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The role of GATA transcription factors in treatment-induced senescence in non-small cell lung cancer

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MDP in Biomedical Sciences, Drug Discovery and Development
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Lung cancer is one of the leading causes of cancer-related deaths. The most common subtype, non-small cell lung cancer (NSCLC), is still mostly uncurable. Therapies targeting driver mutations are effective initially, however, over time therapy resistance inevitably evolves. NSCLC cells surviving targeted therapy have a senescent phenotype and treatment-induced senescence has been suggested to play a role in therapy resistance. Besides their role in embryonic development, GATA transcription factors have also been shown to induce senescence.

The project aimed to assess the role of GATA transcription factors, especially GATA6, in treatment-induced senescence. Doxycycline-inducible expression of GATA transcription factors was successfully established in NSCLC cells, HCC827, using lentiviral vectors. Successful transduction was confirmed with Western blotting. The effects of GATA transcription factor expression on apoptosis during early treatment were assessed by live cell imaging with IncuCyte S3, whereas the effects on cell survival after long-term targeted therapy were determined using a CellTiter-Glo® cell viability assay. Finally, the effects on senescence were studied with senescence-associated β-galactosidase staining and RT-qPCR.

The overexpression of GATA6 decreased cell survival following long-term targeted therapy without inducing apoptosis during early treatment. Additionally, overexpression of GATA6 increased the expression of senescence-related genes, however, senescence-associated β-galactosidase activity was not observed. Silencing of GATA6 both induced apoptosis during early treatment and decreased cell survival following long-term targeted therapy. GATA6 silencing also decreased the proportion of β-galactosidase-positive cells and the expression of senescence-related genes in long-term assays.

Taken together, GATA6 alone is not sufficient to induce senescence, however, it may have a role in treatment-induced senescence in NSCLC. Further research is needed to assess this phenomenon and elucidate the role of GATA6 in treatment-induced senescence and therapy resistance.

Key words: non-small cell lung cancer, therapy resistance, treatment-induced senescence, GATA6 transcription factor

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1 Introduction

1.1 Non-small cell lung cancer and its treatment with targeted therapies

Lung cancer is one of the leading causes of cancer-related deaths in the world with a 5-year survival rate of only 17% (Sung et al., 2021). Its estimated incidence is 2 million new cases per year worldwide, being the second highest rate in the world (Padinharayil et al., 2022; Suster and Mino-Kenudson, 2020). The most common histological lung cancer subtype is non-small cell lung cancer (NSCLC), which accounts for 85% of all lung cancer subtypes (Padinharayil et al., 2022). NSCLC is further divided into subcategories, of which the most common groups are adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Suster and Mino-Kenudson, 2020). The most prominent risk factor for lung cancer is smoking, and over 95% of all lung cancers in non-smokers are NSCLC cases (Dias et al., 2017; Padinharayil et al., 2022; Suster and Mino-Kenudson, 2020).

Multiple pathways can be involved in the pathogenesis of NSCLC, and the mechanisms include both genetic factors, such as somatic mutations, and nongenetic factors, such as histone modifications and deoxyribonucleic acid (DNA) methylation (Suster and Mino-Kenudson, 2020). Driver mutations are defined as mutations that drive and promote the development of cancer (Lee et al., 2018). The two most common driver mutations in NSCLC are mutations in *Kirsten rat sarcoma (KRAS)* and *epidermal growth factor receptor (EGFR)* genes (Rotow and Bivona, 2017). Smokers harbor more often mutations in KRAS, whereas non-smokers are more likely to have a mutated EGFR, indicating that risk factors may influence pathogenesis pathways (Fujimoto and Wistuba, 2014; Padinharayil et al., 2022).

EGFR or ErbB family of receptor tyrosine kinases consists of four members: EGFR or ErbB1, human epidermal growth factor receptor 2 (HER2) or ErbB2, HER3 or ErbB3, and HER4 or ErbB4 (**Figure 1**) (Roskoski, 2014). Upon dimerization and ligand binding, ErbB receptors can mediate their effects on, for example, proliferation and cell survival via multiple signaling pathways, including phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR), Rat sarcoma-mitogen-activated protein kinase (RAS-MAPK), Janus kinase-signal transducer and activator of transcription (JAK-STAT), and phospholipase C (PLCγ) pathways (Roskoski, 2014; Rotow and Bivona, 2017). ErbB receptors have 11 different ligands, and HER2 is the only receptor family member that does not need a ligand for activation (**Figure 1**) (Roskoski, 2014). About 16% of patients with

advanced NSCLC harbor mutations in EGFR, and EGFR mutations are common especially in females and patients of East Asian origins (Fujimoto and Wistuba, 2014; Rotow and Bivona, 2017).

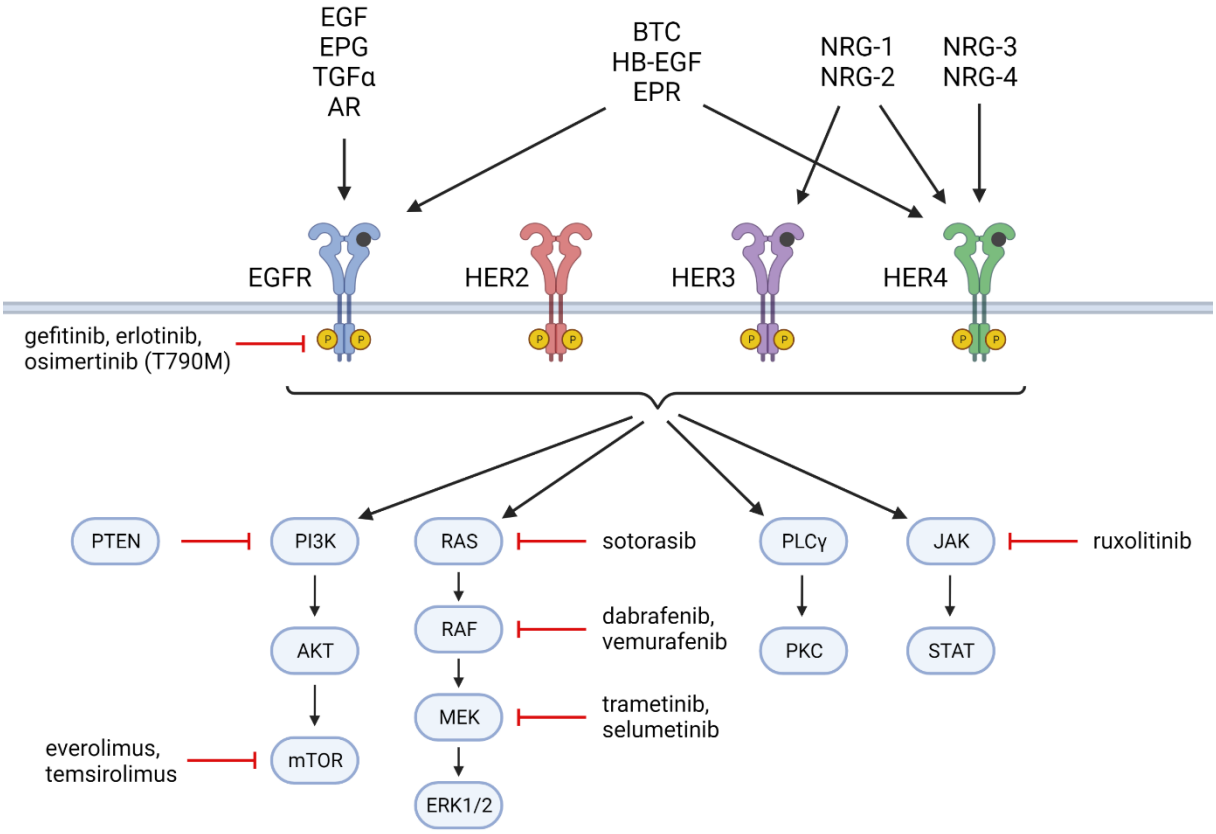

Figure 1. A schematic diagram of EGFR signaling and therapies targeting it. ErbB receptors have a total of 11 ligands and upon dimerization and ligand binding, downstream signaling pathways can be activated (Padinharayil et al., 2022; Roskoski, 2014; Yarden and Sliwkowski, 2001). Dimers can also be formed from two different ErbB monomers. EGFR tyrosine kinase inhibitors, mTOR inhibitors, one RAS inhibitor, RAF inhibitors, MEK inhibitors, and one JAK inhibitor are indicated in the figure. EGF = epidermal growth factor, EPG = epigen, TGFα = transforming growth factor-α, AR = amphiregulin, BTC = betacellulin, HB-EGF = heparin-binding EGF-like growth factor, EPR = epiregulin, NRG = neuregulin, PTEN = phosphatase and tensin homolog, RAF = Raf proto-oncogene, serine/threonine kinase, MEK = mitogen-activated protein kinase kinase, ERK = mitogen-activated protein kinase, PKC = protein kinase C. The figure is created by Juuli Hietarinne with BioRender.com.

Targeted cancer therapies are designed to target driver mutations that are essential, for example, to tumor growth and cancer cell survival (Lee et al., 2018). In 2009, Mok and colleagues demonstrated for the first time that EGFR tyrosine kinase inhibitors (TKIs) provided better outcomes compared to cytotoxic therapy in NSCLC patients with activating EGFR mutations. Nowadays, EGFR TKIs are already used as a standard of care for NSCLC patients harboring mutations in EGFR, and other targetable pathways in NSCLC include RAS-MAPK, JAK-STAT, neurotrophic tyrosine kinase-ROS proto-oncogene 1 (NTRK-ROS1), and PI3K/AKT/mTOR pathways (Padinharayil et al., 2022).

Most common activating EGFR mutations, i.e. mutations that are targetable with EGFR TKIs, are in-frame deletions in exon 19 and substitution mutations of leucine to arginine at position 858 (L858R) in exon 21, which together account for 85-90% of all EGFR mutations in NSCLC patients (Rotow and Bivona, 2017). About 4% of EGFR mutations are exon 20 insertions and 0.5% substitution mutations of threonine to methionine at position 790 (T790M), which both possess intrinsic resistance to EGFR TKIs (Rotow and Bivona, 2017). The most common second-site mutations that confer acquired resistance to targeted therapies include secondary T790M mutations, that are harbored by over 50% of patients treated with first-generation EGFR TKIs, and C797S mutations at the covalent binding site of osimertinib (Rotow and Bivona, 2017; Thress et al., 2015).

Erlotinib and gefitinib are first-generation EGFR TKIs. They compete with adenosine triphosphate (ATP) by reversibly binding to the tyrosine kinase domain of EGFR (Chan and Hughes, 2015). Second-generation EGFR TKIs, such as afatinib, dacomitinib, and neratinib, in turn, irreversibly bind to EGFR, as well as to other kinase-competent members of the receptor family (HER2, HER4) (Baraibar et al., 2020). The T790M mutation renders EGFR capable of escaping the treatment with first-generation EGFR TKIs (Padinharayil et al., 2022). Second-generation EGFR TKIs were developed to overcome this resistance mechanism, however, therapeutic doses cannot be reached in the clinical setting due to the toxicity caused by the binding to the non-mutated EGFR (Baraibar et al., 2020). Third-generation EGFR TKIs, such as osimertinib and rociletinib, irreversibly covalently target the T790M mutation without binding to the non-mutated form, thus providing a selective inhibition of EGFR with activating mutations (Jänne et al., 2015; Padinharayil et al., 2022). In addition to EGFR TKIs, the EGFR signaling pathway can also be targeted with other targeted therapies, such as mitogen-activated protein kinase kinase (MEK) inhibitors and mTOR inhibitors, in combination with EGFR TKIs (**Figure 1**). For example, combined EGFR and MEK inhibition has been shown to prevent drug resistance emerging via mitogen-activated protein kinase (ERK) reactivation, thus leading to greater apoptotic responses (Tricker et al., 2015). This therapy combination has also been studied in clinical trials (NCT03392246; NCT03516214).

1.2 Resistance to targeted therapies and residual disease

Even though different kinase inhibitors have been effective in the treatment of multiple cancer types with targetable oncogenes, therapy resistance towards TKIs almost always evolves,

hindering the long-term efficacy of such treatments (Botting et al., 2015). Resistance can be divided into two subgroups: intrinsic and acquired resistance (Hammerlindl and Schaider, 2018). Intrinsic or primary resistance refers to the situation where resistance-causing alterations are present already at the time of treatment initiation, and thus, the treatment is ineffective from the beginning. In acquired or secondary resistance, an effective response to treatment is initially observed. However, over time resistance develops, for example through the resistance-causing adaptations that cells develop to survive under the pressure of targeted therapy (Hammerlindl and Schaider, 2018; Qin et al., 2020). Adaptations include both genetic or nongenetic changes that can, for example, render a drug target itself insensitive to therapy, activate downstream pathway targets even in the absence of a normal inducer, or develop alternative pathways to activate downstream pathways (Konieczkowski et al., 2018).

It has been suggested that before the development of the irreversible drug-resistant state, a small population of cancer cells enter a so-called drug-tolerant state (Sharma et al., 2010). These drug-tolerant cells, or drug-tolerant persisters (DTP), have reversibly arrested the cell cycle, usually in a G_0 or G_1 state (Recasens and Munoz, 2019; Sharma et al., 2010). DTPs are developed under therapy pressure through epigenetic changes, such as chromatin remodeling and changes in histone methylation patterns, and they can start to proliferate again upon drug withdrawal (Sharma et al., 2010). DTPs form a so-called residual disease (RD), referring to a situation in which patients do not achieve a complete response after targeted therapy (Bivona and Doebele, 2016). The remaining tumor cells, DTPs, can over time acquire resistance-causing changes, for example, mutations that eventually lead to the development of irreversible therapy resistance, relapse, and cancer progression (Luskin et al., 2018; Ramirez et al., 2016).

1.3 Cellular senescence

Classical cellular senescence is a program that prevents abnormal cells from proliferating by inducing permanent cell cycle arrest (Campisi and D'Adda Di Fagagna, 2007). The first time, cellular senescence was described when Hayflick and colleagues proposed that cells, in this case, fibroblasts, cannot replicate infinitely (Hayflick, 1965). This limited ability of normal cells to proliferate was defined as replicative senescence, and it has been associated closely with normal aging processes (Campisi and D'Adda Di Fagagna, 2007). Moreover, senescence also has tumor-suppressive functions, and it can attenuate cancer cell proliferation. This is important for two reasons – on one hand, increased cell proliferation is a prerequisite for

tumor development, and, on the other hand, rapidly dividing cells are also more likely to acquire mutations (Campisi, 2001; Campisi and D'Adda Di Fagagna, 2007). Two main pathways driving classical senescence are the p53/p21 pathway and the $p16^{INK4a}$/pRB (retinoblastoma protein) pathway, triggered by telomere shortening or various stress factors, such as hypoxia, hydrogen peroxide (H_2O_2), and genotoxic agents, including different drug treatments (Campisi and D'Adda Di Fagagna, 2007; Kang et al., 2015; Liu et al., 2021; Rebbaa, 2005).

