TESIS DE DOCTORADO

RADIORESISTANCE IN LUNG CANCER: A MOLECULAR APPROACH

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Título da tese: **Radioresistance in lung cancer: a molecular approach.**

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FIGURE INDEX

3D-CRT	Three-dimensional conformal radiation therapy
53BP1	Tumor suppressor p53-binding protein 1
ABC	ATP binding casette
ABCB1	ATP binding cassette subfamily B member 1
ABCC1	ATP binding cassette subfamily C member 1
ABCG2	ATP binding cassette subfamily G member 2
AFI	Average flourescence intensity
AKT	Protein kinase B
ALDH1	Aldehyde dehydrogenase 1
ALK	Anaplastic lymphoma kinase
ATM	Ataxia telangiaectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
ATXN3	Spinocerebellar Ataxia Type 3 Protein
BCR	Breakpoint clusterr region protein
BER	Base excision repair
BRAF	v-Raf murine sarrcoma viral oncogene homolog B
BRCA1	Breast cancer type 1 susceptibility protein
CDK	Cyclin-dependent kinase
CDK4	Cyclin-dependent kinase 4
CSC	Cancer stem cell
CT	Computerized tomography
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL12	Stromal cell derived factor 1
CXCR4	Chemokine receptor type 4
DBS	Double strand breaks
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DNA-SCARS	DNA segments with chrromatin alterations reinforcing senescence
DSBR	Double strand breaks repair
EGFR	Epidermal growth factor receptor
EML4	Echinoderm microtubule-associated protein-like 4
EMT	Epithelial to mesenchymal transition
ERBB3	Epidermal growth factor receptor 3
ERCC1	Excision repair cross-complementation group 1
ERK	Extracellular signal-regulated kinases
EXO1	Exonuclease I

FEN1	Flap structure-specific endonuclease 1
FI	Fluorescence intensity
GDP	Guanosine diphosphate
GRP130	Glycoprotein 130
GTP	Guanosine triphosphate
Gy	Gray
H2AX	H2A histone family member X
HER2	Epidermal growth factor receptor 2
HGF	Hepatocyte grow factor
HIF1	Hypoxia-inducible factor 1
HMGB1	High mobility group box 1
HR	Homologous recombination
HSP90	Heat shock protein 90
IL-6	Interleukin 6
IR	Irradiation
JAK	Janus kinase
KIF5B	Kinesin family member 5B
KRAS	Kirsten rat sarcoma viral oncogene homolog
LC3	Microtubule-associated proteins 1A/1B light chain 3B
LIG3	DNA ligase 3
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MET	Hepatocyte grow factor receptor
MLH1	MutL homolog 1
MMR	Mismatch rerpair
MRE11	Meiotic recombination 11 protein
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
NBS1	Nibrin
NEIL1	Endonuclease VIII-like 1
NER	Nucleotide excision repair
NF-kB	Nuclear factor kappa B
NHEJ	Non-homologous end joining
NSCLC	Non-small cell lung cancer
NTRK	Neutrophic tropomyosin-related kinase
OCT4	Octamer-binding trranscription factor 4
OCT	Optimal cutting temperature
PBS	Phosphate buffered saline
PD-L1	Programmed cell death ligand 1

PD1	Programmed cell death protein 1
PDGFB	Platelet-derived growth factor B
PDGFR	Platelet-derived growth factor receptor
PET	Positron emission tomography
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphoinositide-3-Kinase, Catalytic, Alpha Polypeptide
Pix	pixels
PNKP	Polynucleotide kinase 3´-phosphate
PTEN	Phosphatase and tensin homolog
RAD18	E3 ubiquitin-protein ligase RAD18
RAD50	DNA repair protein RAD50
RAD51	RAD51 recombinase
RAD51AP1	RAD51 associated protein 1
RAD51B	RAD51 Paralog B
RAD51C	RAD51 Paralog C
RAD51D	RAD51 Paralog D
RAD54L	DNA Repair And Recombination Protein RAD54-Like
RAP80	Ubiquitin Interaction Motif Containing 1
RB	Retinoblastoma-associated prrotein
RBBP8	Retinoblastoma binding protein 8
RET	Rearranged during transfection
RNA	Ribonucleic acid
ROS	Reactive oxygen species
ROS1	ROS proto-ocogene 1
RPA3	Replication protein A3
RTK	Receptor tyrosin kinase
SBRT	Stereotactic body radiation therapy
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SDF-1	Stromal cell-derived factor 1
SLUG	Snail Family Transcriptional Repressor 2
SNAIL1	Snail Family Zinc Finger 1
SOX2	Sex determining region Y box 2
ssDNA	Single strand DNA
STAT3	Signal transducer and activator of transcription 3
STK11	Serine/threonine kinase 11
TBNA	Transbronchial needle aspiration

TBS	Tris buffered saline
TGF-b	Transforming growth factor beta
TKI	Tyrosine kinase inhibitor
TP53 or p53	Tumor protein 53
TTF-1	Thyroid transcription factor 1
UNG	Uracil DNA Glycosylase
VEGFR	Vascular endothelial grow factor receptor
WIF1	WNT inhibitor factor 1
XRCC1	X-Ray Repair Cross Complementing protein 1
XRCC2	X-Ray Repair Cross Complementing protein 2
XRCC3	X-Ray Repair Cross Complementing protein 3
XRCC4	X-ray repair cross-complementin protein 4
ZEB1	Zinc Finger E-Box Binding Homeobox 1

O cancro de pulmón é responsable de mais do 20% das mortes producidas por cancro en todo o mundo e o cancro de pulmón de células non pequenas é a causa do 85% das fatalidades en doentes diagnosticados de cancro de pulmón. A supervivencia a cinco anos en doentes cun diagnóstico temperán está arredor do 50%, sen embargo, a maioría dos casos son diagnosticados en estadios avanzados da enfermidade e nestes enfermos a supervivencia non supera o 4%. A mediana da idade de diagnóstico do cancro de pulmón está en 70 anos, sendo unha doenza pouco frecuente en persoas menores de cincuenta anos.

A aparición de cancro de pulmón pode estar asociada a diferentes causas como alteracións xénicas hereditarias ou producidas pola exposición a factores ambientais (contaminación ambiental, radón ou asbestos) e malos hábitos dietéticos. Sen dúbida, a principal causa asociada a aparición de cancro de pulmón é a inhalación de fume de tabaco. Un fumador pode ter ata dez veces mais probabilidades de sufrir dun cancro de pulmón ca un non fumador.

As opcións de tratamento dispoñible para atallar o cancro de pulmón hoxe en día son mais amplas que anos atrás, principalmente debido ós avances producidos grazas ós estudios xenómicos do cancro. A pesar disto, a cirurxía, a radioterapia e a quimioterapia seguen a ser os tres piares do tratamento do cancro de pulmón. Nos últimos anos desenvolvéronse novas terapias como os diferentes inhibidores dos receptores tirosin-quinasa, que son usados en tumores con alteracións xénicas específicas; ou mais recentemente, a inmunoterapia.

A pesar destes avances as recorrencias son frecuentes en doentes que foron tratados contra o cancro de pulmón. Nestes casos é habitual que os novos tumores aparezan con resistencia ós tratamentos, o que fai moito mais complicado o manexo da enfermidade.

Unha hipótese posible para explicar estas resistencias xorde coa aparición do concepto de células nai tumorais. Estas células constituirían unha subpoboación no tumor con características particulares, como a maior capacidade de sobrevivir ós tratamentos tumorais, desta forma, os tratamentos eliminarían a maioría do tumor deixando atrás as células nai tumorais. As células nai tumorais son capaces de establecer un novo tumor, pero tamén de adquirir a capacidade de separarse do tecido orixinal, mobilizarse e establecerse nun novo nicho, dando lugar a metástases. Este proceso polo que unha célula adquire a capacidade de migrar coñécese como transición epitelio-mesénquima, proceso que se inicia coa expresión de diferentes factores de transcrición como SNAIL1, TWIST, SLUG ou ZEB1, producindo unha represión da expresión de xenes asociados a epitelios como E-caderina ou citoqueratinas ao mesmo tempo que se expresan outros xenes característicos de liñaxes mesenquimais como a N-caderina ou a fibronectina. Estes cambios na expresión xénica resulta na pérdida da polaridade baso-lateral e as unións entre células potenciando a capacidade migratoria e invasora das células tumorais. Ademais, estes cambios no patrón de expresión xénica poden producir cambios importantes na fisioloxía celular que lle permitirían a adaptación a novas condicións ambientais. Nos últimos anos a distinción entre células nai tumorais e células que sofren un proceso de transición epitelio-mesénquima volveuse difusa, xa que numerosas investigacións suxiren que se trata dun proceso onde as células nai tumorais existen nun equilibrio de expresión variable de características epiteliais e mesenquimais. Esta plasticidade das células nai tumorais suporían unha vantaxe de supervivencia aos diferentes tratamentos antitumorais.

Amplos estudos no campo da resistencia a diferentes terapias proporcionaron algunhas respostas de como as células tumorais poderían estar evadindo os efectos antiproliferativos das terapias antitumorais. No caso de quimioterapéuticos, a sobreexpresión dos transportadores ABC, que poden segregar compostos ao espazo extracelular diminuíndo a súa eficacia; cambios na resposta a danos no ADN, que poden producir resistencias a drogas que funcionan producindo danos no ADN celular; ou no caso de inhibidores de receptores de tirosin quinasas,

é común que se produzan mutacións nas proteínas diana destas terapias que fan que o tratamento non teña efecto.

Unha das ferramentas principais no tratamento de cancro de pulmón segue a ser a radioterapia que aínda que é efectiva, a miúdo se producen recorrencias con tumores que se volven resistentes á radiación. Esta radioresistencia pode explicarse polas catro "R" da radiobioloxía: 1) Reparación do dano no ADN, 2) Repoboación das células tumorais, 3) Reosixenación do tumor, 4) Redistribución das células en distintas fases do ciclo celular. As alteracións na maquinaria de reparación do ADN nas células tumorais, poderían supoñer unha vantaxe, permitindo que estas células reparen os danos producidos pola radiación. A miúdo os tumores son diagnosticados cun volume relativamente grande, o que requiriría de doses altas de radiación para poder eliminar as células tumorais no interior do tumor, pero a dose está limitada pola tolerancia dos tecidos sans que rodean o tumor. Ademais, os tumores ben osixenados son mais sensibles á radiación debido á formación de especies reactivas de osíxeno, polo que os novos tumores, que con frecuencia ocorren nunha situación de hipoxia terían mais resistencia á radioterapia. Así mesmo, as células en mitose son mais sensibles á radiación que outras fases do ciclo celular.

A maiores, existen outros mecanismos xerais de resistencia a tratamentos antitumorais como a desregulación da apoptose, a activación da autofaxia, ou a activación de rutas de sinalización que promoven a proliferación celular.

Debido ás resistencias que se producen cos tratamentos antitumorais actuais, é necesario explorar novos tratamentos que permitan atallar esta problemática.

