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Review

# EMT: A mechanism for escape from EGFR-targeted therapy in lung cancer

Eugene Tulchinsky[a,b,*], Oleg Demidov[c], Marina Kriajevska[a], Nickolai A. Barlev[b,c], Evgeny Imyanitov[d]

[a] Leicester Cancer Research Centre, Leicester University, UK
[b] Moscow Institute of Physics and Technology, Dolgoprudny, Moscow, region, 117303, Russia
[c] Instutute of Cytology, Russian Academy of Sciences, Saint-Petersburg 194064, Russia
[d] Petrov Institute of Oncology, Saint-Petersburg, Russia

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ABSTRACT

Epithelial mesenchymal transition (EMT) is a reversible developmental genetic programme of transdifferentiation of polarised epithelial cells to mesenchymal cells. In cancer, EMT is an important factor of tumour cell plasticity and has received increasing attention for its role in the resistance to conventional and targeted therapies. In this paper we provide an overview of EMT in human malignancies, and discuss contribution of EMT to the development of the resistance to Epidermal Growth Factor Receptor (EGFR)-targeted therapies in non-small cell lung cancer (NSCLC). Patients with the tumours bearing specific mutations in *EGFR* have a good clinical response to selective EGFR inhibitors, but the resistance inevitably develops. Several mechanisms responsible for the resistance include secondary mutations in the *EGFR* gene, genetic or non-mutational activation of alternative survival pathways, transdifferentiation of NSCLC to the small cell lung cancer histotype, or formation of resistant tumours with mesenchymal characteristics. Mechanistically, application of an EGFR inhibitor does not kill all cancer cells; some cells survive the exposure to a drug, and undergo genetic evolution towards resistance. Here, we present a theory that these quiescent or slow-proliferating drug-tolerant cell populations, or so-called "persisters", are generated via EMT pathways. We review the EMT-activated mechanisms of cell survival in NSCLC, which include activation of ABC transporters and EMT-associated receptor tyrosine kinase AXL, immune evasion, and epigenetic reprogramming. We propose that therapeutic inhibition of these pathways would eliminate pools of persister cells and prevent or delay cancer recurrence when applied in combination with the agents targeting EGFR.

## 1. Introduction

**Intratumoural heterogeneity and epithelial mesenchymal transitions.**

Cancer heterogeneity is considered as the major obstacle to the success of different therapeutic approaches. There are two major contributing factors to the intratumoural heterogeneity, genetic diversity and phenotypic plasticity. Mutations in genes safeguarding genome integrity result in a cascading increase in the frequency of random mutations providing material for tumour evolution. Studies combining new generation sequencing with the mathematical modelling demonstrated that in the tumours from treatment-naïve patients, neutral evolution is prevalent in cancer progression [1–3]. Therapeutic interventions impose selective pressure on tumour cells, which results in a shift from neutral to non-neutral tumour evolution mode, discrimination of some genotypes and outgrowth of novel clones. Under these conditions, tumour evolution is driven by Darwinian selection and clonal expansion, in line with the linear model of tumour progression proposed by Fearon and Vogelstein nearly three decades back [4]. The second factor contributing to the intratumoural heterogeneity is phenotypic plasticity or phenotype switching, a phenomenon whereby cancer cells do not acquire new mutations, but transit between different phenotypes in response to environmental cues. Cells featuring high plasticity undergo positive selection when selective pressure is imposed [5].

Phenotypic plasticity occurs at different levels and involves morphological transformation, transition from the proliferative towards migratory and invasive phenotypes, metabolic reprogramming. These phenomena are largely regulated by reversible epithelial mesenchymal (EMT) and mesenchymal epithelial transition (MET) genetic programs. In the course of EMT, cells lose their epithelial characteristics, apicobasal polarity, intercellular adhesion complexes and cytoskeletal

* Corresponding author.
*E-mail addresses:* et32@le.ac.uk (E. Tulchinsky), evgeny@imyanitov.spb.ru (E. Imyanitov).

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architecture specific for epithelial tissues. Concurrently, cells acquire mesenchymal type of cell polarity, the ability to migrate individually and the invasive capacity. Plastic nature of tumour cells postulates that they do not undergo terminal differentiation, but rather remain in intermediate stages [6,7]. For the purpose of convenience, we will use terms “epithelial” or “mesenchymal” to designate cells that are closer to the epithelial or mesenchymal ends of the EMT/MET scale. The EMT/MET-driven cellular plasticity plays a critical role at different stages of embryonic development, such as neural crest delamination and formation of new tissues and organs [8]. Embryonic EMT/MET plasticity is hijacked by cancer cells and contribute to the main steps in the development of malignant tumours. Mesenchymal features are required for overriding cellular failsafe programs, intravasation and extravasation. Cells of growing carcinomas maintain epithelial characteristics in most cases; extravasated cells re-establish epithelial phenotype in target organs to give rise to macrometastases [9,10]. Significance of EMT/MET plasticity was demonstrated in prostate and squamous epidermal cancer cells: induction of extreme irreversible EMT programs led to the formation of tumours incapable to accomplish metastatic process [11,12]. Relevant to this review, EMT/MET plasticity is an important factor in immune escape and therapy resistance.

## 2. EMT/MET plasticity in cancer: Causes and consequences

### 2.1. Molecular mechanisms underpinning EMT/MET in cancer

EMT/MET plasticity is affected by various factors. Mutational activation of the signalling pathways may induce EMT or EMT-like programs in carcinoma or neural crest-derived tumours. Likewise, the wide variety of microenvironmental stimuli (growth factors and cytokines, hypoxia, composition and stiffness of the extracellular matrix, immune responses or exposure to anticancer therapeutics) regulate EMT/MET balance in tumour cells [6]. The mutational or microenvironment-dependent activation of the signalling pathways lead to the up-regulation or posttranslational modifications of embryonic transcription factors (termed EMT-TFs), which execute transcriptional reprogramming. EMT-TFs comprise several groups of Zn finger, helix-loop-helix or forkhead transcriptional repressors, with ZEB1 (ZEB1 and ZEB2), SNAIL (SNAIL1 and SNAIL2) and TWIST (TWIST1 and TWIST2) families playing central role in EMT in cancer. EMT-TFs recruit transcriptional co-repressor complexes and histone-modifying enzymes to repress transcription of target genes encoding E-cadherin and other proteins implicated in epithelial adhesion, cell polarity and cytoskeletal architecture [8,13].