Senescent cells have both morphological and phenotypical differences compared to normally dividing cells. Senescent cells remain metabolically active and have an increased expression of cell cycle inhibitors, such as $p16^{INK4a}$, $p21^{\text{wild-type activating fragment-1 (WAF1)}}$, and $p27^{\text{kinase inhibitor protein (KIP)}}$, leading to failures in DNA replication and cell cycle arrest (Liu et al., 2021; Rebbaa, 2005). The senescent phenotype can be recognized as a flattened cell morphology with an enlarged nucleus (Liu et al., 2021). Additionally, senescent cells have also other special features, such as apoptosis resistance, dysfunctional mitochondria, unfunctional lysosomes, and increased senescence-associated β-galactosidase (SA-β-Gal) activity (Campisi and D'Adda Di Fagagna, 2007; Kang et al., 2015; Liu et al., 2021; Rebbaa, 2005). The most commonly used senescence marker, SA-β-Gal, has been shown to be derived from lysosomal β-D-galactosidase due to the increased lysosomal activity in senescent cells, thus being a marker of senescence rather than a requirement for the development of it (Lee et al., 2006). Furthermore, senescence is also characterized by changes in the protein secretion of cells, also known as the senescence-associated secretory phenotype (SASP), which is defined as increased secretion of pro-inflammatory cytokines, chemokines, growth factors, and different proteases (Coppé et al., 2010).

1.4 Treatment-induced senescence

A senescent phenotype can also be caused by therapies, such as chemotherapy or targeted therapies. Treatment-induced senescence or therapy-induced senescence (TIS) is also characterized by a stable cell cycle arrest but unlike in classical cellular senescence, cells can escape cell cycle arrest and start proliferating again upon drug withdrawal (Fitsiou et al., 2022). The phenotype of senescent drug-tolerant cancer cells is similar to normal senescent cells. Due to the well-known senescence characteristics, including apoptosis resistance, TIS has been suggested to play a role in cancer cell dormancy and possibly even in evolving therapy resistance (Liu et al., 2021; Recasens and Munoz, 2019). Moreover, it has been

recently shown that following targeted therapy, surviving drug-tolerant NSCLC cells acquire a senescent phenotype (Kurppa et al., 2020). When treated with EGFR TKIs alone, a small population of cells escaped the treatment by re-activating the MAPK pathway. Consequently, MEK inhibitors were added, resulting in the establishment of a more stable senescent phenotype characterized by activation of the yes-associated protein/transcriptional enhancer activator domain (YAP/TEAD) complex.

In the cancer context, TIS and the SASP have been observed to have both tumor-suppressive and tumor-promoting effects – depending on the cell type, tissue, and causative factors (Chambers et al., 2021). On one hand, SASP molecules can stimulate an immune response by attracting immune cells to clear senescent cancer cells (Ewald et al., 2010; Pérez-Mancera et al., 2014). On the other hand, it has been shown that SASP factors secreted by senescent cells in the tumor microenvironment can induce cancer cell reprogramming – in both autocrine and paracrine manner (Liu et al., 2021). Thus, cancer cells can escape cell cycle arrest and senescence, resulting in increased proliferation, invasion, and migration. In addition, the SASP has been reported to induce epithelial-mesenchymal transition (EMT), immunosuppression, angiogenesis, and stemness (Liu et al., 2021). Due to the observed tumor-promoting role of senescence in cancer, senescent cells were recently deemed as an emerging hallmark of cancer (Hanahan, 2022).

1.5 GATA transcription factors

GATA-binding factor (GATA) transcription factors are zinc-finger DNA-binding proteins that are essential in embryonic development by controlling cell differentiation and tissue morphogenesis (Zheng and Blobel, 2010). GATA transcription factor family members exclusively bind to a target consensus sequence (A/T)GATA(A/G), which is recognized by their zing-finger domains (Tremblay et al., 2018). The mechanistic principle of GATA transcription factor functions is based on changes in chromatin accessibility and cooperation with other factors, like the friend of GATA 1/2 (FOG1/2) or E-proteins (**Figure 2**) (Tremblay et al., 2018).

Traditionally, GATA transcription factors have been divided into two subgroups, GATA1–3 and GATA4–6 (Lentjes et al., 2016). The former are associated with the development of mesoderm- and ectoderm-derived tissues, such as hematopoietic tissues, whereas the latter are suggested to have a crucial role in the development of endoderm- and mesoderm-derived

tissues, such as lungs and heart. Although all GATA transcription factor family members bind the same consensus sequence, minor changes in the sequence are possible (Molkentin, 2000). In addition, specific bases surrounding the consensus sequence may be preferred more by some family members than others, enabling the differential tissue distribution among GATA transcription factors (Molkentin, 2000). GATA transcription factors can also affect each other's expression with the so-called GATA switch, meaning that when one GATA transcription factor is bound to the chromatin but replaced by another one, the gene expression of the replaced one is causatively inhibited (Tremblay et al., 2018).

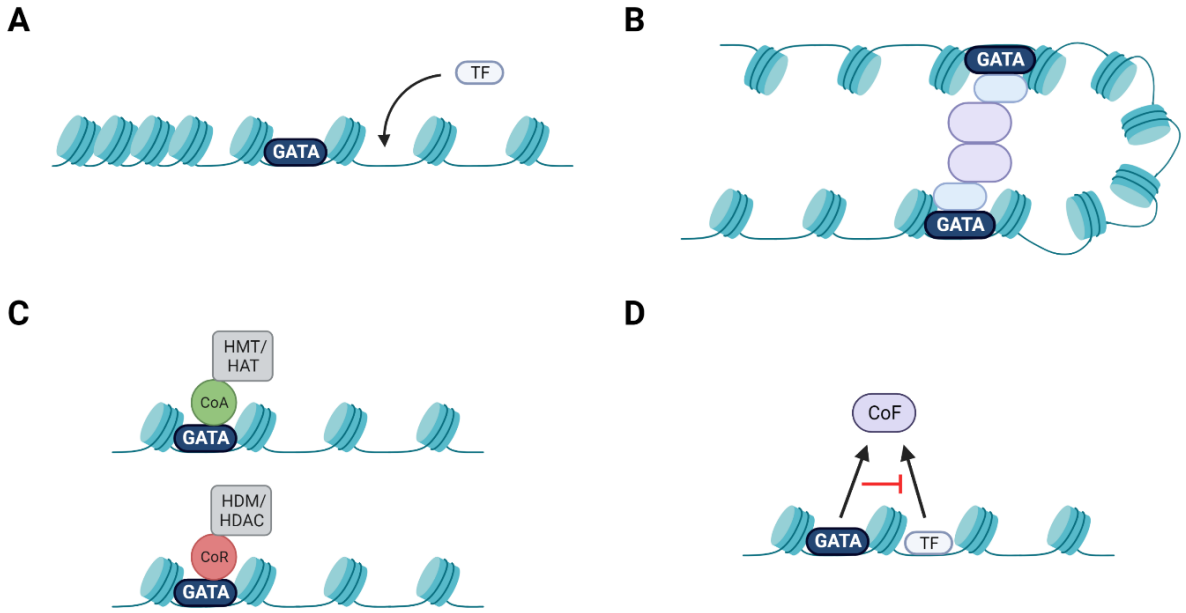

Figure 2. Mechanisms of action of GATA transcription factors. (A) GATA transcription factors can aid in the opening of chromatin, thus, enabling the binding of other transcription factors to chromatin. (B) GATA transcription factors can contribute to chromatin looping which enables distant regulatory elements to come closer to each other and interact. (C) By binding with co-activators (CoA) or co-repressors (CoR), GATA transcription factors can influence histone methylation and acetylation patterns via histone methyl transferases (HMT), histone acetyl transferases (HAT), histone demethylases (HDM), and histone deacetylases (HDAC). This can further activate or inactivate gene expression depending on the resulting change in patterns. (D) By competitively binding to co-factors (CoF) that are required for the activation of other transcription factors, GATA transcription factors can inhibit the function of those transcription factors. TF = transcription factor. The figure is modified from Tremblay et al., 2018 and created with BioRender.com.

1.5.1 GATA transcription factors in development

GATA transcription factors have an important role in the embryonic development of different organs. GATA1, GATA2, and GATA3 contribute to the development of mesoderm- and ectoderm-derived tissues, such as hematopoietic tissues (Lentjes et al., 2016). The crucial role of GATA transcription factors in these organs has previously been assessed by, for example, mouse models. The total absence of GATA1 has led to the development of immature

erythroid cells, whereas the lack of GATA2 and GATA3 have caused anemia as well as brain and central nervous system deformities, correspondingly (Fujiwara et al., 1996; Pandolfi et al., 1995; Tsai et al., 1994). The tissue distribution of GATA transcription factors in organogenesis is depicted in **Figure 3**.

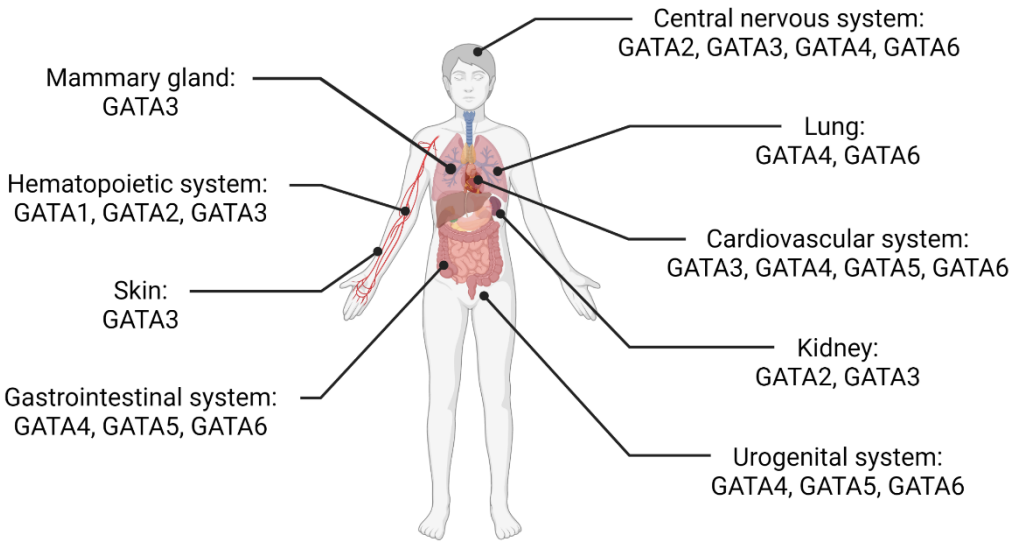

Figure 3. The tissue distribution of GATA transcription factors during organ development. GATA1–3 are expressed primarily in the hematopoietic system and central nervous system, whereas GATA4–6 are commonly expressed in the cardiovascular system, urogenital system, and gastrointestinal system (Lentjes et al., 2016; Tremblay et al., 2018). The figure is created by Juuli Hietarinne with BioRender.com.

Another GATA transcription factor subfamily consists of transcription factors GATA4, GATA5, and GATA6, which play a role in the development of mesoderm- and endoderm-derived tissues, such as lungs and heart (Lentjes et al., 2016). Mouse models lacking GATA4–6 have indeed developed severe deformities, for example, in cardiovascular, genitourinary, and gastrointestinal tissues (Kuo et al., 1997; Molkentin et al., 2000; Zhao et al., 2005).

1.5.2 GATA transcription factors in lung development

Some GATA transcription factor family members, especially GATA6, have been tightly associated with lung development. The lung development process consists of five stages: embryonic, pseudograndular, canalicular, saccular, and alveolar stages (Nikolić et al., 2018). During embryonic and pseudograndular stages, trachea and primary bronchi are developed from lung bud, and more mature bronchi are further formed (Ahmed et al., 2018). Smaller airways, bronchioles, are generated during the canalicular stage and further expanded and matured in the saccular stage (Ahmed et al., 2018). Primary alveoli are also formed in the

saccular stage, and they are further multiplied, enlarged, and finally matured during the alveolar stage, even postnatally (Ahmed et al., 2018; Joshi and Kotecha, 2007).

Lung compartments consist of different cell types, such as ciliated, secretory, basal, and alveolar cells (Koutsourakis et al., 2001). The epithelial cells lining the respiratory tract are divided into airway and alveolar types (Nikolić et al., 2018). Alveolar epithelial cells consist of two main types: type 1 (AT1) and type 2 (AT2) alveolar cells. Flat AT1 cells comprise 95% of the alveolar area and they have a crucial role in the gas exchange between lungs and blood circulation. AT2 cells, in turn, are cuboidal and have an essential role in producing surfactant proteins to decrease surface tension. AT2 cells can differentiate into AT1 cells, and they also share some characteristics with stem cells, including injury resistance and self-renewal capacity (Abdelwahab et al., 2019; Nikolić et al., 2018). Following certain triggering stimuli, AT1 cells can also de-differentiate back to AT2 cells (Nikolić et al., 2018).

GATA6 is predominantly associated with the embryonic, saccular, and alveolar stages of lung development (Joshi and Kotecha, 2007; Liu et al., 2002b). During embryonic development, GATA6 has a role in the formation of foregut and primary lung structures, as well as in branching morphogenesis (Caldeira et al., 2021; Joshi and Kotecha, 2007; Tremblay et al., 2018). GATA6 has been suggested to be a key player in both epithelial cell differentiation and alveoli maturation (Liu et al., 2002b; Tremblay et al., 2018). It also induces the expression of Muc5b, which is an essential mucin glycoprotein to maintain normal respiratory tract function (Jonckheere et al., 2011). GATA6 has been reported to be the only GATA transcription factor that is expressed in epithelial cells during lung development (Liu et al., 2002b).

One suggested mechanism through which GATA6 contributes to lung development is wingless-related integration site (Wnt) signaling. GATA6 is a transcriptional regulator of Wnt, and Wnt signaling has been shown to regulate the number of bronchioalveolar stem cells (BASCs), differentiation of epithelial cells, alveolar morphogenesis, and the transdifferentiation process of AT2 cells to AT1 cells (Abdelwahab et al., 2019; Botting et al., 2015; Lentjes et al., 2016; Mucenski et al., 2003; Y. Zhang et al., 2008). Loss of GATA6 has been linked to the upregulation of canonical Wnt signaling (Ahmed et al., 2018; Y. Zhang et al., 2008).

Furthermore, GATA6 also regulates other factors that have been associated with epithelial cell differentiation in AT2 cells. GATA6 has been shown to increase the transcriptional activity of surfactant protein A (SP-A), surfactant protein C (SP-C), and thyroid transcription

factor-1 (TTF-1) (Liu et al., 2002b). In addition to their role in epithelial cell differentiation, SP-A is also essential in alveolar tubular myelin formation, whereas TTF-1 has a crucial role in surfactant protein production and overall lung morphogenesis (Bruno et al., 2000; Liu et al., 2002a). In addition, GATA6 has been observed to be co-expressed with the Clara cell secretory protein (CCSP), which is also relevant for epithelial cell differentiation (Liu et al., 2002b). In addition to GATA6, GATA4 has also been proposed to participate in lung development, for example, by contributing to the development of pulmonary structures (Ackerman et al., 2007).

