL/6J and BALB/cJ male mice treated with 144 mg/kg of AG1478, in comparison to their corresponding controls (Figure 2.12).

Discussion

Our results showed that the BALB/cJ and C57BL/6J genetic backgrounds had an inherent tendency toward cardiomegaly and dilated cardiomyopathy (DCM) although the BALB/cJ background showed a greater propensity. This is consistent with previous studies showing that the BALB/cJ and C57BL/6J mouse strains are at high risk for developing DCM when exposed to known contributing factors of DCM, such as doxorubicin and Angiotensin II-dependent hypertension (Liu et al., 2012; Peng et al., 2012, Peng et al., 2016). Furthermore, previous studies also indicate that BALB/cJ male mice fare worse than C57BL/6J males in terms of development of DCM and ensuing mortality rates. We saw a 2-fold increase in *Timp1* and a 10-fold increase in *Spp1* gene expression. *Timp1* has been reported to be overexpressed by at least 500% in human hearts with severe DCM (Thomas et al., 1998). *Spp1* is known to play a role in inflammation and autoimmunity and to be highly expressed in the DCM heart. *Spp1* expression has been shown to induce inflammation and fibrosis, which are permissive to the development of DCM and heart failure (Renault et al., 2010).

The BALB/cJ strain was the only genetic background with a statistically significant difference between treatment (144 mg/kg) and control, in which the treatment group had exacerbated accumulation of LVM. Global longitudinal strain (GLS) and strain rate, used to detect subclinical changes in myocardial movement during anti-cancer treatment (Thavendiranathan et al., 2014; Zeglinski et al., 2011) did not reveal

significant trends between control and treatment groups for any of the genetic backgrounds (data not shown). Our results indicate that individuals at risk for DCM (or have pre-existing DCM) had more severe accumulation of left ventricular mass (LVM) in conjunction with use of AG1478 as evidenced by M-mode 2D echocardiography. The consensus in the literature and medical field is that small molecule inhibitors of EGFR do not show significant cardiotoxicity or if there is toxicity it is too subtle to report. Here we demonstrate that a subgroup in the population, those at risk of DCM, may experience severe adverse effects to EGFR inhibitors.

Cardiotoxicity induced by chemotherapeutics are most common with pre-existing cardiovascular conditions. DCM is a common cause of heart failure and has a strong genetic component, with approximately 20-35% of cases being of familial origin (Maron et al., 2006). The population incidence of DCM is estimated to be 4.5 cases per 100,000 people (Rakar et al., 1997). DCM has mixed causes, which include genetic mutations, autoimmune disorders, chemotherapeutic agents (doxorubicin and daunorubicin), and metabolic disorders. Li et al. (2012) showed that in a mouse model of human inherited DCM (deletion of Lys210 in cardiac troponin T) that utilized the BALB/cJ and C57BL/6J mouse strains, the greater severity of DCM in the BALB/cJ background was partly mediated by targeting the BALB/cJ-specific loss of function mutation in the tryptophan hydroxylase-2 gene (C1473G) involved in serotonin regulation. The serotonin reuptake inhibitor, paroxetine, reduced the BALB/cJ elevated levels of *Akt*, *p38*, and *Serca2a* (which can be used as a proxy of ERK activity) myocardial gene expression to levels comparable to C57BL/6J and improved overall symptoms of heart

failure (Li et al., 2012). AKT is known to be involved in physiological response to stress and exercise by compensatory cardiac hypertrophy (Kenessey & Ojamaa, 2006; Kinugawa et al., 2005), p38 MAPK is involved in pathological hypertrophic response, and ERK has been shown to be protective against eccentric hypertrophy (Kehat et al., 2011). The propensity of BALB/cJ to upregulate pathways that are part of the canonical EGFR signal transduction cascade may indicate hyperactivity of EGFR, also potentially linked to the fact that poxvirus (which maintains EGFR activity by prevention of receptor recycling) is a known contributor to DCM development. The fact that the AG1478 EGFR inhibitor in our study exacerbates the DCM phenotype suggests that BALB/cJ is able to activate potent alternative pathways in the presence of EGFR inhibition.

During baseline echocardiographic measurements, we observed consistent differences between mouse strains, indicating the significance of genetic background in determining normality (Figure 2.1; Table 2.1). Our results show that there is significant variability in the values of heart dimension and function among mouse strains and that separation by strain is an important factor to consider during analysis for each experiment. The aging process can also impact sensitivity to dosage and is in itself indicative of underlying differences in genetic architecture to how the heart responds to stress – an indication that there is an inherent difference in how the heart might respond to treatment in relation to changes due to aging.

Differences due to strain and sex were reduced when normalized to lean mass, but some differences remained, highlighting the importance of genetics and sex in

animal modeling. Males had higher absolute CO than females, but the relationship was inversed when normalized to lean mass. Overall, lean mass was more highly correlated with heart parameters than either fat mass and body weight, a similar trend to what has been reported in other studies (Collis et al., 2001), given that lean mass is metabolically active and therefore correlated to the oxygen demands that influence CO.

Histopathological examination did not reveal any significant dose-dependent toxicological patterns. Overall, these findings support the idea that EGFR TKIs pose little cardiotoxicity risk in chronic clinical use. However, individuals with dilated cardiomyopathy should be closely monitored for signs of aggravated hypertrophy while receiving EGFR-targeted treatment.

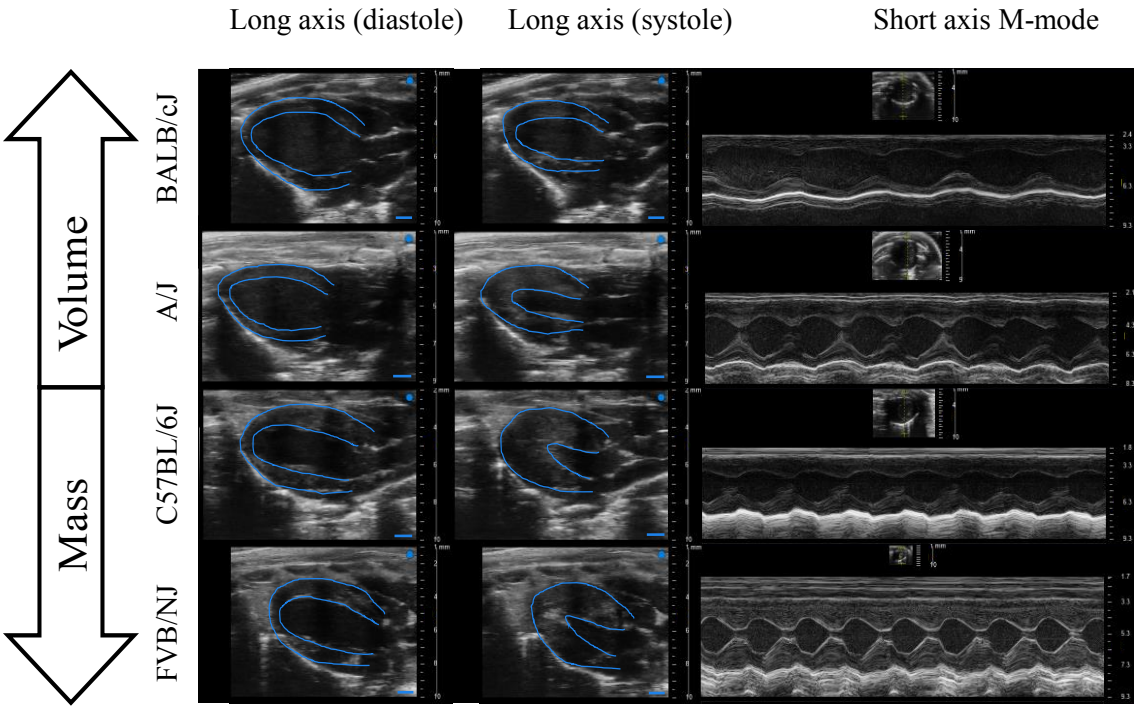
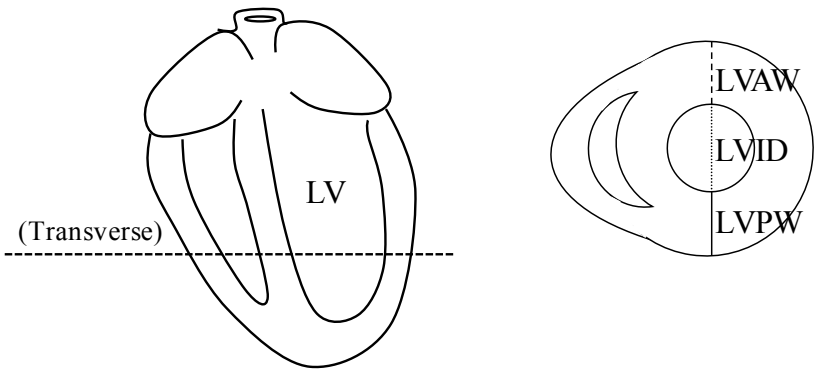


Figure 2.1. Genetic background-dependent differences in baseline echocardiographic parameters. Echocardiography B-mode and M-mode imaging of a representative male per mouse strain. The left ventricular endo and epicardium are traced in blue. The scale bar signifies 1 mm, and the dot indicates the orientation of the transducer (the blue dot is closer to the cranial end). The two-sided arrow indicates a phenotypic spectrum in cardiac lumen volume and wall thickness, in which the top two mouse strains have greater left ventricular diameters and blood volume while the bottom two mouse strains have smaller left ventricular diameters and thicker left ventricular walls.

a



b

	Percent change (%)			
Variable	A/J	BALB/cJ	C57BL/6J	FVB/NJ
LVM (mg)	100	216	195	94
LVID (mm)	27	92	110	0
LVAW (mm)	19	43	38	33
LVPW (mm)	15	8	0	25
CO (mL/min)	18	59	100	-21
SV (uL)	29	50	145	-20
EF (%)	-3	-24	-15	-2

c

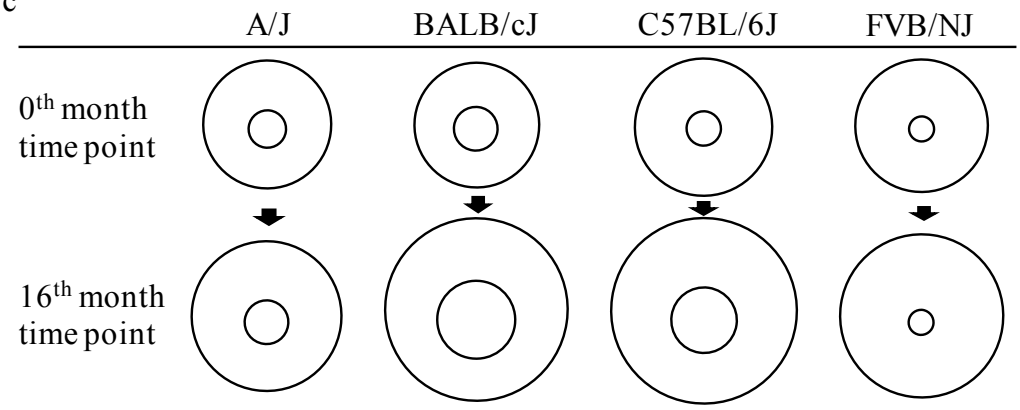


Figure 2.2. Age-related cardiovascular changes vary by genetic background. (a) Cross sectional and transverse views used to analyze left ventricular structure and function. (b-c) Changes in cardiac structure and function among control mice from baseline to the 16th-month time point.