MicroRNA genes represent a distinct group of important EMT-TFs targets. ZEB1 and ZEB2 proteins are repressors of two microRNA clusters, miR-200b/miR-200a/miR-429 and miR-200c/miR-141, expressed as polycistronic transcripts. These homologous microRNAs in turn target both ZEB family members establishing double negative regulatory feedback loops, which define ZEB expression levels [14,15] (Fig. 1). Similar reciprocal repression is the basis of negative feedback loops established between miR-34a and SNAIL1, and miR-186 and TWIST1 [16–18]. A cross talk between these regulatory loops occurs via downregulation of miR-34 by ZEB1 and ZEB1 by miR-186 [19,20]. In addition, FRA1 a transcriptional activator of ZEB genes [21], is a target of miR-34 [22] (Fig. 1). The existence of this self-enforcing regulatory network suggests that tumour cells may amplify EMT or MET-inducing signals, and cells adopt epithelial or mesenchymal states until new stimuli from the microenvironment or new mutations shift the EMT/MET equilibrium.

EMT-TFs have also a potential to activate transcription of mesenchymal genes through interactions with other factors, such as SMADs or TEAD/YAP1 complexes [13,23,24]. Availability of cell-specific EMT-TF-interacting transcription factors, co-repressors or co-activators is a determinant of cellular response to EMT-inducing stimuli.

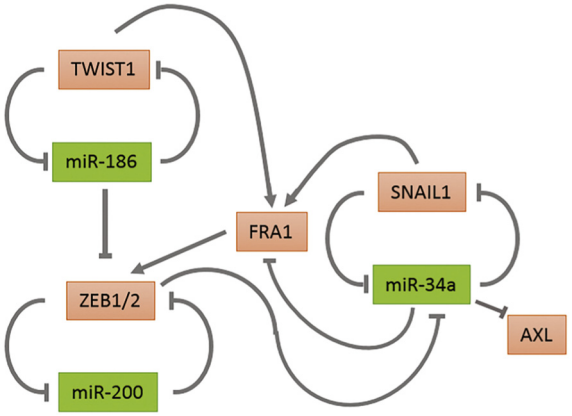


**Fig. 1.** EMT-TFs/microRNA network regulates EMT/MET equilibrium in carcinoma cells. EMT-TFs and indicated microRNAs mutually repress each other forming interconnected double negative feedback loops. Transcription factor FRA1 is incorporated in this network and brings together regulatory loops formed between EMT-TFs and their targets.

### 2.2. EMT, cancer stem cells (CSCs), and drug resistance

The canonical CSC concept is based on the assumption that tumours are organised in a hierarchical mode [25]. CSCs are highly tumourigenic, and possess key characteristics of normal stem cells; they have a self-renewal potential and produce more differentiated progeny that forms the bulk of a tumour. CSCs exhibit enhanced resistance to conventional therapies; it is widely accepted that patients often relapse after many years post-therapy because of the outgrowth of remaining resistant CSCs. Significant efforts have been made to unveil molecular mechanisms underpinning drug resistance in CSCs. Firstly, as they shuttle between slow-proliferating and quiescent states, CSCs are resistant to common chemo- and radiotherapy regimens targeting cells in S and G2/M phases of the cell cycle. Secondly, compared to the CSC-depleted cell populations, CSCs have a more efficient activation of DNA damage checkpoint proteins (ATM, Rad17, Chk1 and Chk2) in response to ionising radiation [26]. Moreover, CSCs maintain decreased levels of DNA damage by producing proteins involved in ROS scavenging, and CSCs contain lower concentrations of ROS than their mature progeny cells [27]. Thus, decreased level of DNA damage and increased propensity to repair DNA are responsible for chemo- and radiotherapy resistance in CSCs. In addition, CSCs express enhanced levels of ATP-binding cassette (ABC) drug transporters, such as *ABCB1* and *ABCG2* genes, which are responsible for multidrug resistance [28].

An association between EMT and CSCs was first established in the seminal studies carried out in the labs of R. Weinberg and A. Puisieux [29,30]. The results demonstrated that stem-like cells isolated from mammary carcinoma express mesenchymal markers and EMT-TFs; and SNAIL1 and TWIST1 confer stem cell-like properties to immortalised epithelial cells. The interrelationship between EMT-TFs and stemness is more complex in normal tissues. SNAIL2 is required for the maintenance of stem cell state in the mammary gland as well as in breast cancer [31]. On the other hand, in other normal adult and embryonic tissues EMT-TFs drive exit from stemness and control differentiation in several lineages during haematopoiesis, maturation of melanocytes, Schwann cells, etc. [32].

EMT-TFs promote resistance to radio- and chemotherapeutics in cell lines derived from the most types of epithelial tumours [33–38]. An effect of chemotherapy on the EMT/MET status of breast and pancreatic adenocarcinoma has been analysed in mouse models. Treatments resulted in the enrichment for mesenchymal tumour cells exhibiting reduced sensitivity to conventional therapeutics [39,40]. Accumulated data indicate that EMT-driven resistance to conventional therapies relies on the same mechanisms, which operate in CSCs. Cells experiencing

an EMT in vitro become quiescent or slowly proliferating [41–43], which renders them insensitive to the antiproliferative treatments. Akin to CSCs, cells in EMT express enhanced levels of ABC transporters, which results in multidrug resistance [44]. EMT-TF ZEB1 activates mechanisms maintaining genome integrity, which are similar to those acting in CSCs, namely efficient DNA repair and ROS scavenging. Mechanistically, in response to the DNA damage, ZEB1 binds deubiquitylating enzyme USP7 and enhances its ability to deubiquitylate and stabilize CHK1. This pathway activates DNA damage checkpoint, promotes DNA repair via homologous recombination and ensures resistance to ionising radiation [45]. In the course of RAS-mediated transformation of mammary cells, ZEB1 overturns oncogene-induced senescence by activating an anti-oxidant defence program, which includes up-regulation of genes encoding ROS-detoxifying enzymes [46]. Thus, there is an obvious commonality in the mechanisms of drug resistance activated by EMT-TFs and those operating in CSCs. On the other hand, there is an apparent contradiction between the reversible nature of EMT programs and the standard hierarchical model that envisages irreversible unidirectional generation of cellular progeny from CSCs. However, recent lineage tracing studies and targeted ablation of CSCs in mouse models and human organoids provided evidence that CSCs hierarchy is not as rigid as previously anticipated at least in some cancer types. In response to microenvironmental cues such as WNT or NF-kB-activating pathways, nonstem cells have a capacity to undergo dedifferentiation to regenerate CSC pools. Neutral competition for a tumour niche sustaining CSC phenotype has been proposed as a determinant of this process [47,48]. A role for EMT/MET reprogramming in CSC plasticity is yet to be determined.