Dende fai anos as chaperonas ou proteínas de choque térmico, concretamente HSP90 foron vistas como unha posible diana terapéutica que permita atallar a progresión do cancro. As chaperonas son proteínas que aumentan a súa expresión cando a célula está sometida a condicións de estres (calor, hipoxia, falta de nutrientes, exposición a axentes químicos, etc) axudando á supervivencia celular. As células cancerosas existen nun constante estado de estrés celular pola rápida proliferación e o ambiente no que medran, polo que as proteínas de choque térmico atópanse a miúdo sobreexpresadas nos tecidos tumorais.

A importancia da proteína HSP90 atópase no feito de que moitas da proteínas importantes para a supervivencia celular usan esta chaperona como estabilizador durante o proceso de pregado proteico. Algunhas destas proteínas son AKT, RB1 ou p53, importantes moduladores do ciclo celular, entre outras moitas.

Neste traballo de investigación pretendemos explorar os efectos que a o tratamento con radiación ten nas células de cancro de pulmón de células non pequenas e dan lugar a maiores resistencias a este tratamento, ao mesmo tempo que investigamos a utilidade de bloquear a actividade da chaperona HSP90 para controlar a progresión tumoral.

Primeiramente quixemos estudar como se relaciona a radiación coa aparición de células nai tumorais. Para isto usamos dúas liñas celulares de cancro de pulmón de células non pequenas ben coñecidas, A549 e H460. Observamos que cando estas liñas celulares son tratadas con radiación, teñen maior capacidade de medrar en suspensión, unha característica propia de células nai tumorais. Ademais, analizamos a expresión de diferentes proteínas recoñecidas na literatura por ser marcadores de células nai tumorais. Así atopamos que as células previamente irradiadas que medran en suspensión teñen unha maior expresión de marcadores como CD24 e CD44 en ambas liñas e de CD166 en A549, ademais de factores de transcrición chave para a células nai tumorais como β-catenina, OCT4 e SOX2. Ao mesmo tempo, as mesmas poboacións de células expresan marcadores de transición epitelio-mesénquima como SNAIL,

TWIST, Vimentina ou Caderina N. Así mesmo, as células que sobreviviron ao tratamento con radiación presentan unha maior expresión de receptores celulares asociados á resistencia a tratamentos tumorais como CXCR4, importante no mantemento das propiedades das células nai; e PDGFR-β, que activa unha ruta de sinalización celular que promove a transición epitelio-mesénquima.

Ao observar este incremento en expresión do receptor PDGFR-β, que é un receptor tirosin-quinasa, quixemos avaliar os efectos de combinar a radioterapia xunto con coñecidos inhibidores de receptores tirosin-quinasa, Axitinib e Dasatinib. Os resultados demostraron que estes inhibidores aumentan a letalidade producida pola radioterapia *in vitro.* A pesar destes prometedores resultados, é sabido que este tipo de inhibidores dan lugar a resistencias en doentes inducindo a aparición de mutacións nas proteínas diana. Por este motivo, consideramos apropiado avaliar a viabilidade como tratamento da inhibición da actividade da proteína de choque térmico HSP90. Para isto usamos o inhibidor Ganetespib (STA-9090) como ferramenta para bloquear a acción de dita chaperona.

O primeiro paso foi avaliar os efectos antiproliferativos da droga Ganetespib en liñas celulares de cancro de pulmón de células non-pequenas. Observamos que esta droga inhibe a proliferación celular de liñas celulares ben estudadas como A549 e dúas liñas derivadas de tumores de doentes (T2821 e T2851). Estes efectos ocorren tanto en células medrando como monocapa, e dicir en condicións de adhesión, como en esferas tumorais derivadas das mesmas liñas celulares, é dicir, células medrando en suspensión. Incluso, o inhibidor de actividade de HSP90 foi capaz de limitar a mobilidade celular das tres liñas celulares nun ensaio de peche de ferida.

Para descartar que os efectos do Ganetespib na capacidade migratoria fosen debidos a procesos de apoptose, realizouse unha tinción con anexina V, a cal se une á fosfatidilserina na membrana das células que comezan o proceso de apoptose. Non observamos que a doses e tempos similares ós utilizados nos ensaios de migración se producise un aumento significativo da proporción de células apoptóticas. Sen embargo, ao analizar o estadio do ciclo celular no que se atopan as células tratadas, atopamos que se produce un bloqueo do ciclo na fase G2.

Por outra banda, estudamos a posibilidade de que as células estivesen entrando nun proceso de autofaxia, analizando a expresión de LC3 e HMGB1 dous dias despois de cultivar as células con diferentes doses de Ganestespib. Só se observou un aumento da expresión de ambos marcadores a altas doses (300nM). O mesmo sucedeu ca análise da expresión de p53, p21 e p27, que se acumulan cando as células son tratadas a altas doses de Ganestespib. Sen embargo, ao analizar o tamaño celular despois de cinco dias de exposición ao tratamento, vemos que o tamaño nuclear aumenta significativamente, incluso a doses cen veces mais baixas (3nM). Como estes datos son indicativos de que as células poden estar entrando en senescencia, realizamos unha tinción de β-galactosidasa. Con este ensaio vimos que o número de células tinguidas aumenta ao ser expostas a Ganetespib, e que o efecto aumenta co aumento da dose.

Unha vez establecido que a inhibición da actividade de HSP90 con Ganetespib ten efectos antiproliferativos, o seguinte paso era comprobar se a combinación deste tratamento coa radiación supón algunha mellora nos efectos desta última. En primeiro lugar, analizamos a expresión de HSP90 nas liñas celulares logo de que foron expostas a diferentes doses de radiación para asegurar de que a expresión da proteína non desaparece co tratamento. Logo, comprobamos que a tempos de ata 72h, obsérvase un efecto aditivo da radiación co Ganetespib nas tres liñas celulares (A549, T2821, T2851).

Seguidamente, comprobamos mediante un ensaio de colonias o diferente efecto de usar Ganetespib como pre- ou post-tratamento á radiación. Aínda que nos dous casos se observa un potenciamento do efecto inhibitorio da radiación, a inhibición da actividade de HSP90 24h

antes da irradiación ten efectos moito mais dramáticos á hora de reducir a capacidade de producir colonias das liñas celulares. Estes datos poderían suxerir que o bloqueo da actividade de HSP90 afecta á capacidade de reparación de ADN das células tratadas. Para comprobar esta hipótese, estudamos o efecto de Ganetespib sobre os focos de reparación de ADN producidos despois da exposición a radiación. Nestes experimentos, observamos que Ganetespib por si só non induce a formación de focos de reparación de ADN, medidos polo número medio de focos de pH2AX. Sen embargo, Ganetespib produce un retardo na resolución dos focos de pH2AX xerados tras a radiación. Ademais, observamos que a inhibición de HSP90 reduce a fosforilación inducida pola radiación de proteínas importantes no proceso de reparación do ADN como ATM e ATR.

Os datos obtidos ata o momento son consecuentes coa formación de "DNA-SCARS" (segmentos de ADN con alteracións da cromatina que reforzan a senescencia, polas súas siglas en inglés). Para corroborar isto, analizamos o efecto de Ganestespib en combinación con radiación na resolución dos puntos de reparación de ADN medindo os focos de pH2AX, pATM e 53BP1. Despois de cinco días da exposición á radiación o número de focos de pH2AX e pATM foron significativamente mais altos que nas células que non foron expostas a Ganetespib. Ademais, a tinción destas mesmas mostras para β-galactosidasa revelou que o número de células con tinción positiva era maior nas células que foron sometidas ao dobre tratamento.

Vistos todos os resultados expostos anteriormente, consideramos oportuno comprobar que estes efectos eran trasladables a unha poboación de células radioresistentes. Para realizar estes ensaios, xeramos liñas radioresistentes a partir das liñas derivadas de doentes por exposición continuada a radiación e nomeáronse como T2821/R e T2851/R, derivadas das liñas parentais coa mesma numeración.

En primeiro lugar observamos cambios morfolóxicos nas liñas celulares radioresistentes comparadas coas súas parentais. As células resistentes presentaban unha morfoloxía alargada que parecía ser indicativo dun proceso de transición epitelio-mesénquima, o que puidemos corroborar analizando a expresión de xenes asociados a este proceso. Así comprobamos que a expresión de xenes como SNAIL, Vimentina ou a Fibronectina estaba incrementada en comparación coas liñas parentais. A maiores, atopamos incrementados nas liñas celulares resistentes os niveis de pAKT, a expresión de CXCR4 e o seu ligando, SDF1, xunto con outros factores de crecemento coma PDGFB e IL-6, suxerindo a posibilidade da existencia dun bucle de sinalización proliferativa autocrina ou paracrina.

Tamén estudamos o estado e resposta dos mecanismos de reparación do ADN nestas liñas celulares resistentes. Curiosamente, ao realizar unha análise da expresión de xenes involucrados neste mecanismo, atopamos alteracións na expresión en ambas liñas celulares comparadas coas súas respectivas liñas parentais, pero ao mesmo tempo cada unha con diferentes alteracións. Mentres que T2851/R só mostrou cambios en xenes asociados coa reparación de roturas da dobre hélice, T2821/R exhibiu alteracións nos mecanismos de reparación de excisión de bases e de nucleótidos ademais das roturas da dobre hélice. Estas alteracións na expresión xénica indican que estas liñas radioresistentes teñen alteracións importantes na maquinaria responsable da reparación do ADN. Hipótese que analizamos seguindo a resolución dos focos de reparación do ADN logo de ser sometidas a radiación. Os focos de pH2AX e 53BP1 formados tras o tratamento con radiación foron significativamente menores nas células resistentes que nas respectivas liñas parentais. Do mesmo xeito, a fosforilación dos efectores da reparación do ADN, ATM e ATR , foi significativamente menor nas liñas resistentes.

Unha vez caracterizadas as liñas celulares resistentes a radiación verificamos que estas células non perderan a expresión da chaperona HSP90 e que o inhibidor Ganetespib tiña os

efectos antiproliferativos observados previamente. Ademais, o tratamento con Ganetespib foi capaz de inhibir a mobilidade celular das células resistentes como xa viramos en experimentos anteriores coas liñas parentais. Do mesmo xeito, o tratamento co inhibidor é capaz de reducir a fosforilación de AKT, importante efector da ruta de sinalización PI3K/AKT que induce a proliferación celular. Ao mesmo tempo, observamos que a inhibición da actividade da chaperona HSP90 reduce a produción da interleuquina IL-6, limitando a posible sinalización autocrina ou paracrina que podería estar axudando á supervivencia celular.

Cando avaliamos os efectos da inhibición de HSP90 en combinación coa radiación, observamos que en ambas liñas parentais, T2821 e T2851, practicamente perden a capacidade de formar clons cando son tratadas con doses de 4 Gy de radiación e 3nM de Ganetespib. Estes efectos aditivos do inhibidor xunto cos efectos da radiación mantéñense no caso das liñas resistentes, aínda que para conseguir a total eliminación dos clons, a dose de Ganetespib foi de 6 nM en combinación con 4 Gy de radiación.