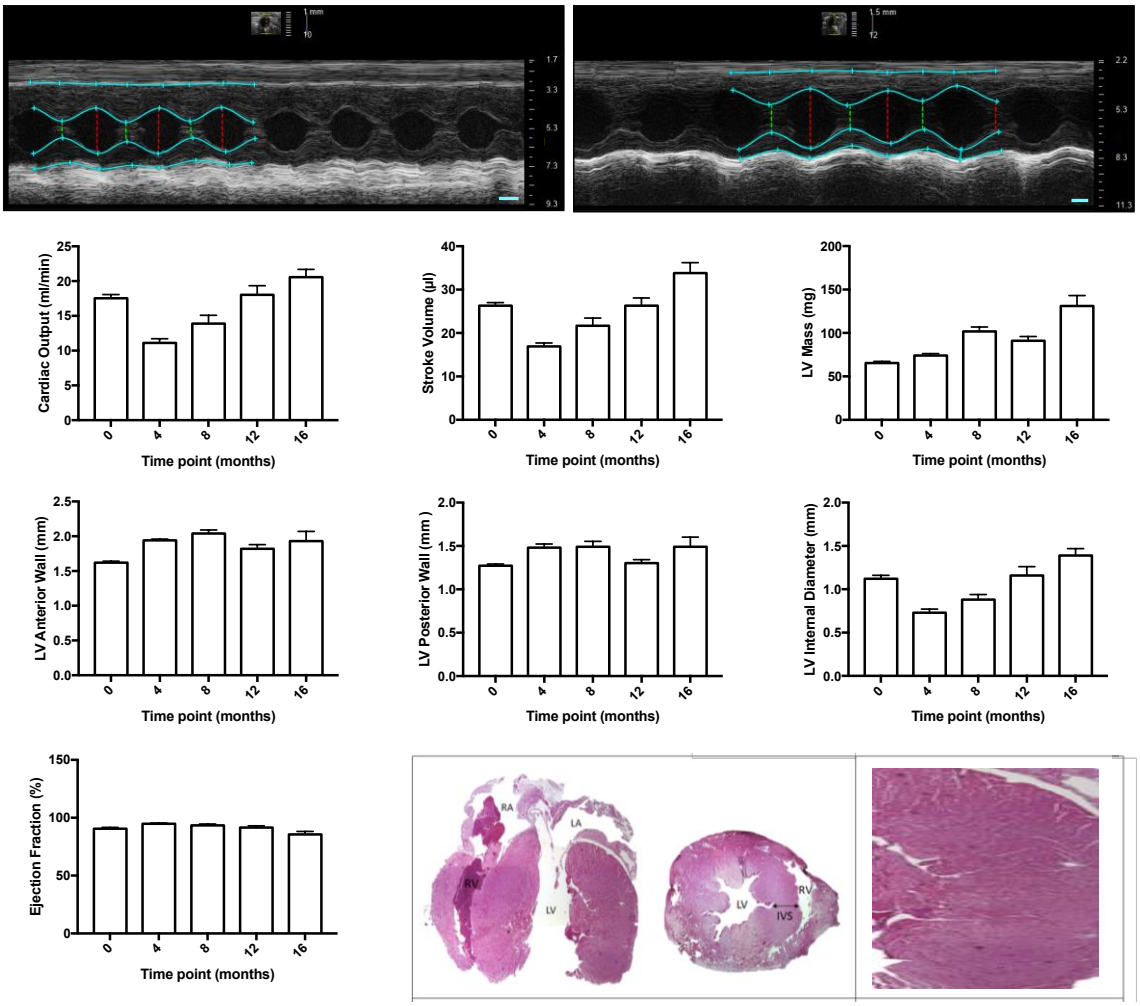


Figure 2.3. Age-related changes in cardiac function and structure in the A/J genetic background. Control mice with males and females grouped together.

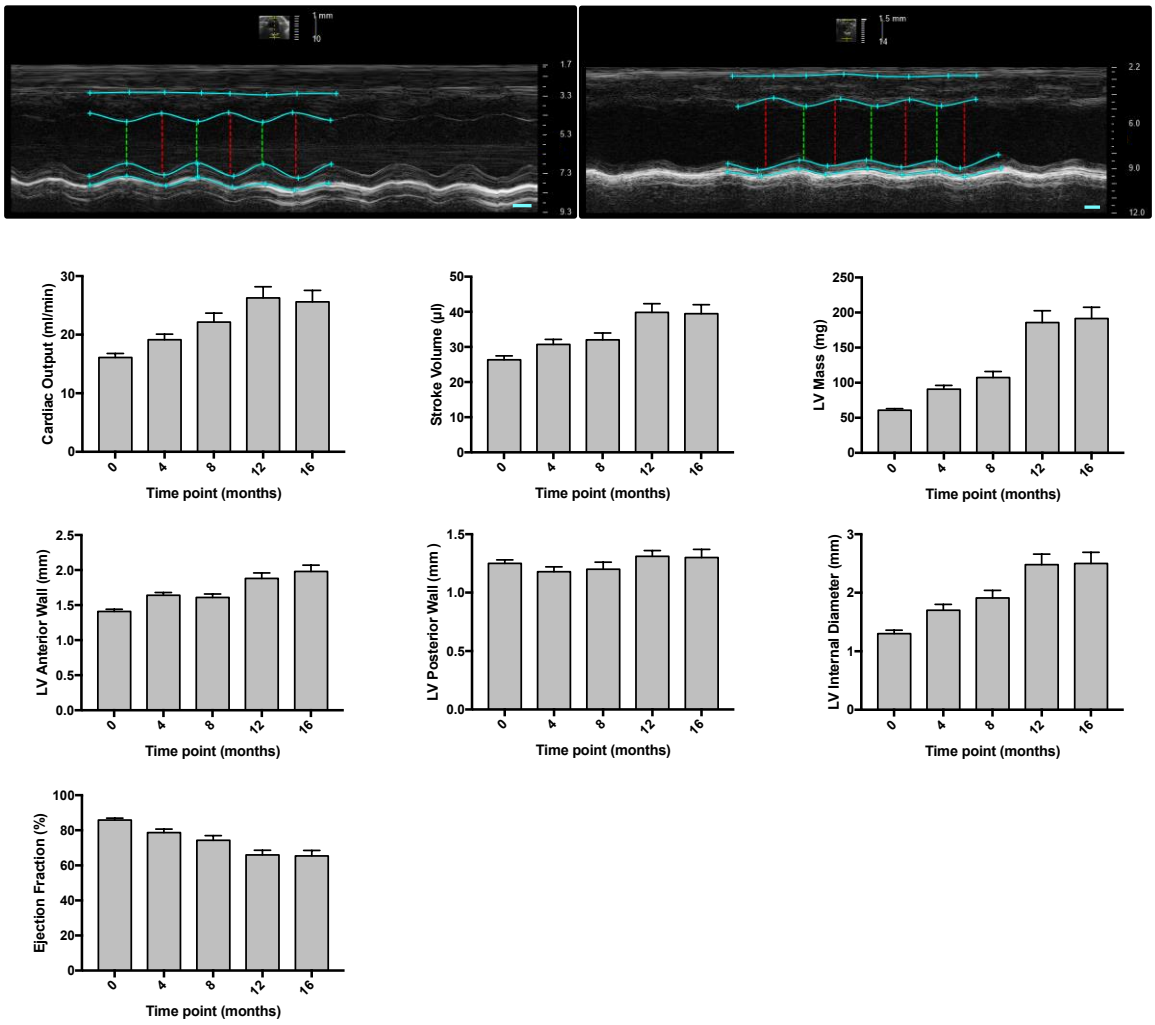


Figure 2.4. Age-related changes in cardiac function and structure in the BALB/cJ genetic background. Control mice with males and females grouped together.

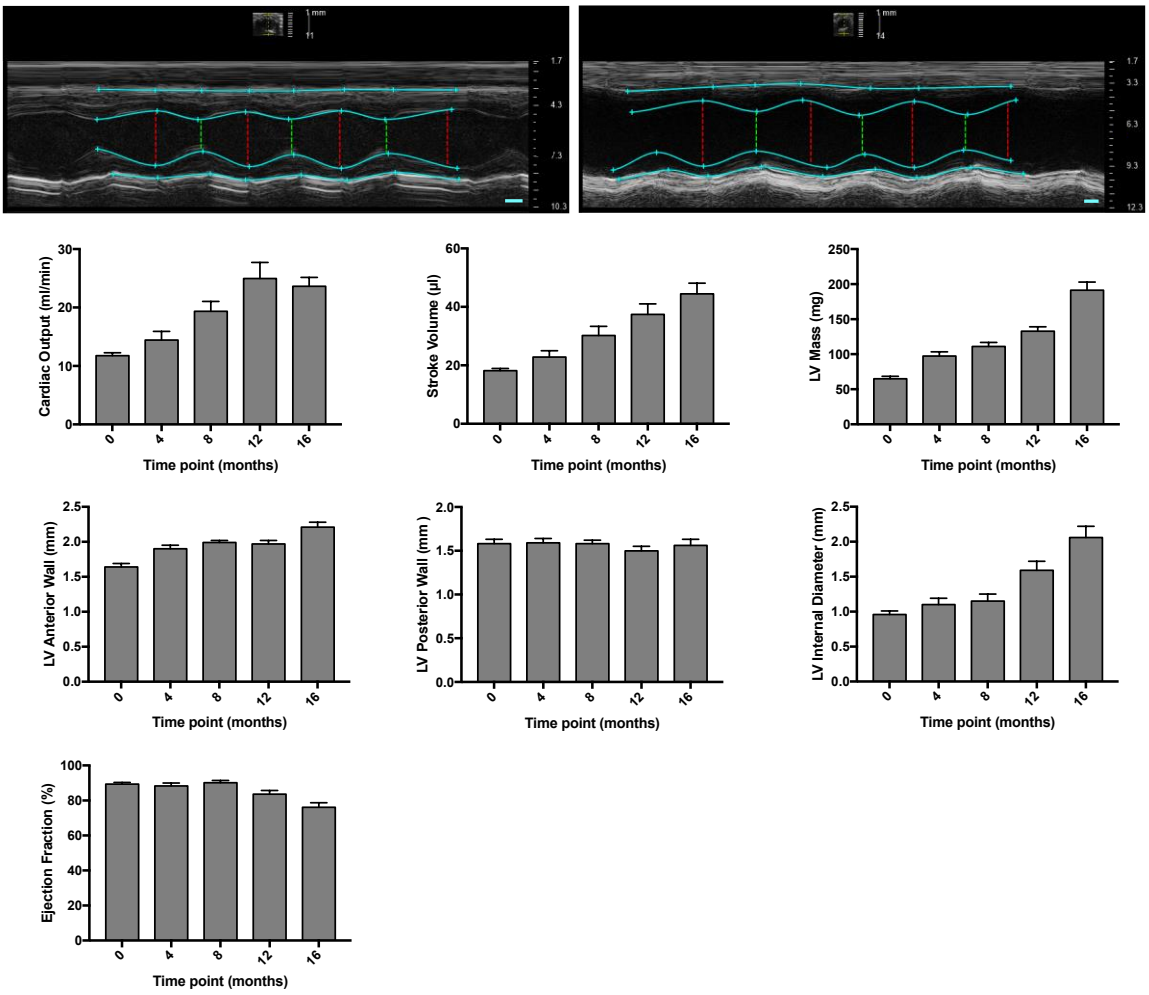


Figure 2.5. Age-related changes in cardiac function and structure in the C57BL/6J genetic background. Control mice with males and females grouped together.

