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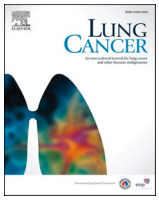

Review

Pre-clinical modelling of *ROS1*+ non-small cell lung cancer

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A B S T R A C T

Non-small cell lung cancer (NSCLC) is a heterogeneous group of diseases which accounts for 80% of newly diagnosed lung cancers. In the previous decade, a new molecular subset of NSCLC patients (around 2%) harboring rearrangements of the *c-ros oncogene 1* was defined. *ROS1*+ NSCLC is typically diagnosed in young, nonsmoker individuals presenting an adenocarcinoma histology. Patients can benefit from tyrosine kinase inhibitors (TKIs) such as crizotinib and entrectinib, compounds initially approved to treat *ALK*-, *MET*- or *NTRK*- rearranged malignancies respectively. Given the low prevalence of *ROS1*-rearranged tumors, the use of TKIs was authorized based on pre-clinical evidence using limited experimental models, followed by basket clinical trials. After initiating targeted therapy, disease relapse is reported in approximately 50% of cases as a result of the appearance of resistance mechanisms. The restricted availability of TKIs active against resistance events critically reduces the overall survival. In this review we discuss the pre-clinical *ROS1*+ NSCLC models developed up to date, highlighting their strengths and limitations with respect to the unmet clinical needs. By combining gene-editing tools and novel cell culture approaches, newly developed pre-clinical models will enhance the development of next-generation tyrosine kinase inhibitors that overcome resistant tumor cell subpopulations.

1. Introduction

1.1. c-Ros oncogene 1 (ROS1)

The *c-ros oncogene 1*, hereafter referred as *ROS1,* is an evolutionarily conserved gene [1] located on chromosome 6q22 in humans [2]. It codes for a receptor tyrosine kinase (RTK) belonging to the insulin receptor family and its orthologues are *sevenless* and *ROL-3* in *D. melanogaster* and *C. elegans* respectively [3,4]. ROS1, a single pass transmembrane protein has two main human isoforms [5]. Although its physiological role in humans still remains unknown, the presence of fibronectin III-like repeats suggests that ROS1 might couple mechanical extracellular interactions with intracellular signaling pathways [6]. Expression studies in chicken, rat and mice linked the presence of ROS1 exclusively to epithelial tissues [7]. Additionally, the importance of ROS1-mediated signaling during the mesenchymal-epithelial transition was revealed by Liu *et al.* during murine renal development [8]. Finally, concomitant analyses revealed the contribution of ROS1 signaling for the proper differentiation of epididymal epithelium [9]. Pathways known to be activated by ROS1 include the MAP kinase, PI3K, Akt and JAK/STAT pathways, which promote cell proliferation and survival, increase transcriptional capacity and enhance the expression of anti-apoptotic genes respectively [6]. Taken together, these results point to a role of ROS1 during growth and development.

1.2. Role of ROS1 in oncogenesis

The presence of *ROS1* rearrangements was initially discovered in 1987 by Birchmeier *et al.*, when abundant *ROS1* expression was reported in the glioblasoma U-118 MG cell line [10,11]. In parallel, the oncogenic transformation of chicken embryo fibroblasts by avian sarcoma virus UR2 (v-ros) containing a *ROS1* isoform lacking the sequence coding for the extracellular domain was observed [12]. The hypothesis of chromosomal rearrangements being the underlying oncogenic mechanism was supported by the association of the cytogenetic location of *ROS1* and tumor-specific rearrangements involving these loci [2]. In subsequent years, several cell lines were reported to harbor *ROS1* rearrangements such as HCS-2/8, derived from human chondrosarcoma [13] or HCC78, obtained from a lung adenocarcinoma [14]. Today, more than 25 different fusion partners have been reported in *ROS1*+ NSCLC patients, with *CD74*, *SLC34A2*, *TPM3*, *SDC4* or *EZR* being the most prevalent ones. Fig. 1 depicts some of the fusions and the diversity in terms of their subcellular localization; determined by the amino-terminal domain of each fusion partner. The presence of coiled-coil domains in certain fusion partners like *EZR*, *FIG* and *TPM3* might indicate that dimerization leads to the constitutive activation of ROS1 oncogenic fusions; while the activation mechanism remains elusive for the other fusion partners [15]. Once in their active state, the majority of ROS1 fusions converge in the activation of the MAPK pathway by interacting with the SHP2 protein. However, the CD74-ROS1 fusion

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present in the endoplasmic reticulum is unable to interact with SHP2, suggesting the presence of alternative signaling mechanisms based on the localization of the fusion protein [16].

Although the aetiology of *ROS1*+ NSCLC remains unclear, the Radon France Study revealed a significantly higher prevalence of oncogenic-driven NSCLC cases in French regions where the risk of being exposed to this radioactive gas was classified as intermediate or high [17]. It is thought that α-radiation originated during radon decay might induce DNA double-strand breaks, which upon repair via the canonical nonhomologous end-joining (c-NHEJ) pathway, will result in chromosomal rearrangements [18,19].