1.6 GATA transcription factors in cancer

GATA deficits have been associated with malignant transformation due to the failure of cells to exit the cell cycle (Zheng and Blobel, 2010). However, some GATA transcription factors have also been shown to promote tumorigenesis, which further highlights the dual role of GATA transcription factors in cancer (Lentjes et al., 2016; Zheng and Blobel, 2010).

GATA transcription factor alterations have been observed in multiple cancers (**Table 1**). Due to their essential role in the development of hematopoietic tissues, GATA1–3 alterations are mainly associated with different types of leukemia, whereas alterations in GATA4–6 have been found in cancers prominent in gastrointestinal and urogenital tissues (Lentjes et al., 2016). In addition to genetic changes, such as mutations, insertions, and deletions, also nongenetic changes in GATA transcription factors have been observed. For example, CpG methylation in the promoter region has been shown to reduce transcriptional activation of GATA transcription factor family members, thus leading to tumorigenesis (Lentjes et al., 2016). Despite revealing multiple associations between GATA transcription factors and cancer, mechanisms behind these relationships remain to be fully elucidated.

Table 1. GATA transcription factor alterations in different cancers.
Modified from Lentjes et al., 2016.

	Related cancer	Alteration	Source
GATA1	acute megakaryoblastic leukemia (in Down syndrome patients)	frameshift insertion & deletion, nonsense mutation, splice site mutation	Nikolaev et al., 2013; Wechsler et al., 2002; Yoshida et al., 2013
GATA2	chronic myeloid leukemia	missense mutation, frameshift deletion	S. Zhang et al., 2008
	acute myeloid leukemia	missense mutation, frameshift insertion, full gene deletion	Hahn et al., 2011; Niimi et al., 2013; Ostergaard et al., 2011

	Related cancer	Alteration	Source
GATA3	breast cancer	missense & nonsense mutation, frameshift insertion & deletion	The Cancer Genome Atlas Network, 2012; Usary et al., 2004; Wheler et al., 2014
	T-cell acute lymphoblastic leukemia	missense mutation, frameshift deletion, in-frame deletion	Zhang et al., 2012
	B-cell acute lymphoblastic leukemia	single nucleotide polymorphism (SNP)	Perez-Andreu et al., 2013
	urothelial carcinoma & renal cell carcinoma	CpG methylation	Cooper et al., 2010
GATA4	gastrointestinal cancers	CpG methylation, amplification	Akiyama et al., 2003; Chia et al., 2015; Guo et al., 2006; Hellebrekers et al., 2009; Wen et al., 2010
	glioblastoma multiforme	CpG methylation, frameshift insertion & deletion	Agnihotri et al., 2011
	ovarian cancer	hypoacetylation, loss trimethylation, CpG methylation	Caslini et al., 2006; Wakana et al., 2006
	other cancers, including lung cancer and diffuse large cell B-cell lymphoma	CpG methylation	Guo et al., 2004; Pike et al., 2008
GATA5	gastrointestinal cancers & renal cell carcinoma	CpG methylation	Akiyama et al., 2003; Guo et al., 2006; Hellebrekers et al., 2009; Wen et al., 2010
GATA6	ovarian cancer	hypoacetylation, loss trimethylation, upregulation	Caslini et al., 2006
	gastrointestinal cancer	amplification, CpG methylation	Akiyama et al., 2003; Chia et al., 2015
	pancreatobiliary cancer	amplification	Kwei et al., 2008
	pediatric rhabdomyosarcoma	CpG methylation	Mahoney et al., 2012
	glioblastoma multiforme	CpG methylation	Martinez et al., 2009

Some GATA transcription factor family members, especially GATA6, have been associated closely with lung cancer. GATA6 has been shown to have both tumor-suppressive and tumor-promoting actions (W. Chen et al., 2020). In EGFR-mutant NSCLC patients, high GATA6 expression was correlated with both shorter overall survival as well as progression-free survival after the treatment with EGFR TKIs (Ma et al., 2019). The GATA6 expression was also correlated with the autophagy marker microtubule-associated protein 1A/1B-light chain 3-phosphatidylethanolamine conjugate (LC3-II), suggesting that GATA6 upregulation might confer resistance to tyrosine kinase inhibitors via autophagy. Contrarily, in treatment-naïve

resected lung adenocarcinomas, GATA6 expression was associated with the absence of invasion and metastasis as well as with moderate-to-well differentiated tumors, indicating that higher GATA6 expression may be related to a better prognosis (Nakajima et al., 2018). GATA6 is suggested to inhibit the metastatic process by regulating epithelial differentiation together with other transcription factors, such as homeodomain-only protein homeobox (HOPX). The simultaneous inhibition of GATA6 and HOPX has been shown to cause abnormal differentiation and increased lung cancer cell invasion *in vitro*, and consistently, overexpression of GATA6 alone or in combination with HOPX has also been reported to inhibit metastatic processes *in vivo* (Cheung et al., 2013). Additionally, Cheung and colleagues have also observed that low GATA6 and HOPX expression results in poor prognosis in patients with lung adenocarcinoma. Consistent with these observations, GATA6 overexpression caused by retinoic acid (RA) has been shown to lead to the inhibition of EGFR repression and Wnt signaling, further inducing the G_0/G_1 cell cycle arrest in gefitinib-resistant NSCLC cell lines *in vitro* (Zito et al., 2017).

In addition to GATA6, also other GATA transcription factor family members have been suggested to play a role in lung cancer. For example, GATA2 expression has been observed to be essential for the survival of KRAS-mutant NSCLC cells and the tumor development in both KRAS- and EGFR-mutant xenografts, and consistently, its inhibition has been shown to lead to tumor regression (Kumar et al., 2012). GATA4, in turn, has been proposed to act as a tumor suppressor by inducing senescence in lung cancer cells, but its activation has also been causatively linked to metastatic processes of lung adenocarcinoma (Castro et al., 2013; Gao et al., 2019).

1.7 GATA transcription factors in senescence

In addition to their role in cancer, GATA transcription factors have also been reported to be associated with senescence. For example, GATA4 has been previously shown to drive senescence in fibroblasts in the absence of normal senescence pathways, p53/p21 and $p16^{INK4a}$/pRB, by activating nuclear factor kappa B (NF-κB) and the SASP (Kang et al., 2015). Recently its senescence-inducing abilities have also been observed in the context of lung cancer, where GATA4 was shown to regulate senescence via the downregulation of Wnt signaling (Gao et al., 2019). Furthermore, overexpression of GATA6 has also been found to induce senescence in NSCLC cells *in vitro* via classical senescence-inducing pathways, by

upregulating p53 and p21 and inhibiting AKT activation to stabilize p21 (W. Chen et al., 2020).

The association between the enrichment of GATA transcription factor motifs in open chromatin and a senescent phenotype has been previously observed in drug-tolerant EGFR-mutant NSCLC cell lines, including PC9, HCC827, and HCC4006 (Kurppa et al., 2020). Kurppa and colleagues have also observed that treatment-induced senescence developed regardless of functional classical senescence pathway mediators, for example, in NSCLC cells with a mutated p53 and a negligible expression of p21 and p16, indicating that other senescence pathways should exist.

1.8 Summary and aims of this research

Even though targeted therapies have been shown to prolong the survival of NSCLC patients in the short term, the long-term efficacy of treatments is often hindered by acquired resistance. Treatment-induced senescence has been proposed to play a role both in tumor dormancy and resulting therapy resistance. Alterations in GATA transcription factors have been observed in multiple cancers, and some GATA transcription factor family members have also been associated with senescence. Previous research has shown that drug-tolerant, dormant EGFR-mutant NSCLC cells acquire a senescent phenotype following targeted therapy (Kurppa et al., 2020). Moreover, GATA transcription factor motifs were observed to be enriched in open chromatin in those cells. Furthermore, preliminary data from the Kurppa laboratory shows that the expression of GATA6 is increased in several dormant, drug-tolerant EGFR-mutant NSCLC cell lines after combined EGFR and MEK inhibition. Because GATA transcription factors, especially GATA4 and GATA6, have been shown to drive senescence in other contexts, they might play a role in treatment-induced senescence in non-small cell lung cancer as well.

The overall goal of this research project was to determine whether GATA6 has a role in treatment-induced senescence in EGFR-mutant NSCLC. Two main goals were to find out whether the GATA6 overexpression alone is sufficient in inducing a senescent phenotype in EGFR-mutant NSCLC cells and whether GATA6 is necessary for the development of treatment-induced senescence. GATA2 was hypothesized to have no role in treatment-induced senescence, and thus, to function in this study only as a control from the same transcription factor family.

Taken together, we hypothesized that GATA6 induces a senescent phenotype in EGFR-mutant NSCLC cells *in vitro*. The specific aims for this master's thesis research project were as follows:

Aim 1: Establish doxycycline-inducible expression of GATA2 and GATA6 transcription factors in the EGFR-mutant NSCLC cell line

Aim 2: Investigate the role of GATA2 and GATA6 expression in treatment-induced senescence

Aim 3: Study the effect of GATA2 and GATA6 overexpression and GATA6 silencing on the expression of SASP genes

2 Results

2.1 Doxycycline-inducible overexpression of GATA2 and GATA6 and shRNA-mediated silencing of GATA6

To establish doxycycline-inducible overexpression of GATA2 and GATA6 as well as shRNA-mediated silencing of GATA6 in EGFR-mutant NCSLC cells, HCC827 cells were transduced with plasmids containing complementary DNAs (cDNAs) coding GATA2 and GATA6 transcription factors or GATA6-targeting short hairpin ribonucleic acid (shRNA) using 3rd generation lentiviral vectors. Successful transduction was confirmed with Western blotting, using escalating doses of doxycycline for 24 h (**Figure 4**). Silencing of GATA6 was performed with only one doxycycline concentration (200 ng/ml) for 24 h and 48 h.

The results showed that the transduction of HCC827 cells to establish doxycycline-inducible overexpression of GATA2 (**Figure 4A**) and GATA6 (**Figure 4B**) was successful when compared to controls without doxycycline treatments as well as to cells transduced with empty vectors (**Figure 4C**). Doxycycline induced overexpression of GATA2 and GATA6 in corresponding cell lines even with the lowest concentration (50 ng/ml), and protein expression levels had no remarkable differences between different doxycycline concentrations. Doxycycline-inducible shRNA-mediated silencing of GATA6 was also successful when compared to cells without doxycycline treatments and to cells transduced with empty vectors, as GATA6 expression decreased after 24 h with 200 ng/ml doxycycline (**Figure 4D**).

Successful overexpression and silencing were further confirmed with reverse transcription-quantitative polymerase chain reaction (RT-qPCR), quantifying GATA2 and GATA6 mRNA expression levels after 24 h of 200 ng/ml doxycycline treatment. Doxycycline induced a 120-fold increase in GATA2 expression (**Figure 4E**) and a 60-fold increase in GATA6 expression (**Figure 4F**) when compared to controls with no doxycycline treatment. GATA6 expression was also decreased to less than half when compared to the control without doxycycline treatment, suggesting that GATA6 silencing is working, however, this decrease was not statistically significant ($p = 0.055146$) (**Figure 4G**). In control cell lines transduced with empty vectors, doxycycline did not cause changes in the expression of GATA transcription factors (**Figure 4E, F, G**). Taken together, these data confirmed that lentiviral transduction of HCC827 cells was successful.

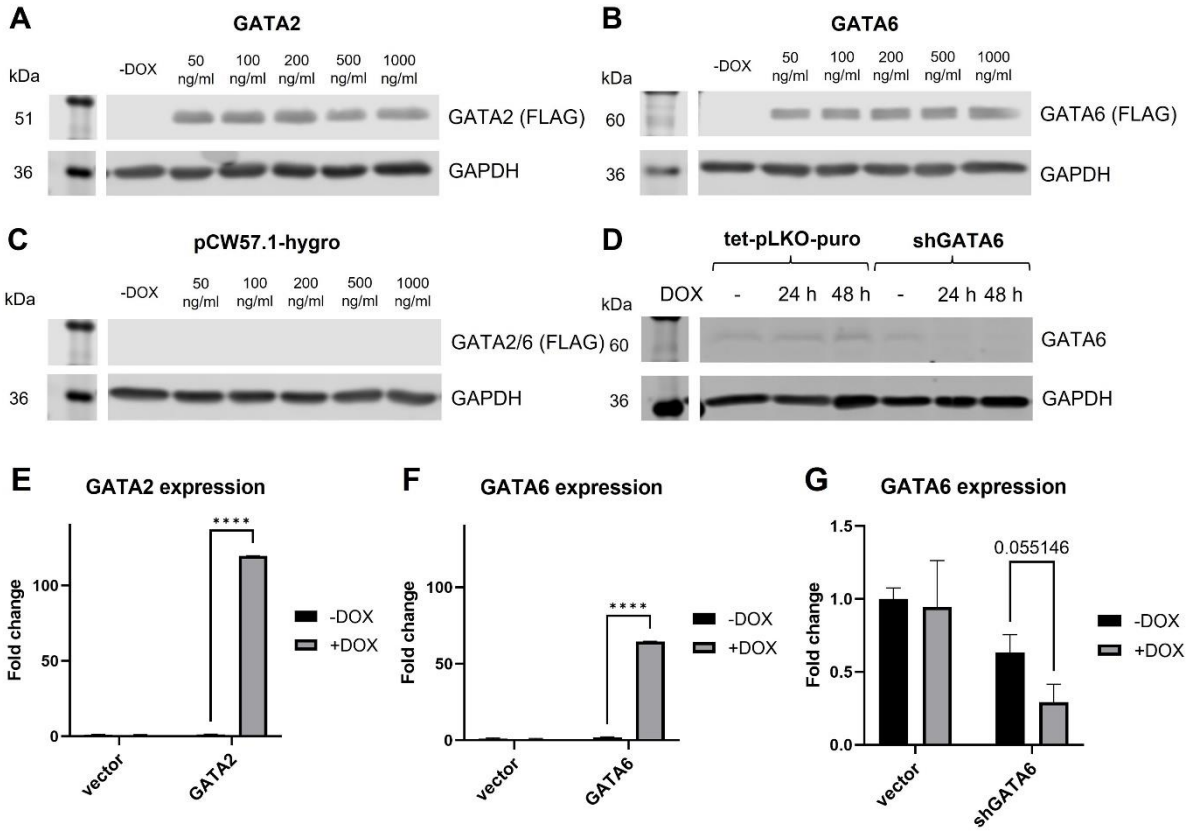

Figure 4. **Doxycycline-inducible overexpression of GATA2 and GATA6 and shRNA-mediated silencing of GATA6.** GATA transcription factor expression levels were immunoblotted from (A) pCW57.1-hygro-3X-Flag-GATA2 cells, (B) pCW57.1-hygro-3X-Flag-GATA6 cells, and (C) pCW57.1-hygro control cells using a FLAG antibody. Before cell lysis, cells were treated with escalating doses of doxycycline (0 ng/ml – 1000 ng/ml) for 24 h. (D) GATA6 expression levels were immunoblotted from tet-pLKO-puro and tet-pLKO-puro-shGATA6 cells using a GATA6 antibody, after treatments of 200 ng/ml doxycycline for 24 h and 48 h. GAPDH was used as a loading control. RT-qPCR was performed to confirm doxycycline-inducible (E) overexpression of GATA2, (F) overexpression of GATA6, and (G) silencing of GATA6. In cell line names, vector refers to pCW57.1-hygro or tet-pLKO-puro depending on the figure, GATA2 refers to pCW57.1-hygro-3X-Flag-GATA2, GATA6 to pCW57.1-hygro-3X-Flag-GATA6, and shGATA6 to tet-pLKO-puro-shGATA6. One-way analysis of variance (ANOVA), ****p<0.0001. DOX = doxycycline.