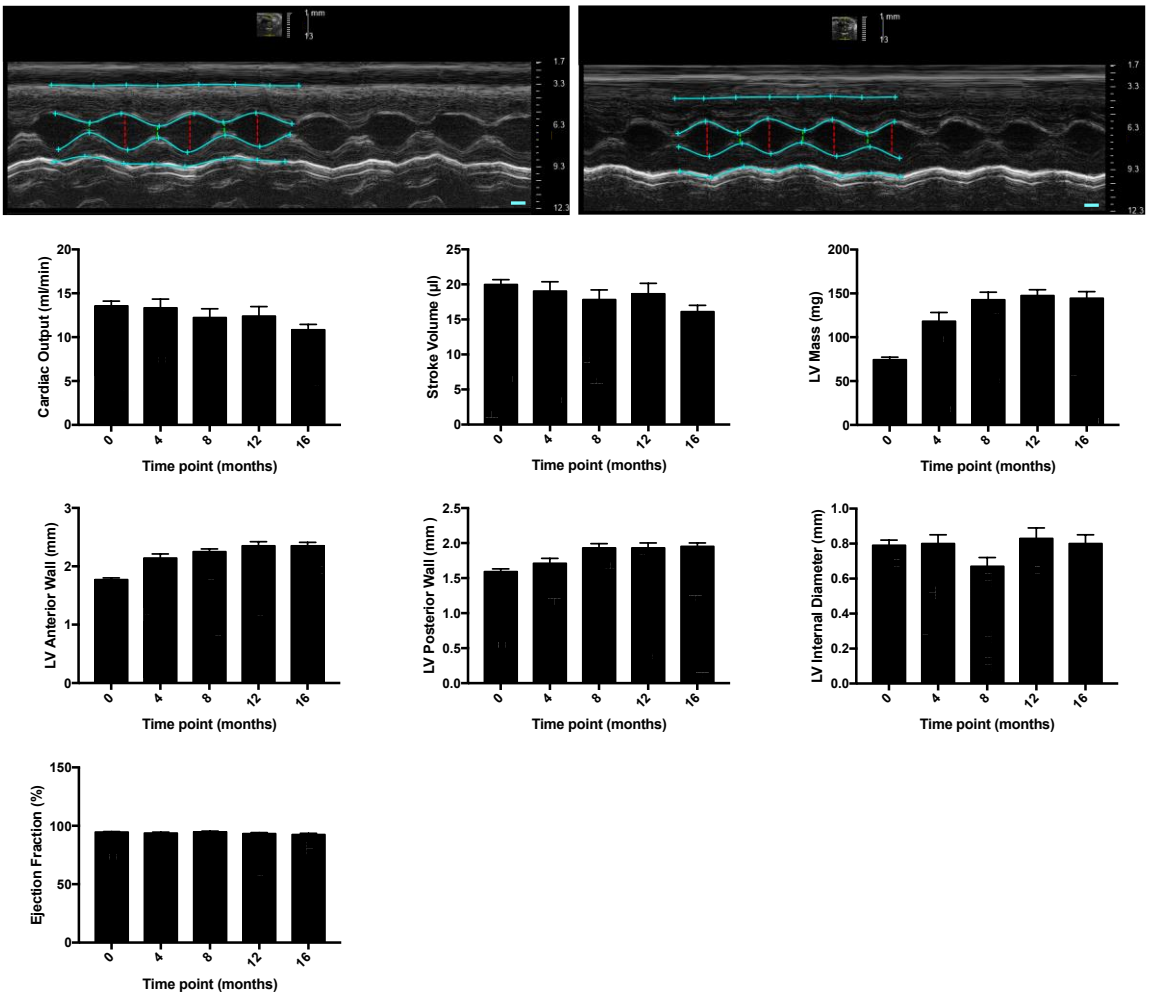


Figure 2.6. Age-related changes in cardiac function and structure in the FVB/NJ genetic background. Control mice with males and females grouped together.

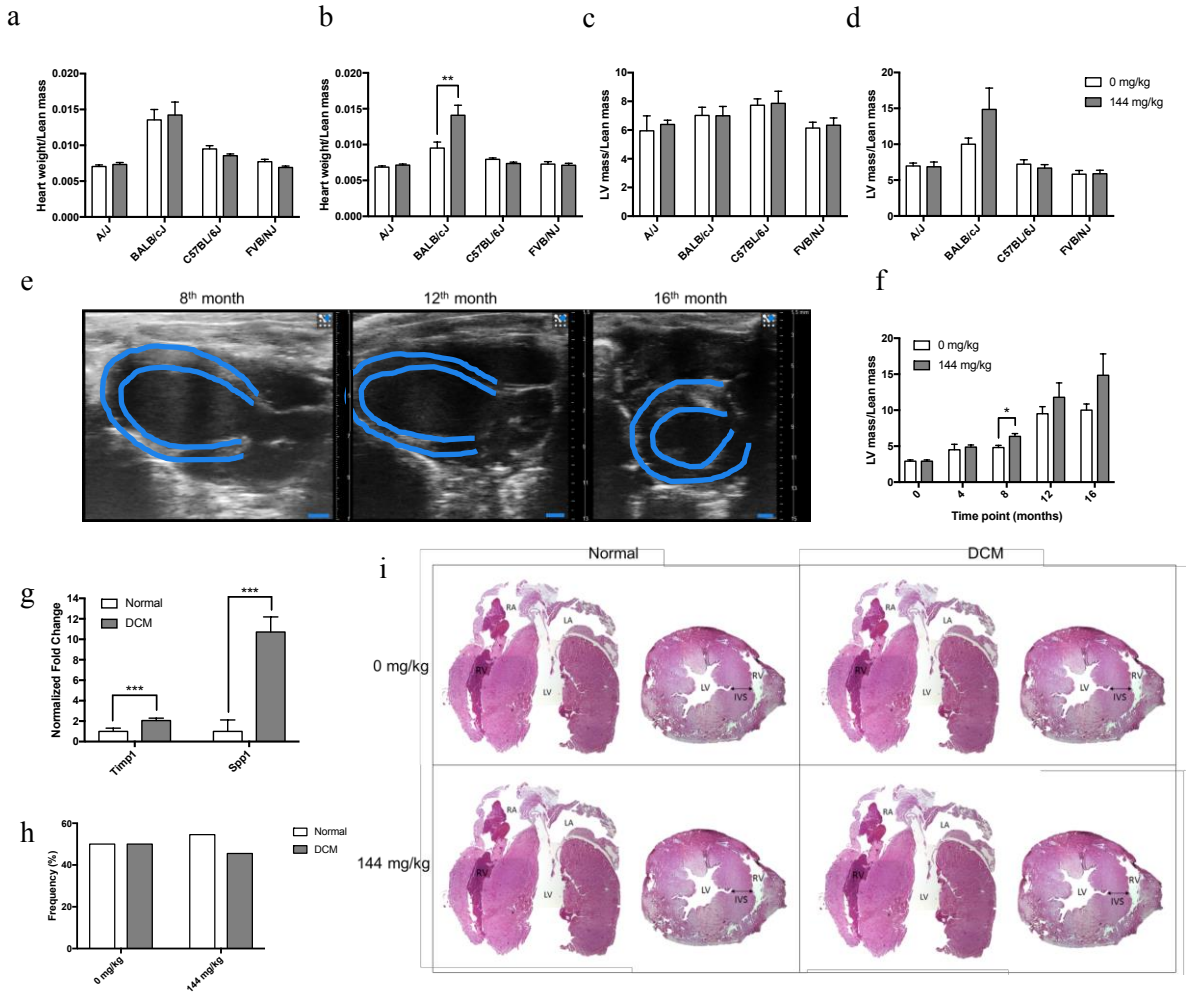


Figure 2.7. Treatment with AG1478 exacerbates severity of dilated cardiomyopathy. Ratio of heart weight to lean mass at necropsy for (**a**) female (n ≥ 11) and (**b**) male (n ≥ 8) mice. Ratio of left ventricular mass (obtained by echocardiography at the 16th-month time point) to lean mass for (**c**) female (n ≥ 5) and (**d**) male (n ≥ 5) mice. (**e**) Echocardiography B-mode imaging of the longitudinal axis of a representative BALB/cJ male (144 mg/kg treatment group) across three time points, displaying progression of dilated cardiomyopathy. The blue scale bar indicates 1 mm. (**f**) Changes in the ratio of left ventricular mass to lean mass across time points. BALB/cJ male mice in the control and 144 mg/kg treatment groups are compared (n ≥ 5). (**g**) Fold change in expression of *Timp1* and *Spp1* for BALB/cJ male hearts grouped by normal versus dilated cardiomyopathy phenotypes (n ≥ 5). (**h**) Frequency of the dilated cardiomyopathy phenotype among BALB/cJ males in the control and 144 mg/kg treatment groups (n ≥ #). (**i**) H&E stained longitudinal and short axis sections of heart tissue from BALB/cJ male mice. * $p < 0.5$, ** $p < 0.01$, ** $p < 0.001$

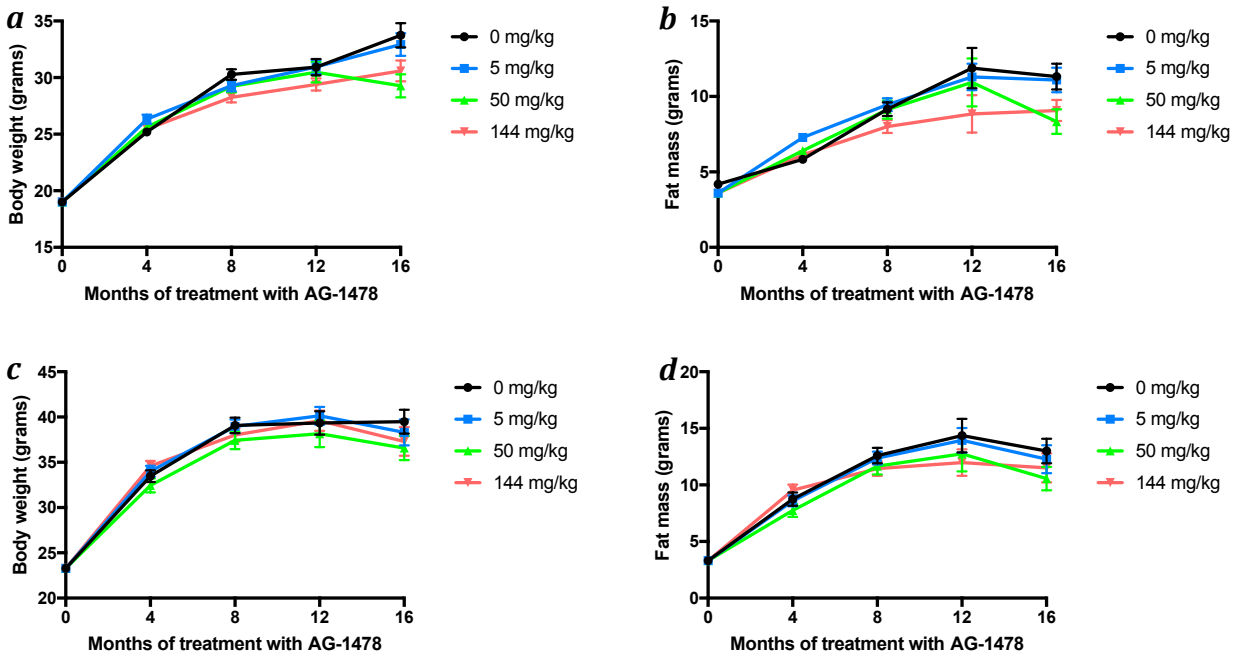


Figure 2.8. No significant changes in body and fat mass. Comparison of control versus treatment groups, separated by sex. Changes in body weight and fat mass (±SEM) for (a-b) all female mice combined (n = 78 -80) and (c-d) all male mice combined (n = 74-80).

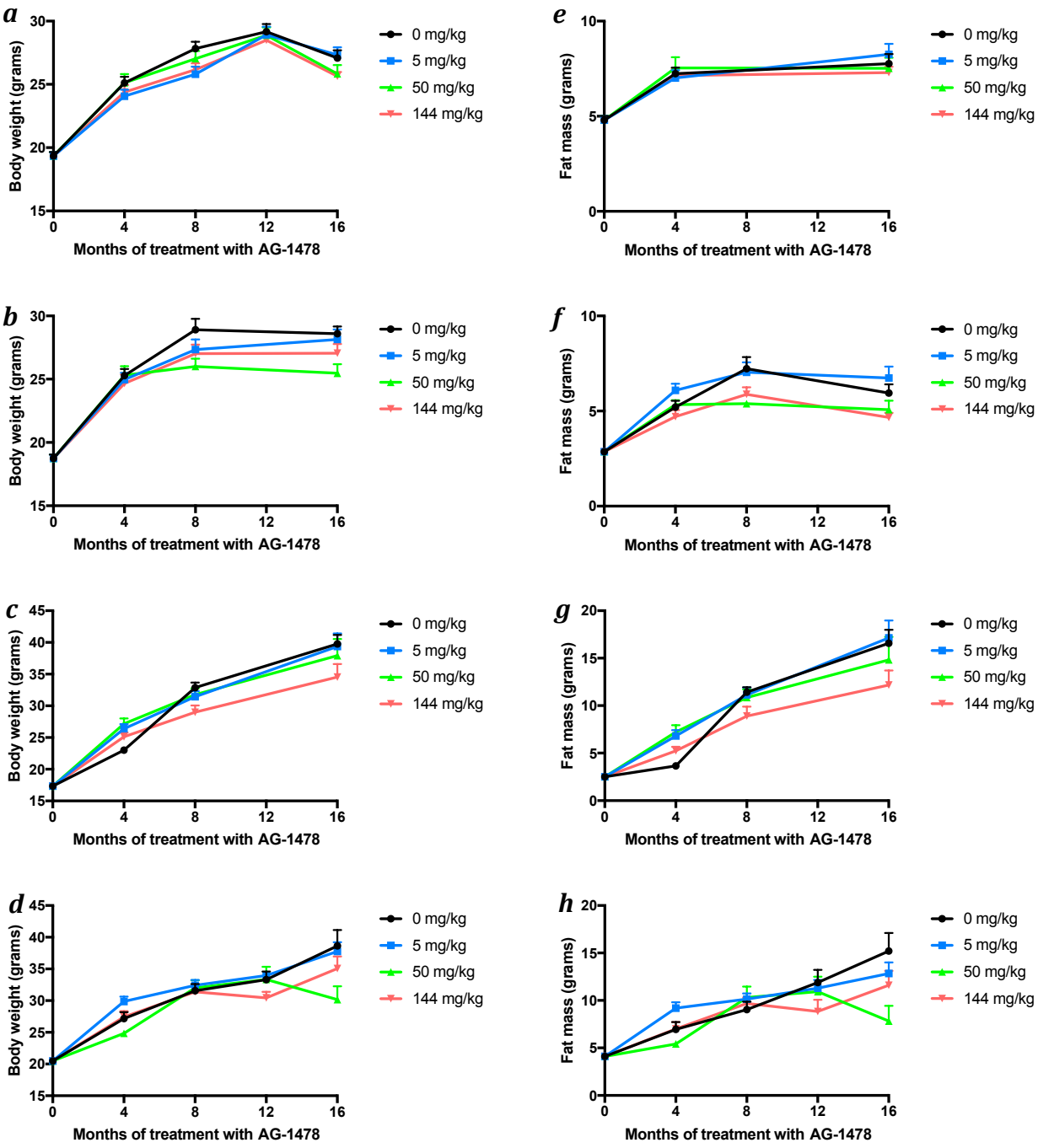


Figure 2.9. No significant differences in body weight and fat mass among female mice separated by strain.