Por último, quixemos avaliar a combinación de radiación e a inhibición de HSP90 nun modelo *in vivo*. Con este obxectivo, establecéronse tumores subcutáneos da liña T2821 en ratos inmunodeprimidos e foron divididos en catro grupos de tratamento (vehículo, radiación, Ganetespib e Ganetespib en combinación con radiación). Os ratos foron inoculados co inhibidor dous dias antes de recibir unha única dose de radiación localizada de 5 Gy e os tratamentos de Ganetespib continuaron dúas veces por semana ata a fin do experimento. A análise do volume individualizado dos tumores demostrou que o tratamento en combinación do inhibidor coa radiación é mais efectivo ca calquera dos dous tratamentos como monoterapia, en reducir o crecemento dos tumores.

Unha vez concluído o experimento, seccións histolóxicas dos tumores foron tinguidas para β-catenina, xa que esta é parte importante da ruta WNT e polo tanto pode ser indicativo da presenza de células con características de células nai tumorais ou células que están nun proceso de transición epitelio-mesénquima. As análises mostraron que a radiación aumenta a expresión de β-catenina e que o tratamento con Ganetespib é capaz de reducir a expresión desta proteína inducida nos tumores pola radiación.

Os nosos resultados demostran que as células de cancro de pulmón de células non pequenas que sobreviviron á radiación presentan características de células nai tumorais, como a incrementada capacidade de medrar en suspensión, sobreexpresan marcadores de células nai tumorais (como son CD44 e CD166) e factores de transcrición asociados a células nai como OCT4, SOX2 ou β-catenina. Ademais, as mesmas poboacións de células expresan marcadores de transición epitelio-mesénquima como SNAIL, TWIST, Vimentina ou Caderina N.

Cos datos expostos neste documento demostramos que as células que sobreviviron á radiación poden ser eliminadas mediante a inhibición da actividade de HSP90, unha chaperona involucrada no pregado e estabilización de moitas proteínas de cascadas de sinalización esenciais para a célula. A inhibición de HSP90, combinada coa radiación, diminuíu a proliferación celular mediante a desregulación dos mecanismos de reparación do ADN, abocando ás células á parada do ciclo celular e á senescencia, ao mesmo tempo que bloquea rutas de sinalización que promoven a proliferación, como é PI3K/AKT, e inhibe a produción de citoquinas e factores de crecemento que poden estar xerando unha sinalización autocrina ou paracrina.

Mediante a comparación de células radioresistentes coas súas parentais, provamos que as células radioresistentes manteñen a expresión de xenes asociados á transición epitelio-mesénquima, maquinaria de reparación do ADN alterada e activación da sinalización que activa a proliferación.

Os nosos datos demostran, en modelos *in vivo*, que o bloqueo da chaperona HSP90 en combinación con radioterapia inhibe o crecemento tumoral e que a inhibición de HSP90 reduce a expresión de marcadores de células nai tumorais e transición epitelio-mesénquima como demostra a redución en expresión de β-catenina nos tumores tratados.

Lung cancer is responsible for more than 20% of cancer deaths worldwide and non-small cell lung cancer is the cause of 85% of fatalities in patients diagnosed with lung cancer. The five-year survival in patients with early diagnosis is around 50%, however, most cases are diagnosed in advanced stages, and the survival does not exceed 4%. The median age of diagnosis of lung cancer is 70 years, being a rare disease in people under fifty.

The onset of lung cancer may be associated with different causes such as hereditary or gene alterations caused by exposure to environmental factors (environmental pollution, radon, or asbestos) and poor dietary habits. Undoubtedly, the main cause associated with the onset of lung cancer is the inhalation of tobacco smoke. A smoker may be up to ten times more likely to suffer from lung cancer than a non-smoker.

The treatment options available to treat lung cancer today are broader than years ago, mainly due to advances in cancer genomic studies. Despite this, surgery, radiation therapy, and chemotherapy remain the three pillars of lung cancer treatment. In recent years, new therapies have been developed such as tyrosine kinase inhibitors, which are used in tumors with specific gene alterations; or more recently, immunotherapy.

Despite these advances, recurrences are common in patients who have been treated for lung cancer. In these cases, it is usual that the new tumors display resistance to treatments, which makes it much more complicated to handle the disease.

One possible explanation for these resistances arises with the emergence of the concept of tumor stem cells. These cells constitute a subpopulation in the tumor with particular characteristics, such as the greatest ability to survive the tumor treatments, thereby treatments eliminate the bulk of the tumor cells leaving behind the tumor stem cells. Tumor stem cells can establish a new tumor, but also acquire the ability to separate the original tumor, mobilize and establish themselves in a new niche, leading to metastasis. This process by which a cell acquires the ability to migrate is known as the epithelial-mesenchymal transition, a process that begins with the expression of different transcription factors such as SNAIL1, TWIST, SLUG, or ZEB1, producing repression of the expression of epithelial-associated genes such as E- cadherin or cytokeratins while other genes characteristic of mesenchymal lineages, such as N-cadherin or fibronectin, are expressed. These changes in gene expression result in loss of basal-lateral polarity and cell to cell junctions enhancing the migratory and invasive capacity of tumor cells. In addition, these changes in the expression pattern of genes can produce major changes in cell physiology that allow them to adjust to new conditions. In recent years the distinction between stem cells and tumor cells that undergo a process of epithelial-mesenchymal transition became fuzzy because numerous research suggests that this is a process where cancer stem cells exist in a balance of variable expression of epithelial and mesenchymal features. This plasticity of tumor stem cells would provide a survival advantage to different antitumor treatments.

Extensive studies in the field of resistance to different therapies have provided some answers on how tumor cells might be evading the antiproliferative effects of tumor therapies. In the case of chemotherapeutics, overexpression of ABC transporters, which can secrete compounds into the extracellular space decreasing their effectiveness; changes in the response to DNA damage, which can result in resistance to drugs that work by causing damage to cellular DNA; or in the case of tyrosine kinase receptor inhibitors, it is common for mutations in the target proteins to occur that cause the treatment to have no effect.

One of the main tools in treating lung cancer remains to be radiation that despite its effectiveness, recurrences often appear with tumors that become radiation-resistant. This radioresistance can be explained by the four “Rs” of radiobiology: 1) Repair of DNA damage, 2) Repopulation of tumor cells, 3) Reoxygenation of the tumor, 4) Redistribution of cells in different phases of the cell cycle. Alterations in the DNA repair machinery in tumor cells could

be an advantage, allowing these cells to repair the damage caused by radiation. Many of the tumors are diagnosed with a relatively large volume, which requires high doses of radiation to eliminate tumor cells within the tumor, but the dose is limited by the tolerance of the healthy tissue surrounding the tumor. In addition, tumors well-oxygenated are more sensitive to radiation due to the formation of reactive oxygen species, so that new tumors, which frequently occur in hypoxic environments have higher resistance to radiotherapy. Moreover, cells in mitosis are more sensitive to radiation than other phases of the cell cycle.

Besides, other mechanisms generate resistance to cancer treatments such as deregulation of apoptosis, the activation of autophagy, or activation of signaling pathways that promotes cell proliferation.

Due to the resistance that occurs with current tumor treatments, it is necessary to explore new therapies to address this problem.

Since years ago chaperones or heat shock proteins, specifically HSP90, were seen as a possible target therapy that allows tackling cancer progression. Chaperones are proteins that boost their expression when the cell is under conditions of stress (heat, hypoxia, starvation, exposure to chemicals, etc.) helping the cell survive. Cancer cells exist in a constant state of cellular stress due to their rapid proliferation and the environment in which they grow, so the heat shock proteins are often over-expressed in tumor tissues.

The importance of the HSP90 protein lies in the fact that many of the proteins important for cell survival use this chaperone as a stabilizer during the protein folding process. Some of these proteins are AKT, RB1, or p53, important cell cycle modulators, among many others.

In this research work, we aim to explore the effects that radiation treatment has on non-small cell lung cancer cells and lead to increased resistance to this treatment, and at the same time, we investigate the usefulness of blocking the activity of chaperone HSP90 to control tumor progression.

We first wanted to study how radiation relates to the appearance of tumor stem cells. For this, we used two well-known non-small cell lung cancer cell lines, A549 and H460. We observe that when these cell lines are treated with radiation, they have a greater ability to grow in suspension, a characteristic of tumor stem cells. In addition, we analyzed the expression of different proteins recognized in the literature to be markers of cancer stem cells. Thus we found that previously irradiated cells growing in suspension have higher expression of markers such as CD24 and CD44 in both lines and of CD166 in A549, in addition to key transcription factors for cancer stem cells such as β-catenin, OCT4, and SOX2. At the same time, the same cell populations express epithelial-mesenchymal transition markers such as SNAIL, TWIST, Vimentin, or N-cadherin. Likewise, cells that have survived radiation treatment exhibit higher expression of associated cellular receptors linked to resistance to tumor treatments such as CXCR4, important in maintaining the properties of stem cells; and PDGFR-β, which activates a cellular signaling pathway that promotes the epithelial-mesenchymal transition.

By observing this increase in expression of the PDGFR-β receptor, which is a tyrosine-kinase receptor, we wanted to evaluate the effects of combining radiotherapy along with known tyrosine kinase receptor inhibitors, Axitinib and Dasatinib. The results showed that these inhibitors increase the lethality produced by radiotherapy *in vitro.* Despite these promising results, it is known that these types of inhibitors result in resistance in patients by inducing the appearance of mutations in the target proteins. For this reason, we considered it appropriate to assess the feasibility of treatment by inhibiting the activity of heat shock protein HSP90. For

this, we used the inhibitor Ganetespib (STA-9090) as a tool to block the action of this chaperone.

The first step was to evaluate the antiproliferative effects of the drug Ganetespib on non-small cell lung cancer cell lines. We observed that this drug inhibits cell proliferation of well-studied cell lines such as A549 and two patient-derived lines (T2821 and T2851). These effects occur both in cells growing as a monolayer, i.e. under adhesion conditions, as well as in tumorspheres derived from the same cell lines, i.e., cells growing in suspension. Moreover, the inhibitory activity of HSP90 was able to limit the motility of the three cell lines in a wound assay.

To rule out that the effects of Ganetespib on the migratory capacity were due to processes of apoptosis, we performed an annexin V staining, which binds to phosphatidylserine on the membrane of cells that begin the process of apoptosis. There we noted that at doses and times similar to those used in the migration assays there was not a significant increase in the proportion of apoptotic cells. However, when analyzing the phase of the cell cycle in treated cells, we found that a blockage occurs in the G2 phase of the cell cycle.

On the other hand, we studied the possibility that the cells were entering an autophagy process, analyzing the expression of LC3 and HMGB1 two days after culturing the cells with different doses of Ganestespib. Only an increase in the expression of both markers was observed at high doses (300nM). The same was true for analysis of p53, p21, and p27 expression, which accumulated when cells were treated at high doses of Ganestespib. However, when analyzing cell size after five days of treatment exposure, we saw that nuclear size increases significantly, even at doses one hundred times lower (3nM). As these data are indicative that cells may be entering senescence, we performed a β-galactosidase staining. With this test, we saw that the number of stained cells increased when exposed to Ganetespib and that the effect increases with increasing dose.