2.2 Overexpression of GATA6 decreased the proliferation of HCC827 cells

The effect of GATA transcription factors on cell proliferation and morphology was determined with the IncuCyte S3 live cell imaging system, taking images every other hour for 72 h (**Figure 5**). Overexpression of both GATA2 (**Figure 5A**) and GATA6 (**Figure 5B**) decreased the proliferation of HCC827 cells regardless of the doxycycline concentration when compared to the vector control cell line, in which only the highest doxycycline concentration (1000 ng/ml) decreased cell proliferation (**Figure 5C**). shRNA-mediated silencing of GATA6 decreased the proliferation of HCC827 cells statistically significantly only with the two highest doxycycline concentrations (500 ng/ml and 1000 ng/ml) (**Figure 5D**). Doxycycline did not affect the proliferation of tet-pLKO-puro vector control cells (**Figure 5E**).

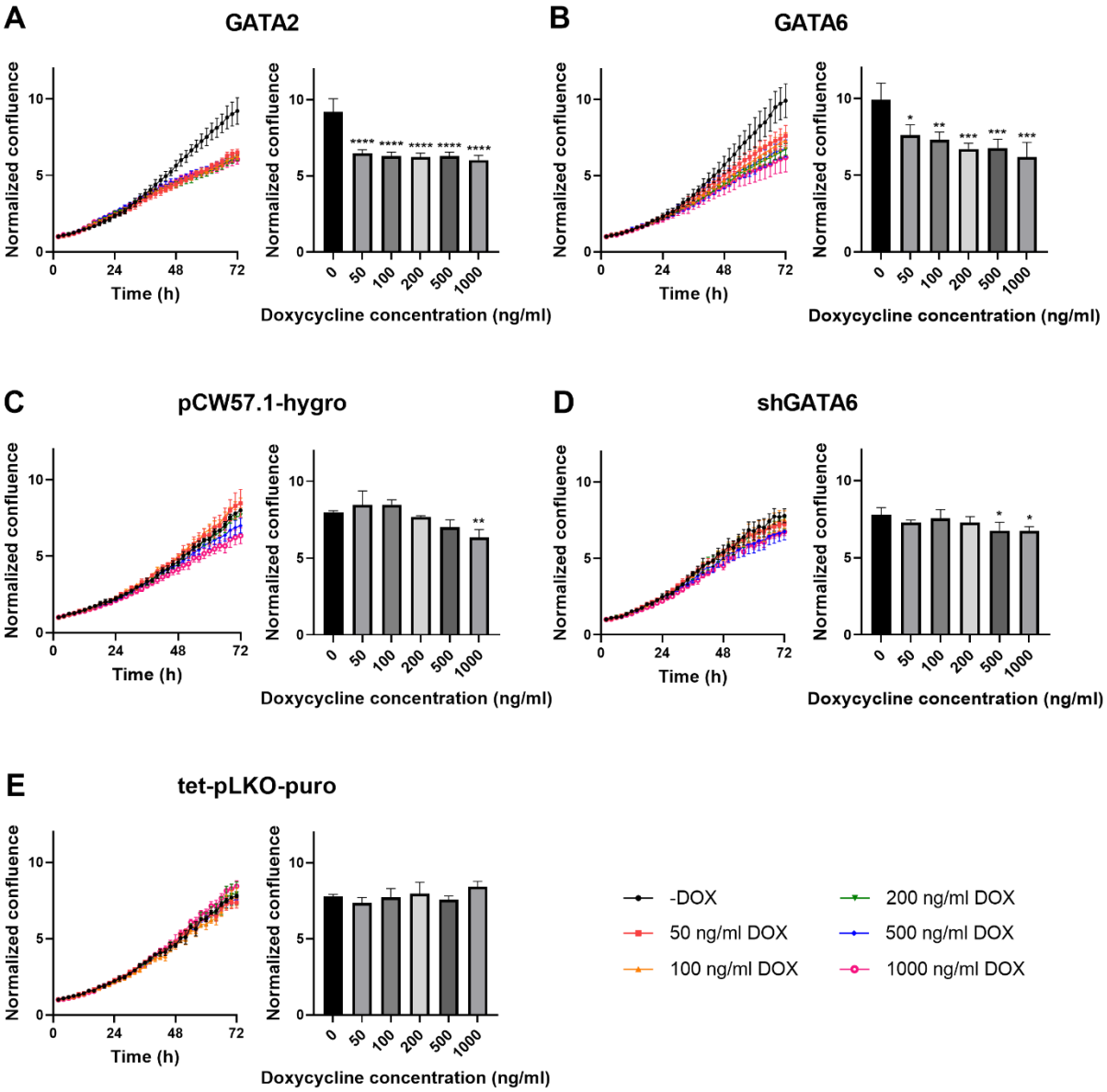

Figure 5. The effect of GATA transcription factor expression on cell proliferation. The cell proliferation of transduced cell lines was followed with the IncuCyte S3 live cell imaging system for 72 h, taking images every other hour. Cell confluences were normalized to the 2 h time point, and one-way ANOVA was used to compare cell confluences at the 72 h time point. (A) ****p<0.0001; (B) *p=0.0104, **p=0.0043, ***p=0.008, 0.0010, 0.0002; (C) **p=0.0060; (D) *p=0.0399, 0.0383; (E) No significant differences. Representative data was chosen among three independent replicates and shown as mean ± standard deviation (SD).

Taken together, these results suggested that overexpression of GATA2 and GATA6 significantly decreased the proliferation of HCC827 cells, while silencing of GATA6 did not have remarkable effects on cell proliferation.

2.3 Both overexpression and silencing of GATA6 decreased cell survival following targeted therapy

To assess the effect of overexpression and silencing of GATA transcription factors on cell survival after long-term targeted therapy, HCC827 cells were treated with 100 nM osimertinib alone or in combination with 30 nM trametinib (OT) for 10 days, after which cell viability was measured using a CellTiter-Glo® assay (**Figure 6**). Overexpression of both GATA2 and GATA6 statistically significantly decreased the cell viability after both osimertinib (**Figure 6A**) and OT treatments (**Figure 6B**). Additionally, shRNA-mediated silencing of GATA6 also significantly decreased the viability of both osimertinib (**Figure 6C**) as well as OT-treated cells (**Figure 6D**). When GATA6 was silenced, more cells survived osimertinib treatment than OT treatment whereas with the overexpression cell lines, the effect was the opposite. In other words, when GATA2 or GATA6 was overexpressed, a bigger proportion of cells survived OT treatment than osimertinib treatment.

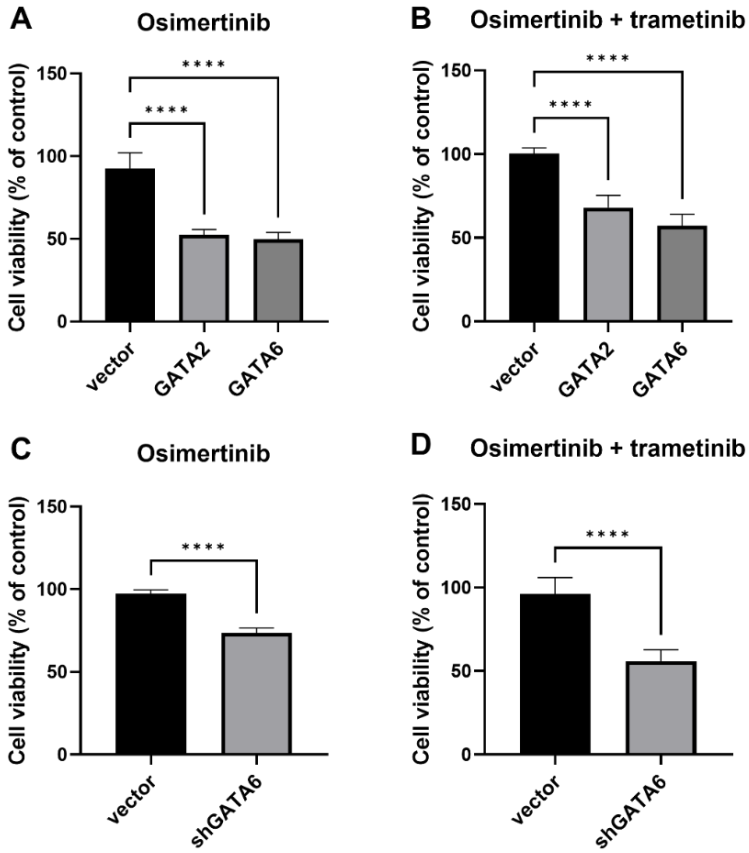

Figure 6. The effect of GATA transcription factor expression on cell survival after targeted therapy. Cells were treated for 10 days, after which cell viability was measured with the CellTiter-Glo® cell viability assay. Overexpression cell lines and their vector control were treated with (A) osimertinib or (B) OT, and differences were analyzed with one-way ANOVA. The GATA6-silenced cell line and its vector control were treated with (C) osimertinib or (D) OT, and differences were analyzed with an unpaired t-test. The representative data was chosen among three independent replicates and represented as mean ± SD. ****p<0.0001. control = -DOX, OT = osimertinib + trametinib.

To conclude, these data suggested that overexpression of GATA2 and GATA6 as well as silencing of GATA6 significantly decreased the viability of HCC827 cells following long-term targeted therapy with osimertinib alone and in combination with trametinib.

2.4 Overexpression of GATA6 decreased and silencing of GATA6 increased apoptosis following targeted therapy

As both overexpression as well as silencing of GATA6 significantly decreased cell viability following long-term targeted therapy, the effect of GATA6 expression on apoptosis during early treatment was further studied with IncuCyte S3 live cell imaging and CellEvent™ Caspase-3/7 Green Reagent (**Figure 7**).

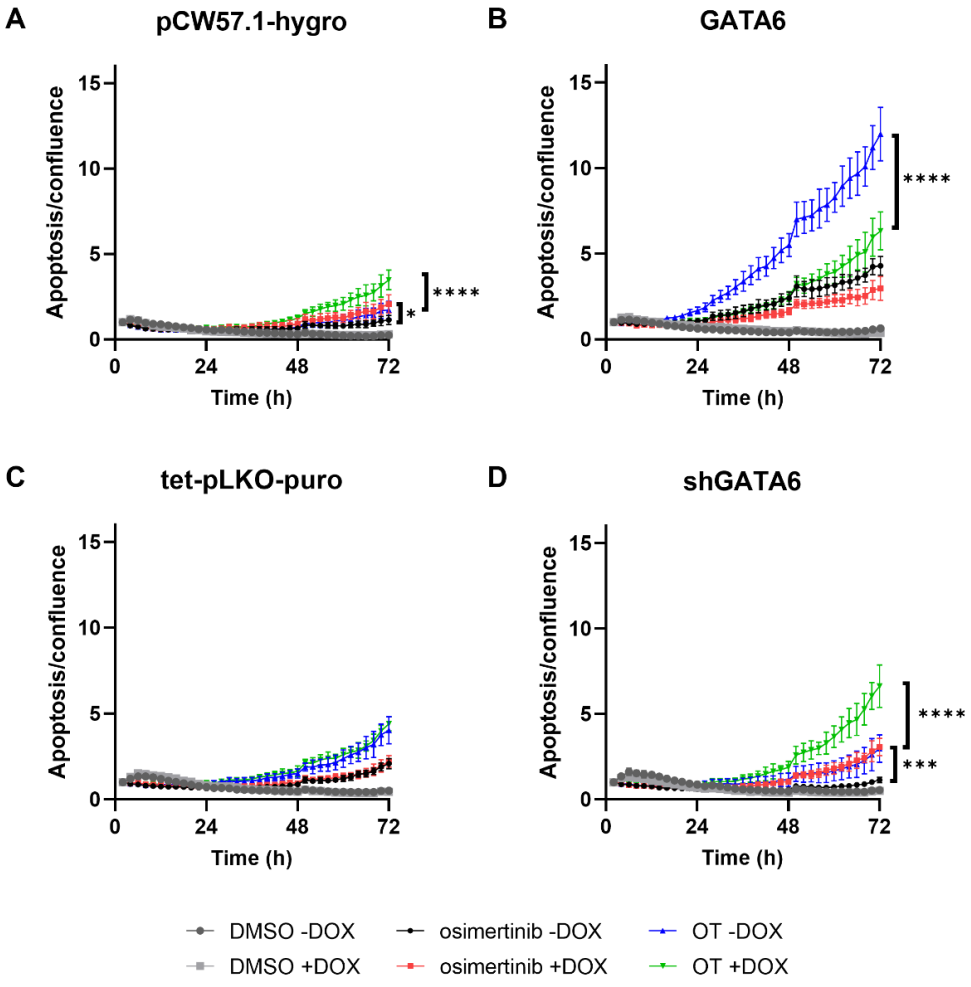

Figure 7. The effect of GATA6 expression on apoptosis. (A) pCW57.1-hygro control, (B) GATA6 overexpression, (C) tet-pLKO-puro control, and (D) GATA6-silenced cells were treated with 100 nM osimertinib or OT (100 nM osimertinib + 30 nM trametinib) in the presence and absence of 200 ng/ml doxycycline (DOX). The Caspase-3/7 Green Reagent was added, and cells were followed with IncuCyte S3 for 72 h. The rate of apoptosis was deemed as the green object count/confluence ratio per image and normalized to the initial value (2 h). One-way ANOVA at 72 h, *p=0.0121, ***p=0.0007, ****p<0.0001. Representative data was chosen among three independent replicates, and shown as mean ± SD.

In the vector control cell line pCW57.1-hygro, doxycycline induced apoptosis after both osimertinib and OT treatments (**Figure 7A**). It is also noteworthy that when compared to other cell lines in this experiment, pCW57.1-hygro vector control cells were consistently more resistant to apoptosis with each treatment condition. Overexpression of GATA6 induced remarkably higher overall apoptosis rates when compared to the vector control cell line, and overexpression of GATA6 decreased the apoptosis rate after osimertinib and OT treatments, although its effect on osimertinib-treated cells was not statistically significant (**Figure 7B**). As can be seen in **Figure 7C**, doxycycline did not cause any differences in apoptosis rates of tet-pLKO-puro vector control cells, while silencing of GATA6 increased apoptosis following both treatments (**Figure 7D**). The apoptosis assay was also conducted using the GATA2 overexpression cell line, but consistent results could not be obtained in three individual replicate experiments.