Mean body weight and mean fat mass (±SEM) for (a) A/J females (n = 19-20), (b) BALB/cJ females (n = 19-20), (c) C57BL/6J females (n = 19-20), and (d) FVB/NJ females (n = 20).

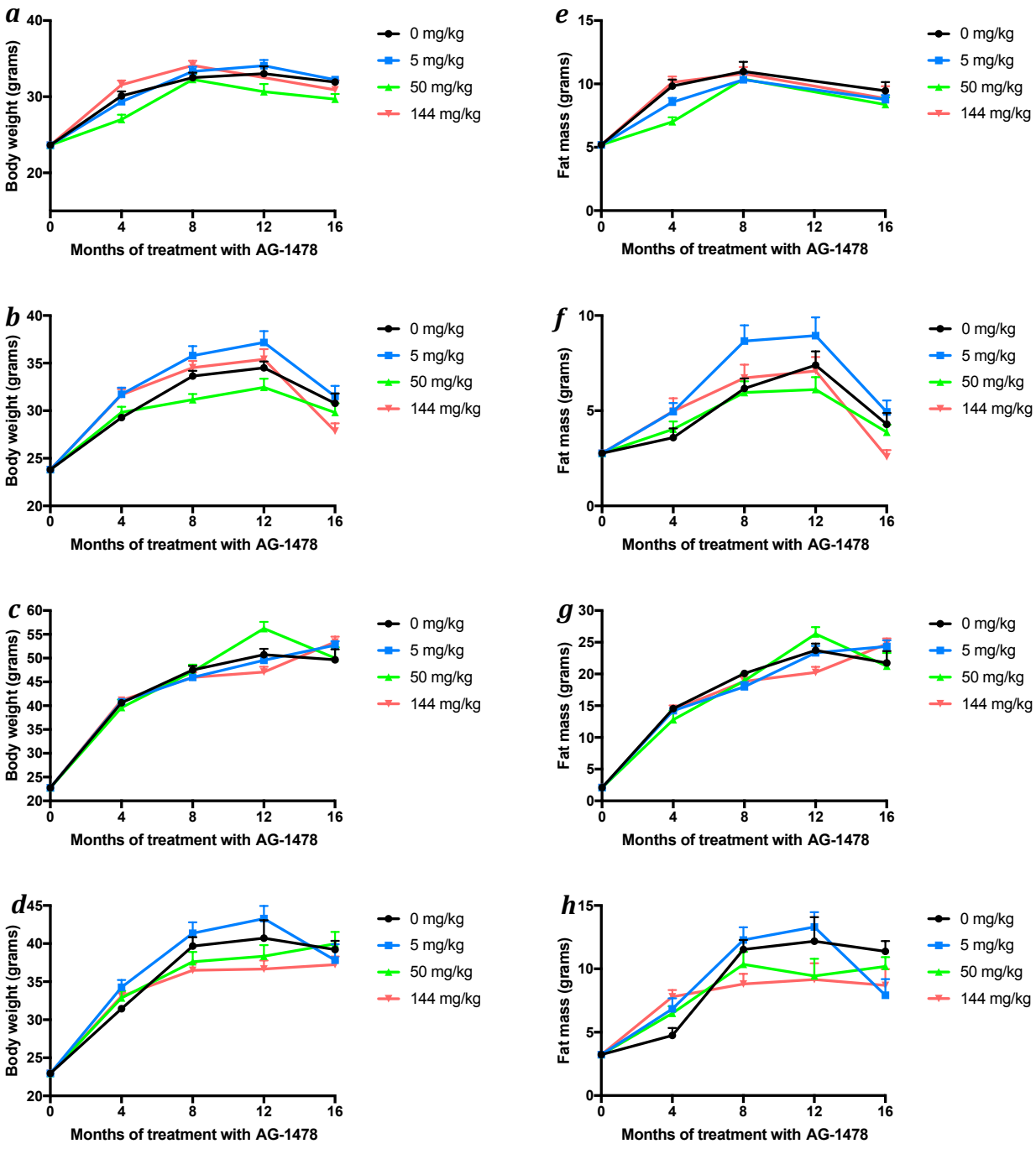


Figure 2.10. No significant differences in body weight and fat mass among male mice separated by strain.

Mean body weight and mean fat mass (±SEM) for (a-b) A/J males (n = 19-20), (b) BALB/cJ males (n = 18-20), (c) C57BL/6J males (n = 20), and (d) FVB/NJ males (n = 17-20).

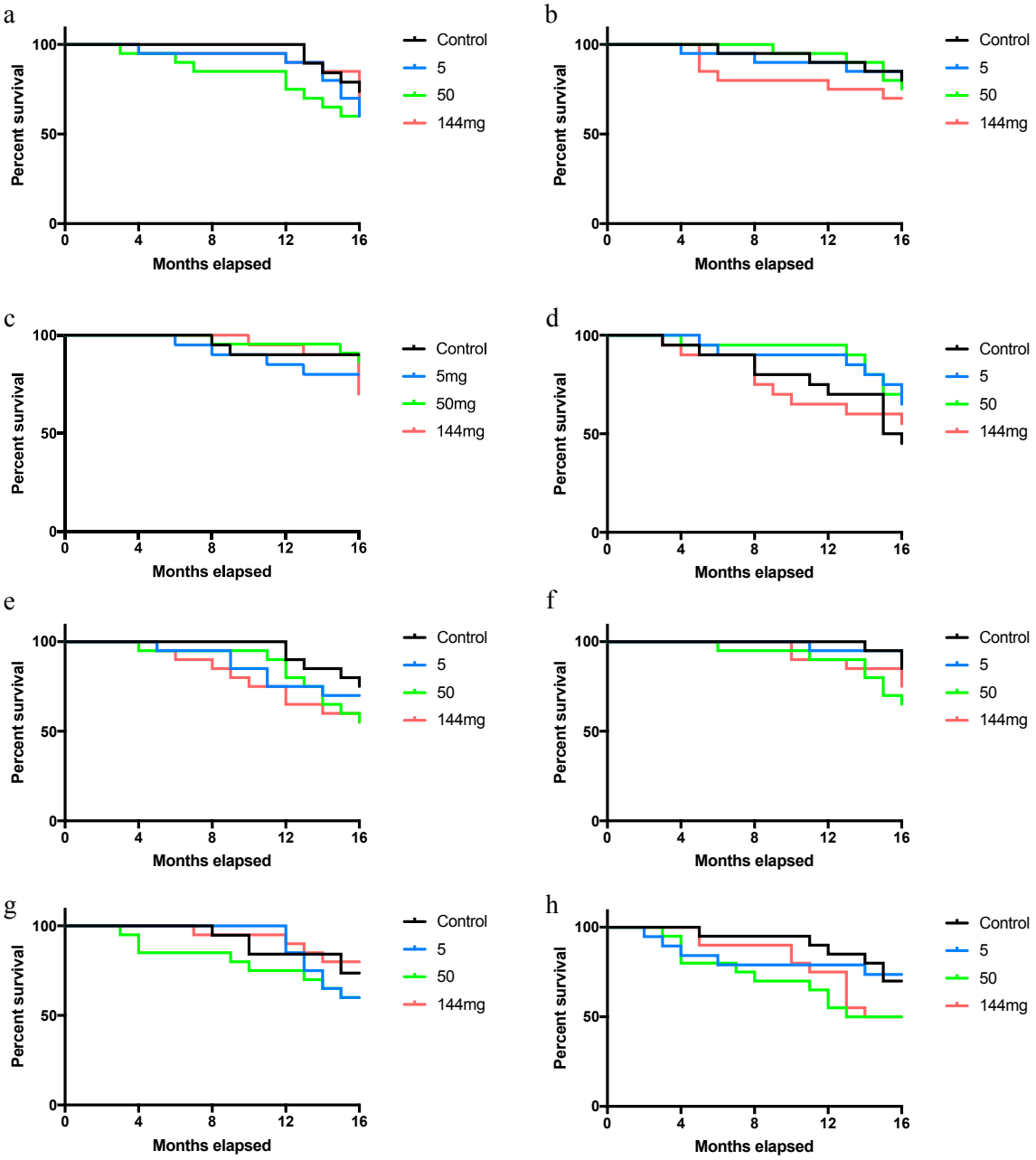


Figure 2.11. Kaplan-Meier survival curves for mice separated by strain and sex. (a-b) A/J female and male mice, respectively. (c-d) BALB/cJ, (e-f) C57BL/6J, (g-h) FVB/NJ.

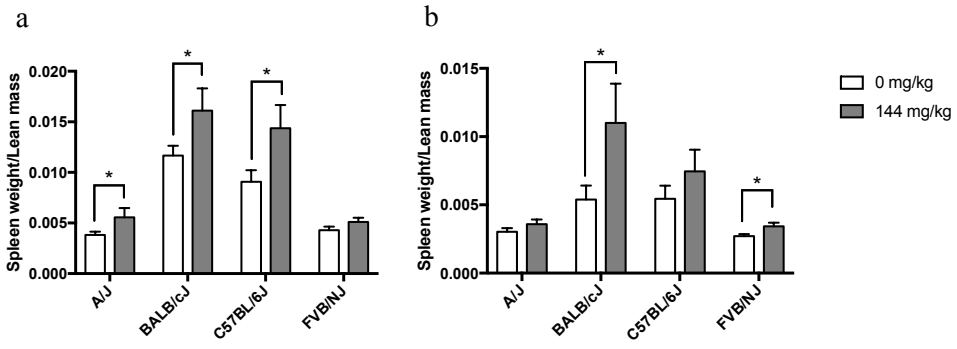


Figure 2.12. Increased splenomegaly among treated mice. Comparison between treated and control mice among (a) female and (b) male mice. * $p < 0.5$