Once we established that inhibition of HSP90 activity with Ganetespib has antiproliferative effects, the next step was to test whether the combination of this treatment with radiation involves some improvement in the effects of the latter. First, we analyzed the expression of HSP90 in cell lines after they were exposed to different doses of radiation to ensure that the protein expression does not disappear with the treatment. Then we observed that at 72h, Ganetespib has an additive effect on radiation in the three cell lines (A549, T2821, T2851).

Next, we verified by a colony formation assay the effect of using Ganetespib as pre- or post-treatment to radiation. Although in both cases a potentiation of the radiation inhibitory effect is observed, inhibition of HSP90 activity 24h before irradiation has much more dramatic effects in reducing the ability of cell lines to produce colonies. These data could suggest that blocking HSP90 activity affects the DNA repair ability of treated cells. To test this hypothesis, we studied the effect of Ganetespib on DNA repair foci produced after radiation exposure. In these experiments, we found that Ganetespib by itself does not induce the formation of pockets of DNA repair, measured by pH2AX foci. However, Ganetespib produces a delay in resolving pH2AX foci generated after radiation. Furthermore, we observed that inhibition of HSP90 reduces radiation-induced phosphorylation of important proteins in the DNA repair process such as ATM and ATR.

The data obtained so far are consistent with the formation of "DNA-SCARS" (DNA segments with chromatin alterations reinforcing senescence). To corroborate this, we analyzed the effect of Ganestespib in combination with radiation on the resolution of DNA repair by measuring foci of pH2AX, pATM, and 53BP1. After five days of radiation exposure, the number of pH2AX foci were significantly higher than in cells that were not exposed to

Ganetespib. In addition, staining of these same samples for β-galactosidase revealed that the number of cells with positive staining was higher in cells that underwent double treatment.

With all the results presented above, we considered it appropriate to check that those results were transferable to a population of radioresistant cells. To test this, we generated radioresistant lines from the patient-derived cell lines by continued exposure to radiation and were named T2821/R and T2851/R, derived from the parental lines with the same numbering.

We first observed morphological changes in the radioresistant cell lines compared with their parental. Resistant cells had an elongated morphology that appeared to be indicative of an epithelial-mesenchymal transition, which we were able to corroborate by analyzing the expression of genes associated with this process. Thus we found that the expression of genes such as SNAIL, Vimentin, or Fibronectin were increased compared to the parental lines. In addition, we found increased levels of pAKT, CXCR4 expression, and its ligand, SDF1, in resistant cell lines, along with other growth factors such as PDGFB and IL-6, suggesting the possibility of an autocrine or paracrine signaling loop.

We also studied the status and response of DNA repair mechanisms in these resistant cell lines. Interestingly, when performing an analysis of the expression of genes involved in this mechanism, we found alterations in expression in both cell lines compared to their respective parental lines, but at the same time each with different alterations. While T2851/R only showed changes in genes associated with double-strand break repair, T2821/R exhibited changes also in the mechanisms of base and nucleotide excision repair. These alterations in gene expression indicate that these radioresistant lines have important alterations in the machinery responsible for DNA repair. We analyzed this hypothesis by following the resolution of DNA repair foci after being subjected to radiation. The foci of pH2AX and 53BP1 formed after radiation treatment were significantly lower in the resistant cells than in the respective parental lines. Similarly, phosphorylation of DNA repair effectors ATM, and ATR, was significantly less in resistant lines.

Once the radioresistant cell lines were characterized we verified that these cells had not lost the expression of HSP90 chaperone and that the inhibitor Ganetespib had the antiproliferative effects observed previously. In addition, treatment with Ganetespib was able to inhibit cellular mobility of resistant cells as we had seen in previous experiments with parental lines. In the same way, treatment with the inhibitor can reduce the phosphorylation of AKT, an important effector of the n PI3K/AKT signaling pathway that induces cell proliferation. At the same time, we observed that inhibition of chaperone HSP90 activity reduces IL-6 interleukin production, limiting possible autocrine or paracrine signaling that could be aiding cell survival.

When we evaluated the effects of HSP90 inhibition in combination with radiation, we observed that in both parental lines, T2821 and T2851, they virtually lose the ability to form clones when treated with doses of 4 Gy of radiation and 3nM of Ganetespib. These additive effects of the inhibitor along with the effects of radiation are maintained in the case of resistant lines, although to achieve complete elimination of the clones, the dose of Ganetespib was 6 nM in combination with 4 Gy of radiation.

Finally, we wanted to evaluate the combination of radiation and the HSP90 inhibition in an *in vivo* model. With this aim, subcutaneous tumors of T2821 were established in immunocompromised mice and were divided into four treatment groups (vehicle, radiation, Ganetespib, and Ganetespib in combination with radiation). The mice were inoculated with the inhibitor two days before receiving a single dose of localized radiation of 5 Gy, and treatments with Ganetespib continued two times a week until the end of the experiment. Analysis of

individualized tumor volume showed that the combination of the inhibitor with radiation is more effective than either treatment as monotherapy in reducing tumor growth.

Once the experiment concluded, histologic sections of the tumors were stained for β-catenin, since that it is an important part of the WNT pathway and therefore may indicate the presence of cells with stem cell characteristics or tumor cells that are in an epithelial-mesenchymal transition. The analysis shown that radiation increases the expression of β-catenin and that treatment with Ganetespib can reduce the expression of this protein induced in tumors by radiation.

Our results show that non-small cell lung cancer cells that have survived radiation exhibit cancer stem cell traits, like the increased ability to grow in attachment independent conditions, and overexpress stem markers (such as CD44 and CD166) and stem related transcription factors, like OCT4, SOX2, or β-catenin. Moreover, the same populations expressed epithelial to mesenchymal transition markers, such as SNAIL, TWIST, Vimentin, or N-cadherin.

With the data exposed in this document, we showed that cells that survived radiation can be eliminated by inhibiting HSP90, a chaperone involved in the correct folding and stabilization of many essential signaling pathway proteins. The inhibition of HSP90, combined with radiation, impaired tumor cell growth by dysregulating DNA damage repair pathways, leading the cells to cell cycle arrest and senescence at the same time that switches off important prosurvival signaling such as PI3K/AKT.

By comparing radioresistant cell lines with their parental ones, we proved that radioresistant cell lines maintain expression of epithelial to mesenchymal associated genes, altered DNA repair machinery, and activated prosurvival signaling like PI3K/AKT.

Our data also proves, *in vivo*, that blocking the chaperone HSP90 in combination with radiation therapy inhibits tumor growth and that HSP90 inhibition can abrogate the expression of cancer stem cells and epithelial to mesenchymal markers as seen by the reduction of β-catenin expression on treated xenografted tumors.

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Introduction

1. Epidemiology of lung cancer

Lung cancer represents the leading cause of cancer death worldwide accounting for a quarter of all fatalities. It has been the most common form of cancer since 1985, and it is estimated that it will continue to be one of the more prevalent cancers globally. Patients with early diagnoses have a five year survival rate of around 50%, however, in most cases, lung cancer is not detected until the disease is already advanced. In its advanced stages, the five year survival rate falls below 4% [1].

Lung cancer is relatively rare before the age of 50, but the risk increases with every age thereafter due to biologic factors like DNA alterations and environmental elements[2]. Consequently, the median age for lung cancer diagnoses is 70 years old for both men and women[3].

Smoking is the most common exposure related to lung cancer, causing more than 80% of the cases. An average smoker can have ten times more risk of being diagnosed with lung cancer than a non-smoker, and the risk increases proportionately with the amount of tobacco used. Cigarette use became extremely popular after World War I, and it wasn´t until the 60s that tobacco use was linked with an increased incidence of lung cancer and other respiratory diseases. Since then, the incidence of smoking has been slowly decreasing as a result of tobacco control initiatives, which led to a decrease in deaths due to lung cancer for men and women[1,4].

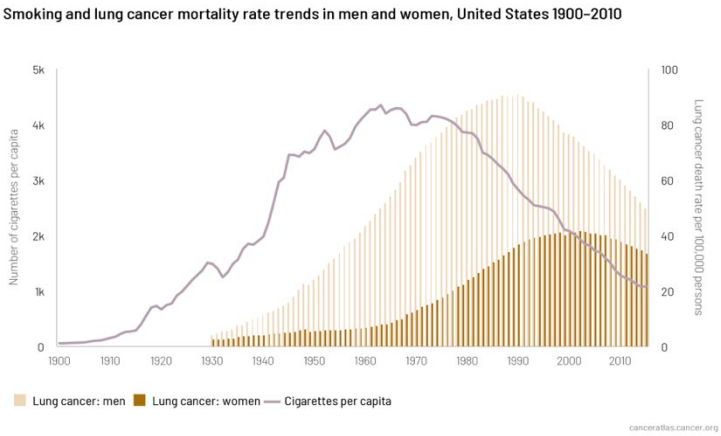

Figure 1. Lung cancer deaths and cigarettes use in USA 1900-2010.(Reproduced with permission of IARC Publications)[5].

Apart from the use of tobacco products, other exposure risks have been linked to increased risk of developing lung cancer like exposure to radon, asbestos, or air pollution, among others, but these represent a small proportion compared to the cases attributable to smoking. Nevertheless, only 15% of smokers end up developing lung cancer, suggesting a genetic susceptibility to the disease. In fact, genome association studies have identified chromosome regions associated with increased risk of lung cancer, and nowadays we know tumor driver mutations in different cell signaling pathways which govern cell proliferation or cell death that provide a susceptibility to developing a tumor[3,6–8].

2. Diagnosis of lung cancer

The most common clinical manifestations in lung cancer patients are cough, chest pain, hemoptysis, dyspnea, and other more unspecific symptoms like weight loss or fatigue [2,9]. However, symptoms are rare in the early stages of the disease allowing the tumor to develop silently, and in most cases the first clinical manifestations that the patient experiences are a result of metastasis[9].

A few clinical studies have assessed the potential benefit of lung cancer screenings in the general population using chest radiography, computerized tomography, or sputum cytology. Despite finding a higher number of resectable lung tumor cases in the population, none of the studies demonstrated an increase in survivability. For this reason, there is no operating screening program for lung cancer in the general population and most diagnoses are made after doing a chest X-ray or CT scan due to suspicious symptoms or sometimes for unrelated clinical reasons.

There are a few techniques that help physicians diagnose and determine the stage of tumors of the lung:

- Chest radiography

X-ray of the chest is the most commonly used imaging technique to diagnose tumors of the lung because of its simplicity, low-cost, and widespread availability. Chest radiography can be used to differentiate benign and cancerous growths in the lung by assessing the tumor doubling time. It can also help with the staging of the tumor by detecting pleural effusion and in some cases extrapulmonary spread[10,11].

- Computerized tomography (CT)

The CT scan uses a series of X-ray images to form cross-sectioned images of the body that can be processed to render a three-dimensional image of the inside of the body. CTs allow for evaluation of the primary tumor, mediastinal lymph nodes, and to detect distant metastasis[11,12].