### 2.3. EMT/MET plasticity, targeted therapy and oncogene addiction

The principle of targeted therapy is based on the concept of oncogene addiction. This theory stipulates that survival and proliferation of cancer cells depend on one particular oncogenic event and downstream pathways [49]. This conception inspired hope that the identification and targeting the “Achilles' heel” of a particular cancer with a relatively non-toxic pharmacological agent would be highly beneficial for the patients. Development and application of targeted therapeutics indeed have shown promise in various cancer types, but the majority of patients developed resistance later, and tumours became refractory to the treatment. Investigation of the molecular mechanisms underlying the resistance became the central theme in cancer research in the last decade.

Predictably, EMT/MET plasticity appeared to be a key feature permitting oncogene-addicted cancer cells to escape cytotoxicity and develop resistance. Indeed, a shift to a mesenchymal phenotype involves reorganisation of the whole signalling network that governs cell survival, proliferation and homeostasis, making tumour cells to rely on new molecular pathways, and insensitive to targeted therapeutics. An example of such shift is EMT-induced stimulation of PDGFR-PKCα-FRA1 pathway on the expense of EGFR-c-FOS signalling in transformed mammary epithelial cells HMLE [50]. Noteworthy, constitutively active autocrine PDGFR signalling has been described in tumours originating from neural crest-derived mesenchymal tissues expressing EMT-TFs [51]. The ability to override oncogene addiction seems to be a common feature of EMT-TFs. In vitro studies have shown that epithelial pancreatic and lung carcinoma cells harbouring mutations in the *KRAS* oncogene are KRAS-addicted, whereas mesenchymal are not; and ZEB1 is the factor overriding the addiction [52]. In MMTV-Her2/Neu Doxycycline-inducible mouse model, Doxycycline withdrawal led to the regression of tumours, and the formation of multiple mesenchymal Her2-negative, but Snail1-positive tumours after a period of latency. Data suggest that Snail1-driven EMT allowed tumour cells to overcome addiction to Her2 [53].

In the next sections we will discuss the role of phenotypic plasticity in the escape of oncogene-addicted cancer cells from targeted therapy using a clinically important example: development of the resistance to EGFR-Tyrosine Kinase Inhibitors (EGFR-TKIs) in lung cancer patients with mutant *EGFR*.

## 3. Pathways to EGFR-TKI resistance in lung cancer cross on EMT?

### 3.1. Lung cancer and EGFR targeted therapy

Lung cancer is a leading cause for oncological morbidity and mortality worldwide, being responsible for 1.8 million new cancer cases and 1.6 million deaths every year. Even small progress in lung cancer treatment results in enormous number of saved lives. Clinical development of EGFR inhibitors dates back to 1980s, being based on the fact that a large proportion of carcinomas is characterized by overexpression of EGFR, and EGFR-TKIs were initially hoped to become a “universal” drug against cancer [54]. However, the results of the clinical trials on the use of EGFR-TKIs for treating NSCLC patients showed a high degree of inconsistency. For example, Ranson and colleagues documented evident tumour reduction in 4 out of 16 NSCLC patients, which significantly outperformed all available treatment options for lung cancer [55]. On the other hand, the results of the phase III trials considered the use of EGFR-TKIs in combination with chemotherapy in the first-line setting were absolutely disappointing, as the addition of the TKI to the conventional drugs failed to improve treatment outcomes [56]. However, all clinical trials involving NSCLC continued to occasionally observe very deep and durable tumour responses in some patients. The resolution of this puzzle came in the year 2004, when three independent research groups sequenced the *EGFR* gene in tumour tissues obtained from EGFR-TKI responders and revealed by then unknown intragenic somatic mutations (in-frame deletions in exon 19 or L858R substitution) [57–59]. *EGFR* mutational analysis produced an unprecedented level of predictive accuracy: while the presence of mutation actually guaranteed evident clinical benefit from EGFR-TKI, the wild-type EGFR receptor served as a very strong indicator of the non-response to the drug [60,61].

Although administration of EGFR inhibitors ceases tumour growth in virtually all patients with *EGFR* mutations, the duration of this effect is relatively short: the median progression-free survival reported by various studies is usually within the range of 8–12 months [62].

Mechanisms of acquired resistance to EGFR-TKIs were intensively studied, and three main routes have been dissected: i) mutational activation of alternative pathways bypassing the EGFR pathway addiction; ii) acquisition of additional mutations in the *EGFR* gene; and iii) phenotypic metaplasia, whereby EGFR-TKI-resistant recurrent tumours exhibit mesenchymal features, or transdifferentiate to a less common lung cancer subtype, small cell lung cancer (SCLC). Emerging evidence suggests that all three routes may use common mechanisms to establish the resistance.

### 3.2. “Drug-tolerant persisters” and development of EGFR-TKI resistance

Acute exposure of NSCLC cells to TKIs does not result in the eradication of all the cells in the culture. A small subpopulation of drug-tolerant cells maintain viability, and these “drug-tolerant persisters” (DTP) are detected at a frequency higher than random gene mutations would cause [63]. These DTP cells express CSC markers, and are constantly generated from drug-sensitive cell populations. Propagation of drug-tolerant cells in drug-free conditions re-established EGFR-TKI sensitivity indicating that the process of the generation of DTP is reversible [63]. Presence of these viable cells is the basis for subsequent genetic evolution of the resistant clones.