	Female				Male			
Variable	A/J	BALB/cJ	C57BL/6J	FVB/NJ	A/J	BALB/cJ	C57BL/6J	FVB/NJ
BW (g)	19.3 ± .3 [a]	18.7 ± .3 [a]	17.3 ± .2 [b]	20.5 ± .2 [c]	23.6 ± .4	23.8 ± .4	22.8 ± .4	22.9 ± .2
Fat mass (g)	4.8 ± .2 [a]	2.9 ± .1 [b]	2.5 ± .1 [b]	4.1 ± .1 [c]	5.2 ± .3 [a]	2.8 ± .1 [b]	2.1 ± .1 [c]	3.2 ± .1 [d]
Lean mass (g)	13.8 ± .1 [a,b]	14.2 ± .2 [a]	13.5 ± .2 [b]	14.8 ± .1 [c]	17.3 ± .2 [a]	19.6 ± .3 [b]	19.4 ± .4 [b]	18.2 ± .2 [c]
n (body composition)	20	20	18	19	20	18	20	20
CO (mL/min)	16.58 ± .71 [a]	14.77 ± .87 [a,b]	12.36 ± .60 [b,c]	11.58 ± .66 [c]	18.53 ± .78 [a]	17.50 ± 1 [a,b]	11.02 ± .80 [c]	15.47 ± .56 [b]
CO/BW (mL/min/g)	1.00 ± .06 [a]	.94 ± .08 [a]	.72 ± .04 [b]	.50 ± .05 [c]	.96 ± .09 [a]	.59 ± .09 [b]	.51 ± .04 [b]	.67 ± .06 [b]
SV (uL)	24.80± .89 [a]	24.39 ± 1.43 [a]	18.87 ± .92 [b]	17.71 ± .92 [b]	27.92 ± .90 [a]	28.42 ± 1.65 [a]	17.34 ± 1.20 [b]	22.15 ± .77 [c]
SV/BW (uL/g)	1.47 ± .07 [a]	1.67 ± .11 [a]	1.10 ± .06 [b]	.79 ± .07 [c]	1.42 ± .12 [a]	.97 ± .21 [a,b]	.80 ± .06 [b]	.93 ± .08 [b]
Heart rate (beats/min)	666 ± 10 [a]	609 ± 12 [b]	656 ± 7 [a]	657 ± 13 [a]	661 ± 12 [a]	621 ± 15 [b]	634 ± 18 [a,b]	699 ± 7 [c]
LVID,s (mm)	1.02 ± .04 [a]	1.21 ± .08 [b]	.90 ± .09 [a,c]	.76 ± .05 [c]	1.23 ± .06 [a]	1.40 ± .09 [a]	1.05 ± .05 [b]	.81 ± .04 [c]
LVID,d (mm)	2.70 ± 04 [a]	2.67 ± .08 [a]	2.43 ± .06 [b]	2.31 ± .05 [b]	2.88 ± .04 [a]	2.88 ± .10 [a]	2.33 ± .07 [b]	2.49 ± .04 [b]
EF (%)	90.54 ± 1.14 [a]	87.13 ± 1.33 [b]	90.95 ± 1.16 [a,b]	94.74 ± .52 [c]	88.15 ± 1.06 [a]	84.57 ± 1.75 [a]	87.47 ± 1.04 [a]	94.52 ± .55 [b]
LVM (mg)	64.52 ± 2.69	58.06 ± 3.33	58.79 ± 5.28	67.26 ± 3.30	66.17 ± 2.12 [a]	63.48 ± 3.24 [a]	72.36 ± 3.28 [a,b]	81.25 ± 3.85 [b]
LVM/BW	3.80 ± .23 [a]	3.72 ± .39 [a,b]	3.42 ± .33 [a,b]	2.89 ± .24 [b]	3.04 ± .24 [a,b]	2.39 ± .16 [a]	3.35 ± .19 [b]	3.10 ± .08 [b]
LVAW,s (mm)	1.67 ± .03 [a]	1.40 ± .04 [b]	1.61 ± .07 [a]	1.70 ± .03 [a]	1.56 ± .03 [a]	1.43 ± .04 [b]	1.67 ± .05 [a]	1.84 ± .04 [c]
LVAW,d (mm)	.94 ± .03 [a]	.77 ± .03 [b]	.92 ± .07 [a]	.98 ± .02 [a]	.85 ± .02 [a]	.80 ± .03 [a]	1.09 ± .05 [b]	1.07 ± .03 [b]
LVPW,s (mm)	1.28 ± .03 [a]	1.26 ± .03 [a]	1.55 ± .07 [b]	1.56 ± .04 [b]	1.27 ± .03 [a]	1.24 ± .05 [a]	1.61 ± .06 [b]	1.61 ± .06 [b]
LVPW,d (mm)	.67 ± .01 [a]	.72 ± .02 [a]	.76 ± .04 [a]	0.93 ± .04 [b]	.69 ± .01 [a]	.66 ± .03 [a]	.90 ± .06 [b]	.92 ± .04 [b]
n (echocardiography)	25	19	10	19	24	18	8	20
n (echocardiography/BW)	15	5	10	5	9	3	8	5

Table 2.1. Genetic background-dependent differences in baseline body composition and echocardiographic parameters.

BW, body weight; CO, cardiac output; SV, stroke volume; LVID,s, left ventricular internal dimension at systole; LVID,d, left ventricular internal diameter at diastole; EF, ejection fraction; LVM, left ventricular mass; LVAW,s left ventricular anterior wall at systole; LVAW,d, left ventricular anterior wall at diastole; LVPW,s, left ventricular posterior wall at systole; LVPW,d, left ventricular posterior wall at diastole. The table displays the mean for each variable along with the standard error of the mean. Mouse strains were tested for significant differences between genetic background by the Wilcoxon signed rank test. Males and females were analyzed separately. The superscript letters indicate statistical groupings, denoting significant differences ($p \leq 0.05$) between groups with dissimilar letters.

Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Cardiac ouput	—	**.95**	**.11**	**.87**	**-.56**	**.47**	-.22	**-.21**	-.10	**.21**	.12	**.38**	**.59**	.15
2. Stroke volume	**.95**	—	-.10	**.92**	**-.60**	**.52**	-.24	**-.19**	-.05	**.23**	.09	**.45**	**.65**	.10
3. Heart rate	**.23**	-.05	—	-.07	.06	-.15	.03	**-.17**	-.11	.14	.18	-.08	-.07	.17
4. LV internal diameter	**.85**	**.89**	-.03	—	**-.83**	**.51**	-.25	**-.28**	-.06	**.25**	.04	**.43**	**.69**	.06
5. Ejection fraction	**-.52**	**-.55**	.05	**-.84**	—	**-.37**	.27	**.30**	.15	**-.19**	.05	**-.30**	**-.58**	.03
6. LV mass	**.45**	**.53**	-.17	**.50**	**-.33**	—	**.43**	**.38**	-.08	**.21**	**.18**	**.51**	**.50**	.03
7. LV anterior wall	-.22	-.19	-.07	-.22	**.23**	**.46**	—	.06	.08	.10	.08	.18	-.13	-.09
8. LV posterior wall	**-.18**	**-.13**	-.15	**-.27**	**.34**	**.46**	.10	—	-.07	**-.20**	.04	-.02	**-.16**	.08
9. Mean arterial pressure	.28	.23	**.32**	.23	-.28	**.31**	.15	-.01	—	.28	.23	.16	.21	.03
10. Body weight	**.29**	**.29**	-.02	**.38**	**-.34**	**.28**	.06	-.06	.27	—	**.86**	**.71**	**.37**	-.12
11. Fat mass	.15	**.16**	-.04	**.26**	**-.24**	**.17**	-.02	.05	.34	**.86**	—	**.48**	**.09**	-.16
12. Lean mass	**.36**	**.42**	-.17	**.48**	**-.40**	**.43**	.16	.04	.16	**.73**	**.46**	—	**.46**	**-.18**
13. Time point	**.51**	**.60**	**-.21**	**.57**	**-.39**	**.69**	.14	.15	.17	**.26**	.09	**.41**	—	.05
14. AG-1478 dose	.15	.15	.08	.15	-.09	.07	.14	**-.12**	.12	.08	.14	.10	.07	—

Table 2.2. Correlation coefficients (r) for the A/J mouse strain across four time points (4, 8, 12, and 16-month points). Coefficients above the diagonal represent correlations for the female mice (n = 183) and coefficients below the diagonal correspond to the male mice (n = 174). LV stands for left ventricular; coefficients in bold are significant ($p < 0.05$), as tested by the Spearman's ρ test.

Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Cardiac ouput	—	**.92**	.16	**.60**	**-.25**	**.36**	-.05	-.05	.18	.17	.18	.16	.11	-.07
2. Stroke volume	**.96**	—	**-.23**	**.75**	**-.41**	**.46**	-.03	-.14	.21	.15	**.17**	**.17**	.16	-.01
3. Heart rate	**.26**	-.02	—	**-.41**	**.42**	-.23	-.03	**.26**	-.08	-.01	-.01	-.05	-.09	-.15
4. LV internal diameter	**.59**	**.70**	**-.27**	—	**-.90**	**.52**	-.08	**-.37**	.30	**.22**	**.17**	**.28**	**.27**	.10
5. Ejection fraction	**-.28**	**-.38**	**.29**	**-.90**	—	**-.43**	**.11**	**.40**	-.28	**-.23**	**-.14**	**-.31**	**-.31**	-.12
6. LV mass	**.46**	**.54**	-.14	**.61**	**-.50**	—	**.70**	**.32**	.35	**.22**	.08	**.39**	**.53**	.06
7. LV anterior wall	.18	.18	.03	.12	-.05	**.77**	—	**.27**	.13	.09	.00	**.19**	**.31**	.04
8. LV posterior wall	**-.20**	**-.24**	.06	**-.41**	**.37**	**.20**	**.29**	—	.01	.06	-.03	**.18**	**.25**	-.14
9. Mean arterial pressure	.12	.08	.18	.15	-.20	**.19**	**.27**	.10	—	.05	-.08	**.45**	**.60**	-.02
10. Body weight	**.22**	**.20**	**.13**	**.21**	**-.19**	**.16**	.03	.12	**.41**	—	**.85**	**.69**	**.22**	**-.13**
11. Fat mass	.13	.10	.12	.11	-.10	.11	.05	.13	**.34**	**.93**	—	**.45**	.03	**-.17**
12. Lean mass	**.25**	**.23**	.11	**.29**	**-.30**	**.18**	-.01	.07	**.40**	**.83**	**.58**	—	**.45**	-.06
13. Time point	**.19**	**.21**	-.13	**.36**	**-.39**	**.46**	**.36**	**.22**	**.50**	-.09	-.13	.06	—	.01
14. AG-1478 dose	.02	.07	**-.18**	-.06	.13	.19	**.20**	**.23**	-.04	.02	-.04	-.02	.01	—

Table 2.3. Correlation coefficients (r) for the BALB/cJ mouse strain across four time points (4, 8, 12, and 16-month points). Coefficients above the diagonal represent correlations for the female mice (n = 201) and coefficients below the diagonal correspond to the male mice (n = 184). LV stands for left ventricular; coefficients in bold are significant ($p < 0.05$), as tested by the Spearman's ρ test.

Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Cardiac ouput	—	**.94**	.01	**.84**	**-.37**	**.74**	**.20**	**.13**	-.04	**.56**	**.47**	**.70**	**.72**	-.03
2. Stroke volume	**.69**	—	-.30	**.93**	**-.50**	**.72**	.11	.03	.04	**.61**	**.54**	**.70**	**.71**	-.06
3. Heart rate	**.26**	**-.48**	—	**-.39**	**.46**	-.09	**.23**	**.29**	-.31	-.22	-.25	-.09	-.04	.09
4. LV internal diameter	**.46**	**.87**	**-.62**	—	**-.77**	**.73**	.07	-.09	.07	**.58**	**.51**	**.64**	**.66**	-.07
5. Ejection fraction	-.11	**-.51**	**.60**	**-.86**	—	**-.47**	**.07**	**.27**	-.12	**-.24**	-.21	**-.26**	**-.27**	.03
6. LV mass	**.18**	**.48**	**-.44**	**.61**	**-.57**	—	**.63**	**.30**	-.12	**.59**	**.51**	**.69**	**.70**	.03
7. LV anterior wall	**-.16**	**-.23**	.11	**-.24**	**.18**	**.49**	—	.11	-.13	**.22**	.18	**.33**	**.33**	.12
8. LV posterior wall	-.12	**-.26**	**.18**	**-.30**	**.24**	.22	**.08**	—	-.21	**.25**	.21	**.27**	**.24**	-.07
9. Mean arterial pressure	-.31	-.29	-.26	-.05	-.26	-.18	-.25	-.19	—	.07	.11	-.13	**-.28**	**-.26**
10. Body weight	**.27**	**.48**	**-.30**	**.45**	**-.30**	**.41**	-.06	.06	-.52	—	**.98**	**.82**	**.75**	-.11
11. Fat mass	**.23**	**.49**	**-.36**	**.44**	**-.30**	**.36**	-.08	-.01	-.52	**.97**	—	**.70**	**.69**	-.13
12. Lean mass	.14	**.35**	**-.30**	**.42**	**-.36**	**.45**	-.02	.18	-.53	**.75**	**.58**	—	**.83**	-.08
13. Time point	.09	**.41**	**-.48**	**.53**	**-.53**	**.55**	.08	.01	-.02	**.51**	**.48**	**.62**	—	-.06
14. AG-1478 dose	-.09	-.04	-.08	-.06	.06	-.02	.08	-.04	.01	-.09	-.04	-.03	.01	—

Table 2.4. Correlation coefficients (r) for the C57BL/6J mouse strain across four time points (4, 8, 12, and 16-month points). Coefficients above the diagonal represent correlations for the female mice (n = 159) and coefficients below the diagonal correspond to the male mice (n = 129). LV stands for left ventricular; coefficients in bold are significant ($p < 0.05$), as tested by the Spearman's ρ test.

Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Cardiac ouput	—	**.98**	**.19**	**.88**	**-.28**	**.45**	**.24**	-.11	.04	.18	.19	**.22**	-.04	-.03
2. Stroke volume	**.97**	—	-.01	**.90**	**-.28**	**.50**	**.29**	-.06	.03	.22	.21	**.26**	.02	.00
3. Heart rate	**.42**	**.22**	—	-.02	.03	**-.21**	**-.23**	**-.24**	.05	**-.15**	-.09	**-.19**	**-.32**	-.11
4. LV internal diameter	**.80**	**.86**	.04	—	**-.64**	**.50**	**.27**	-.06	-.06	**.22**	.21	**.30**	.10	.06
5. Ejection fraction	-.14	**-.20**	.21	**-.60**	—	**-.20**	-.07	.11	**.24**	-.11	-.10	-.17	-.08	-.13
6. LV mass	**.27**	**.33**	**-.17**	**.36**	**-.24**	—	**.85**	**.68**	**.14**	**.50**	**.46**	**.55**	**.49**	**-.02**
7. LV anterior wall	.01	.04	-.20	.06	-.07	**.78**	—	**.48**	.15	**.33**	**.27**	**.43**	**.53**	-.03
8. LV posterior wall	**-.17**	**-.14**	**-.22**	-.12	.02	**.69**	**.36**	—	**.32**	**.55**	**.51**	**.55**	**.56**	-.05
9. Mean arterial pressure	.18	.18	.08	.13	.08	.12	.04	.14	—	.29	.25	**.35**	**.24**	**-.21**
10. Body weight	.10	.14	**-.19**	.14	-.05	**.54**	**.29**	**.56**	.22	—	**.96**	**.89**	**.47**	-.06
11. Fat mass	.06	.11	**-.17**	.09	-.02	**.44**	**.23**	**.46**	**.24**	**.94**	—	**.77**	**.37**	-.07
12. Lean mass	.11	.16	**-.19**	.19	-.10	**.57**	**.34**	**.59**	.12	**.79**	**.55**	—	**.59**	.02
13. Time point	**-.15**	-.08	**-.38**	.06	-.16	**.28**	**.25**	**.36**	.08	**.33**	**.28**	**.45**	—	.04
14. AG-1478 dose	-.02	-.03	.01	-.03	-.02	-.07	.01	-.13	-.14	-.23	-.17	**-.30**	**-.15**	—

Table 2.5. Correlation coefficients (r) for the FVB/NJ mouse strain across four time points (4, 8, 12, and 16-month points). Coefficients above the diagonal represent correlations for the female mice (n = 176) and coefficients below the diagonal correspond to the male mice (n = 169). LV stands for left ventricular; coefficients in bold are significant ($p < 0.05$), as tested by the Spearman's ρ test.

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CHAPTER III

INHIBITION OF EGFR BY AG1478 REDUCES INCIDENCE OF SPONTANEOUS PULMONARY ADENOMAS IN A/J FEMALE MICE

Introduction

The epidermal growth factor receptor (EGFR) is one of four members of the ERBB family of tyrosine kinase receptors, which consists of EGFR (ERBB1/HER1), ERBB2 (HER2), ERBB3 (HER3), and ERBB4 (HER4). The ERBB receptors are critical for tissue development and cell maintenance due to their roles in mediating cellular proliferation, differentiation, inhibition of apoptosis, and migration. EGFR stands out from its family members in that it has full activation and auto-inhibitory functions (unlike ERBB2 and ERBB3) and has its unique set of ligands and tyrosine residue docking sites. This unique genetic and biochemical makeup of EGFR makes it a potent promoter of tumorigenesis. Overexpression of EGFR and/or its activity is present in many types of cancers including lung squamous-cell carcinoma (85% of cases), colorectal (75%), breast (>50%), prostate (41-100%), glioblastoma (60%), and epithelial head and neck cancers (80-100%) (Chakraborty et al., 2014; de Castro-Carpeño et al., 2008; Di Lorenzo et al., 2002; Eze et al., 2017; Masuda et al., 2012; Vavala, 2017; Xu et al., 2017). Given that carcinomas account for 80-90% of cancers and that EGFR misregulation is prevalent in about one-third of carcinoma cases (Mendelsohn, 2002), there has been much effort in targeting EGFR for anti-cancer therapy. Treatment strategies include antibodies that target the receptor's ligand binding domain, small

molecular tyrosine kinase inhibitors that compete with ATP at the kinase domain, and EGF-targeted immunotherapy. The prevalence of aberrant EGFR activity in human cancers has made EGFR-targeted mono or combinatorial therapies common strategies for inhibiting tumor growth (Han et al., 2005; S. Wang et al., 2018). With lung cancer in particular, EGFR-targeted therapy has become a front-line treatment.

Lung cancer is the leading cause of cancer deaths worldwide. EGFR activating mutations are particularly common in both non-small cell lung carcinoma (NSCLC) and squamous cell carcinoma, which combined account for 85-90% of lung cancer cases. Approximately 10% of lung tumors in the North American population have an *EGFR* mutation (Graham et al., 2018). This frequency can be much higher in other regions, with approximately 50% of tumors in Southeast Asian patients having mutations in the *EGFR* gene (Graham et al., 2018; Shi et al., 2014; Shigematsu et al., 2005; Wieduwilt & Moasser, 2008). In a study encompassing several countries, Shigematsu et al. (2005) reported that 21% of NSCLC samples had *EGFR* activating mutations. Non-smoking East Asian females with adenocarcinoma have a particularly high incidence of activating *EGFR* mutations (Shigematsu et al., 2005; Sholl et al., 2009) and this group tends to respond favorably to EGFR-targeted therapy compared to other ethnicities (Yang et al., 2014).

EGFR-targeted treatments, such as with tyrosine kinase inhibitors (TKIs), eventually fail due to the emergence of additional mutations that are resistant to the original treatment. For instance, NSCLC patients eventually develop resistance to first generation EGFR TKIs (such as gefinitib and erlotinib). The *T790M* EGFR missense

mutation (at exon 20) is responsible for more than 60% of resistant cases (Yu et al., 2013). The *T790M* residue confers a greater affinity to ATP and is able to compete with EGFR TKIs and thus evade their inhibitory actions. Even with third generation TKIs (such as osimertinib and olmutinib) that are designed to overcome *T790M* resistance by forming covalent bonds at the residues, resistance typically re-emerges via the *C797S* missense mutation which disrupts the covalent bonds. Fortunately, the *C797S* mutation is sensitive to first generation TKIs and combinatorial therapy of first and third generation TKIs has been shown to be effective in treating *T790M/C797S* dual mutants (Kobayashi & Mitsudomi, 2016; Li et al., 2017).

Initially effective treatments may eventually fail due to late detection of cancer and corresponding tumor heterogeneity (Casás-Selves & DeGregori, 2011; Frank & Nowak, 2004; Merlo et al., 2006). Chance mutations can confer non-regulated growth and proliferation of a cell subpopulation. Additional mutations (that discourage apoptosis, evade the immune system, and enhance proliferation) are more likely to accumulate as the cell line expands rapidly and undergoes more mitotic events. By the time that the tumor is detected and treatment commences, subclonal groups of cells resistant to treatment may already exist. Targeted-therapies may initially treat a large portion of the cancer cell population. However, treatment may inadvertently add selective pressure that results in rapid adaptation of the tumor in favor of the resistant clones. Conversely, early stage cancers tend to be more homogeneous and respond more favorably to treatment. Overall, early detection and preventative methods are key to avoiding the risks associated with tumor heterogeneity.

There is some evidence that EGFR may be a good target for cancer prevention. Among cases of lung adenocarcinomas with EGFR mutations, 43% of patients also had *EGFR* mutations in adjacent normal lung epithelium (Tang et al., 2005), suggesting that *EGFR* mutations are present in the early stages of tumorigenesis and that preventative treatment may suppress expansion of these rogue cells. Several studies have reported significant reductions (50-90%) in tumor load in mouse models of colorectal cancer when EGFR activity was inhibited (Rinella & Threadgill, 2012; Roberts et al., 2002; Torrance et al., 2000). This is in stark contrast to efficacy rates seen in the clinic, where about 10% of colorectal cancer patients respond to EGFR-targeted treatment (Troiani et al., 2016). We hypothesize that the timing of treatment explains the discrepancy between preclinical and clinical success rates. Resistance to EGFR-targeted treatment has multiple causes, including upregulation of redundant pathways (such as activating mutations in the *KRAS* gene or overexpression of the insulin growth factor receptor 1) and the emergence of mutant EGFR receptors that have acquired resistance to the particular chemotherapeutic (such as the *T790M* mutation in NCSLC). Unlike in preclinical trials, EGFR-targeted therapy commences in the clinic after tumors are established. This difference in timing may correspond to the decreased effectiveness observed in the clinic and indicates potential use of EGFR inhibition for cancer prevention. The topic of cancer prevention by targeted therapy has been explored in breast cancer prevention research (den Hollander et al., 2013). Tamoxifen and raloxifene in particular, which target the estrogen receptor, were tested in several clinical trials with women who did not have breast cancer in order to evaluate its potential use as a

chemopreventative agent. Trials were largely beneficial at preventing ER-positive cancer (reviewed by den Hollander et al., 2013) and it is now used clinically for chemoprevention in high risk individuals. The EGFR/ERBB2 dual inhibitor lapatinib and the EGFR TKI gefinitib were shown to greatly delay development of ERBB2-overexpressing mammary tumors in mice, in which gefitinib delayed onset of cancer to 310 days of age compared to 140 days observed in non-treated mice (Lu et al., 2003). Taken together, targeting the commonly disrupted EGFR network could be immensely beneficial since rogue cells would have a reduced chance of gaining access to uncontrolled growth and thus have a smaller chance of acquiring additional mutations that confer a selective advantage.

Preclinical studies have shown that AG1478 is a promising candidate for therapeutic use due to its high specificity to EGFR and its efficacy at reducing tumor growth in cancer cell lines and mouse models (Ellis et al., 2006; Johns et al., 2003; Rinella & Threadgill, 2012). Furthermore, even a subtherapeutic dose of AG1478, in combination with radioimmunotherapy, was shown to be effective in inhibiting growth of human squamous cell carcinoma cells *in vitro* (Lee et al., 2005). In the current study, we tested the effectiveness of AG1478 in preventing the spontaneous occurrence of various neoplasia (as modeled by four inbred mouse strains with different genetic predispositions to various cancers).

Materials and Methods

Animals and Husbandry

We obtained mice that were 6-to-8-weeks-old (A/J, BALB/cJ, C57BL/6J, and FVB/NJ strains) from The Jackson Laboratory (Bar Harbor, ME). There were 160 mice per strain, 80 males and 80 females, to equal a total of 640 animals on study. Baseline measurements were taken after one week of acclimation to the facility and treatment commenced at ages 8-10 weeks. Animals were measured for all parameters at 4-month intervals, yielding a total of 5 time points (0, 4, 8, 12, and 16 months), with some time points omitted for particular parameters as noted in the corresponding sub-methods sections. The mice were housed 5 per cage, in Nextgen filtered cages, at 22° C under a 12-hour light/dark cycle. In all our experiments, we adhered to Animal Use Protocols approved by the Texas A&M University Institution Animal Care and Use Committee. Mice were euthanized by carbon dioxide asphyxiation and immediately necropsied. Tissues were either flash frozen in liquid nitrogen or fixed in formalin.