1.3. Therapeutical challenges in ROS1+ NSCLC

With the identification of the shared homology between ROS1 and other well studied kinases such as the anaplastic lymphoma kinase (*ALK*), MET proto-oncogene (*MET*) or the neurotrophic tyrosine receptor kinase (*NTRK*), *ROS1*+ NSCLC patients can also benefit from TKIs against the aforementioned rearranged oncogenes [20]. Due to the low prevalence of *ROS1* translocations in lung cancer, the approval of TKIs targeting ROS1 has often been granted based on single-arm phase I/II or basket clinical trials [21–24].

Inevitably, the majority of patients will experience disease progression due to the appearance of tumor resistance mechanisms. A first group of observed strategies consist in bypassing the ROS1-mediated signaling by acquiring a wide range of mutations such as *MET* amplification, $KRAS^{G12C}$, $BRAF^{V600E}$ [25,26], activation of signaling pathways like EGFR, RAS, and KIT or histological transformation to small cell lung cancer [27–29]. The second group of events consist in point mutations located in the ROS1 kinase domain which modify the conformation of its active site, preventing drug binding without impairing the capacity to phosphorylate the downstream targets. *ROS1* p.G2032R is the most commonly observed mutation, followed by D2033N, L2026M and S1986Y/F, among others [30,5,31]. The inefficacy of the first-line TKI crizotinib towards these mutant clones explains tumor progression [32]. Alternatively, cabozantinib, a MET, RET, VEGFR2, ALK and ROS1 TKI also bypasses the resistant point mutations [33,34]. The efficacy of compounds like lorlatinib, repotrectinib or NVL-520 against wild type ROS1 and some of its on-target mutations has also been demonstrated [23,35–37]. However, they have not been approved by the regulatory agencies, restricting their use to clinical trials, off-label or compassionate use.

In consequence, therapy selection should be carefully evaluated considering the components of the translocation, the acquired resistance mechanisms and compound availability. As *ROS1*+ NSCLC is a rare cancer type, real-world evidence on drug efficacy is equally scarce, and solid preclinical models are critical to unravel the suitability of a compound or a combination, towards a specific resistant scenario.