### 3.3. Acquisition of mesenchymal morphology in relapsed tumours is a distinct pathway to EGFR-TKI resistance

DTP cells express CSC markers and their formation is reversible

suggesting that EMT/MET programs might be involved in their generation and maintenance. This view is supported by the reports documenting mesenchymal phenotypes of recurrent tumours in the patients who underwent EGFR-TKI-based therapy. Three out of twelve NSCLC cases that developed erlotinib resistance in the absence of secondary mutations in the *EGFR* gene displayed mesenchymal morphology and reduced levels of E-cadherin [64]. A switch to mesenchymal morphology was demonstrated in 20% of NSCLC cases in a different study investigating matched EGFR-mutant specimens obtained from the patients both before treatment with erlotinib or gefitinib and after the resistance was developed [65]. Experimental studies and case reports documenting EMT in EGFR-TKI-resistant NSCLC investigated also genetic alterations, which commonly occur in relapsed tumours and activate alternative pathways bypassing EGFR addiction. Some of the secondary genetic events associated with the resistance, *c-MET* amplification, activating mutations in *PIK3CA*, *BRAF* and *KRAS*, loss of *PTEN* or inactivating mutations of *LKB1* have been implicated in the induction or maintenance of mesenchymal morphology in different carcinoma backgrounds. Some of these genetic alterations, such as mutations of *LKB1*, *BRAF* or *KRAS* genes are common in treatment-naïve tumours, but in most cases mutually exclusive with the mutant *EGFR* [66–68]. However, there is no evidence that these genetic alterations are associated with the acquisition of mesenchymal phenotype in EGFR-TKI resistant NSCLC tumours [64,65,69]. It remains currently unclear which molecular pathways are utilised by NSCLC cells do develop mesenchymal morphology. One could propose that epigenetic mechanisms are activated in DTP cells exposed to EGFR-TKI; they shift the EMT/MET balance to the mesenchymal end to give rise to the mesenchymal clones.

### 3.4. EMT and acquisition of secondary mutations in EGFR

Acquisition of mesenchymal phenotype in EGFR-TKI resistant clones is relatively infrequent [70]. In contrast, secondary point mutation in exon 20 of the *EGFR* gene (T790 M) is the most common mechanism of resistance to the first generation EGFR-TKIs accounting for 50–60% of all cases [71,72]. Mechanistically, the T790 M mutation confers resistance by preventing TKI binding to the ATP-pocket of mutant EGFR by steric hindrance. Although most of the tumours with mesenchymal characteristics do not harbour $EGFR^{T790M}$ mutations, there are examples of EMT co-occurring with the secondary mutations in the *EGFR* gene [65]. Recent work shed light on this phenomenon, and provided an evidence that EMT promotes de novo development of gefitinib resistance via secondary mutations in the *EGFR* gene. Treatment of NSCLC cells with escalating concentrations of gefitinib allowed to model evolutionary paths leading to the resistance to EGFR-TKIs [73]. Early evolving resistance was caused by the secondary $EGFR^{T790M}$ mutation and occurred as the result of the expansion of pre-existing rare $EGFR^{T790M}$-mutant clones. This mechanism, however, appears to have limited relevance to the naturally occurring lung tumours: indeed, pre-existing $EGFR^{T790M}$-mutant cells can be reliably detected only in 3% of treatment-naïve NSCLCs [74]. Another path (late resistance) reflected evolution of DTP, in which $EGFR^{T790M}$ mutation was initially not present, towards gefitinib-resistant phenotype harbouring $EGFR^{T790M}$. Importantly, late resistance was associated with the reduced response to the apoptosis-inducing agents. In contrast to the early resistant clones, late $EGFR^{T790M}$ clones as well as their drug-tolerant precursors displayed an EMT gene expression signature [73] **(**Fig. 2**)**. Mechanistically, reduced apoptotic propensity of drug-tolerant and late resistant cells was caused by the direct transcriptional repression of the *BCL2L11* gene by ZEB1 [75]. *BCL2L11* encodes the BH3-only protein BIM that plays a key role in the initiation of the intrinsic cell death pathway via induction of BAX/BAK oligomerization on mitochondria. Thus, ZEB1-BIM axis allowed DTP cells to evade strong selective drug pressure at the initial stage of the treatment. Acquisition of secondary *EGFR* gene mutations during clonal evolution provided these cells with additional selective advantage **(**Fig. 2**)**. These findings suggest that use of the BH3 mimetics in combination with EGFR-TKIs may help to tackle de novo development of $EGFR^{T790M}$-harbouring tumours from initially $EGFR^{T790M}$-negative drug-tolerant cells.

### 3.5. Resistance to the first and third generation inhibitors may involve NSCLC/SCLC transition: A hypothetical role for EMT

As secondary mutations in the *EGFR* gene appear as the most common mechanism of acquired resistance to the first generation inhibitors, much effort has been devoted to developing compounds which target both *EGFR* initial and $EGFR^{T790M}$ mutations. These third generation irreversible inhibitors possess much lower activity against wild-type EGFR, which underlies their relatively low toxicity [76]. Some of those agents such as osimertinib and rociletinib, produced response in approximately 60% of the patients with tumours harbouring secondary $EGFR^{T790M}$ mutations acquired during the first-line treatment with the first generation EGFR-TKIs [77,78]. When used for the first-line treatment, osimertinib was more efficient than the first generation inhibitors. The response rate was 80% and median progression-free survival 18.9 months (vs. 10.2 months for first generation inhibitors), but acquisition of the resistance was inevitable [79,80]. Resistance was attained via a tertiary mutation in *EGFR*, or amplification of the *EGFR ex19del* mutant gene accompanied with the loss of $EGFR^{T790M}$ [81]. In a proportion of tumours the resistance involved EGFR-independent routes, mutations or amplification of other RTK-encoding genes (*c-MET*, *FGFR*, *HER2*, *IGF1R)* or genetic alterations in other oncogenes (*BRAF*, *PIK3C*, *CTNNB1*) or tumour suppressor genes (*PTEN*) [80,81]. Alike the first generation EGFR-TKIs, the third generation EGFR-TKIs-resistant tumours showed evidence of EMT in some cases [82–85], or histological transformation of tumour cells to small cell lung cancer (SCLC) phenotype [86,87].

Small Cell Lung Cancer (SCLC) is a highly aggressive subtype of lung cancer which develops along the neuroendocrine lineage and is characterized by frequent mutations in *TP53* and *RB1* genes [88]. There are several considerations in support of the hypothesis that EMT-TFs are drivers of NSCLC/SCLC transdifferentiation **(**Fig. 2**)**. Firstly, neuroendocrine tumours originate in tissues derived from the neural crest, and EMT-TFs play a key role in the formation, development and differentiation of embryonic neural crest [8]. Sex determining region Y-box 2 (SOX2), a key regulator of pluripotency, is amplified in approximately 27% of SCLC cases [89]. SOX2 is implicated in neural differentiation, and crosstalks between SOX2 and EMT-TF/miR networks have been documented in cancer and in the context of neural differentiation [90–93]. Secondly, Notch and Hedgehog signalling pathways are known mediators of CSC self-renewal and drivers of the EMT/MET plasticity. They are implicated in the invasion and maintenance of CSC properties in neuroendocrine tumours and hyperactivated in SCLC [88,94]. In agreement with this notion, the proportion of CSCs in SCLC is unusually high and may reach up to 50% of all cells within a tumour [95]. Thirdly, a hallmark of EMT is activation of the retinoblastoma-mediated G1 checkpoint [41–43]. Notably, mutations or loss of the *RB1* gene was observed in 90–100% of the SCLC samples analysed [95,96], and RB1 was lost also in all EGFR-TKI-treated tumours undergoing NSCLC/SCLC transdifferentiation [97]. It is tempting to speculate that the inactivation of the G1 checkpoint by loss of RB1 permits the expansion of EMT-TF-expressing DTP cells resulting in NSCLC/SCLC transdifferentiation (Fig. 2).