Diet and Delivery of AG1478

Tryptosin AG-1478 was purchased from LC Laboratories (Woburn, MA) and incorporated into Western Diet mouse chow (D12079B) by Research Diets, Inc. (New Brunswick, NJ). The therapeutic-level dose chosen was 144 mg of AG1478/kg of food, shown to be sufficient to inhibit EGFR phosphorylation (Barrick et al., 2008; Rinella & Threadgill, 2012). The 50 mg/kg sub-therapeutic dose was selected due to its efficacy at inhibiting cancer cell growth at an equivalent dose *in vitro* (Lee et al., 2005), with the

lowest dose chosen to be $1/10^{th}$ of the 50 mg/kg dose (i.e. 5mg/kg). The control chow (0 mg/kg) consisted of the Western diet alone. Food dyes were added for ease of feeding purposes, and mice received food and water *ad libitum*.

Histopathology

Lungs were removed and inflated with 10% formalin by injected into the trachea and were fixed in formalin for 48 hours before being stored in 70% ethanol until further processing and paraffin embedding. Five μm transverse sections were stained with hematoxylin and eosin (H&E). Soft tissue sarcomas, which were attached to surrounding muscoloskeletal tissue, were detached by scissors and then but by scalpel into three pieces so as to obtain a middle section that was representative of both the center and outer edges of the mass. Sarcomas were sectioned at 5 μm along the same plane as the dissection cut sites and H&E stained.

Results

Reduced incidence of pulmonary adenoma in A/J female mice.

The presence of surface pulmonary neoplasia was readily at necropsy. The frequency of neoplasia occurrence was based on the presence or absence of nodules upon visible examination during necropsy, regardless of nodule size or quantity (Figure 3.1). Generally, a nodule-positive lung contained 1-3 nodules, each with a diameter of ≤1 mm. Regions of neoplasia were identified as pulmonary adenomas by histological examination. Abnormal cells were generally cuboidal or columnar shaped and were

located on fibrovascular stromal tissue. Cells had irregularly oval nuclei, with dispersed chromatin and inapparent nucleoli, and no evidence of mitoses. The pulmonary adenomas were of benign nature. Other pathologies observed included pulmonary epithelial hyperplasia and bronchus-associated lymphoid tissue hyperplasia. FVB/NJ neoplasia frequencies were comparable to other reports showing that mice aged to 14 months had a 13% and 26% frequency of pulmonary neoplasia among males and females, respectively (Mahler et al., 1996). BALB/cJ had moderate frequencies of neoplasia, as expected. C57BL/6J is widely reported to be resistant to developing lung neoplasia. Accordingly, we did not observe lung neoplasia among C57BL/6J mice. In general, the frequency of lung neoplasia was not affected by preventative treatment with AG1478 (Tables 3.1-3.3). However, the A/J female group treated with the therapeutic level dose of AG1478 (144 mg/kg) had a significant reduction in neoplasia frequency, with nodules observed in 18.8% of treatment mice and in 47.7% of control mice (Spearman's $p = 0.04$). It is possible that the relatively low frequency of nodules in the other mouse strains (as compared to A/J) were insufficient for adequate statistical analysis and that continued aging was necessary to increase neoplasia frequency.

The occurrence of soft tissue sarcoma was unaffected by preventative treatment with AG1478.

Soft tissue sarcomas, which were obsereved only in the A/J mouse strain, were of an aggressively invasive and highly proliferative nature. Neoplastic masses were fast growing and generally measured about 2.5 cm in diameter at the time of necropsy and

occurred preferentially in the region of the hind limbs. Neoplastic tissue was observed infiltrating and replacing the local skeletal muscle and adipose tissue (Figure 3.2). These neoplasms were characterized histologically as being unencapsulated, poorly demarcated, and invasive. Neoplasm was composed of spindle cells arranged in haphazard streams at right angles supported by a scant amount of preexisting stroma, and often interposed between remaining skeletal muscle fibers and adipocytes. Cells had a small amount of pale eosinophilic cytoplasm, indistinct cellular borders, and oval to irregularly shaped nuclei with finely stippled chromatin and 1-3 large, prominent, basophilic nucleoli. Anisocytosis and anisokaryosis were marked and there were occasional very large multinucleated cells with more abundant eosinophilic cytoplasm and up to approximately 30 oval nuclei, often arranged around the periphery of the cell. Mitoses ranged from 4 to >10/hpf (400x), indicating high levels of proliferation. We observed neoplasms that were well vascularized with irregular capillaries in several areas and large areas of necrotic debris. The frequency of spontaneous soft tissue sarcoma did not significantly vary by preventative treatment with AG1478 (Table 3.4).

Discussion

The observed pulmonary adenomas had benign features of well delineated demarcation and few to no mitoses. This type of adenoma was unlikely to have progressed to malignancy (Meuwissen & Berns, 2005; Nikitin et al., 2004). Epithelial hyperplasia was also observed, often within the same sample containing an adenoma. Reports in the literature have suggested that hyperplasia and benign adenomas can be a

continuum along a shared process (Derwahl & Studer, 2002), possibly explaining why we frequently observed the two pathologies within the same tissue.

The present study demonstrates a proof of concept that a genetic background highly susceptible to pulmonary neoplasia can benefit from chemopreventative targeted therapy. We observed high instances of pulmonary adenomas among A/J mice, more moderate incidence in BALB/cJ, much less pronounced frequency in FVB/NJ, and no cases of adenomas among the C57BL/6J mice. These findings are in agreement with existing literature that susceptibility to developing both spontaneous and induced lung neoplasia is highly mouse strain-dependent, involving complex interactions among many genes (Lemon et al., 2002; Meuwissen & Berns, 2005). A/J female mice are also highly susceptible to developing adenocarcinoma (Meuwissen & Berns, 2005; Gordon & Bosland, 2009) and have been the subject of several prevention studies (Patlolla et al., 2015; Rahmani et al., 2015). Overall, A/J female mice are a good model for individuals at high risk for various cancers.

Our findings indicate that EGFR activity may be involved in the development of pulmonary adenoma in the susceptible A/J genetic background. It is clear that there are genetic factors that contribute to lung cancer risk. EGFR variant alleles have been reported to be involved in familial cases of NSCLC (Kanwal et al., 2017). Furthermore, upregulation in expression and/or activity of EGFR, KRAS, and downstream ERK are common features of adenocarcinoma that are also observed in mouse models (Mervai et al., 2018; Yamakawa et al., 2016; Sartori et al., 2008).

There are currently no effective targeted treatments for soft tissue sarcomas. Treatment strategies consist mainly of surgical removal of tumor in conjunction with chemo and radiotherapies. *EGFR* and other genes in the EGFR network are highly expressed in human soft tissue sarcoma, but EGFR inhibitors have not been an effective mode of treatment for these cancers. A recent study showed that phosphorylated EGFR and downstream phosphorylated ERK were correlated with sarcoma progression whereas overexpression of these and other EGFR pathway genes were not correlated (Yang et al., 2017). Our findings demonstrate that while A/J mice are prone to developing fast growing soft tissue sarcomas, preventative EGFR-targeted treatment did not have a significant effect on tumor frequency. Although the outcome was unexpected, our findings are in agreement with human data indicating that soft tissue sarcomas are largely irresponsive to EGFR inhibitors. The A/J mouse strain appears to be a good model for sarcoma. Our findings support the utility of this mouse model as a way to explore treatment options for soft tissue sarcoma.

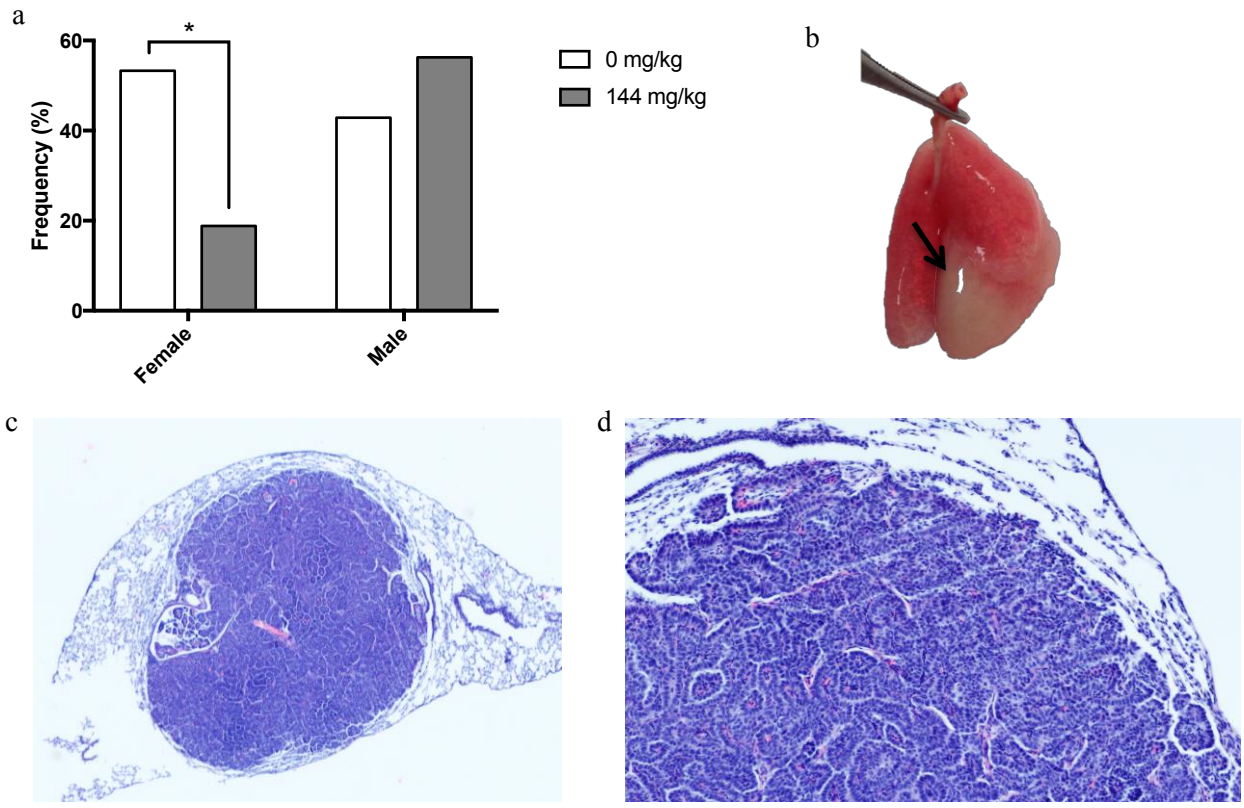

Figure 3.1. Reduced incidence of pulmonary adenoma in A/J female mice. (**a)** Frequency of pulmonary adenomas after 16 months of treatment with 144 mg/kg of AG1478. Tumor frequencies were based on **(b)** visible nodules recorded during necropsy. **(c-d)** An approximately 1mm diameter, well demarcated unencapsulated expansile adenoma on a moderate amount of fibrovascular stroma. Images magnified at 20x and 100x, respectively.