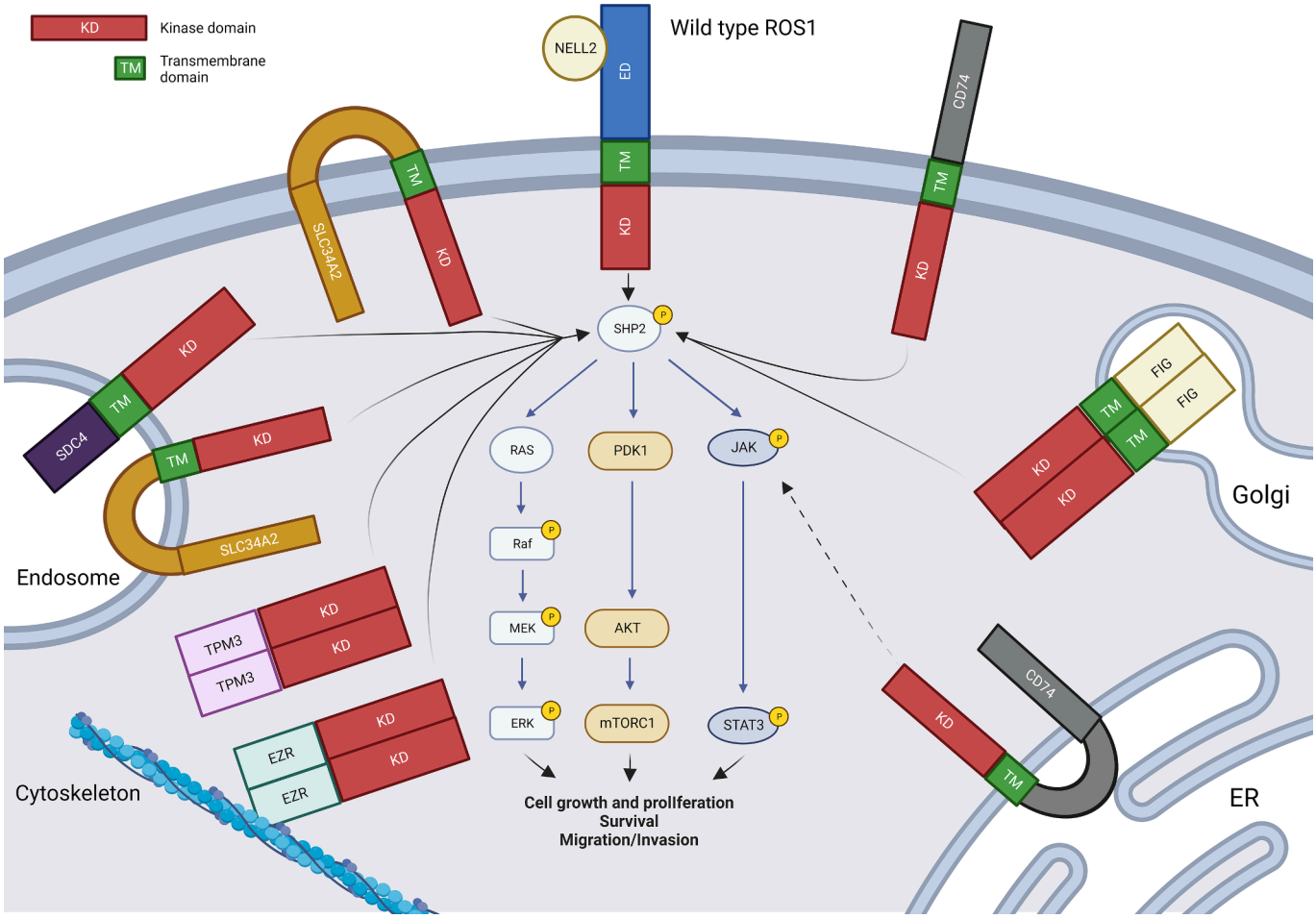

Fig. 1. Diversity of oncogenic ROS1 fusions in NSCLC. Wild type ROS1 is localized in the plasma membrane and interacts with the soluble ligand NELL2. The ROS1 fusion partners activate constitutively its kinase domain by dimerization such as TPM3-ROS1, EZR-ROS1 and FIG-ROS1, but he dimerization of wild type ROS1 and other oncogenic fusions remains unknown. The amino-terminal domain of each fusion partner determines its subcellular localization and the subsequent signaling cascade. The dotted arrow indicates that the CD74-ROS1 fusion localized in the endoplasmic reticulum does not activate the MAPK pathway via SHP2 and it might act through an alternative pathway.

2. Pre-clinical *ROS1*+ NSCLC experimental models

2.1. In vitro models

Cell lines are classically the first option to functionally validate the impact of genetic alterations. They become more relevant when studying such mutations within the context of cancer, in which there is an interplay between signaling pathways determining the dynamics of neoplastic cells. With the aim to avoid bias introduction in an experimental model, a thorough selection of a cell line based on its origin will facilitate the extrapolation of experimental results to more complex systems. Table 1 summarizes the main advantages and limitations of each cellular model discussed hereby.

2.1.1. Ba/F3 cells

Ba/F3 is a murine, interleukin 3-dependent, hematopoietic cell line widely used in high-throughput screening of TKIs. Cultured in suspension, they have a fast proliferation rate and can be easily transfected by electroporation [38]. Infecting Ba/F3 cells with retroviral particles containing the cDNA sequence of *ROS1* fusions, followed by a selection of transformants using antibiotics is the most extended engineering approach, allowing the expression of fusion proteins either wild type or mutant for the ROS1 kinase domain [25,39,40]. In parallel, changes in gene expression or cell morphology can be easily monitored upon treatment with TKIs. Several Ba/F3 lines exogenously expressing genetic fusions such as *CD74-ROS1*, *TPM3-ROS1* or *SDC4-ROS1* have been established. They have been of great importance in pre-clinical studies to demonstrate the *in vitro* efficacy of crizotinib, entrectinib, cabozantinib or lorlatinib [34,41–43].

According to Koga *et al.* up to 68% of resistance mutations against several TKIs observed in patients were also detected in assays using Ba/F3 cells [44]. Despite being a remarkable proportion, the remaining mutations were not observed *in vitro* presumably by the following reasons: firstly, the lymphoid origin of Ba/F3 cells conditions their patterns of adhesion, which result in growth in suspension. This feature does not represent the epithelial tissue, whose architecture relies on different types of cell–cell adhesion structures like desmosomes, or tight junctions. Because the role of adhesion molecules has been highlighted as crucial for the epithelial-mesenchymal transition, adherent cell lines might provide more fruitful insights when assessing morphological changes in response to treatment [45]. Secondly, the plasmid transfection to induce the expression of fusion constructs does not recapitulate the altered genetic landscape of *ROS1*+ NSCLC. The use of different promoters present in the cloning vectors could be a source of bias not only among experimental models, but also with respect to *in-vivo* strength of promoters of *ROS1* fusion partners like *CD74, SDC4* or *SLC34A2*. Additionally, the random plasmid integration can result in the functional disruption of relevant genes or a variable expression of the construct due to copy number differences. Finally, the murine genetic background of the Ba/F3 cells might also interfere with transcriptomic analyses due to inter-specific differences.

2.1.2. NIH3T3 cells

NIH3T3 is a spontaneously immortalized mouse embryonic fibroblastic cell line. They have been proven to be practical for DNA transfection studies due to their susceptibility to be infected by murine leukemia retroviruses [46]. NIH3T3 cells were engineered using retroviral plasmids to induce the overexpression of *CD74-ROS1* or *SLC34A2-ROS1* fusions by Sato *et al.* Although there is no evidence of their direct *in vitro* use in literature, they served to generate a heterotopic *ROS1*+ allograft model by subcutaneously injecting edited NIH3T3 cells suspended in Matrigel in the flanks of immunocompromised mice [47]. The main limitation of this cell line is linked to their culture conditions. Xu *et al.* highlighted the contribution of high density seeding of NIH3T3 cells to spontaneous transformation. Mechanistically, it can be explained as a trade-off between growth rate and differentiation that emerges as a

Table 1
Main characteristics of the preclinical *ROS1*+ experimental models in literature.