- Positron emission tomography (PET)

A PET scan is an imaging technique that uses radioisotopes to visualize metabolic pathways in the tissues. Flourodeoxyglucose is commonly used in oncology for PET imaging. Cancerous cells have higher glucose metabolism than normal cells, thus this modified D-glucose molecule is trapped inside the cells at a level proportionate to the glucose metabolic rate, allowing for the detection of accumulated radioisotopes. PET scans are more sensitive detecting lymph node invasion and distal metastasis than CT scans[10,11].

- PET/CT scan

Nowadays, PET is combined with CT to enhance the sensibility of the imaging. PET provides metabolic information and CT gives anatomical information. This allows for detection of lesions not detected by either of the independent techniques, precise localization and delineation of the lesions, and better characterization [10,11].

Apart from imaging techniques, sampling of the tumor is important to allow for accurate diagnosis of the disease. The most common techniques used to collect biopsies are:

- Transbronchial needle aspiration (TBNA):
  The sample is collected with a beveled needle inserted in the channel of the bronchoscope[10,13].

- Endobronchial ultrasound needle aspiration:
  To solve the problem of blinded sampling in TBNA, an ultrasound transducer is integrated into the bronchoscope. In this way, the sample can be collected by direct imaging of the needle once it penetrates the tissue. On occasion, this same technique is performed through the esophageal wall to access certain lymph nodes, and this is called transesophageal ultrasound needle aspiration[10,13].

- Thoracentesis:
  A needle is inserted in the pleural space through the chest wall to sample the pleural fluid[13].

- Thoracotomy:
  An incision is made in the intercostal space to access the chest cavity and collect a sample[13].

3. Classification of lung cancer.

3.1. Histological classification

Lung cancers have been traditionally divided into two main groups according to the histological appearance of the tumor cells: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).

- Non-small cell lung cancer (NSCLC)
  Non-small cell lung cancer accounts for 80 to 85% of patients with lung cancer. This group includes three major subtypes: adenocarcinomas, squamous cell carcinomas, and large cell carcinomas. Even though the cellular origin of these subtypes is different, they are grouped together because the treatment and prognosis are often similar[14,15]

  - Adenocarcinoma: Is the most common subtype of NSCLC representing 40% of lung cancers and 60% of the NSCLC. Adenocarcinomas develop from transformed alveolar cells in the small airway epithelium and are characterized by the production of mucin. Thyroid transcription factor (TTF-1) and Napsin A are immunohistochemical markers used to identify adenocarcinomas. Many subtypes of adenocarcinoma have been defined by the World Health Organization based on pathology and clinical features of the tumors[14,16,17].

  - Squamous cell carcinoma (SCC): represents around 20% of the lung tumors. These are epithelial tumors that usually appear in the central part of the lung, in the bronchial epithelium. They are characterized by the keratinization of the cells and intercellular bridges[15–17]. When a morphological differentiation of the tumor is not possible, markers like cytokeratin 5 and 6, p40, and desmoglein-3 are used to help confirm the diagnosis [18].

- Large cell carcinoma: Represents around 9% of lung cancers. These tumors are undifferentiated NSCLC tumors that lack any of the immunophenotypic characteristics of adenocarcinomas, SCCs, or neuroendocrine tumors. Large cell carcinomas are composed of big cells with ample cytoplasm and large nuclei[14–17].

- Small cell lung cancer (SCLC).

Small cell lung cancers represent around 15% of lung tumors. These epithelial tumors are characterized by small cells with scant cytoplasm, showing a high mitotic rate and undefined cell borders. SCLC is a highly aggressive disease and patients are often diagnosed with advanced metastasis[15,16].

The World Health Organization has established a more detailed and complex histological classification of lung tumors using the acquired knowledge gained from years of clinical practice, allowing for better management of the diseases. There are many more types and subtypes of lung cancer, however many of those have low prevalence in the population.

Adenocarcinoma	◦ Lepidic adenocarcinoma ◦ Acinar adenocarcinoma ◦ Papillary adenocarcinoma ◦ Micropapillary adenocarcinoma ◦ Solid adenocarcinoma ◦ Invasive mucinous adenocarcinoma ◦ Colloid adenocarcinoma ◦ Fetal adenocarcinoma ◦ Enteric adenocarcinoma ◦ Minimally invasive adenocarcinoma ◦ Adenocarcinoma in situ
Squamous cell carcinoma	◦ Keratinizing squamous cell carcinoma ◦ Nonkeratinizing squamous cell carcinoma ◦ Basaloid squamous cell carcinoma ◦ Squamous cell carcinoma in situ
Neuroendocrine tumors	◦ Small cell carcinoma ◦ Large cell neuroendocrine carcinoma ◦ Carcinoid tumors ◦ Preinvasive lesion

Table 1. Classification of epithelial tumors of the lung. (Modified from WHO classification of tumours of the lung, pleura, thymus and heart. 4th edition, 2015. With permission of IARC publications)[16]

3.2. Staging of lung cancer

- TNM staging

The classification of tumors based on anatomic disease extent is useful for appropriate treatment and prognosis. The TNM classification was developed and maintained by the Union for International Cancer Control (UICC) and it is used globally for the staging of cancers.

The TNM classification is an anatomical system used to record the primary and nodal extent and the presence of metastasis.

- T describes the status of the primary tumor.
- N describes the spread of the disease to regional lymph nodes.
- M describes the presence or absence of distal metastasis.

The numbers and letters accompanying the T, N, and M indicate the grade, with the higher numbers indicating the more advanced disease progression[19].

- Stage grouping

TNM staging provides a great deal of information, but in the clinical setting it is common to group the TNM stages in less detailed groups for simplification, and these are named: occult carcinoma; 0; IA; IB; IIA; IIB; IIIA; IIIB; IIIC; IVA; and IVB.

AJCC Stage	Stage grouping	Stage description
Occult cancer	**TX N0 M0**	The main tumor can't be assessed or its location can't be determined.
0	**Tis N0 M0**	The tumor has not invaded other lung tissues. The cancer has not spread
IA	**T1 N0 M0**	The tumor is no larger than 3 cm. The cancer has not spread to nearby lymph nodes or to distant parts of the body.
IB	**T2a N0 M0**	The tumor is between 3 and 4 cm. It might have spread to the pleura or is clogging the airways. The cancer has not spread.
IIA	**T2b N0 M0**	The tumor is between 4 cm and 5 cm.The cancer has not spread to nearby lymph nodes or to distant parts of the body.
IIB	**T1 N1 M0** **T2 N1 M0** **T3 N0 M0**	The tumor is up to 5 cm and has spread to lymph nodes within the lung. The cancer has not spread to distant parts of the body. Or has no lymph node invasion but has invaded other chest cavity structures.
IIIA	**T1 N2 M0** **T2 N2 M0** **T3 N1 M0** **T4 N0/N1 M0**	The tumor is up to 5 cm and has invaded lymph nodes in the center of the chest. Or it is between 5 and 7 cm with more than one tumor. Or there is invasion of chest structures.
IIIB	**T1 N3 M0** **T2 N3 M0** **T3 N2 M0** **T4 N2 M0**	The cancer is no larger than 5 cm and has spread to lymph nodes outside the chest or on the other side of the body. Or has spread to major chest structures.
IIIC	**T3 N3 M0** **T4 N3 M0**	The tumor is between 5 and 7 cm, has spread to lymph nodes outside the chest, there is more than one tumor, has invaded major structures in the chest, neck or the spine.
IVA	**Any T** **Any N** **M1a/M1b**	The cancer can be any size with or without lymph node invasion and has spread to the other lung or cancer cells are found in pleural effusion. Or has spread to a single tumor outside the chest.
IVB	**Any T** **Any N** **M1c**	The cancer can be any size but has spread as more than one tumor outside the chest.

Table 2. Stage grouping in lung cancer[20].

4. Molecular alterations in lung cancer

Since the completion of the human genome project, a list of genes has been implicated in the development and maintenance of different types of cancer. Prior to our knowledge of genomic alterations related to lung cancer, patients were indiscriminately assigned to chemotherapy treatments, rendering poor results and producing bad side effects in patients that in most cases are diagnosed with advanced stages of the disease and therefore already have a debilitated health status. Thus, the development of next-generation sequencing tools has made possible the analysis and identification of oncogenic genetic alterations (also known as "driver mutations"), which help clinicians identify a more efficient treatment path, expanding the field of precision cancer medicine[21,22].

NSCLC has been the catalyst in the process of identification of driver mutations based on the discovery of an increasing number of genetic alterations that are involved in the tumor development and treatment response. Some of the genetic alterations have been well established and studied, such as the Epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral

oncogene homolog (KRAS), and anaplastic lymphoma kinase (ALK) rearrangements. Many others are still arising from the multiple genomic studies done in patient samples[23,24].

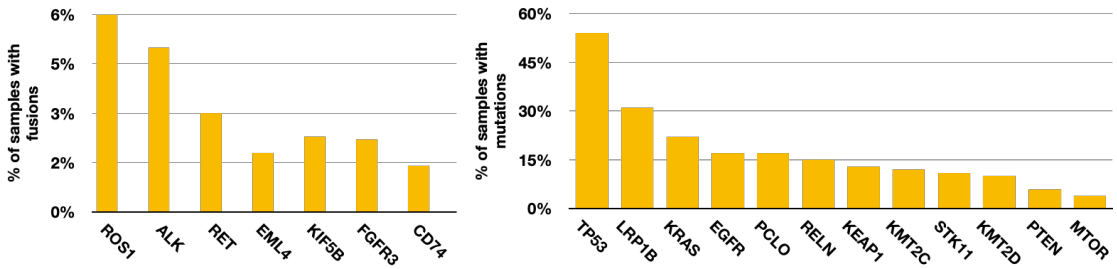

Figure 2. Genetic alterations in NSCLC. (A) Frequency of NSCLC samples with mutations in the most commonly altered genes. **(B)** Percentage of NSCLC samples with the most common fusion alterations. (Data extracted from The Cancer Genome Atlas)

4.1. Epidermal growth factor receptor (*EGFR*)

EGFR encodes a transmembrane tyrosine kinase with an extracellular binding domain and an intracellular tyrosine kinase domain[25]. Binding of the ligand induces signaling transduction through PI3K/AKT/mTOR, RAS/RAF/MAPK, and JAK/STAT signaling pathways playing an important role in cell proliferation, survival, differentiation, and invasion [26,27]. Mutations in the tyrosine kinase domain produce a constitutively active kinase domain which dysregulate cell proliferation [28,29]. This gene has many known mutations, but almost 90% of the ones occurring in NSCLC are in-frame deletions in exon 19 and a point mutation in exon 21 (L858R)[30]. The T790M mutation has been observed in close to 50% of patients that initially responded to treatment with tyrosine kinase inhibitors[31]. Other mechanisms of increased signaling through EGFR have been reported in lung cancer, such as protein overexpression or gene amplification[32,33].