Thus, application of either first or third generation EGFR-TKIs may lead to the resistance associated with either EMT or NSCLC/SCLC transdifferentiation of DTP cells, or acquisition of additional mutations in the *EGFR* gene. As implication of EMT/MET plasticity in DTP maintenance is highly likely, understanding EMT-mediated drug tolerance mechanisms is important for the development of the approaches to eradicate DTP cells and to tackle *EGFR*-mutant NSCLC by combination therapies.

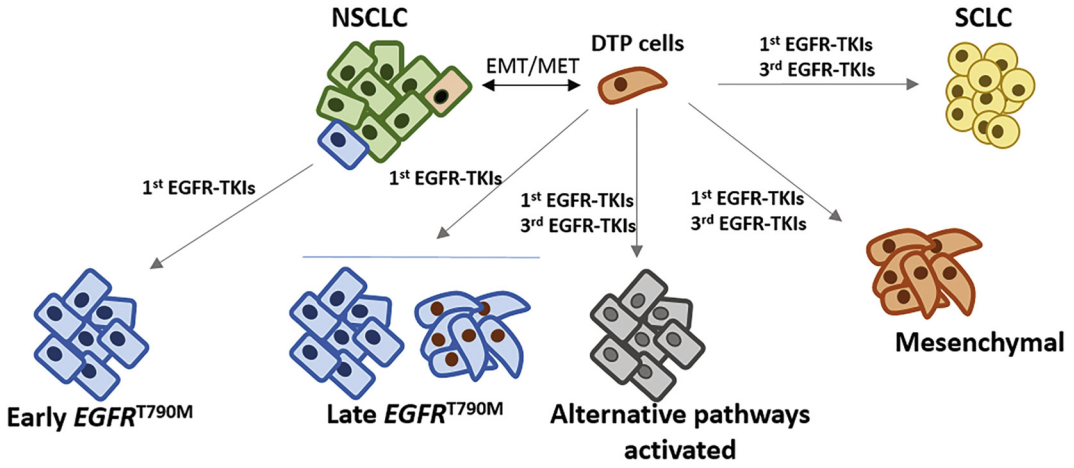


**Fig. 2.** Hypothetical model illustrating development of the resistance to the first and third generations EGFR-TKIs (indicated as 1st EGFR-TKIs and 3rd EGFR-TKIs respectively) in patients with NSCLC harbouring primary mutations in the *EGFR* gene. Cells with primary mutations are shown in green. Clones with the acquired secondary mutations ($EGFR^{T790M}$; shown in blue) develop either from rare cells pre-existing in therapy-naïve tumours (early resistance path), or from drug-tolerant persister cells (DTP) generated via EMT (late resistance path) [73]. DTP cells may give rise also to mesenchymal $EGFR^{T790M}$-negative EGFR-TKI-resistant tumours, tumours with genetically activated alternative pathways, or to the NSCLC/SCLC transition.

## 4. Mechanisms of EGFR-TKI tolerance imposed by EMT in NSCLC; therapeutic implications

In addition to the decreased sensitivity to apoptosis via down-regulation of BIM discussed before, EMT opens other avenues for tumour cells to overcome the EGFR pathway addiction.

### *4.1. Loss of E-cadherin and resistance to EGFR-TKIs*

Earlier studies have shown that mesenchymal phenotype predisposes NSCLC cells to intrinsic resistance to EGFR-TKIs. EMT gene expression signature determined low sensitivity of NSCLC cells to erlotinib in cell lines and in tumour xenografts [98,99]. Moreover, presence of E-cadherin in tumours positively correlated with a significantly longer time to progression in patients enrolled in a randomized NSCLC clinical trial, in which erlotinib was administered in combination with chemotherapy [99]. In line with these data, re-expression of E-cadherin in the mesenchymal NSCLC cell lines increased apoptotic response to gefitinib therapy [100]. E-cadherin is known to interact with EGFR through their extracellular domains resulting in the augmentation of the pathway activation by EGFR ligands [101]. Consistently, implementation of the 76-gene EMT gene signature has shown that epithelial NSCLC cell lines expressed markers of the activated EGFR pathway; and mesenchymal cell lines were more resistant to the compounds inhibiting EGFR [102]. Therefore, loss of functional E-cadherin complexes may cause the reduced dependence of mesenchymal cells on EGFR signalling for growth and survival.

In addition to E-cadherin loss, several other EMT-activated molecular mechanisms reduce cellular sensitivity to EGFR-TKIs.

### *4.2. EMT-associated RTK AXL*

RTK AXL plays a major role in resistance to conventional and targeted therapies in different cancer types including NSCLC. AXL belongs to the TAM (TYRO3, AXL, MER) subfamily of RTKs. Binding the vitamin K-dependent protein ligand, growth arrest-specific gene 6 (GAS6), activates the intrinsic AXL tyrosine kinase and downstream signalling pathways, which control different cellular functions, survival, proliferation and migration [103,104]. AXL is a part of EMT gene expression signatures in NSCLC [102] and other carcinomas [105]. Zhang and colleagues have demonstrated that activation of AXL was necessary and sufficient for acquired resistance to the first generation EGFR-TKIs in NSCLC cell lines. Activation of AXL was associated with EMT features in erlotinib-resistant tumour samples and occurred either through its overexpression or via upregulation of GAS6. Genetic or pharmacological inhibition of AXL restored sensitivity to erlotinib in erlotinib-resistant cell lines and tumour xenografts [65].

AXL is not only a downstream effector of EMT, but it has also been shown to drive EMT programs and maintain a mesenchymal state. In breast epithelial cells, ectopic expression of AXL induced an EMT, and AXL inhibition reversed phenotype of mesenchymal mammary carcinoma cells [106,107]. Likewise, silencing AXL in mesenchymal subtype of ovarian cancer cells reversed their morphology and activated expression of epithelial markers indicating a common role of AXL in safeguarding mesenchymal state in carcinoma cells [108].