Experimental model	Advantages	Limitations	References
In vitro models			
Ba/F3 cells	Easy to genetically engineer Useful for high throughput compound screening	Non-human cells Non-epithelial lineage	[23,35–37]
HEK293T cells	Human origin Well established transfection protocols	Undifferentiated cells Non pneumocytes Partial p53 hijack by the viral antigen SV40T	[46–48]
HBECp53 cells	Human origin Consistent growth Proven to be engineered using CRISPR/Cas9 technology	Constitutive expression of cyclin-dependent kinase 4 (*Cdk4*) and human telomerase reverse transcriptase (*hTERT*) Expression of a dominant negative p53 protein isoform	[49–52]
A549 cells	Consistent growth Alveolar cells Adenocarcinoma	Not driven by *ROS1* rearrangement Additional $KRAS^{G12S}$ variant	[54–61]
YU lines	*ROS1*+ NSCLC lines	All were isolated from pleural effusions Unknown presence of additional mutations	[73]
HCC78 cells	*ROS1*+ NSCLC line Genetically characterized Consistent growth Monoclonally expandable Additional $TP53^{S241F}$ variant	Complex karyotype	[14,62,63]
CUTO lines	Different *ROS1* fusion partners available Spontaneously immortalized lines Preservation of neoplastic genetic background	Difficult to expand monoclonally High patient-associated heterogeneity	[67,69,70,71]
ADK-VR2 cells	*ROS1*+ NSCLC line Spontaneously immortalized Useful to study the genotype of pleural effusion-specific neoplastic cells	Unknown driver alteration of primary resistance to crizotinib	[96]
Tumoroids	Recapitulation of tridimensionality Preservation of neoplastic genetic background	Laborious protocol In absence of *TP53* mutations, removal of normal airway organoids is manually done Difficult to edit genetically	[74]
In vivo models			
Allograft mice	Relatively easy to generate Easy monitoring of tumor size Tumor can be serially explanted	ExpensiveHeterotopic model (tumor in flanks, not in lungs) Humanized tumor in murine microenvironment Immunocompromised mice	[44,77]
Patient-derived xenografts (PDXs)	Original stromal cells included Tumor can be serially explanted	Expensive Heterotopic model Immunocompromised mice	[81]

(*continued on next page*)

Table 1 (*continued*)

Experimental model	Advantages	Limitations	References
	Easy monitoring of tumor size	Months required until obtaining a PDX Variable engraftment rate	
Genetically engineered mice (GEM)	Recapitulate partially *ROS1*+ pathophysiology Immunocompetent mice	Expensive Difficult to establish Transgene copy number variation Lack of temporal control of transgene expression Magnetic resonance-based imaging required to assess lesions	[44,83]

response to growth-inhibiting conditions [48]. Thus, cell density should be considered during the experimental design involving NIH3T3 cells to exclude any oncogenic effect not linked to the presence of a *ROS1* fusion.

2.1.3. HEK293T cells

HEK293T is the second most commonly used cell line to obtain *ROS1*+ NSCLC models. From the parental line HEK29, a derived line, named HEK293T was generated expressing a temperature-sensitive allele of the SV40 T antigen, known to form a complex with p53 and inhibiting it [49]. HEK293T cells have been used for the study of the downstream phosphorylation targets of the rearranged ROS1 kinase following a similar approach as described with Ba/F3 cells for cloning and expression of fusion construct purposes [50]. Bergethon *et al.* included *CD74-ROS1*-lipofected HEK293T cells in their experiments to study changes in phosphorylation-mediated ROS1 kinase activation in presence of crizotinib [51]. The robustness of protein synthesis and folding in HEK293T cells allowed Neel *et al.* to demonstrate that different subcellular localization of *ROS1* fusions modulates their oncogenic signaling [16]. Generally, HEK293T cells have been used as a tool for the synthesis and assembling of adenoviral particles used for the integration of *ROS1* fusion constructs in other cell lines [25,34,40,41]. In the case of HEK293T cells, the alteration of p53 function compromises the stability of their genome, adding another layer of complexity when studying malignancies like *ROS1*+ NSCLC, which typically account for a low tumor mutational burden. *De novo* variants that can appear due to the partial hijack of p53 by the viral antigen SV40 T -a TP53 inactivation not observed in patients- could be wrongly attributed to the expression of a *ROS1* fusion construct. Thus, the genetic background of a chosen experimental model should be carefully considered. Despite being a human cell line, its embryonic phenotype does not necessarily recapitulate the stemness of neoplastic cells. Hence, interpreting HEK293T-derived results should be done carefully, especially when extrapolating results to other cell lines or organisms.

2.1.4. HBECp53 cells

Human bronchial epithelial line (HBEC) was collected from a 65-year-old woman without cancer. It was immortalized after a retroviral infection leading to the constitutive expression of cyclin-dependent kinase 4 (*Cdk4*) and human telomerase reverse transcriptase (*hTERT*) genes [52]. HBEC is the parental line of HBECp53, in which *TP53* is knocked down as a consequence of the expression of a small interfering RNA in which the inhibition of p53 activity promotes genomic instability being a suitable background to trigger neoplastic transformation [53]. HBECp53 cells were edited by Sato and colleagues using the CRISPR/Cas9 technology to induce chromosomal translocations [54]. By co-expressing two different guide RNAs (gRNAs), both targeting the chromosomal regions involved in the *EZR-ROS1* rearrangement, a fraction of the double-strand breaks induced by the nuclease Cas9 will be repaired through the canonical non-homologous end-joining (c-NHEJ) pathway, resulting in the fusion of interest [55]. HBECp53-based models reproduce more faithfully the genomic context of the driving alteration since the expression of the fusion transcripts will be subject to the regulatory elements of the fusion partner, in this case *EZR*. Physiologically, this is a relevant factor since the *in vitro* models expressing exogenous *ROS1* fusions might yield higher levels of the fusion protein due to effects of the available promoters (fusion partner-specific promoter vs cytomegalovirus CMV standard promoter in expression vectors). This observation should not be neglected, especially when studying TKI-addicted lines, which have shown that high ROS1 kinase activity triggers apoptosis *per se* in the absence of TKIs [56]. Overall, *EZR-ROS1*-rearranged HBECp53 cells seem a consistent *ROS1*+ disease model despite its limitations such the constitutive expression of a TP53-targeting siRNA and the potential CRISPR/Cas9 off-target effects.