4.2. Kirsten rat sarcoma viral oncogene homolog (*KRAS*)

KRAS is the most commonly mutated oncogene in adenocarcinomas of the lung. *KRAS* is part of the *RAS* proto-oncogenes and encodes a G-protein that plays a crucial part in signal transduction. Upstream activation of growth factor receptors induces a switch from GDP- to GTP-bound KRAS that produces the activation of RAS/RAF/MAPK or PI3K/AKT/mTOR signaling pathways regulating cell proliferation, differentiation, and survival [34,35]. Mutations that produce a constitutive activation of the KRAS protein avoid the need for upstream receptor activation. Alterations in this gene occurring in NSCLC are single amino acid substitutions in limited hotspots, the most common one being codon 12: G12C, G12V, and G12D, from most to least commonly occurring. Other less frequent sites of mutations like codons 13 and 61, have also been identified [36].

4.3. Anaplastic lymphoma kinase (*ALK*)

ALK is a tyrosine kinase receptor that belongs to the superfamily of insulin receptors. Alterations in this gene are frequent in lung adenocarcinomas, but not so much in other NSCLC[37]. The alterations that occur in the *ALK* gene are a result of chromosome rearrangements leading to a fusion oncogene of the tyrosine kinase domain of ALK and another

portion of a partner gene. The most common fusion oncogene is that produced by the fusion of the amino-terminal portion of the Echinoderm microtubule-associated protein-like 4 (EML4) and the tyrosine kinase domain of ALK (EML4-ALK) [38,39]. This fusion results in constitutive activation of the kinase domain and promotes cell proliferation through the activation of PI3K/AKT/mTOR, RAS/RAF/MAPK, and JAK/STAT signaling pathways [40,41].

4.4. v-Raf murine sarcoma viral oncogene homolog B (*BRAF*)

BRAF encodes a serine/threonine-protein kinase that is the downstream effector of KRAS and activates the MAPK pathway by phosphorylating MEK1 and MEK2. Mutations in BRAF produce increased kinase activity with effects on cell proliferation. Mutations in this gene occur in 3% of the NSCLC cases, almost always in adenocarcinomas, and can happen in the kinase domain or the G-loop of the activation domain. The most common mutation observed is V600E[42–44].

4.5. Epidermal growth factor receptor 2 (*HER2*)

The *HER2* gene encodes a membrane tyrosine kinase receptor belonging to the same family as EGFR. This receptor forms homodimers or heterodimers with other activated receptors of the same family. Alterations on this gene can happen by overexpression, gene amplification, or activating mutations, producing induction on PI3K/AKT/mTOR, RAS/RAF/MAPK, and JAK/STAT signaling pathways. HER2 overexpression appears in 20% of NSCLC cases, with other modifications being less frequent[45,46].

4.6. Hepatocyte grow factor receptor (*MET*)

MET encodes a tyrosine kinase receptor for the hepatocyte growth factor (HGF). After binding of the ligand, the receptor homodimerizes and activates the kinase function, producing the activation of ERK/MEK/MAPK and PI3K/AKT signaling pathways. *MET* is amplified in 1-7% of NSCLC cases[47]. It has been proven that *MET* amplification is associated with constitutive activation of the kinase domain and activation of the PI3K/AKT signaling pathway[48,49]. Mutations in this gene have been also reported, but only in around 3% of lung adenocarcinomas[50].

4.7. Tumor protein 53 (*TP53*)

TP53 encodes a nuclear phosphoprotein that identifies and binds DNA damaged regions and induce cell cycle arrest to undertake a DNA repair process or lead the cell to apoptosis. Alterations on *TP53* are one of the most common genetic alterations in human cancers, usually by hemizygous deletion of the chromosomal region 17p13, and the second allele altered most often by inactivating mutation[51]. These mutations are mostly located in the DNA binding domain of the protein[52]. Inactivation of TP53 is an early event in the development of NSLC that can coexist with other driver mutations and happens in about 65% of the NSCLC tumors[53,54].

4.8. Cyclin-dependent kinase inhibitor 2A ($p16^{INK4a}$)

$p16^{INK4a}$ inhibits the phosphorylation of RB1, one of the first tumor suppressors described in lung cancer, by cyclin D1, preventing the progression of the cell cycle from G1 to S phase. $p16^{INK4a}$ is inactivated in about 80% of NSCLC, mostly through homozygous deletion[55,56].

4.9. Phosphatase and tensin homolog (*PTEN*)

PTEN encodes a protein and lipid phosphatase that acts on phosphatidylinositol 3,4,5-trisphosphate, preventing the activation of the PI3K/AKT/mTOR signaling pathway. Therefore inactivation of PTEN produces an unrestricted activation of the signaling and promotion of cell proliferation[57]. Loss of PTEN has been described by allelic loss and promoter methylation in 75% of NSCLC[58]. Mutations in the gene have been described, but only with an incidence of around 5%[59].

4.10. Serine/threonine kinase 11 (*STK11*)

STK11 encodes a serine/threonine kinase that inhibits mTOR. STK11 can be inhibited by mutations or deletions that produce a truncated protein[60,61]. The frequency of inactivation of STK11 in lung adenocarcinomas has been reported in a range of 11% to 30%[62,63].

4.11. Rearranged during transfection (*RET*)

The proto-oncogene *RET* encodes a receptor tyrosine kinase belonging to the cadherin superfamily. The described translocation fuses the kinase domain of RET to KIF5B and has been reported in 1 to 2% of lung adenocarcinomas[64–66].

4.12. ROS proto-oncogene 1 (ROS1)

ROS1 encodes a tyrosine kinase receptor belonging to the superfamily of the insulin receptor. With high homology with ALK in the kinase domain, this receptor promotes proliferation via activation of PI3K/AKT/mTOR, STAT3, and RAS/MAPK[67]. Fusion proteins of the kinase domain of ROS1 with other partner genes have been described in NSCLC, with an incidence of less than 2%[68].

5. Treatment of NSCLC

Due to the complex heterogenicity of NSCLC, treatment regimens are personalized and established by taking into consideration the stage of the tumor, the health condition of the patient, the histological type, and the molecular profile of the tumor.

5.1. Surgery

Surgery is the recommended treatment approach in stages I and II of NSCLC. Lobectomy is the generally accepted procedure for early-stage tumors[14,69]. In other patients, the clinical team can decide to perform a sublobar resection, although the data from some clinical trials

show a higher rate of recurrence[70,71]. Sometimes sleeve resection, the removal of a portion of a major airway where the tumor is located, is recommended. Pneumonectomy, the removal of a complete lung, is less frequent due to the consequences on the quality of life of the patient. Regardless of the extent of resection, it is crucial for the success of the surgical procedure to dissect mediastinal lymph nodes[72].

Lung lobectomies and sublobar resections are performed by video-assisted thoracoscopy, as it is minimally invasive and improves the postoperative performance of the patient. Nowadays this same procedure is being done by robotic-assisted thoracoscopy in some hospitals, which improves the maneuverability and the precision of movements of instruments[69].

5.2. Radiation

Radiation is used in early-stage NSCLC when surgery is not applicable or the patient refuses it. It is also used in more advanced tumors in combination with chemotherapy and surgery[73]. Traditionally, radiation treatment consisted of 60 Gy to 70 Gy delivered to the middle plane of the tumor in fractions of 2 Gy per day. With the latest technological advances, stereotactic body radiation therapy (SBRT) became the standard used for radiation treatment, using high doses of radiation in fewer fractions (10 Gy to 18 Gy delivered in 3 to 5 sessions) achieving a higher effective dose compared to traditional radiotherapy[74]. SBRT needs precision delimitation and localization of the tumor, therefore the development of new imaging techniques, like the PET/CT, is incredibly important in the implementation of this precision technology. Moreover, new techniques of radiation have been developed by combining SBRT and live imaging techniques, like the three-dimensional conformal radiation therapy (3D-CRT), in which the tumor is mapped by computer analysis allowing for better aiming at the tumor and reduced damage to surrounding tissues. Newer experimental techniques have been developed to improve radiotherapy like protons therapy, which uses protons instead of photons, or the MRI-Linac, which combines the powerful imaging of MRI to guide radiation therapy[75].

5.3. Chemotherapy

Chemotherapy can be applied in different regimens depending on the stage of the tumor. The treatment consists of a platinum-based drug (cisplatin or carboplatin) used alone or in combination with a second drug (paclitaxel, docetaxel, gemcitabine, etoposide, vinorelbine, or pemetrexed)[14].

In instances, neoadjuvant chemotherapy is used to reduce the size of the tumor prior to surgery, and on occasion in combination with radiation therapy. This schedule of treatment might allow for early treatment of metastasis, complete resection of the primary tumor after its shrinkage, and possibly better tolerance than post-surgery administration. However, the advantages compared to adjuvant chemotherapy have yet to be proven[14,76].

Adjuvant chemotherapy is the standard recommended for stage II and IIA patients with two chemotherapeutic drugs. It is also used in early-stage resected patients to avoid the appearance of metastasis[14,73].

For tumors in an advanced stage that have been determined as non-resectable, chemotherapy is applied in combination with radiation. Usually, cisplatin as a single drug is used in chemoradiation[73].

5.4. Targeted therapies

The advances in tumor genotyping have led to the identification of lung cancer-associated mutations which produced advances in personalized treatments, improving the survival rates of NSCLC patients[77].

- EGFR inhibition

EGFR activation leads to downstream signaling concluding in the promotion of cell proliferation, survival, invasion, and angiogenesis[78]. Heterozygous mutations around the ATP binding site of the kinase domain might lead to constitutive activation of the receptor and activation of linked signaling pathways[79,80]. The most common mutations related to sensitivity to tyrosine kinase inhibitors (TKIs) are deletions on exon 19 and the missense mutation, L858R, on exon 21[81].

The first-generation TKIs like gefitinib and erlotinib, which are reversible competitive ATP inhibitors, showed improved results in survival and tumor response compared to cytotoxic agents[82]. Acquired resistance to these TKIs is usually linked to the appearance of a secondary mutation on exon 20 (T790M)[83,84]. Other known resistance mechanisms are amplifications in *HER2* or mutations in *MET, BRAF, or PIK3CA*[85]. Nowadays, third generation TKIs are available that can overcome the resistance acquired with the T790M mutation, like Osimertinib, but new mutations have arisen, like the C797S, that, together with the sensitizing mutations, confer resistance to these new inhibitors[86]. If the three mutations occur at once (sensitizing mutation, T790M, and C797S), the tumor becomes resistant to all three generations of TKIs[87], and new treatment strategies are being studied for those cases[88,89].

Alternatively, blocking monoclonal antibodies for EGFR can be used. A few anti-EGFR antibodies have been developed since the appearance of the first one, cetuximab. However, only necitumumab has shown a relevant improvement in survival when added to the chemotherapeutic treatment in clinical trials. For this reason, necitumumab is only approved to treat metastatic stage IV NSCLC in combination with chemotherapy[90].

- ALK inhibition

Patients with NSCLC carrying *ALK* rearrangement are responsive to treatment with TKIs. The first inhibitor approved to treat NSCLC positive for ALK fusions was crizotinib, a competitive ATP inhibitor, that also has an inhibitory effect of MET and ROS1[91]. Second generation ALK inhibitors (ceritinib, alectinib, brigatinib) have shown to be beneficial in NSCLC patients previously treated with crizotinib. Moreover, alectinib has been established as the first line of treatment in ALK-positive patients after two randomized trials showed an improvement in survival and response rates compared to crizotinib[92,93].