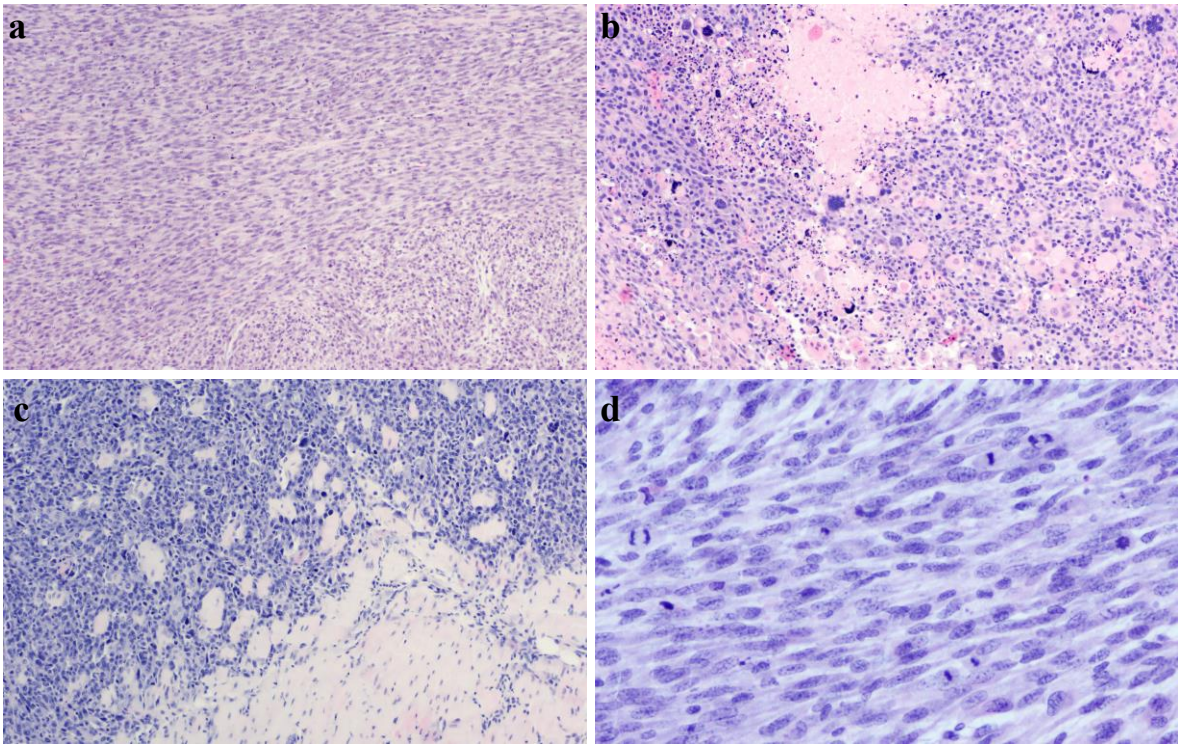

Figure 3.2. Fast growing smooth muscle sarcoma in a representative A/J mouse. **(a)** Spindle cells arranged in perpendicular streams and bundles (100x). **(b)** Large areas of necrosis characterized by complete loss of cellular detail and replacement with pale eosinophilic granular material. Large multinucleated cells are visible (100x). **(c)** Neoplastic cells infiltrating and replacing the normal soft tissues (100x) **(d)** High level of mitoses, >10/hpf (400x).

	Females			Males		
Dose (mg/kg)	*N*	Neoplasia (%)	p-value	*N*	Neoplasia (%)	p-value
0	15	47		14	43	
5	12	50	0.86	16	56	0.77
50	12	50	0.86	17	35	0.67
144	16	19	**0.04**	16	56	0.77

Table 3.1. Pulmonary adenoma frequency for A/J mice. Tumor frequencies were based on visible nodules recorded during necropsy. Dose groups were compared to their corresponding control group and tested for significant differences via Spearman's ρ test.

	Females			Males		
Dose (mg/kg)	*N*	Neoplasia (%)	p-value	*N*	Neoplasia (%)	p-value
0	18	22		10	40	
5	16	19	0.8	13	46	0.77
50	17	18	0.74	14	43	0.89
144	17	12	0.41	11	64	0.28

Table 3.2. Pulmonary adenoma frequency for BALB/cJ mice. Tumor frequencies were based on visible nodules recorded during necropsy. Dose groups were compared to their corresponding control group and tested for significant differences via Spearman's ρ test.

	Females			Males		
Dose (mg/kg)	*N*	Neoplasia (%)	p-value	*N*	Neoplasia (%)	p-value
0	14	21		14	7	
5	12	8	0.36	14	7	1.00
50	12	42	0.27	10	10	0.80
144	16	31	0.54	11	36	0.07

Table 3.3. Pulmonary adenoma frequency for FVB/NJ mice. Tumor frequencies were based on visible nodules recorded during necropsy. Dose groups were compared to their corresponding control group and tested for significant differences via Spearman's ρ test.

	Females		Males	
Dose (mg/kg)	*N*	Neoplasia (%)	*N*	Neoplasia (%)
0	19	26	16	19
5	18	22	18	11
50	19	26	17	6
144	17	18	19	14

Table 3.4. Sarcoma frequency for A/J mice. Tumor frequencies were based on visible nodules recorded during necropsy. Dose groups were compared to their corresponding control group and tested for significant differences via Spearman's ρ test.

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CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The epidermal growth factor receptor (EGFR) is essential for cell growth and survival. EGFR signaling is widely misregulated in diseases, often by overexpression of the *EGFR* gene or by hyperactivity of the receptor. Abberant EGFR signaling is implicated in early stages of human breast and lung cancers (den Hollander et. al., 2013; Tang et. al., 2005) as well as in mouse models of colorectal cancer (Roberts et al., 2002). Since early stage cancers are often homogenous and more readily treatable, we sought to to understand whether EGFR-targeted treatment was effective for chemoprevention. Our animal model consisted of genetic strains (A/J, BALB/cJ, C57BL/6J, FVB/NJ) with familial histories of high rates of spontaneous neoplasia. Our animal models reflected the situation in which groups of people have strong familial histories of developing cancer but do not have detectable mutations or pre-existing lesions.

Overall, our animal models had lower rates of neoplasia than expected, compared to historical frequencies reported in the Jackson Laboratory mouse database. We conclude that mouse strains likely experienced genetic drift before the standardization of cryopreservation techniques at animal facilities and that our findings represent current neoplasia rates. We measured the frequencies of neoplasia and compared the treatment groups to the non-treated control for each mouse strain. Our analyses focused on pulmonary adenomas and soft tissue sarcomas since these neoplasias occurred at the

highest rates. Our findings indicate that EGFR-targeted treatment did not reduce incidence of spontanous lung adenoma or soft tissue sarcoma. We conclude that preventative treatment with an EGFR pharmaceutical inhibitor is not effective at reducing cancer rates in a model of familial predisposition toward cancer. Our findings contrast with previous research with the $Apc^{Min/+}$ colorectal cancer mouse model in which reduced EGFR activity effectively reduced the number of small intestinal polyps (Roberts et. al., 2002). We conclude that although EGFR-targeted therapy is not beneficial for groups with familial predispositions to cancer, individuals with specific mutations, such as in the *APC* gene, may benefit from chemopreventative treatment.

As EGFR-targeted treatment becomes more widely used in the clinic, there is an increasing need to understand how differences in genetic background contribute to treatment response. We do not currently know the clinical rate of cardiotoxicity response to EGFR inhibitors. Our findings demonstrate that a genetic background predisposed to developing dilated cardiomyopathy, the BALB/cJ mouse strain, is at risk of adverse effects from treatment with the EGFR inhibitor AG1478. BALB/cJ male mice had an exacerbated increase in left ventricular mass and weight, suggesting that EGFR plays a role in the heart's adaptation to stress. There is a need for future experiments focused on how allelic differences between mouse strains determine cardiotoxicity outcome in response to EGFR-targeted therapy. Such a direction would aid in identifying risk factors present in the BALB/cJ genetic background that may be translatable to identifying humans at risk of cardiotoxicity.

Future Directions

As EGFR-targeted therapies are becoming more commonly used in the clinic for treatment of various cancers, there is an increasing need to understand potential adverse consequences to treatment. Despite the fact that EGFR mediates intracellular signaling pathways known to be involved in cardiomyopathies (Peng et al. 2016; Thomas et al., 2002; Kagiyama et al., 2002; De Pasquale et al., 2018; Schreier et al., 2013; Haq et al., 2001; Howes et al., 2006), cardiotoxicity response rates to EGFR-targeted treatments are currently unknown. We report that individuals at risk of developing dilated cardiomyopathy (DCM), the BALB/cJ male mice, may also be at risk for further increase in heart mass and cardiomyopathy progression. Our central hypothesis for a future study is that polymorphisms in the BALB/cJ genome combined with reduced EGFR signaling is conducive to progression of dilated cardiomyopathy. We propose to test the hypothesis by the following aims: 1) Generate mice with genetically inducible reduction in EGFR activity, 2) chemically induce DCM in mice from Aim 1 and compare mouse strains for severity of DCM phenotype, and 3) identify quantitative trait loci (QTL) in the BALB/cJ genome that contribute to sensitivity to reduced EGFR signaling.

The goal of Aim 1 is to generate a genetic model of pharmaceutical inhibition of EGFR. The creation of a genetic model is justified because it would eliminate the confounding variable of off-target effects from pharmaceutical inhibitors, in which inhibitors may bind to other tyrosine kinases in addition to EGFR. The genetic model would consist of mice heterozygous for EGFR variants: the hypomorphic allele *EGFR*^{wa2}

and the inducible cre-lox allele *EGFR*^{tm1Dwt}. The focus of Aim 2 is to induce DCM in two strains of male mice with potentially contrasting responses, BALB/cJ and C57BL/6J (Figure 4.1). Other groups have shown that DCM can be chemically induced by treatment with doxorubicin or, alternatively, by angiotensin-II (Liu et at., 2012; Peng et al., 2011). When comparing DCM outcome between the two mouse strains, we expect BALB/cJ *EGFR*$^{tm1Dwt/wa2}$ mice treated with cre-recombinase to have a more severe DCM phenotype, with greater left ventricular mass, compared to the other experimental groups (Figure 4.1). The goal of Aim 3 is to identify QTLs involved in DCM outcome that are also dependent on EGFR activity level. The long-term goal is to identify genes that interact with EGFR within a network that mediates how the heart responds to stress in terms of dilated cardiomyopathy progression. Identification of these genes and polymorphisms that contribute to network interactions would aid in developing genetic markers for cardiotoxicity risk in response to EGFR-targeted treatment.

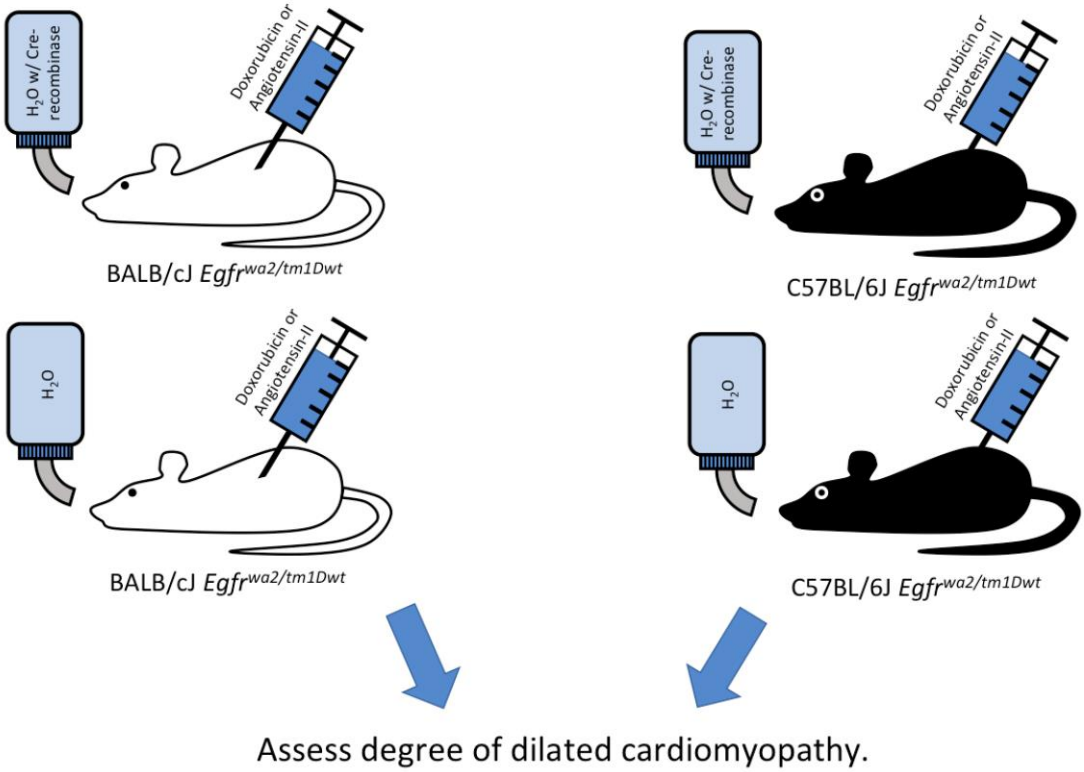

Figure 4.1. Diagram for future works. BALB/cJ and C57BL/6J mice have a inducible reduction in EGFR activity via a combination of the hypomorphic *EGFR*^{wa2} allele with the *EGFR*^{tm1Dwt} conditional knockout allele. One group of mice per strain is treated with cre-recombinase. Dilated cardiomyopathy is induced among all mice by injections with doxorubicin, or alternatively, with angiotensin-II.

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