2.1.5. A549 cells

A549 is a lung adenocarcinoma cell line with type-II alveolar cell features which was established from an explanted lung tumor from a 58 year-old man [57,58]. It has been used not only as a disease model, but also as a system to study the metabolic pathways involved in the synthesis of phospholipids and proteins which constitute the pulmonary surfactant [59,60]. The mutational landscape of A549 cells includes the homozygous variant $KRAS^{G12S}$ [61], responsible for increasing the levels of GTP-bound KRAS and the subsequent overactivation of its downstream targets like MAPK and PI3K-AKT pathways [62]. A549 cells served as a *ROS1*+ NSCLC model after being transfected with the plasmid p.DNA3.1 containing the *CD74-ROS1* insert to elucidate the molecular mechanisms behind migration, invasion and drug resistance [63]. When choosing the A549 line as experimental model, one should consider that the concomitant *KRAS* p.G12S mutation might probably be the oncogenic driver in A549 cells [64] Therefore, appropriate controls should be included in assays oriented to the study of extrinsic resistance mechanisms in *ROS1*+ NSCLC because the preexisting overactivation of the Kras kinase can lead to non-deterministic interactions with the oncogenic ROS1-mediated signaling.

2.1.6. HCC78 line

The HCC78 cell line was established from a pleural effusion of a 65 year-old male patient diagnosed with a lung adenocarcinoma [65]. Importantly, the $p53^{S241F}$ inactivating variant has been reported in HCC78 cells, serving as a model for concomitant *TP53*-mutant *ROS1*+ NSCLC, a molecular subclass associated with poorer progression-free survival [66]. It was not until 2007 when Rikova *et al.* unraveled the presence of a *SLC34A2-ROS1* rearrangement while profiling the phosphotyrosine signaling landscape in NSCLC [14]. HCC78 cells have been pivotal for the development of ROS1-targeting TKIs like crizotinib, entrectinib, or cabozantinib among others [67–69]. In parallel, TKI-resistant lines have also been generated from HCC78 cells such as the HCC78-TR and HCC78R lines. They were obtained independently by Davies et al. and Kato et al. respectively. After continuously culturing HCC78 cells in crizotinib, the resulting resistance mechanism consisted of an overactivation of the EGFR pathway, rendering both lines sensitive to gefitinib, an EGFR inhibitor [69,70]. Ku et al. obtained the entrectinib-resistant HCC78ER line following also a long-term culture in the presence of the kinase inhibitor. The amplification of *KRAS* and *FGF3* together with the $KRAS^{G12C}$ variant bypassed the ROS1 blockade [71]. In summary, the HCC78 cell line contributed remarkably to elucidate the biology of ROS1 fusions in NSCLC and served as the first *ROS1*+ NSCLC line to model TKI-resistant variants.

2.1.7. CUTO lines

The unit of thoracic oncology of the University of Colorado has an ongoing collaboration with the *ROS1*+ patient organization to develop new patient-derived xenografts and cell lines. This international and patient-driven project, termed the ROS1 Cancer Model Project, resulted in twelve NSCLC *ROS1*-rearranged cell lines so far, helping to address

the unmet need of *ROS1*+ experimental models [72]. These lines include nine *CD74-ROS1*, one *SDC4-ROS1*, one *TPM3-ROS1* and one *SLC34A2-ROS1* so far [73]. Some of the CUTO lines have been crucial not only to characterize the *in vitro* anti-proliferative effect of compounds like entrectinib but also to unveil extrinsic TKI resistance mechanisms such as the activation of the EGFR pathway [70,74]. The diversity of translocation types of the established cell lines might be pivotal to determine the contribution of the ROS1 fusion partner in the patterns of metastases. Gainor *et al.* reported that oncogenic-driven tumours like *ALK*+ NSCLC show higher brain metastatic rates when compared to *ROS1*+ lung malignancies [75], suggesting that the patterns of neoplastic cell migration are differentially modulated based on the resulting kinase fusion. Per contra, such lines are often hard to culture since they have become spontaneously immortal, without any induced modification that ensures their sustained proliferation. Moreover, some of the patient-derived lines are not treatment-naïve, which can introduce a bias in their analysis.