How does AXL upregulation help NSCLC cells to escape EGFR-targeted therapies? AXL activates cell survival pathways via several mechanisms: AXL-induced canonical PI3K pathway leads to the survival response through the AKT/S6K axis. AXL triggers an NF-kB-dependent increase in the expression of the antiapoptotic protein BCL-XL in mouse fibroblasts [109], upregulates BCL2, and represses caspase 3 in HUVEC cells [110]. Although it has been documented that several genes encoding BCL2 family members are direct targets of EMT-TFs [75,111,112], the role of EMT-TFs in AXL-induced antiapoptotic response is unknown.

A recent report described molecular underpinning of AXL-mediated resistance of NSCLC cells to the EGFR-blocking antibody cetuximab [113]. The study demonstrated that AXL induced nuclear trafficking of EGFR through activation of SRC family kinases YES and LYN. Nuclear EGFR functions as a kinase that stimulates DNA repair leading to cell survival in response to the treatment with DNA-damaging agents [114] (Fig. 3). Interestingly, nuclear translocation of EGFR is associated also with gefitinib resistance in breast cancer cells [115] suggesting that AXL-mediated EGFR nuclear trafficking may represent a common mechanism of acquired resistance to EGFR targeted therapies.

Another explanation for AXL-mediated resistance to EGFR-TKIs came from the reports displaying AXL as a key determinant of rewiring RTK signalling networks in cells undergoing an EMT. In mesenchymal breast and ovarian cancer cells, AXL is engaged in interactions with the EGFR family members, PDGFR and c-MET [108,116]. In these cells, AXL has a capability to recruit and activate interacting receptors through clustering (Fig. 3). The latter results in the diversification of downstream signalling, as well as reducing cellular specialisation and addiction to a particular RTK, such as EGFR.

Characterisation of AXL as a key molecule implicated in EMT in NSCLC cells opens new treatment perspectives involving combination

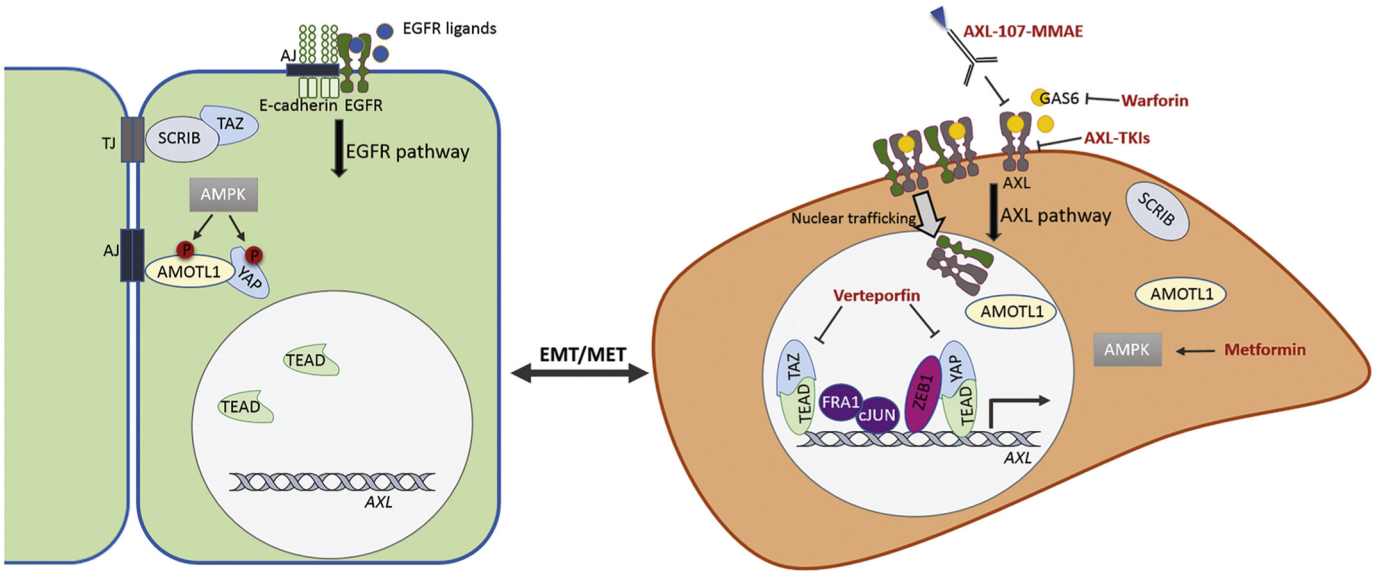


**Fig. 3.** Activation of AXL signalling in cells undergoing an EMT. In mesenchymal cells, *AXL* gene transcription is regulated by cooperative action of FRA1/cJUN, TAZ/TEAD, and ZEB1-YAP-TEAD complexes. In epithelial cells YAP and TAZ are inactivated via inhibitory interactions with proteins associated with adherens junctions (AJ) and tight junctions (TJ). In epithelial cells, E-cadherin/EGFR interactions augment EGFR signalling pathway. In mesenchymal cells, co-clustering of AXL and other RTKs including EGFR rewires RTK signalling. Direct and indirect approaches for inhibiting AXL signalling in mesenchymal cells are shown.

therapies with EGFR and AXL inhibitors [117]. A number of AXL-TKIs have been developed, with one selective inhibitor, BGB324 (R428) is currently being used in Phase II trial in combination with erlotinib in patients with Stage IIIb or IV NSCLC. Recently, Boshuizen et al. designed an antibody-drug conjugate, AXL-107-MMAE, in which a human AXL antibody was chemically linked to the microtubule toxin monomethyl auristatin E (AXL-107-MMAE). AXL-107-MMAE effectively eliminated AXL-positive cells in different types of patient-derived xenograft models including lung carcinoma [118] (Fig. 3).