Treatment with ALK inhibitors eventually produces acquired resistance in the tumor. This can be due to *ALK* mutations and amplifications, or bypass activation of downstream signaling pathways through EGFR, MET, KIT, or others[94].

- ROS1 inhibition

ROS1 is a tyrosine kinase receptor that shows high homology in its sequence with ALK. For this reason, drugs with anti ALK activity, have proven effective inhibiting ROS1 as well. No drug with specific anti-ROS1 activity has been approved for treatment yet, therefore compounds with multikinase inhibition activity are used for treatment. Thus crizotinib has been approved as the first line of treatment for NSCLC patients with ROS1 rearrangement and lorlatinib may be used as the second line of treatment[95,96]. However, the development of new

inhibitors and treatment regimens are necessary to overcome the newly acquired resistances. Some clinical trials are showing promising results with new TKIs like entrectinib, cabozantinib, or repotrectinib[97].

- RET inhibition

Similar to what happens with the treatment of *ROS1* rearrangements, there are no approved specific inhibitors for RET, and therefore inhibitors with activity against multiple kinases are being used to target RET. Recently, some RET specific inhibitors have shown promising results in clinical trials, like selpercatinib or pralsetinib, that can change the treatment strategy followed in NSCLC tumors harboring RET alterations[98].

- BRAF inhibition

NSCLC tumors with the V600E mutation in BRAF can be targeted with specific drugs like vemurafenib or dabrafenib[99,100]. However, when these compounds have no effect, other players in the signaling cascade can be targeted, as it is the case of MEK, which can be inhibited with trametinib, in combination with dabrafenib[101].

- MET inhibition

The only specific MET inhibitor available is capmatinib, which can be used in advanced NSCLC in tumors with MET amplification and exon 14 mutations[102].

- NTRK inhibition

A small portion of NSCLC (0.2% to 3%) present fusions of neurotrophic tropomyosin-related kinases (*NTRK*), however data obtained from TKIs clinical trials have postulated that targeting of TRKs could be a beneficial strategy[103]. Two drugs have been approved to treat tumors with *NTRK* fusions after limited clinical trials: Larotrectinib and entrectinib[104]. Entrectinib is a multi-kinase inhibitor that has an effect on NTRKs, while larotrectinib is the only approved selective inhibitor for NTRKs. Other selective inhibitors are currently in development and evaluation, like selirectinib or reprotectinib, among others[105].

- VEGFR inhibition

Some studies have shown that blocking angiogenesis through vascular endothelial growth factor receptor inhibition can be beneficial in the management of NSCLC. Two monoclonal antibodies that are anti-VEGFR have been approved to use in combination with chemotherapy: bevacizumab and ramucirumab[106,107].

5.5. Immunotherapy

The activation of the host immune system to destroy cancer cells has been a pursued idea for long time. Only recently were we able to bring this idea to the clinic to provide patients with another treatment option.

The immune system can recognize neoplastic cells and eliminate them before they grow uncontrolled. To avoid problems with healthy tissues, this is a highly regulated process with activation and inhibitory mechanisms. Cancer cells can take advantage of these inhibitory mechanisms in order to escape immune surveillance. The approved immunotherapies in NSCLC try to modulate these immune checkpoints to make the immune system more effective at recognizing and removing cancer cells from the host. Thus, the approved immunotherapy for lung cancer aims at regulating said immune checkpoints[108,109].

- Programmed cell death protein 1 (PD1) and programmed cell death ligand (PD-L1)

PD1, a membrane receptor expressed in T cells, is the receptor for PD-L1, expressed in many tissues. The interaction of ligand and receptor leads to the inactivation of T cells. Therefore, cancer cells often overexpress PD-L1 to evade the immune activity in the tumor[109,110]. This interaction can be blocked with antibodies against PD1 (nivolumab and pembrolizumab) and PD-L1 (atezolizumab, durvalumab), and is a beneficial treatment strategy in different types of cancer, including NSCLC[109,111]. These antibodies have been approved as a treatment for advanced NSCLC patients whose tumors progress with cytotoxic therapy. Sometimes these antibodies are given together with cytotoxic agents, as the combination was shown to improve the tumor response and survival[112].

- Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)

CTLA-4 is expressed in T cells and acts as another immune checkpoint. When it is activated, sends an inhibitory signal that prevents the action of T-cells[113113,114]. Similar to the PD1 axis, anti-CTLA-4 antibodies prevent the binding of the ligand and CTLA-4 and allowing the immune surveillance to continue. The approved antibody to target CTLA-4, ipilimumab, has not shown improved results as a monotherapy, and has been a benefit only when combined with the PD1 inhibitor, nivolumab, with or without chemotherapy and is used to treat some advanced NSCLC[113,114].

6. Cancer stem cells (CSC)

Stem cell populations have been described in almost all types of mature tissues. Their role is to replenish and renew the aged and damaged cells in the tissue, maintaining homeostasis. These cells can self-renew and generate lineage-specific cells for the tissue in which they belong[115].

Tumors are a heterogeneous mix of cells, among those, a subset population has the ability to self-renew and generate the different lineages of cells that form the tumor[116] and those are known as cancer stem cells (CSCs). This subset of tumor cells has been identified in many different tissues, including those in the lung[117].The origin of these CSCs, however, is not clear. CSCs have the same functional characteristics as normal stem cells therefore it is plausible to hypothesize that CSCs are derived from normal tissue stem cells. In this scenario, normal stem cells undergo a series of oncogenic mutations producing their transformation into cancer stem cells[118]. Alternatively, CSCs might be derived from normal non-stem cancer cells that acquire stem properties[119,120].

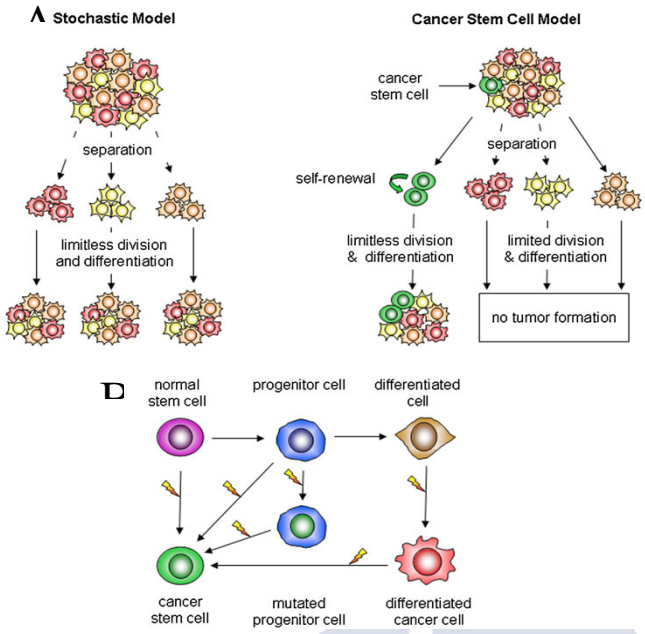

Figure 3. Cancer stem cell model[121]. **(A)** In the stochastic model of tumor progression, all cells have the same ability to generate a tumor. In the cancer stem cell model, there is a hierarchy in which a subset of cells are able to divide asymmetrically to self-renew and produce generations of tumor cells with limited capacity of proliferation. **(B)** Cancer stem cells could originate from normal stem cells or by regaining of stem properties by differentiated tumor cells. (Reproduced with permission from Springer Nature)

6.1. Signaling pathways in CSCs

CSCs make use of the same signaling pathways regulating self-renewal, proliferation, and differentiation in normal stem cells. WNT, NOTCH, and Hedgehog are important signaling axes in the maintenance of self-renewal and proliferation[122].

- The WNT pathway is a highly conserved one that encompasses two signaling pathways: canonical, mediated by β-catenin, and non-canonical, which is independent of β -catenin. These two pathways are important in the embryonic development of many tissues[123,124]. In general, the canonical pathway regulates proliferation and survival, while the noncanonical WNT pathway regulates asymmetrical cell division, polarity, and migration. Nonetheless, cross-talk can occur between the two arms of the signaling pathway. The role of WNT signaling in CSCs regulation is supported by experimental research that has shown that β-catenin overexpression can promote CSC survival and tumorigenesis *in vitro* and *in vivo*[125], and activation of the WNT pathway has been associated with tumor progression in lung tumors[126]. Moreover, secreted WNT antagonists, like WNT inhibitory factor 1 (WIF1), have been shown to inhibit NSCLC growth[127]. In addition to tumorigenesis, WNT signaling has been associated with metastasis in cancer[128].

- NOTCH plays an important role in defining cell fate and self-renewal. When the NOTCH receptor is activated, the intracellular domain is released and translocated into the nucleus

of the cell activating gene transcription. It is a highly evolutionary conserved pathway important in the regulation of stem and progenitor cells in embryonic development[129–131]. In adults, this pathway has been shown to regulate the stem cell functions in healthy tissues. NOTCH is a pathway commonly dysregulated in cancer[132] and many studies have demonstrated its importance in the maintenance of CSCs[133,134].

- The Hedgehog pathway is also indispensable during embryonic development[135]. Although altered signaling has been reported in many different types of cancer, it is not clear the role of the Hedgehog pathway in lung CSCs[136]. Different studies have shown that blocking Hedgehog signaling hampers proliferation and self-renewal capacity of tumor cells, at the same time that expression of genes associated with stemness (NANOG, SOX2, OCT4) was reduced[137–139].

- Other signaling pathways are altered in CSCs and therefore can contribute to the maintenance of the stemness characteristics. Signaling pathways important in pro-survival like JAK/STAT[140], PI3K[141], or NF-κB, also important in regulating the inflammatory process and immune response[142,143].

6.2. Cancer stem cell markers

The identification of CSCs has relied on their similarity with embryonic and adult stem cells. Thus different surface proteins and transcription factors have been used to help identify this tumor cell population.

- CD133: is a transmembrane glycoprotein involved in cell growth and development[144]. It is one of the most common CSC markers across many tumor types and cells that express CD133 show stem characteristics such as self-renewal capacity, higher tumorigenicity, and expression of other stemness associated proteins than cells with no detectable expression of CD133[145,146].

- CD44: is a transmembrane glycoprotein, a receptor that binds to hyaluronic acid and mediates cell-cell communication and signaling with implications in cell division, migration, and adhesion[147–149]. CD44 expression is regulated by WNT/β -catenin[150] and has known interactions with other proteins implicated in tumor progression and metastasis[151]. CD44 has been identified in many epithelial cancers[152,153], including lung, in which the expression of this glycoprotein in tumors correlates with survival[154].

- CD24: is an adhesion molecule important for cell-cell and cell-matrix interactions and has been shown to play a role in tumor growth and invasion, facilitating the passage of tumor cells into the bloodstream[155,156]. In NSCLC, expression of CD24 was correlated with the aggressive tumor behavior and metastasis[157].