2.1.8. YU lines

In 2019, Kim *et al.* established a collection of 23 patient-derived cell lines from pleural effusions of advanced lung adenocarcinoma patients. Five of the cell lines were driven by *ROS1* translocations: three of them expressing *SLC34A2-ROS1* (YU-1082, YU-1083 and YU-1085), one carrying the *TPM3-ROS1* fusion (YU-1081) and another driven by *CD74-ROS1* rearrangement (YU-1080). Some of them were generated from TKI-naïve lesions that progressed upon chemo/radiotherapy (YU-1082 and YU-1083); whereas lines YU-1081 and YU-1085 originated from crizotinib-treated patients [76]. Among cell-based pre-clinical models, YU lines represent a robust choice, because the expression of the *ROS1* genetic fusion is regulated by the promoter of its fusion partner. They also offer valuable information about the original genetic background of the neoplastic cells. In addition, establishing cell lines with different fusion partners will be important to study if the impact of the partner gene has further implications in the course of the disease.

2.1.9. ADK-VR2 cell line

The most recently published *SDC4-ROS1* patient-derived cell line is ADK-VR2. Derived from a pleural effusion from a patient who developed crizotinib resistance, its growth in 2D culture was poorly inhibited by lorlatinib, entrectinib and taletrectinib. Interestingly, when growing in 3D, ADK-VR2 cells were strongly inhibited by crizotinib, entrectinib and lorlatinib. A crizotinib-resistant line (ADK-VR2 AG143) was derived after culturing cells in 3D in presence of crizotinib. Interestingly, it was refractory to lorlatinib, entrectinib and taletrectinib. The tumorigenic and metastatic abilities were studied in immunocompetent mice after being intravenously injected with ADK-VR2 cells. Metastatic lesions were detected but they were not significantly reduced upon crizotinib administration [96]. Thus, this novel cell line constitutes a great asset to explore the biology of ROS1 fusions.

2.2. ROS1+ tumoroids

Tumoroids are organotypic structures derived from neoplastic cells. They are 3D structures exclusively composed by tumor specimens initially cultured in presence of elements of the basal membrane and extracellular matrix. Yamatsuji and colleagues obtained a tumoroid (PDT-LUAD#19) derived from a lung adenocarcinoma patient harboring a *TPM3-ROS1* fusion. Interestingly, a concomitant $TP53^{K120Sfs*3}$ mutation was identified [77]. While reproducing the three-dimensional structure of neoplasias, the growth of the *TPM3-ROS1* PDT-LUAD#19 tumoroid (kinase wild type) was significantly suppressed upon treatment with crizotinib or entrectinib, being a highly valuable 3D cell system. However, the concomitant truncating *TP53* variant present in the model might result in misleading findings as mentioned with HEK293T cells. Thus, tumoroids are disease avatars that offer promising applications to assess targeted therapies. However, they rely on the availability of patient material and the genetic edition to model resistant variants of the tumor should be adapted to this novel culture system.

2.3. In vivo models

The use of organisms for disease modelling offers a more integrative, yet complex, vision about the physiological alterations responsible for each pathology. Up to five *ROS1*-rearranged strains are available in the literature. Important differences between mice and human lungs that must be considered when studying pathologies affecting such organ. They can be classified in two main categories: developmental and differential genetic background. It is known that mouse lungs develop faster and the formation of alveoli occurs after birth. In contrast, human lungs undergo several branching cycles before alveoli are constituted [78]. In terms of genetic backgrounds, a remarkable amount of mutant murine models that failed to develop an expected phenotype revealed that the answer for modelling human diseases might rely in a specific combination of the mutation of interest and a particular genetic background [79].

2.3.1. Allograft mice

Engineered NIH3T3 cells expressing a *EZR-ROS1* fusion were subcutaneously injected in the flanks of immunocompromised BALB/cnu/nu mice by Arai *et al.* and the formation of tumors was only observed in mice carrying the wild type *EZR-ROS1* rearrangement. In consequence, this experimental model unraveled the function of the ezrin FERM domain for the activation of the ROS1 kinase and the pro-tumorigenic role of the EZR-ROS1 fusion [47]. Following a similar approach, Davare *et al.* obtained allograft mice bearing *FIG-ROS1*-driven murine cholangiocarcinoma cell lines to demonstrate the superiority of foretinib versus crizotinib *in vivo* [80]. The use of heterotopic models allows easy measurement of subcutaneous tumors with a caliper, especially when compared to the quantification of lesions affecting intern organs, which require MRI technology. Despite this technical advantage, their major biological limitation relies on the interaction of neoplastic cells with the peritoneal or subcutaneous environment, which can have a different impact in the tumor physiology due to their different cellular composition. Additionally, the tumor architecture plays a crucial role in the development of the disease. Previous studies showed that the incorporation of extracellular matrix for the injection of engineered or tumor cells improve the cell viability in other intraperitoneal cancer models [81,82].

2.3.2. Patient-derived xenografts (PDXs)

Patient-derived xenografts offer valuable information of the tumor biology since they include the stroma, a crucial component that determines the dynamics of neoplastic cells [83]. Kang *et al.* established a *CD74-ROS1* NSCLC PDX murine model by injecting subcutaneously a fraction of a patient's primary tumor in severely immunocompromised mice (YIHM-1005 PDX). The engrafted tumor was subsequently re-implanted until the third mouse generation. Targeted deep sequencing revealed the presence of two missense variants in *PI3KCA* and *ROS1* respectively besides the chromosomal rearrangement [84]. A study by Xi et al. showed that NSCLC tumors with an elevated tumor-stroma ratio (TSR) have a negative impact in patient overall survival [85]. Thus, preserving the stromal compartment in PDXs contributes to a better recapitulation of *ROS1*-rearranged tumor pathophysiology. Nevertheless, the interaction between a human-derived tumor with a murine immunodefficient host should be taken into account since it can be a limitation in studies involving the role of the immune system in tumor progression. In addition, the establishment of PDXs is a costly and time consuming procedure heavily impacted by the engraftment rate of the human specimens.