Taken together, these results suggested that overexpression of GATA6 decreased and silencing of GATA6 increased apoptosis of HCC827 cells during early treatment with targeted therapies. For some reason, doxycycline increased apoptosis also in pCW57.1-hygro vector control cells, whereas in tet-pLKO-puro control cells, doxycycline treatment did not affect apoptosis.

2.5 Silencing of GATA6 reduced the proportion of β-galactosidase-positive cells and decreased the expression of SASP genes after targeted therapy

To assess the role of GATA transcription factors in senescence, 10-day treatments with doxycycline for pCW57.1-hygro-transduced cells and with OT in the presence and absence of doxycycline for tet-pLKO-puro-transduced cells were performed. The aim was to assess if the overexpression of GATA2 or GATA6 alone induces a senescent phenotype and how silencing of GATA6 affects treatment-induced senescence following OT. After 10 days of treatments, cells were either stained for senescence-associated β-galactosidase (SA-β-Gal) activity and imaged, or lysed for RNA isolation, followed by RT-qPCR (**Figure 8**).

Overexpression cell lines did not have any SA-β-Gal positive cells following β-galactosidase stainings (**Figure 8A, B**), whereas OT-treated tet-pLKO-puro-transduced cell lines had (**Figure 8C**). The ratio of SA-β-Gal positive/negative cells was significantly decreased after shRNA-mediated silencing of GATA6 when compared to cells without doxycycline treatment

as well as to the vector control cell line (**Figure 8D, E**). In addition, doxycycline-treated pCW57.1-hygro vector control cells had SA-β-Gal positive cells (**Figure 8F**).

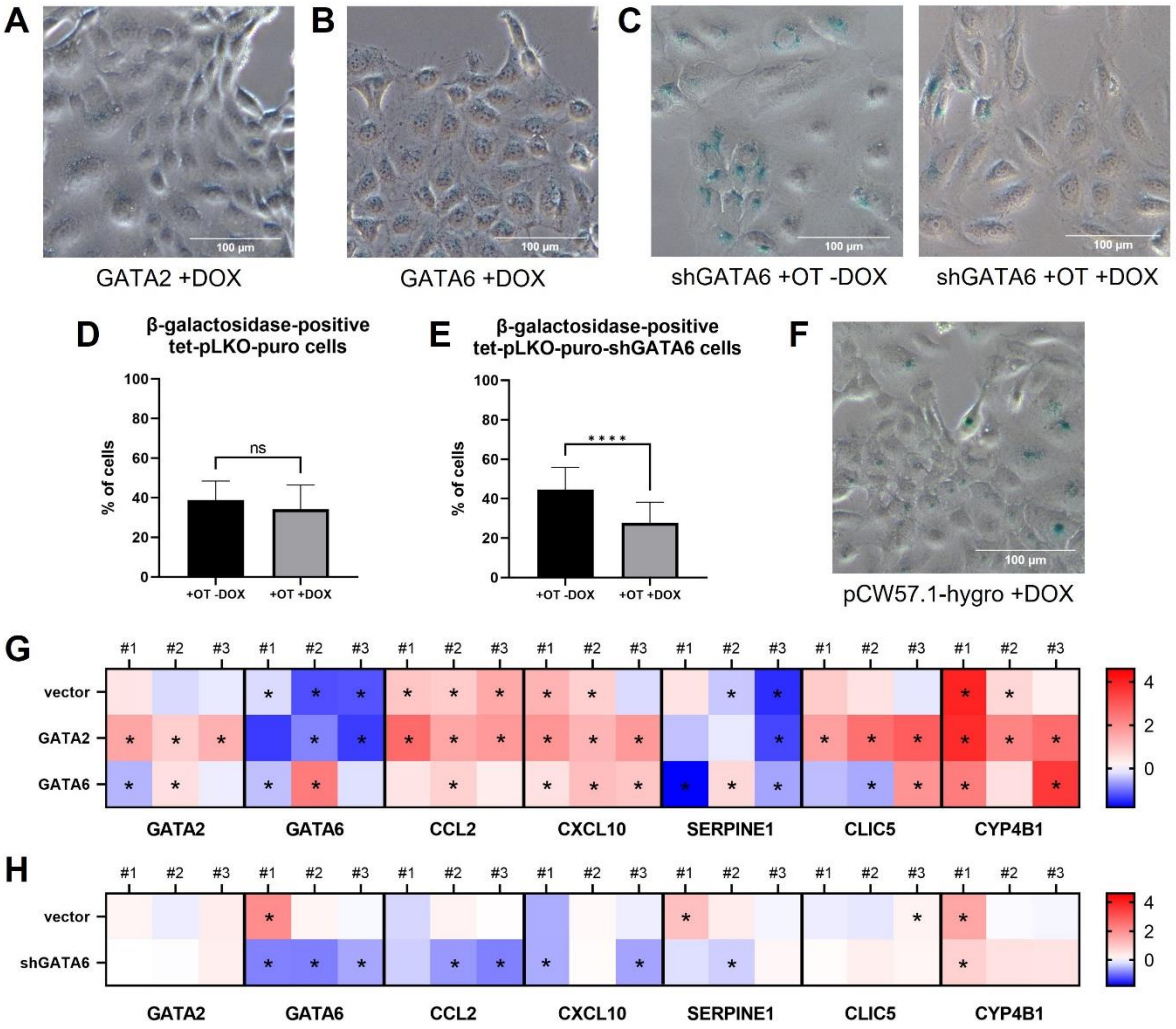

Figure 8. The effect of GATA transcription factor expression on senescence and gene expression. An example image of senescence-associated β-galactosidase stainings when (A) GATA2 or (B) GATA6 were overexpressed. (C) Example images of β-galactosidase staining in the OT-treated tet-pLKO-puro-shGATA6 cell line in the absence and presence of doxycycline. β-galactosidase-positive cells are stained blue. Scale bar = 100 µm. Cells were counted manually, and the ratio of β-galactosidase-positive/negative cells was calculated for (D) tet-pLKO-puro cells and (E) tet-pLKO-puro-shGATA6 cells. Unpaired t-test, ****p<0.0001. (F) An example image of doxycycline-treated pCW57.1-hygro vector control cells. In A-F, representative data was chosen among three independent replicates, and data in D and E are shown as mean ± SD. RT-qPCR was conducted to assess changes in gene expression, and $\log_2$ fold changes of (G) pCW57.1-hygro-transduced cell lines and (H) OT-treated tet-pLKO-puro-transduced cell lines compared to -DOX samples were calculated. Data from all three independent replicates are shown. Unpaired t-test, *p ≤ 0.05. CCL2 = C-C motif chemokine ligand 2, CXCL10 = C-X-C motif chemokine ligand 10, SERPINE1 = serpin family E member 1, CLIC5 = chloride intracellular channel 5, CYP4B1 = cytochrome P450 family 4 subfamily B member 1.

The effect of overexpression of GATA transcription factors and silencing of GATA6 on the expression of SASP genes C-C motif chemokine ligand 2 (CCL2), C-X-C motif chemokine ligand 10 (CXCL10), and serpin family E member 1 (SERPINE1) was assessed with RT-

qPCR. B-glucuronidase (GUSB) was used as a reference gene, and gene expression fold changes relative to samples without doxycycline treatment were calculated using the ΔΔCT method. In addition to GATA transcription factors and SASP genes, qPCR target genes included also AT1 cell markers chloride intracellular channel 5 (CLIC5) and cytochrome P450 family 4 subfamily B member 1 (CYP4B1), because dormant cells have previously been suggested to survive targeted therapy by exhibiting an AT1-like phenotype (Figarol et al., 2022).

Doxycycline induced overexpression of GATA2 (**Figure 8G**) and shRNA-mediated silencing of GATA6 (**Figure 8H**) consistently, whereas effects on the GATA6 gene expression were inconclusive between repeat experiments (**Figure 8G**). Overexpression of GATA2 significantly decreased GATA6 expression, while overexpression or silencing of GATA6 did not cause consistent effects on GATA2 expression (**Figure 8G, H**). As can be seen in **Figure 8G**, overexpression of GATA2 increased the expression of AT1 markers CLIC5 and CYP4B1 and decreased the expression of the SASP gene SERPINE1. Additionally, overexpression of GATA2 clearly increased the expression of other SASP genes, CCL2, and CXCL10. Overexpression of GATA6 also showed trends towards an increased expression of SASP genes CCL2 and CXCL10 as well as of an AT1 marker CYP4B1 (**Figure 8G**). Silencing of GATA6 decreased mRNA expression of CCL2 and CXCL10, as well as SERPINE1 (**Figure 8H**). In the expression of AT1 marker genes, there were no significant differences, although the AT1 marker CYP4B1 expression was slightly increased.

pCW57.1-hygro control cells had an increased expression of SASP genes CCL2 and CXCL10 (**Figure 8G**). Trends towards an increased expression of AT1 markers, CLIC5 and CYP4B1, and a decreased expression of EMT marker SERPINE1 could also be seen. It is noteworthy, that the gene expression of multiple genes, including SASP genes, was already without doxycycline treatment remarkably higher in pCW57.1-hygro control cell lines than in other cell lines (data not shown). In another vector control cell line, tet-pLKO-puro, doxycycline did not induce remarkable differences in gene expression levels (**Figure 8H**).

Taken together, these results suggested that overexpression of GATA2 and GATA6 increased the expression of SASP-related genes CCL2 and CXCL10, whereas silencing of GATA6 decreased the proportion of β-galactosidase-positive senescent cells and the expression of SASP genes following targeted therapy. According to the gene expression data,

overexpression of GATA2 also decreased GATA6 and SERPINE1 expression, while increasing the expression of AT1 genes.

2.6 pCW57.1-hygro control cells exhibited EMT-like phenotype

In pCW57.1-hygro vector control cells, doxycycline induced apoptosis (**Figure 7A**), increased the expression of CCL2 and CXCL10 (**Figure 8E**), as well as induced β-galactosidase activity (**Figure 8F**). Additionally, pCW57.1-hygro cells had an uncommon morphology when compared to other cell lines as they were more isolated and exhibited back-front polarity (**Figure 9A**) when compared to, for example, pCW57.1-hygro-3X-Flag-GATA2 cells that grew attached to each other (**Figure 9B**). Besides these GATA2 overexpression cells, also other cell lines had similar phenotype resembling parental HCC827 cells (**Figure 9C**).

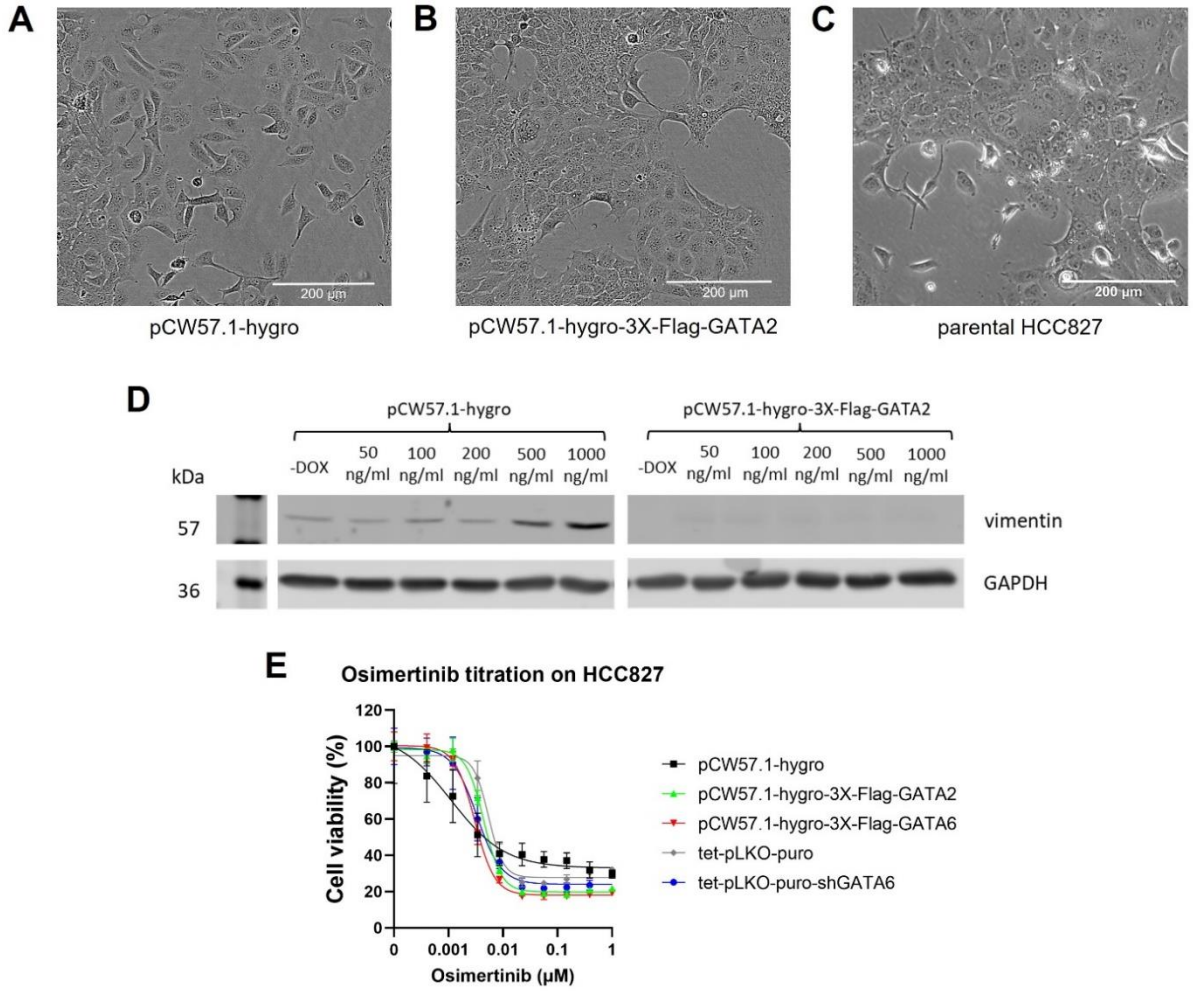

Figure 9. Assessing EMT-like features and osimertinib sensitivity of the pCW57.1-hygro control cell line. Morphology of (A) pCW57.1-hygro, (B) pCW57.1-hygro-3X-Flag-GATA2, and (C) parental HCC827 cells. Scale bar = 200 µm. (D) The expression levels of mesenchymal marker vimentin immunoblotted from pCW57.1-hygro and pCW57.1-hygro-3X-Flag-GATA2 cells. GAPDH was used as a loading control. (E) Osimertinib titration was performed by treating cells with increasing doses (0.5 nM – 1 µM) of osimertinib for 72 h, after which cell viability was measured with the MTT assay.

Due to unexpected results in multiple experiments and the morphological changes resembling epithelial-to-mesenchymal (EMT)-like changes in the pCW57.1-hygro vector control cell line, the protein expression of a common mesenchymal marker, vimentin, was assessed (**Figure 9D**). pCW57.1-hygro control cells expressed vimentin, whereas the GATA2 overexpression cell line used as a control did not. Additionally, higher doxycycline concentrations (500 ng/ml and 1000 ng/ml) induced higher vimentin expression in pCW57.1-hygro cells. When performing osimertinib titration with increasing osimertinib concentrations (0.5 nM – 1 μM), pCW57.1-hygro vector control cells were more resistant to osimertinib treatment when compared to other cell lines (**Figure 9E**). To conclude, the pCW57.1-hygro control cell line exhibited an EMT-like morphology, expressed vimentin, and was more resistant to osimertinib than other cell lines.