### 4.3. Targeting molecular pathways upstream of AXL: Perspectives of drug repurposing?

Two transcription factor complexes implicated in EMT regulate AXL expression. *AXL* was identified as a part of a gene set regulated by the transcription cofactors YAP1 and TAZ. These two paralogue proteins interact with TEAD1–4 transcription factors and are recruited to the *AXL* gene promoter via four TEAD-binding elements [119] (Fig. 3). Moreover, YAP1, but not TAZ, physically interacts with ZEB1 and both factors co-occupy the *AXL* gene promoter and stimulate transcription [24]. TAZ may activate *AXL* expression via a different, but also EMT-related mechanism: in epithelial cells, TAZ is engaged in inhibitory interaction with the cell-polarity determinant Scribble, and EMT activates TAZ-TEAD by disrupting this association [120]. *AXL* gene is a direct target of the FRA1-cJUN transcription factor complexes [121], both components of which belong to the AP-1 family (Fig. 3). FRA1 is a key factor orchestrating EMT programs in breast [21,50] and colorectal cancer [122], and in EMT-like processes and drug resistance in cutaneous melanoma [123,124]. Interestingly, genome-wide analyses of YAP/TAZ-TEAD and AP-1 transcriptomes in breast cancer cells revealed physical association of these complexes at the enhancers of genes driving invasion and growth [125,126]. In addition, *AXL* is a target of miR-34a, one of the miR species which determine the EMT/MET balance via negative regulatory feedback loops involving EMT-TFs [127] (Fig. 1).

Interfering with the molecular pathways upstream of AXL can be considered in the context of overriding resistance to EGFR-TKIs in DTP. A potent inhibitor of YAP/TEAD interaction verteporfin [128] is applied in photodynamic therapy for age-related macular degeneration. Verteporfin increased sensitivity to erlotinib [129] and reduced self-renewal ability of NSCLC cells [130]. AMP-dependent Protein Kinase AMPK is implicated in functional inactivation of YAP1 via its direct phosphorylation and inhibition of YAP1 interactions with TEADs [131]. In addition, AMPK phosphorylates angiomotin family member AMOTL1 that is involved in sequestering YAP in the cytoplasm and its accelerated degradation [132] (Fig. 3). The commonly used antidiabetic drug metformin reduces blood glucose level via activation of AMPK. A study was carried out in EGFR-mutant NSCLC patients with Diabetes Mellitus Type II, who received combined therapy with the first generation EGFR-TKIs and hypoglycemic medications. Remarkably, metformin significantly delayed onset of resistance as compared to the other antidiabetic agents [133]. Another class of drugs reducing YAP/TAZ activity, statins, is commonly used to lower the cellular cholesterol levels and prevent cardiovascular diseases. However, the concentrations of statins required for YAP/TAZ repression significantly exceeded the therapeutically approved doses [134,135]. Implication of YAP/TAZ-TEAD, ERK1/2-AP-1 and miR-34a/SNAIL1 axis in upregulation of AXL in DTP cells remains to be explored.

Functional activity of the AXL ligand GAS6 depends upon γ-carboxylation of glutamic acids within the glutamic acid–rich domain. This modification is vitamin K-dependent and can be effectively blocked by the vitamin K antagonist, anticoagulant warfarin [136,137]. As warfarin inhibits GAS6/AXL pathway at much lower doses than required for anticoagulation effects, vitamin K antagonists could be considered for combination therapy with EGFR-TKIs in patients with NSCLC.

### 4.4. Resistance to EGFR-TKIs, EMT and ABC transporters

Activation of ABC transporters is associated with CSC phenotype and EMT (Fig. 4). Accordingly, enhanced expression of the ABC transporter ABCB1 was detected in those EGFR-TKI afatinib-resistant NSCLC cell lines, which displayed CSC/mesenchymal features and epigenetic silencing of miR-200 [138]. *EGFR*$^{T790M}$–mutant gefitinib-resistant cells with mesenchymal morphology expressed high levels of another transporter, ABCG2. Inhibition of the aberrantly activated Hedgehog signalling pathway in these cells induced a MET and diminished BCRP/ABCG2 expression [139]. In a recent study, an unexpected nuclear function of the ABCG2 protein has been reported. In the nucleus ABCG2

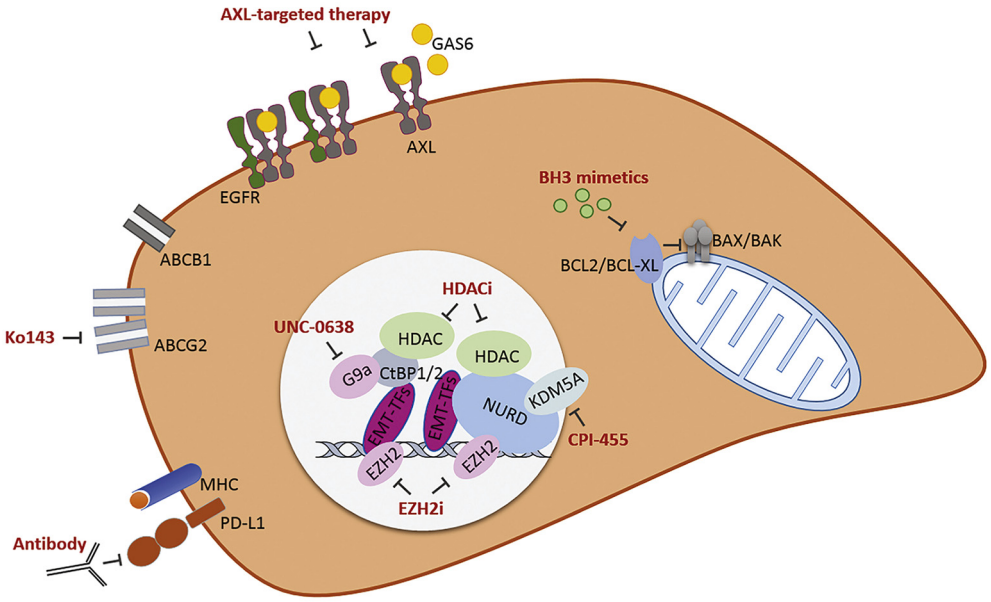


**Fig. 4.** Approaches to eradicate mesenchymal NSCLC cells from tumours developing resistance to EGFR-TKIs. Targetable pathways are discussed in this review.

interacted with the E-cadherin gene promoter and contributed to epithelisation of A549 NSCLC cells. Regulation of ABCG2 nuclear/cytoplasmic shuttling remains poorly understood, but it may contribute to EMT/MET plasticity in lung cancer [140]. Gefitinib may act as an inhibitor of the BCRP/ABCG2 transporter [141]. Therefore, its enhanced expression, nuclear export and membranous localisation may represent an EMT-promoted therapy escape mechanism that acts cooperatively with ZEB1-BIM and AXL pathways to contribute to drug-tolerance (Fig. 4).