2.3.3. Genetically engineered mouse models

Arai *et al.* established four independent transgenic mice strains

expressing *EZR-ROS1* fusion under the control of the *SPC* promoter with different copy number each. Eventually, they focused on a strain harboring four copies of the transgene confirmed to be widely expressed in the lungs. The authors did not detect extrapulmonary metastases, suggesting that additional alterations might be required to promote the migration of neoplastic cells to other organs [47].

Using a similar approach, nine genetically engineered murine strains expressing either *CD74-ROS1* (n = 3) or *SDC4-ROS1* (n = 6) were generated by Inoue *et al.* The desired fusion constructs were subcloned in SPC-iNOS plasmid and after the excision of the iNOS cassette, the resulting construct was injected in pronuclear stage embryos of C57BL/6J mice. Each strain differed in transgene copy number, which correlated with the expression levels of the fusion protein validated upon immunoblotting. The transgenic mice started developing multiple lesions in lungs between 2 and 4 weeks of age [86].

Genetically engineered mouse models of *ROS1*+ NSCLC employ the surfactant protein C (*SPC*) as a tissue-specific promoter targeting alveolar-type II cells, which allows a restricted expression of the construct of interest. However, *SPC*-targeted expression in distal regions of murine airways has been reported to start between 15,5 and 17,5 embryonic days during development [87]. Taking these findings into consideration, *ROS1* fusions are transcribed in a premature stage in mice. This phenomenon differs from the spontaneous somatic translocation observed in *ROS1*+ NSCLC patients, which would be only transcriptionally active once the developmental program of the lung is complete and the tissue microenvironment is fully established. A potential improvement of this system would be the incorporation of the tetracycline-controlled transcriptional expression system. By crossing a *SPC-TetR* responsive element-expressing male with a transgenic female harboring a *ROS1* genetic fusion regulated by the *Tet-O* operator, the resulting litter will specifically express the desired *ROS1* fusion only in the lungs and exclusively in the presence of tetracycline or one of its derivatives. Therefore, by restricting the transcription of the *ROS1* fusions to the adult stage, an undisturbed development of the lungs will be achieved. Although no interactions between tetracyclines and TKIs against hematological malignancies have been reported, it can not be excluded that co-administering compounds lead to unexpected physiological alterations [88].

Another common problem of transgenic mice is the variability in expression of fusion protein and in transgene copy number across the murine strains, which can be explained by the random integration of the expression cassette, leading to undesired alteration of the genetic landscape. Importantly, the authors reported significantly lower lifespans of *ROS1*+ NSCLC transgenic mice compared to the control strains, confirming the early activation of the *SPC* promoter during development. This observation, coupled to a diffuse pattern of lesions across the lungs increase the complexity of the model since a remarkable amount of tumors are simultaneously proliferating, opening the door to a wide variety of clonal dynamics within each lesion. With regard to the absence of extrapulmonary metastases, the fusion partner might determine this observation, although it remains to be elucidated. None of the published mouse models has been reported to develop metastatic lesions, revealing that there are still unknown mechanisms of metastasis to be reproduced. Additionally, other genetically engineered models of *Kras*-driven NSCLC showed poor mitotic activity of alveolar type-2 cells expressing the mutant allele, indicating heterogeneity among this cell type [89]. Collectively, the previous observations warn about the genetic artifacts caused by the transgenesis procedure, which should be accurately characterized upon strain establishment to pinpoint them as potential confounders.

3. Future directions

Refining cell-based disease models becomes almost indispensable nowadays thanks to the popularization of genetic engineering tools like CRISPR/Cas9 technology. With this technique, Choi and Meyerson induced a *CD74-ROS1* rearrangement in non-transformed immortalized lung epithelial cells AALE by targeting in parallel the breakpoint regions observed in patients [90]. Traditional methods like long-term cell culture in progressively increasing concentrations of TKIs followed by a screening of the spontaneous emergence of resistant subclones are costly and time-consuming. Moreover, different resistance mechanisms can simultaneously emerge within a cell population that accumulates a substantial amount of cell passages, ultimately acting as potential confounders. We learnt from Ba/F3 data that extrinsic TKI-resistance mechanisms seem to predominate over the acquisition of ROS1 kinase point mutations *in vitro*. As shown in Fig. 2, the use of Ba/F3 for TKI development predominates together with HCC78 cells. CUTO and YU lines edited with the CRISPR/Cas9 system are a good alternative to generate repositories of monoclonal mutant populations in a rather short period of time without the disadvantages of long-term drug exposure. However, the main limitation, besides low editing efficiency are CRISPR/Cas9 off-target effects. Thus, verifying absence of significant genetic alterations caused by the off-target activity should be considered when validating an experimental model. A major advantage of this approach lies in the generation of multiple lines harboring on-target resistance mutations, in an identical and real-world genetic background. By doing so, the elucidation of factors which explain differences in metastatic sites and differential signaling pathway activation can be achieved. Importantly, every patient-derived cell line represents only one patient's characteristics, and as shown in the HCC78 cell line and EGFR-mediated derived resistance, a model tends to acquire the same resistance mechanism. Therefore, the high variability in patient response during clinical trials must be also addressed by combining experimental models containing a common resistance-conferring mutation. This alternative strategy will elucidate the potential genetic modifiers and the co-occurring alterations likely to modulate the response towards TKIs.