3 Discussion

3.1 Overexpression of GATA6 decreases the proliferation of EGFR-mutant NSCLC cells

Treatment-induced senescence has been proposed to play a role in tumor dormancy and evolving therapy resistance to targeted cancer therapies. Alterations in GATA transcription factors have been observed in multiple cancer types, and their senescence-inducing capabilities have been recently revealed in the cancer context. The objective of this master thesis was to assess the role of GATA transcription factors, especially GATA6, in treatment-induced senescence in EGFR-mutant non-small cell lung cancer. Two main goals were to determine whether overexpression of GATA6 alone is sufficient to establish a senescent phenotype and whether its expression is necessary for treatment-induced senescence in NSCLC.

At first, HCC827 cells were transduced with relevant plasmids using 3rd generation lentiviral vectors to establish doxycycline-inducible overexpression of GATA2 and GATA6 as well as shRNA-mediated silencing of GATA6. As can be seen in **Figure 4**, transduction was successful, and doxycycline induced the overexpression of GATA2 (**Figure 4A, E**) and GATA6 (**Figure 4B, F**) already with a concentration of 50 ng/ml. Because different doxycycline concentrations did not cause significant differences in protein expression levels and silencing of GATA6 succeeded with 200 ng/ml doxycycline after 24 h (**Figure 4D, G**), 200 ng/ml doxycycline was chosen to be used in further experiments.

Overexpression of both GATA2 (**Figure 5A**) and GATA6 (**Figure 5B**) decreased the proliferation of HCC827 cells. Consistent with these results, overexpression of both GATA2 and GATA6 has been shown to reduce cell proliferation also in previous *in vitro* studies. For example, Zhang and colleagues (2022) have observed that GATA2 overexpression decreases the cell proliferation of KRAS-mutant lung adenocarcinoma cell lines A549 and H460. W. Chen and colleagues (2020), in turn, have shown that also GATA6 overexpression reduces the proliferation of A549 cells. Additionally, Sun and colleagues (2021) have observed that overexpression of GATA6 induces G_0/G_1 cell cycle arrest and decreases cell proliferation of lung adenocarcinoma cell lines H1650 and H1299. In all these proliferation experiments, the cell counting kit-8 (CCK8) was used instead of the live-cell imaging that was used in our study.

Unlike overexpression of GATA2 and GATA6, silencing of GATA6 did not have remarkable effects on the proliferation of HCC827 cells, although a minor decrease with the highest doxycycline concentrations could be seen (**Figure 5D**). In previous *in vitro* studies, silencing of GATA2 or GATA6 has caused opposite effects on proliferation when compared to overexpression of transcription factors in question – meaning that overexpression has decreased and silencing has increased proliferation (W. Chen et al., 2020; Zhang et al., 2022). However, when it comes to the silencing of GATA6, contradictory results have also been reported. For example, Cheung and colleagues (2013) have not observed significant differences in the cell growth of KRAS-mutant H2030 cells when GATA6 is silenced, even though a slight decrease similar to our results was shown. Sun and colleagues (2021), in turn, have shown that silencing of GATA6 slightly decreases the proliferation of H1650 and H1299 lung adenocarcinoma cells. Additionally, they have suggested that GATA6 inhibits the expression and activity of sex determining region Y-box protein 12 (SOX12), which has an important role in the cell cycle and proliferation (Sun et al., 2021). On the contrary, overexpression of SOX12 has been shown to increase the proliferation of NCI-H292 human lung mucoepidermoid carcinoma cells, indicating that the decreased GATA6-mediated inhibition of SOX12 should lead to increased proliferation (Wang et al., 2017). Furthermore, Zang and colleagues (2020) have proposed that GATA6 increases the expression of long-noncoding RNA prostate cancer-associated transcript 1 (PCAT1). They have also shown that silencing of PCAT1 decreases the proliferation rate of NSCLC cell lines H226 and A549 as well as increases apoptosis rates, suggesting that GATA6 silencing might decrease PCAT1 levels, thus decreasing proliferation and increasing apoptosis, as seen in our results.

3.2 Overexpression of GATA6 decreases cell survival following targeted therapy without inducing apoptosis during early treatment

Overexpression of GATA2 and GATA6 also decreased HCC827 cell survival after 10 days of targeted therapy with both osimertinib (**Figure 6A**) and OT (**Figure 6B**) treatments. Interestingly, also silencing of GATA6 reduced cell viability following both treatments (**Figure 6C, D**). Due to the contradictory effects of GATA6 overexpression and silencing on cell survival after long-term targeted therapy, the effects on apoptosis during early treatment were further evaluated to reveal possible mechanisms behind the reduction in cell viability rates. Overexpression of GATA6 decreased apoptosis (**Figure 7B**) while silencing of GATA6 increased apoptosis (**Figure 7D**), suggesting that the reduced cell survival following long-

term targeted therapy caused by GATA6 overexpression should have another mechanism than apoptotic cell death during early treatment.

In the previous study by W. Chen and colleagues (2020), overexpression of GATA6 did not cause any effects on apoptosis of lung adenocarcinoma cells A549, measured by fluorescence-activated cell sorting (FACS) analysis of annexin-V and propidium iodide (PI) -stained cells and protein levels of poly (ADP-ribose) polymerase 1 (PARP1) and caspase 3. As mentioned before, GATA6 has been suggested to inhibit SOX12, while SOX12 inhibition has been further proposed to promote apoptosis (Sun et al., 2021; Wang et al., 2017). However, in our results regarding GATA6 overexpression, apoptosis rates were reduced, indicating that overexpression of GATA6 should decrease HCC827 cell proliferation via other possible mechanisms than apoptosis. The reduction in the proliferation of HCC827 cells and the cell survival after targeted therapy may be due to the ability of GATA6 to slow the cell cycle down or to induce a total cell cycle arrest. In previous studies, GATA6 has been proposed to inhibit for example EGFR, Wnt, and hedgehog (Hh) signaling pathways, leading to decreased cell proliferation rates (Xu et al., 2019; Zito et al., 2017). Xu and colleagues (2019) have shown that GATA6 inhibits the activity of the important component of the Hh signaling pathway, Sonic hedgehog (Shh), by binding its promoter region and, thus, leading to decreased cell proliferation and migration of the human lung squamous cell carcinoma (LSCC) cell line SK-MES-1. Zito and colleagues (2017), in turn, have observed that GATA6 inhibits the transcription of EGFR as well as the activation of Wnt signaling in the lung adenocarcinoma H-1975 cell line, further decreasing cell proliferation rates.

After treating cells with osimertinib alone, cell viability of the cells with GATA2 and GATA6 overexpression was higher than the cell viability of the cells with silenced GATA6. It has been previously demonstrated that some cells can escape EGFR TKI treatments by reactivating alternative pathways and starting to proliferate again (Kurppa et al., 2020). If cells have also started to escape osimertinib and OT treatments in this study, decreased cell proliferation of cells with GATA2 and GATA6 overexpression may explain slower re-proliferation rates, and thus also smaller amounts of cell survival after targeted therapy, when compared to the cells with silenced GATA6.

To conclude, overexpression of GATA6 decreased the proliferation of HCC827 cells as well as cell survival following long-term targeted therapy, without inducing apoptosis during early treatment. Thus, GATA6 overexpression most likely decreases the proliferation and survival

of HCC827 cells via some other mechanism, for example, by slowing the cell cycle down. Silencing of GATA6 decreased cell survival following long-term targeted therapy and increased apoptosis during early treatment, suggesting that it is to some extent necessary for HCC827 cells to survive targeted therapy.

3.3 Overexpression of GATA6 is not sufficient to establish a senescent phenotype but GATA6 might have a role in treatment-induced senescence

According to the obtained results from SA-β-Gal stainings after 10 days of doxycycline treatment, overexpression of neither GATA2 (**Figure 8A**) or GATA6 (**Figure 8B**) induced SA-β-Gal activity and, thus, overexpression of neither of them alone is sufficient in inducing a senescent phenotype. Even though overexpression of GATA6 did not induce SA-β-Gal activity (**Figure 8B**), it increased the expression of SASP genes (**Figure 8G**) and another characteristic of senescent cells – apoptosis resistance (**Figure 7B**). However, RT-qPCR results suggest that overexpression of GATA6 succeeded only in one of three independent replicate experiments (**Figure 8G**), which must be considered when interpreting results. In these experiments, GATA6 expression levels were only measured by determining mRNA expression using RT-qPCR, without confirming the protein expression levels of GATA6.

Previously, W. Chen and colleagues (2020) have shown that overexpression of GATA6 induces senescence in A549 cells via classical senescence pathway regulators, p53 and p21. In the same study, GATA6 overexpression was shown to increase the gene expression of CCL2, and similar trends could also be seen in our experiments. However, HCC827 cells used in our experiments have a negligible expression of p21 and a mutated p53 (Kurppa et al., 2020), and the absence of functional factors involved in classical senescence pathways may be a reason for the absence of a senescent phenotype. GATA6 may be unable to induce a senescent phenotype and SA-β-Gal activity via other pathways than the classical ones. The reduction in proliferation and cell survival of HCC827 cells in our study can be due to totally different mechanisms than senescence. For example, Zito and colleagues (2017) have shown that overexpression of GATA6 inhibits Wnt signaling, thus arresting the cell cycle of H-1975 cells. Whether the inhibition of Wnt signaling via GATA6 overexpression also results in cell cycle arrest in HCC827 cells requires further study. If the reduction in cell proliferation and cell survival, however, is due to a partial senescence process, it is possible that GATA6 might need other factors to contribute to establishing a complete senescent phenotype. For example, liver kinase B1 (LKB1), which can be mutated in lung adenocarcinoma, may regulate GATA6

functions and activity via a complex consisting of LKB1, LIM domain only 4 (LMO4), GATA6, and LIM domain-binding protein 1 (LDB1) (Setogawa et al., 2006). Additionally, posttranslational modifications, such as phosphorylation and SUMOylation, have been reported to be essential for the function and activity of GATA6 in cardiovascular tissues – and they can have a role in this context as well (H. Chen et al., 2020; Xie et al., 2015).

Silencing of GATA6 decreased the expression of SASP-related genes CCL2, CXCL10, and SERPINE1 (**Figure 8H**) as well as the proportion of SA-β-Gal positive cells (**Figure 8E**) following 10 days of targeted therapy with OT, suggesting that GATA6 has some role in treatment-induced senescence. Results from other experiments, such as the lack of apoptosis resistance (**Figure 7D**) which is suggested to be one characteristic of senescent cells, are consistent with this conclusion. As can be seen in **Figure 4D**, endogenous GATA6 expression in HCC827 cells is relatively low, which might be a reason why the induced changes in gene expression levels are also relatively small. Additionally, based on the GATA6 mRNA expression analysis, GATA6 expression was not fully inhibited but reduced to half of the endogenous expression level (**Figure 4G**), indicating that some GATA6 activity may remain despite shRNA-mediated inhibition. After 10 days of targeted therapy, some cells survived although GATA6 was silenced (**Figure 6C, D**). This subpopulation could be AT1-like, consistent with previous literature proposing that cell differentiation into AT1-like cells is a potential mechanism for NSCLC cells to survive targeted therapy (Figarol et al., 2022). However, because the increases in AT1 gene expression were only moderate without statistical significance (**Figure 8H**), other mechanisms should most likely exist.

To conclude, overexpression of GATA6 alone was not sufficient in inducing a senescent phenotype measured by SA-β-Gal activity, although the expression of SASP-related genes was increased. Silencing of GATA6 decreased the proportion of β-galactosidase-positive senescent cells and the SASP gene expression after 10 days of targeted therapy with OT, suggesting that GATA6 may have an important role in treatment-induced senescence in EGFR-mutant non-small cell lung cancer.

3.4 Overexpression of GATA2 may convert HCC827 cells to dormant AT1-like cells following targeted therapy

Overexpression of GATA2 decreased the proliferation of HCC827 cells (**Figure 5A**) as well as cell survival following long-term targeted therapy (**Figure 6A, B**). Even though overexpression of GATA2 was not sufficient to establish a senescent phenotype measured by

SA-β-Gal activity (**Figure 8A**), interestingly, it was shown to significantly increase the gene expression of AT1 markers CLIC5 and CYP4B1 (**Figure 8G**). Consistent with this observation, recent research has suggested that a small population of NSCLC cells survives targeted therapy by acquiring an AT1-like phenotype (Figarol et al., 2022). In that study, using the cell cycle dynamics system FUCCI (fluorescence ubiquitination cell cycle indicator), Figarol and colleagues (2022) have shown that a subpopulation of NSCLC cell lines HCC4006, PC9, and HCC827 differentiates towards AT1 phenotype after targeted therapy with EGFR inhibitor osimertinib.

In addition to the increased AT1 gene expression, an increase in the expression of SASP genes CCL2 and CXCL10, as well as a decrease in the expression of SASP gene SERPINE1 could also be seen when overexpressing GATA2. In addition to its role as a SASP-related gene, SERPINE1 can also be used as an EMT marker. Consistent with observations regarding the SERPINE1 gene expression, a recent study has reported decreased EMT in lung cancer cell lines when GATA2 was overexpressed (Zhang et al., 2022). Even though the expression of SASP-related genes CCL2 and CXCL10 was increased, according to the SA-β-Gal activity staining results, GATA2 overexpression alone did not induce a senescent phenotype. SASP markers used in this study are chemokines, and their expression can increase also due to various other reasons. Thus, their gene expression alone cannot be deemed as a reliable senescence marker.

To conclude, even though GATA2 was not a key player in this master's thesis, it was found to possibly convert HCC827 cells into AT1-like cells after targeted therapy. Thus, valuable information on the role of GATA2 in tumor dormancy could be obtained.

3.5 The GATA2/GATA6 switch may regulate the expression and activity of GATA transcription factors

The gene expression analysis after 10 days of doxycycline treatment showed that overexpression of GATA2 decreased the gene expression of GATA6 (**Figure 8G**). This phenomenon may be a so-called GATA switch, which has been previously observed in hematopoietic tissues and trophoblasts between GATA1 and GATA2, as well as between GATA3 and GATA2 (Grass et al., 2003; Ray et al., 2009). However, in both of the aforementioned GATA switches, GATA2 has been replaced and repressed by another GATA transcription factor. On the contrary, in our results, GATA2 may be a replacing agent – meaning that when GATA2 is overexpressed, it can inhibit or downregulate other GATA

transcription factors. In the previous studies regarding GATA switches, the knockdown of a repressing agent, in those cases, GATA1 or GATA3, also led to the induction of GATA2 expression. Thus, further experiments with cell lines with silenced GATA2 should be performed to reveal the functionality of the possible GATA switch. In the cancer context, a GATA2/GATA6 switch has also been previously shown *in vitro* in gastric cancer cell lines (Song et al., 2017). Consistent with our results, overexpression of GATA2 silenced GATA6 expression, more specifically via polycomb repressive complex 2 (PRC2). Additionally, Song and colleagues have also shown that overexpression of GATA6 causes silencing of GATA2 via CpG hypermethylation – which was, however, not seen in our results when GATA6 was overexpressed.