### 4.5. Resistance to EGFR-TKIs, EMT and immune evasion

Evasion from the immune surveillance via activation of the immune inhibitory checkpoints is one of the hallmarks of cancer [142]. Blockade of the cytotoxic T lymphocyte antigen 4 (CTLA-4) receptor and PD-1/PD-L1 inhibitory pathway led to the promising results of clinical trials in patients with advanced NSCLC [143]. In NSCLC, molecular pathways driven by the mutations in the *EGFR* gene lead to the immunosuppression through the induction of several tumour-promoting inflammatory cytokines and PD-1/PD-L1 checkpoint activation. Application of the PD-1-blocking antibody increased the infiltration of the effector T-cells, and improved the survival of mice with EGFR-driven NSCLC tumours [144] (Fig. 4). Gefitinib reduced the expression of the *PD-L1* gene in EGFR-mutant bronchial cell lines, thus reversing EGFR-driven immunosuppression in tumour microenvironment and enhancing antitumour immunity [145].

There is compelling evidence in the literature that EMT may drive immune evasion, thereby causing resistance to the agents, which make tumour cells vulnerable to immune surveillance. Expression of the *PD-L1* gene is strongly up-regulated in lung adenocarcinoma with the high mesenchymal score. Analysis of the *PD-L1* gene regulation has shown that it is a direct target of miR-200 family in NSCLC cells [146], and is controlled by the miR-200/ZEB1 axis also in breast and oesophageal squamous carcinoma [147,148]. Thus, expression of ZEB1 in NSCLC cells regulates the function of tumour-infiltrating CD8+ T cells in PD-L1-dependent manner [146]. In addition to the PD-1/PD-L1 pathway, markers of other targetable immune checkpoints were detected in lung adenocarcinomas that displayed an EMT phenotype [149]. These data suggest that EMT may override the immunosuppression evolving in EGFR-TKI-resistant tumours, and targeting the EMT state may improve the response to treatments combining EGFR-TKIs with the immunotherapy.

### 4.6. Resistance to EGFR-TKIs and epigenetics

Epigenetic regulation modifies chromatin structure, impacts on gene expression without affecting primary structure of DNA, and represents a promising target for therapeutic exploitation. Modifications of histones occur predominantly at their unstructured N-terminal tails at specific lysines and include phosphorylation, acetylation, methylation, phosphorylation and SUMOylation. Combination of these modifications of the chromatin at particular gene promoters form so-called “histone code” that is a determinant of transcriptional repression or activation. Reversibility of the DTP state implies that epigenetic mechanisms rather than mutations are involved in the generation of DTP pools in response to the acute exposure to a drug. Indeed, a comparison of histone modifications in DTP cells with that in parental NSCLC cell population has shown remarkable differences [150]. General levels of histone H3 acetylation at several lysines was much lower in DTP cells, whereas repressive H3K9- and H3K27-tri-methylation was considerably higher. In addition, DTP cells exhibited reduced levels of activating H3K4me3 marks. Moreover, depletion of several H3 methyltransferases, such as EZH2 (catalyses H3K27 methylation), or H3K9 methyltransferases SETDB1 and G9a (EHMT2), specifically reduced the number of DTPs. Likewise, knockdown of the H3K4me2/3 demethylase KDM5A/Jarid1A that is overexpressed in DTP cells explicitly reduced their viability, but was not toxic for erlotinib-sensitive cells [63,150]. Specific requirement of histone-modifying enzymes for the establishment of a metastable chromatin explains the reversibility of drug tolerance.

It has been shown that in the course of EMT, EMT-TFs are engaged in a number of interactions leading to the establishment of the repressive chromatin marks H3K9me2/3 and H3K27me3, at the promoters of epithelial genes [13,151,152]. HDAC1/2 interact with co-repressors CtBP1/2 and nucleosome remodelling and deacetylase (NuRD) repressive complex, which are recruited to target promoters by TWIST and ZEB family members [153–155]. Interestingly, KDM5A, a demethylase implicated in the maintenance of the DTP pools physically interacts with CDH4, a subunit of the NURD complex, and KDM5A and NuRD regulate developmental genes in a cooperative fashion [156]. Likewise, G9a methyltransferase that is required for survival of DTP cells binds CtBP and is co-immunoprecipitated with both ZEB proteins

[153]; and EZH2 regulates neural crest development via direct association with SNAIL2 [157].

A role for EMT-TFs in the establishment of the epigenetic landscape in DTP cells has not been addressed. However, pharmacological HDAC inhibitors, or inhibitors of G9a, EZH2 or KDM5A, specifically reduced the representation of DTPs in parental NSCLC cell populations [63,150,158] and interfere with the biological functions of EMT-TFs. These agents are promising candidate drugs for the combination therapy with EGFR-TKIs (Fig. 4).

## 5. Conclusions

Subpopulations of DTP cells, which are present at low quantities in NSCLC survive the exposure to EGFR-TKIs. They undergo genetic evolution in response to the selective pressure imposed by the first or third generation EGFR-TKIs. This leads to the positive selection of clones capable to override EGFR pathway addiction by turning on alternative mechanisms of cellular homeostasis and survival. Thus, DTP cells give rise to recurrent cancer with altered characteristics as illustrated in Fig. 2. Data indicate that epithelial-mesenchymal cell plasticity is responsible for the generation and survival of DTP cells. Firstly, multiple studies have demonstrated that EMT in cancer generates populations of drug resistant cells with the features of CSCs, and DTP cells express CSC markers [63]. DTP cells are resistant to apoptosis, and exhibit a gene expression signature enriched for EMT-related genes [73]. Secondly, DTP cells can emerge de novo and their phenotype is reversible [63], recapitulating reversibility of EMT. Thirdly, histone marks observed in DTP cells are reminiscent of the modifications imposed by EMT-TFs. Finally, there are examples whereby EGFR-TKI-resistant relapsed tumours exhibit genetically or epigenetically stabilised mesenchymal phenotype.

EMT is emerging as a key target for anticancer therapy in combination with the agents eradicating tumour bulk [159]. In this paper we reviewed the pathways which are activated by EMT in NSCLC cells and represent attractive targets for combination therapies involving EGFR-TKIs. Inhibitors of these pathways are either in clinical use or being tested in clinical trials: ABCG2 transporter inhibitor Ko143; YAP/TAZ-TEAD-AXL-GAS6 pathway inhibitors; BH3 mimetics enhancing apoptosis in BIM-deficient cells; antibodies blocking inhibitory immune checkpoints; epigenetic inhibitors targeting HDAC1/2, EZH2, KDM5A and G9a (Fig. 4). Combination of these inhibitors with EGRF-TKIs may improve survival in patients with NSCLC and transform the field of thoracic oncology.

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