The experimental gap between monolayer cell cultures and xenograft tumors can be bridged by the creation of 3D co-culture spheroid models integrating edited patient-derived tumor cells and fibroblasts [91,92]. Being a highly sophisticated *in vitro* model, 3D co-culture spheroids can be easily genetically edited compared to tumoroids. In order to accomplish this goal, a shift from traditional cell models like Ba/F3 cells or HEK293T cells to *ROS1*+ NSCLC patient-derived cell lines is necessary to upgrade disease models. The fruitful collaboration between patient-driven advocacy associations and the academic setting has proved to be a determining bond to enhance the development of such groundbreaking disease models.

The culture of tumor explants is a field that remains to be explored in *ROS1*+ NSCLC. Recently, Talwelkar et al. presented fresh uncultured cells (FUTCs) for treatment personalization in NSCLC. This approach combines tumor phenotyping and isolation of primary epithelial cells expressing EpCAM to ultimately perform drug sensitivity assays [93]. Sadly, no *ROS1*-rearranged samples were included in the study, most likely due to the limited accessibility of patient samples. Tumor tissue explants offer valuable information concerning the tridimensional organization of the neoplastic cells and the stroma, key to understanding spatial tumor heterogeneity, but their main limitation lies in potential difficulties in preserving the viability of the different cell populations present in each sample [94,95] In summary, a combination of pre-existing experimental models like 3D cultures and modern gene editing technologies like CRISPR/Cas9 is a feasible and relatively affordable option that remains to be exploited.

4. Conclusions

In the recent years, the number of *ROS1*+ NSCLC preclinical models has grown exponentially, ranging from cell-based to murine models. They all have contributed to a better understanding of the biology of such low-prevalent malignancy, accelerating the development of tyrosine kinase inhibitors. Unfortunately, most of the available compounds

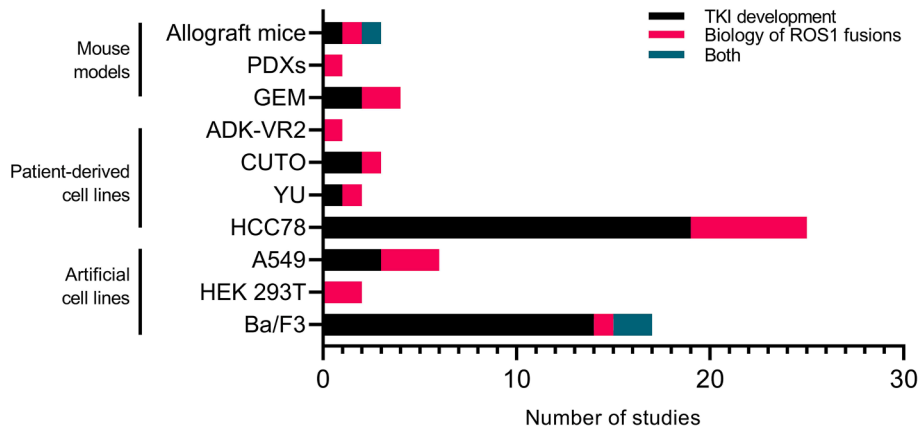

Fig. 2. Distribution of *ROS1*+ NSCLC studies across the different experimental models. (GEM: genetically engineered mice; PDXs: patient-derived xenografts).

approved to treat *ROS1*+ NSCLC fail to inhibit the proliferation of resistant subclonal populations. Hence, an additional effort oriented to, on the one hand, refining the models available today by implementing cutting-edge gene editing technologies and, on the other hand, exploring alternative models will meet the need of a rational clinical decision in refractory cases. This goal can only be achieved with a deeper understanding of the diverse molecular alterations in *ROS1*+ NSCLC which will broaden the spectrum of known molecular subtypes. Taken together, these novel approaches will partially solve the limited access to tumor specimens in *ROS1*+ NSCLC and can be extrapolated to other rare oncogene-driven malignancies.

5. Methods

Search strategy

In order to determine the amount of studies using *ROS1*+ NSCLC preclinical models, two databases (PubMed and Scopus) were used (latest search 14/02/2023). Per each experimental model, the following terms were included: ("ROS1" AND BaF3), ("ROS1" AND "HEK293T"), ("ROS1" AND "A549"), ("ROS1" AND "HCC78"), ("ROS1" AND "YU"), ("ROS1" AND "CUTO"), ("ROS1" AND "Mouse model") and ("ROS1" AND "Xenograft"). The references of the selected articles were also manually searched to find articles not directly shown in the database search. After the manual review of each article, duplicates were excluded and n = 58 publications were included. They were eventually categorized depending on their purpose: study of ROS1 fusions biology, TKI development or both.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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