When it comes to silencing of GATA6, GATA2 did not show any signs of compensation in the gene expression analysis (**Figure 8H**). Therefore, the loss of GATA6 expression might be so critical for cells, for example in terms of surviving following targeted therapy, that GATA2 expression cannot compensate for it. Moreover, it has been previously shown that only total silencing or loss of GATA2 has induced overexpression of GATA6 (Song et al., 2017). Thus, even low remaining expression levels of GATA6 in our GATA6-silenced cell line may prevent compensation by GATA2.

Initially, the concept of the GATA switch may propose the idea that it does not matter which one of the GATA transcription factors is expressed, because one transcription family member can easily replace another one. According to the gene expression data, overexpression of GATA2 and GATA6 caused relatively similar changes in the expression of all target genes, suggesting that the functions of GATA transcription factors may be interchangeable (**Figure 8G**). The reduction of GATA6 expression caused by overexpression of GATA2 might explain some results in different experiments because overexpression of GATA2 resembles silencing of GATA6 – thus leading to similar results to the cells with silenced GATA6. However, in the gene expression analysis, all results were not similar – overexpression of GATA2 and silencing of GATA6 caused opposite effects in SASP gene expression. That suggests that the presence or absence of GATA2 might be in this case critical for the development of mechanisms in surviving HCC827 cells – especially when it comes to the establishment of AT1-like cells.

As mentioned in Section 3.4, overexpression of GATA2 increased the expression of AT1 genes. Previously, it has been reported that a lack of GATA6 induces Wnt signaling, which in

turn has been shown to promote the transdifferentiation process of AT2 cells to AT1 cells (Abdelwahab et al., 2019; Y. Zhang et al., 2008). Thus, the decreased expression of GATA6 caused by overexpression of GATA2 via the GATA switch may have contributed to the transdifferentiation into AT1-like cells via Wnt signaling. Consistently, GATA6 expression has been shown to inhibit Wnt signaling, and thus, increases in the expression of AT1 genes were slighter when GATA6 was overexpressed, although some AT1-restoring GATA2 activity may have remained to compensate for Wnt inhibition. To conclude, GATA2 may be responsible for converting the surviving HCC827 cells into AT1-like cells via the GATA switch by inhibiting GATA6 expression.

Because GATA4 and GATA6 are both critical for the embryonic development of the lung, it may be possible that they cooperate in senescent processes as well – and possibly even function as one GATA switch. GATA4 has been previously proposed to regulate senescence by inhibiting Wnt signaling in NSCLC cells, indicating that GATA4 and GATA6 are involved in the same signaling pathways in lung cancer cells (Gao et al., 2019). Thus, it is possible that they regulate each other's expression and activity, highlighting the need for further research on the role of GATA4 in treatment-induced senescence in NSCLC as well.

Taken together, overexpression of GATA2 decreased the gene expression of GATA6, suggesting that the GATA2/GATA6 switch is prominent in HCC827 cells at least following long-term targeted therapy with OT.

3.6 Study limitations

3.6.1 The vector control cell line pCW57.1-hygro

One of the vector control cell lines, pCW57.1-hygro, showed distinct morphology and behaved differently in comparison to other cell lines, as can be seen from the results presented in Section 2.6. The control cells were more polarized and grew separately (**Figure 9A**), and they also expressed a commonly used mesenchymal marker vimentin (**Figure 9D**). Additionally, pCW57.1-hygro cells showed increased osimertinib resistance (**Figure 9E**), decreased apoptosis (**Figure 7A**), and a higher baseline expression of chemokines when compared to other cell lines (data not shown). Moreover, the proliferation of pCW57.1-hygro cells was also decreased with the highest concentration (1000 ng/ml) of doxycycline (**Figure 5C**). It can be due to the toxicity of the high doxycycline dose, but it can also be due to other doxycycline-induced changes in the cells – which can also be seen in other experiments. A

mesenchymal phenotype with front-back polarity as well as vimentin expression and apoptosis resistance are well-known characteristics of EMT, suggesting that pCW57.1-hygro vector control cells have for some reason undergone EMT (Kalluri and Weinberg, 2009). Because some of the control cells had also stained in SA-β-Gal stainings, they have also acquired increased β-galactosidase activity (**Figure 8F**).

The reason for EMT-like features of pCW57.1-hygro vector control cells could not be identified. One possibility is a transduction failure, however, pCW57.1-hygro vector control cell lines from two independent transduction rounds had similar EMT-like characteristics. Thus, a failure in the transduction process is unlikely – because it should have been repeated in both transduction rounds in the same cell line but not in others. The same pCW57.1-hygro plasmid has also been used to prepare pCW57.1-hygro-3X-Flag-GATA2 and pCW57.1-hygro-3X-Flag-GATA6 plasmids – and they do not have any EMT-like features. The possibility that overexpression of GATA2 and GATA6 inhibits EMT should also be ruled out since the cell lines in question do not have different EMT-like morphology even in the absence of doxycycline. However, in some experiments, we have noticed signs of leakage in overexpression lines, meaning that GATA2 and GATA6 are slightly overexpressed in corresponding cell lines even without doxycycline. That could explain the lack of EMT-like morphology in overexpression lines without doxycycline treatment if GATA2 and GATA6 have an ability to inhibit EMT. Additionally, one possibility is that the pCW57.1-hygro plasmid itself has some EMT-inducing properties. Instead of GATA cDNA found in overexpression plasmids, there is a sequence coding a *ccdB* gene in the pCW57.1-hygro plasmid. During the cloning process, ccdB normally kills bacterial cells that have not successfully received the plasmid, thus improving the cloning efficiency (Bernard, 1996). Thus, the gene should not have any functions in the established pCW57.1-hygro vector control cells. However, it is possible that it has some effect on the pCW57.1-hygro control cells and that the expression of the gene increases due to doxycycline treatments.

Due to the aforementioned EMT characteristics, the use of the pCW57.1-hygro cell line as a control in experiments is not optimal. However, because every experiment also included overexpression lines without doxycycline treatments as internal controls, the results of experiments could be reliably assessed. In further studies, parental HCC827 cells could also be included as a comparison, although they may behave differently to some extent due to the lack of a transduction process. Taken together, even though pCW57.1-hygro control cells had

most likely undergone EMT, results could be reliably assessed due to the use of internal controls of every cell line without doxycycline treatment.

3.6.2 Other cell lines

In addition to the control cell line pCW57.1-hygro, other pCW57.1-hygro-transduced cell lines may have caused some problems in the experiments as well. First, the gene expression levels of GATA2 and GATA6 in corresponding overexpression cell lines were much lower in later experiments (**Figure 8G**) than in the original ones (**Figure 4E, F**). In the first gene expression analysis, doxycycline induced a 120-fold increase in GATA2 expression (**Figure 4E**) and a 60-fold increase in GATA6 expression (**Figure 4F**). However, after 10 days of doxycycline treatment in senescence experiments, corresponding doxycycline-induced fold changes were only about 2-3 in GATA2 – and when it comes to GATA6, overexpression was found to be successful in only one of three independent replicate experiments (**Figure 8G**).

Differences between gene expression levels can be caused by multiple things. Because all pCW57.1-hygro-transduced cell lines experienced some problems or inconsistencies, we cannot rule out the possibility that the doxycycline-inducible cassettes themselves were somehow faulty. In addition to the reduction in fold changes, the cell lines seemed to also have leakages in some of the experiments, strengthening the suspicion towards doxycycline induction. Leakages in doxycycline-inducible expression systems have also been reported in previous studies, although the doxycycline-inducible expression remains to be a widely used research approach (Bersten et al., 2015). Moreover, one possible source of error is the replicative age of cell lines; however, experiments were attempted to be performed with cells with as low a passage number as possible. Even though overexpression of GATA2 succeeded in all gene expression analysis experiments, we cannot exclude the possibility that it did not work in some of the other experiments, for example in apoptosis assay, from which consistent results could not be obtained.

More replicates of experiments, especially in gene expression analysis, could have been done to get a better overview of possible problems and inconsistencies among doxycycline-inducible overexpression cell lines. In addition, establishing cell lines without FLAG-tags could have been considered. It is possible, although not likely, that FLAG-tags themselves affected results somehow – especially because the cell lines had three FLAG-tags. However, FLAG-tags have also been used in previous studies in the establishment of GATA overexpression cell lines, indicating that they should have been a reliable way of establishing

stable cell lines (W. Chen et al., 2020; Xu et al., 2020). Protein expression levels of GATA transcription factors in the cells with GATA overexpression were measured using the FLAG antibody, and thus, endogenous GATA expression was not measured. Thus, the actual levels of GATA protein expression (endogenous + doxycycline-induced) in corresponding cell lines are not known. To conclude, establishing cell lines without FLAG-tags in further research may be considered, however, reliable results could also be obtained using the established FLAG-tagged doxycycline-inducible cell lines.

3.7 Conclusions and future directions

The objective of this master's thesis was to investigate the role of GATA transcription factors, especially GATA6, in treatment-induced senescence in EGFR-mutant non-small cell lung cancer. GATA6 overexpression alone was not found to be sufficient in establishing a senescent phenotype, however, it reduced cell viability following targeted therapy without inducing apoptosis during early treatment. Moreover, the silencing of GATA6 decreased cell viability, the proportion of β-galactosidase-positive senescent cells, and the expression of SASP genes after long-term targeted therapy, suggesting that GATA6 may have an important role in treatment-induced senescence in EGFR-mutant NSCLC. Because all GATA transcription factors bind the same consensus sequence, functions are most likely mediated in collaboration with other factors, and thus, GATA6 may be a critical factor for the development of senescence in combination with some other factors. Results from this study also suggest that, following targeted therapy, overexpression of GATA2 induces HCC827 cell differentiation towards AT1-like phenotype and decreases GATA6 expression – possibly via the GATA switch.

In the future, experiments should be repeated with other EGFR-mutant NSCLC cell lines, for example, PC9 and HCC4006, to validate the results. Additionally, GATA6 could be re-expressed in GATA6-silenced cell lines to see if the effects of GATA6 silencing could be reverted. An analysis of cell cycle phases and the proportion of cells in each phase could also be performed to see how big population of cells has arrested the cell cycle and what is the exact mechanism which mediates the effects of GATA6 overexpression. Finally, further research is needed to investigate possible other factors and pathways contributing to the process and to assess the role of GATA6 in treatment-induced senescence.

Taken together, this study broadens the general knowledge of the role and functions of GATA transcription factors GATA2 and GATA6 in EGFR-mutant NSCLC cells. According to the

results, GATA6 alone is not sufficient to induce senescence, however, it may affect cell survival upon targeted therapies and have a role in treatment-induced senescence in NSCLC. However, further research is needed to elucidate the role of GATA transcription factors in treatment-induced senescence and therapy resistance.

4 Materials and methods

4.1 Reagents

Drugs and antibodies used in this thesis study are collected in **Table 2**.

Table 2. Drugs and antibodies used in this study.

Reagent	Origin
Drugs	
Carbenicillin	Fisher Bioreagents, cat#BP2648-5
Doxycycline hyclate	EMP Millipore, cat#324385-1GM
Hygromycin	Invitrogen, cat#10687010
Osimertinib	Cayman Chemical, cat#16237
Puromycin	Gibco, cat#A1113803
Trametinib	Selleckchem, cat#S2673
Antibodies	
DYKDDDD Tag (9A3) Mouse mAb (FLAG)	Cell Signalling, cat#8146
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) antibody	HyTest, cat#5G4cc
GATA6 (D61E4) XP Rabbit mAb	Cell Signalling, cat#5851
IRDye 680RD donkey anti-mouse	LI-COR, cat#926-6807
IRDye 800CW donkey anti-rabbit	LI-COR, cat#92632213
Vimentin (D21H3) XP Rabbit mAb	Cell Signalling, cat#57415

4.2 Cell lines and cell culture

Human embryonic kidney (HEK293T) cells and EGFR-mutant NSCLC (HCC827) cells were cultured in RPMI-1640 medium (EuroClone, cat#ECB9006L) supplemented with 10% fetal bovine serum (FBS) (Biowest, cat#S181B-500), 2 mM stable L-glutamine (EuroClone, cat#ECB3004D), and 100 U/ml penicillin & 100 mg/l streptomycin (EuroClone, cat#ECB3001D). Cells were maintained in a humified incubator at +37°C with 5% CO_2 and passaged when 70-80% confluent.

4.3 Lentiviral transduction

4.3.1 Plasmid production

To establish doxycycline-inducible overexpression of GATA2 and GATA6 as well as shRNA-mediated silencing of GATA6, HCC827 cells were transduced with 3rd generation

lentiviruses. Used lentiviral expression plasmids and viral packaging plasmids are collected in **Table 3**. pCW57.1-hygro-3X-Flag-GATA2 and pCW57.1-hygro-3X-Flag-GATA6 plasmids were prepared in our laboratory prior to the start of this project. pCW57.1-hygro, produced from two commercial pCW57.1 plasmids (Addgene plasmids #41393 and #80922), and tet-pLKO-puro (Addgene plasmid #21915) were used as control plasmids.

Table 3. Lentiviral expression plasmids and viral packaging plasmids used in this study.

Plasmid		Addgene Plasmid #
Lentiviral expression plasmids	pCW57.1	#41393
	pCW57-MCS1-P2A-MCS2 (Hygro)	#80922
	tet-pLKO-puro	#21915
	tet-pLKO-puro shGATA6	#72615
Lentiviral packaging plasmids	pMDLg/pRRE	#12251
	pMD2.G	#12259
	pRSV-Rev	#12253

Escherichia coli bacterial cells containing relevant plasmids were grown from glycerol stocks in 5 ml LB Broth (Lennox) (Alfa Aesar, cat#H26760) and 100 µg/ml carbenicillin (Fisher Bioreagents, cat#BP2648-5). After rotating incubation at +37ºC for 24 h, plasmids were purified using the NucleoSpin® Plasmid kit (Macherey-Nagel, cat#740588.250) according to the manufacturer's instructions. Plasmid DNA concentrations were measured with Nanodrop ® ND-1000 Spectrophotometer.

4.3.2 Transduction

HEK293T cells were seeded (1.2 x 10^6 cells / T75 flask) and transfected with lentiviral expression plasmids and viral packaging plasmids on the next day. 3000 ng of pMDLg/pRRE, 1500 ng of pMD2.G, and 1500 ng of pRSV-Rev were mixed with 6 µg of expression plasmid per transfection. Opti-MEM™ reduced serum medium (Gibco, cat#31985047) was added up to a final volume of 600 µl, and FUGENE® 6 Transfection reagent (Promega, cat#E2691) at a 6:1 ratio per transfection was added directly into media. After 20 min incubation at room temperature, the final transfection mixture was administered directly to the HEK293T cell culture media. One flask of HEK293T cells was left untransfected in order to have a negative virus control later in the lentiviral test.

After transfection, cells were transferred into the Biosafety Level 2 (BSL2) laboratory. After 48 h incubation, the viral medium was collected from HEK293T cells, and filtered through a

0.45 µm filter. Filtered media was used to transduce HCC827 cells (2.5 x 10^5 cells / T25 flask) and 10 µg/ml polybrene was used to enhance the transduction efficiency. Transduced cells were selected with either 250 µg/ml hygromycin (Invitrogen, cat#10687010) for pCW57.1-hygro-transduced cell lines or 1 µg/ml puromycin (Gibco, cat#A1113803) for tet-pLKO-puro-transduced cell lines. Selection antibiotics were used from this point onwards in cell culturing, but all experiments were performed without the selection antibiotics.

4.3.3 Lentiviral test with qPCR

Before bringing transduced cell lines out of the BSL2 laboratory, a test for replication-competent lentiviruses (RCVs) was conducted using qPCR. After 2-3 medium changes post-transduction, 100 µl medium samples were collected. Medium samples taken from HEK293T cells before transduction were used as positive controls, whereas the sample from untransfected HEK293T cells was used as a negative control. Samples were inactivated by heating at 95ºC for 5-10 min.

The test was conducted using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, cat#A25742) and 10 µM primers KJK172 (FW, 5'-AGCTTGCCTTGAGTGCTTCA-3') and KJK173 (REV, 5'-TGACTAAAAGGGTCTGAGGGA-3'), which bind to the 5' LTR region from HIV-1, thus recognizing the viral DNA of potential RCVs. pCW571.-hygro standard dilution series (10^8-10^{-1} plasmid molecules per 1 µl RPMI-1640 medium) and 2 µl samples were prepared as duplicates, and qPCR was run with QuantStudio™ 3 Real-Time PCR System (Applied Biosystems™ by Thermo Fisher Scientific), using the parameters that are listed in **Table 4**. After negative qPCR test results, cells were allowed to be brought out of the BSL2 laboratory.

Table 4. qPCR parameters used in the lentiviral test with SYBR Green.
Temperatures between steps changed by 1.6ºC/s.

Step	Temperature and duration
Denaturation	95ºC for 10 min
Cycles (40x)	95ºC for 15 sec
	60ºC for 60 sec
Melt curve	95ºC for 15 sec
	60ºC for 60 sec
	95ºC for 15 sec

4.4 Western blot assay

1.5 x 10^5 cells were seeded per well on 6-well plates or 5 x 10^5 cells per 6 cm dish. The day after seeding, doxycycline (EMB Millipore, cat#324385-1GM, 50-1000 ng/ml) was added to induce target gene expression. After 24 h or 48 h, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed. Samples were then centrifuged at 21 000 x g for 10 min at +4ºC, after which cell lysates were transferred into new tubes and stored at -80ºC.

Lysate protein concentrations were determined using the Bradford protein assay (Bio-Rad protein assay dye reagent concentrate, Bio-Rad cat#5000006). 25-40 µg of protein was mixed with 6X sodium dodecyl sulfate (SDS) sample buffer before loading on the gel. Samples were run on a self-made 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane overnight. BlueStar Plus Prestained Protein Marker (Nippon Genetics, cat#MWP04) was used as a molecular weight marker. Membranes were blocked for 30 min in 5% milk in Tris-buffered saline with Tween (TBST), after which they were washed with TBST. Primary antibody solutions were prepared in 5% milk-TBST (1:1000) and membranes were incubated rotating overnight at +4ºC. Used primary antibodies are collected in **Table 2** in Section 4.1. A FLAG antibody was used for immunoblotting GATA transcription levels of pCW57.1-hygro-transduced cell lines, while a GATA6 antibody was used for tet-pLKO-puro-transduced cell lines. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10 000) was used as a loading control.

After primary antibody incubation, membranes were washed with TBST and incubated in a secondary antibody solution (1:15 000, in 5% milk-TBST) for 60 min, covered from light from this point onwards. Used secondary antibodies included IRDye 680RD donkey anti-mouse (LI-COR, 926-6807) and IRDye 800CW Donkey anti-rabbit (LI-COR, 92632213). Membranes were washed with TBST 3 x 5 min and imaged with Odyssey® CLx Imager (LI-COR).

4.5 Osimertinib titration

3 x 10^3 cells per well were seeded on 96-well plates and increasing doses of osimertinib (Cayman Chemical, cat#16237) (0.5 nM – 1 µM) were added with HP D300 Digital Dispenser on the next day. The cell viability was evaluated after 72 h of incubation with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 15 µl of Dye Solution (Promega, cat#G4102) was added per well and formed formazan crystals were

solubilized after three hours using the Solubilization Solution/Stop Mix (Promega, cat#G4101). On the following day, absorbances were measured with the MultiSkan plate reader (ThermoFisher).

4.6 Cell growth and morphology assay

To investigate how the expression of GATA2 or GATA6 affects cell proliferation and morphology, cell growth was followed with the IncuCyte S3 live cell imaging system, taking pictures every other hour for 72 hours. Cells were seeded on 96-well plates, 3 x 10^3 cells per well, and the GATA2 and GATA6 overexpression and GATA6 silencing were induced by adding different concentrations of doxycycline (50 ng/ml – 1 000 ng/ml) 24 hours after plating. Cell growth was analyzed quantitatively from normalized confluences calculated by IncuCyte S3, using parameters listed in **Appendix 1**. Cell morphology was evaluated visually from the pictures taken by IncuCyte S3.

Long-term cell growth experiments were conducted to determine how the expression of GATA2 and GATA6 affects cell survival after osimertinib treatment and the combination treatment of osimertinib and trametinib (OT). On the first day, cells were seeded on 96-well plates at 2 x 10^3 cells per well for osimertinib treatment and 4 x 10^3 cells per well for combination treatment. The next day, 200 ng/ml doxycycline treatment was added to half of the wells and on the third day, 100 osimertinib alone or in combination with 30 nM trametinib (Selleckchem, cat#S2673) was added. Culture media containing doxycycline and drugs was replaced after 5 days of treatment. After 10 days of targeted treatment, cell viability was assessed using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega, cat#G7572) following the manufacturer's instructions, after which luminescent signal was measured with Synergy H1 Multi-Mode Reader (BioTek).

4.7 Apoptosis assay

To study how the GATA2 and GATA6 overexpression or GATA6 silencing affects apoptosis and cell death in dormant cells, an apoptosis assay using the CellEvent™ Caspase-3/7 Green reagent (Invitrogen, ref#R37111) and the IncuCyte S3 live cell imaging system was conducted. 3 x 10^3 cells/well for osimertinib and untreated and 5 x 10^3 cells/well for OT were seeded on 96-well plates. The next day, 200 ng/ml doxycycline treatment was initiated for half of the wells. On the third day, osimertinib (100 nM) treatment or the combination treatment of osimertinib (100 nM) and trametinib (30 nM) was started and 30 µl/ml of the

caspase 3/7 reagent was added. Plates were brought immediately into the Incucyte S3 imaging system, and pictures were taken every other hour for 72 hours with phase and green (300 ms) channels. The green object count per image was normalized to the cell confluency of corresponding wells.

4.8 β-galactosidase senescence stainings

To investigate the role of GATA transcription factors in treatment-induced senescence, cells were stained with a Senescence β-galactosidase staining kit (Cell Signalling, cat#9860) according to the manufacturer's instructions. Cells were seeded as replicates for each treatment on 6-well plates according to **Table 5.** On the following day, 200 ng/ml doxycycline treatments for pCW57.1-hygro-transduced cell lines and OT (100 nM osimertinib and 30 nM trametinib) treatments with and without 200 ng/ml doxycycline for tet-pLKO-puro-transduced cell lines were started. After 5 days, wells were washed with PBS and treatments were refreshed. Two days before stainings, 0.1 x 10^6 cells were seeded on plates as controls for further analysis.

Table 5. **The number of cells seeded for senescence stainings.**

Cell line	Cell number
pCW57.1-hygro	0.008 x 10^6
pCW57.1-hygro-3X-Flag-GATA2	0.008 x 10^6
pCW57.1-hygro-3X-Flag-GATA6	0.008 x 10^6
tet-pLKO-puro	0.15 x 10^6
tet-pLKO-puro-shGATA6	0.15 x 10^6

After 10 days of treatments, wells were washed with PBS and fixed for 15 min at room temperature using the kit's fixing solution. Further staining steps were performed following the kit manufacturer's instructions. After incubation for three days, at least 7 images per well at 20X magnitude were taken with EVOS M5000 Microscope (Thermo Fisher Scientific). β-galactosidase-positive cells were manually counted and calculated as a % of positive cells from the total number of cells. Counting was performed blindly by renaming images with number series.

4.9 Gene expression analysis with RT-qPCR

Gene expression of genes of interest was assessed after either 10-day doxycycline treatments or 10-day OT treatments with and without doxycycline, similar to senescence staining

experiments. Cells were seeded on 10 cm dishes according to **Table 6**, and treatments were started on the following day. After 5 days, treatments were refreshed, and control cells (0.6 x 10^6 cells per 10 cm dish) were seeded two days before cell lysis.

Table 6. The number of cells seeded for RT-qPCR.

Cell line	Cell number
pCW57.1-hygro	0.04 x 10^6
pCW57.1-hygro-3X-Flag-GATA2	0.05 x 10^6
pCW57.1-hygro-3X-Flag-GATA6	0.05 x 10^6
tet-pLKO-puro	1 x 10^6
tet-pLKO-puro-shGATA6	1 x 10^6

After 10-day treatments, cells were lysed and RNA was isolated from cells using the NucleoSpin® RNA purification kit (Macherey & Nagel cat#740955.250) according to the manufacturer's instructions. Purified RNA concentrations were measured with Nanodrop® ND-1000 Spectrophotometer, and 1 µg RNA was used for cDNA synthesis. The cDNA synthesis was performed using the SensiFAST cDNA synthesis kit (Meridian BioScience cat#BIO-65054) according to the manufacturer's instructions, after which cDNA was diluted 1:10 in AccuGENE® (Lonza, cat#BE51200). Purified RNA was stored at -80ºC, whereas cDNA dilutions at -20ºC.

The gene expression of target genes was detected and quantified with qPCR using TaqMan Chemistry (Taqman Universal Master Mix, no UNG, ThermoFisher). The used Taqman assays (ThermoFisher) are collected in **Table 7**.

Table 7. **Taqman assays used in this study.**

Target gene symbol	Target gene	Assay ID
ACTB	actin beta	hs99999903_m1
CCL2	C-C motif chemokine ligand 2	hs00234140_m1
CLIC5	chloride intracellular channel 5	hs00213494_m1
CXCL10	C-X-C motif chemokine ligand 10	hs00171042_m1
CYP4B1	cytochrome P450 family 4 subfamily B member 1	hs01086311_m1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	hs02758991_g1
GATA2	GATA binding protein 2	hs00231119_m1
GATA6	GATA binding protein 6	hs00232018_m1
GUSB	glucuronidase beta	hs00939627_m1
SERPINE1	serpin family E member 1	hs00167155_m1

qPCR was run using QuantStudio™ 3 Real-Time PCR System (Applied Biosystems™ by Thermo Fisher Scientific), and the detailed qPCR cycle information can be found in **Table 8**. Fold changes were calculated using the ΔΔCT method (Livak & Schmittgen, 2001), and results were normalized to the average Ct values of reference genes GAPDH, GUSB, and ACTB. GUSB alone was used as a reference gene in experiments containing OT-treated samples due to its stable expression regardless of treatments.

Table 8. qPCR parameters used in gene expression analysis with Taqman Chemistry.
Temperatures between steps changed by 1.6ºC/s.

	Step	Temperature and duration
Hold stage	Step 1	50ºC for 2 min
	Step 2	95ºC for 10 min
PCR stage (40x)	Step 1	95ºC for 15 sec
	Step 2	60ºC for 1 min

4.10 Statistical analysis

All experiments were repeated three times. The unpaired t-test was used to compare two groups, whereas the one-way analysis of variance (ANOVA) test followed by Dunnett's multiple comparisons test was used for comparing more than two groups. In general, three or four technical replicates (n=3-4) were used, and data was represented as mean ± standard deviation (SD) unless otherwise indicated. Statistical analyses were performed with the GraphPad Prism 9.4.0 for Windows (GraphPad Software) and the significance value (p) threshold of 0.05 was used. For the stars indicating statistically significant differences, p values were as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$. More detailed descriptions of statistical tests performed are described in corresponding figure legends.

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Abbreviations list

AKT	protein kinase B
ANOVA	analysis of variance
AT1	alveolar type 1 cell
AT2	alveolar type 2 cell
BSL2	biosafety level 2
CCL2	C-C motif chemokine ligand 2
CLIC5	chloride intracellular channel 5
CXCL10	C-X-C motif chemokine ligand 10
CYP4B1	cytochrome P450 family 4 subfamily B member 1
DOX	doxycycline
DTP	drug-tolerant persister
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
GATA	GATA-binding factor
GUSB	glucuronidase, beta
HEK	human embryonic kidney
HER	human epidermal growth factor receptor
HOPX	homeodomain-only protein homeobox
JAK	Janus kinase
KRAS	Kirsten rat sarcoma
MEK	mitogen-activated protein kinase kinase
mTOR	mammalian target of rapamycin

NRG	neuregulin
NSCLC	non-small cell lung cancer
OT	osimertinib + trametinib
PCAT1	prostate cancer-associated transcript 1
PI3K	phosphatidylinositol-3-kinase
RAS	rat sarcoma
RT-qPCR	reverse transcription quantitative real-time polymerase chain reaction
SASP	senescence-associated secretory phenotype
SA-β-Gal	senescence-associated β-galactosidase
SERPINE1	serine protease inhibitor clade E member 1
SOX12	sex determining region Y-box protein 12
STAT	signal transducer and activator of transcription
TBST	Tris-buffered saline with Tween
TIS	treatment-induced senescence
TKI	tyrosine kinase inhibitor
Wnt	wingless-related integration site

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Appendices

Appendix 1 IncuCyte S3 parameters

Table S1. IncuCyte S3 parameters used for the analysis of the cell growth assay of pCW57.1-hygro-transduced cells.

Parameter	Value
Segmentation adjustment	1.2
Hole fill (μm^2)	200
Adjust size (pixels)	0
Min area (μm^2)	1000

Table S2. IncuCyte S3 parameters used for the analysis of the cell growth assay of tet-pLKO-puro-transduced cells.

Parameter	Value
Segmentation adjustment	1.1
Hole fill (μm^2)	1000
Adjust size (pixels)	0
Min area (μm^2)	900

Table S3. IncuCyte S3 parameters used for the analysis of the apoptosis assay.

Parameter	Value
Phase	
Segmentation adjustment	0.9
Hole fill (μm^2)	100
Adjust size (pixels)	0
Min area (μm^2)	100
Green	
Segmentation	adaptive
Threshold adjustment (GCU)	1
-	Edge split on
Edge sensitivity	0
Min area	40
Max area	1E+04
Min mean intensity	8.4
Min integrated intensity	900