- CD166: is a transmembrane protein that belongs to the immunoglobulin superfamily participating in the cell-cell adhesion. CD166 overexpression has been reported in different types of cancer, including lung, and has been related to metastasis and evasion of apoptosis[158,159]. Also, CD166 expressing cells have shown higher sphere-forming capacity and ability to establish a tumor[160,161].

- CD117: is a tyrosine kinase receptor and as such is involved in the regulation of cell survival and proliferation. In NSCLC, CD117 expressing tumor cells have shown CSC characteristics[162].

- ALDH1: high activity of aldehyde dehydrogenase, especially ALDH1, is associated with CSCs and regulates cell protection, differentiation, and proliferation. ALDH1 expression in tumor samples also correlates with poor prognosis in lung cancer[163].

- CXCR4: the chemokine CXCL12 and its receptor, CXCR4, regulate cellular adhesion, survival, proliferation, and gene transcription. CXCR4 is overexpressed in many types of cancer and is related to tumor proliferation, invasion, angiogenesis, and therapeutic resistance[164–166].

- OCT4: is a transcription factor important in stem cells. In CSCs, it regulates functions like survival, self-renewal capacity, and resistance to chemo- and radiotherapy[167].

- SOX2: is another transcription factor important in pluripotency preservation. It is implicated in self-renewal, maintenance of stemness, tumor aggressiveness, and therapy resistance[168]. SOX2 expression correlates with patients survival and prognosis in different types of cancer[169–172].

- NANOG: is another crucial transcription factor in development and is shown to be expressed in many cancers. High expression of NANOG promotes epithelial to mesenchymal transition, a process important in metastasis and therapy resistance[173,174].

7. Epithelial to Mesenchymal Transition (EMT)

The transition of cells from epithelial to mesenchymal phenotype is a regulated process that is necessary during embryogenesis[175]. In this process, EMT stimulants like TGF-β and other ligands activate signaling cascades that result in the upregulation of transcription factors like SNAIL1, TWIST, SLUG, or ZEB1. These transcription factors repress the expression of epithelial associated genes (E-cadherin and cytokeratins), accompanied by accumulation of cytoplasmic β-catenin, a participant of the adherens junctions. β-catenin then becomes a nuclear transcription factor, mediated by WNT signaling, that can moderate the expression of mesenchymal lineage genes (fibronectin, vimentin, N-cadherin). This results in loss of epithelial characteristics, like the cell-to-cell junctions or the apical-basolateral polarity, and the acquisition of mesenchymal attributes (elongated morphology and increased capacity of invasion)[176,177].

In pathophysiological conditions, such as tissue damage, inflammation, or tumorigenesis, EMT can be activated in differentiated cells[178,179]. Activation of EMT can produce major changes in the cell physiology that allows the cell to modify the environment and to adapt to a new one. By alteration of pattern expression of hundreds of genes, cells are able to activate the EMT process to various extents, providing the cell with plasticity which allows for adaptation.

It is widely accepted that the EMT process is necessary for cancer cells to be able to migrate and invade distant tissues. For many years, the loss of E-cadherin expression in carcinomas was associated with metastasis and poor prognosis[180], and since the 2000s SNAIL expression was described in metastatic breast carcinoma cells and correlated with tumor dedifferentiation and the appearance of metastasis[181]. Despite all the evidence that seemed to implicate EMT in tumor progression, there has been controversy about this fact, mainly because pathologists were not able to define a clear mesenchymal phenotype in tumor samples from patients[182]. In fact, the complete loss of epithelial traits and gaining of a full spectrum mesenchymal characteristics is rare in cancer. Opposite to what happens during embryogenesis, cancer cells undergo a partial EMT transformation. This can be observed in studies where E-cadherin expression was not repressed completely[180,183] or in the analysis of circulating tumor cells from breast cancer which showed expression of epithelial and mesenchymal mRNA markers at the same time[184].

Besides EMT being activated partially in cancer cells, this program is reversible. Thus, cells that undergo EMT changes in cancer, need to make the reverse process (mesenchymal to epithelial transition, MET) to lose the acquired features that allow them to separate from the original tissue, migrate, and ultimately establish themselves in the new environment. Research about this topic has shown that a process of MET is necessary for disseminated tumor cells to establish a metastatic tumor[185–187].

The molecular process for which this occurs is still mysterious, but the predominant hypothesis is that MET simply occurs by reverting the expression of the transcription factors that produced the EMT[188,189].

7.1. EMT and CSCs

The idea that EMT is responsible for metastasis has been expanded after research demonstrated that induction of EMT produces cancer stem cell like characteristics[173,190]. Similar dedifferentiation has been observed in several systems including NSCLC[191–194], indicating that the hierarchical system established in non-neoplastic tissues (in which only stem cells can produce the entire population of a tissue) does not apply in neoplastic tissues. Moreover, the idea that metastasis are originated from CSCs implies the acquisition of EMT characteristics to be able to migrate from the original tumor and reach distant tissues where to establish a metastatic tumor, and for this, the CSC should have to go through a MET process, regaining epithelial characteristics to establish the new tumor[195,196]. Given all these assumptions and previous data about the plasticity of the EMT process, the tumor cells can be seen as being in a variable state between non-stem and CSCs with the expression of epithelial and mesenchymal features[197].

The molecular mechanisms that explain the interrelation between EMT and CSCs remain unknown. One potential explanation could be the fact that signaling leading to EMT also creates alterations in secreted proteins, activating autocrine signaling loops that activate and maintain stem cell properties. In fact, in mammary cells, activation of EMT produces activation of signaling through TGF-β/SMAD and WNT/β-catenin, both important in the maintenance of stemness[198]. It has also been reported that EMT can produce changes intracellularly, thus, SNAIL reduced the expression of the tumor suppressor p53 by inducing its proteasomal degradation[199].

7.2. EMT, CSCs, and therapeutic resistance

Since the emerging of the CSC hypothesis, it has been proposed that they have increased resistance to chemo- and radiation therapy. Many studies have supported this idea, where tumor cells expressing CSC markers have shown to better survive the effects of different treatments[200,201]. This resistance has been attributed to different mechanisms: increased expression of drug transporters and anti-apoptotic proteins, altered DNA repair pathways; or the slower proliferation rate[122,202–207]. However, the acquired knowledge of EMT as a regulator of the CSC phenotype has provided some experimental results supporting the association of EMT-associated genes and therapeutic resistant phenotype in CSCs[208,209].

8. Resistance to cancer therapies

As we reviewed in section 5, chemotherapy and radiotherapy remain the foundation of lung cancer treatment. However, the efficacy of the treatment approaches is still limited due to resistance within the tumor. Even with the raising of target therapeutics, which allow for outcome improvement in subsets of lung cancer patients, the benefit is often limited. This failure for the therapies used can be motivated by the phenotypic heterogenicity of cells that compose the tumor. Thus, some cells might have an intrinsic resistant mechanism, meaning that tumor cells already arise with those resistant tools, or it can be an acquired resistance, developed after the existence of the tumor. This is a field in which a lot of investigation is still needed in order to improve the efficacy of the therapeutics because for what is known so far, the mechanisms behind therapy resistance can be complex and heterogeneous even within the same tumor[197,210].

8.1. Mechanisms of resistance to chemotherapy

- Drug efflux: many membrane transporters related to the movement of compounds to the extracellular space have been linked to resistance to chemotherapeutics. The ATP-binding cassette (ABC) family of transporters is the main mechanism of multidrug resistance, which uses the energy of ATP to efflux drugs from the cell, protecting them from the cytotoxic effects[211,212]. ABCB1, ABCC1, and ABCG2 are the most studied ones and have been found to be overexpressed in different types of cancer, correlating with the chemoresistance of the tumor[213–215]. Clinical trials aiming at blocking these transporters have shown disappointing results, suggesting that the importance of drug transporters in chemoresistance is relative[216,217].

- Drug activation or inactivation: some drugs can be inactivated by the intracellular components, for instance, platinum drugs can be inactivated by thiol glutathione[218]. In the case of antimetabolites, their conversion into the more active form does not occur if there is not the required enzymatic activity[219–221].

- Alterations in drug targets: changes in the expression or mutations in the target protein of the drug, can result in the inefficiency of the treatment. A well-known example of this is the acquired resistances to TKIs like erlotinib, crizotinib, or imatinib, where arising mutations in the kinases (EGFR, ALK, and BCR-ABL1 respectively) produce resistance to the

inhibitors after a period of positive response[222–226]. In other cases, the drug target is simply overexpressed, as happens in the blockade of androgen receptors in prostate cancer, reducing the efficacy of the treatment, as more molecules of the target have to be inhibited[227].

- DNA damage response: many chemotherapeutic drugs work by inducing DNA damage (i.e. platinum-based drugs or topoisomerase inhibitors). Therefore the efficiency of DNA damage repair has a major impact on the efficacy of these treatments. Consequently, modifications in the DNA repair machinery that enhance their efficiency might produce resistance to chemotherapeutics. For example, overexpression of ERCC1, a key player in nucleotide excision repair, in ovarian and gastric cancers has been related to resistance to cisplatin treatment[228,229]. Similarly, methylation of MLH1, part of the machinery of mismatch repair, causes resistance to cisplatin and carboplatin[230].

8.2. Mechanisms of resistance to radiotherapy

Radiation therapy is administered as fractionated doses, as we described in section 5, assuming the four "R" of radiobiology:

- Repair of sublethal DNA damage
- Repopulation of tumor cells
- Reoxygenation of hypoxic tumor areas
- Reassortment of cells within the cell cycle.

Ionizing radiation works by producing DNA double-strand breaks (DSB), however irreparable DSB are produced randomly in a population of cells of similar radiosensitivity and most induced damage is not lethal since cells are able to repair those breaks at low doses. When the dose accumulates or is increased, the probability that all the cells in a population get hit with lethal DNA damage increases. The assumption is that normal cells are able to repair the DNA damage in between doses of radiation, while cancer cells are not, due to the alterations they often have in the DNA repair machinery[231,232]. Thus, the radiosensitivity of a tumor can be determined by the proportion of cells with an enhanced DNA repair machinery, allowing them to generate a quick and effective repair of damages to the DNA in between radiation doses. This would allow for the surviving tumor cells to proliferate in between treatment doses and it has been shown that those clones proliferate at a much higher rate than the untreated tumor, providing a potential mechanism to escape the anti-tumor effect of ionizing radiation[233]. This implies that radiation doses applied over large periods yield poorer results than smaller doses applied over a shorter time[234].

Tumor volume is another important factor to consider in tumor radiosensitivity. It is known that NSCLC tumors are generally large when diagnosed and that radiation therapy, even when combined with chemotherapy, has a failure rate of at least 30%[235,236]. Additionally, evidence suggests that the larger size of the tumor correlates with lower local tumor control[237–239]. This would imply that larger doses of radiation are needed, but this is limited to the tolerance of the surrounding tissue as shown by previous clinical experimentation[240].

The oxygenated status of the tumor is also important for the effect of radiation therapy. Hypoxic tumor cells are more radioresistant[241]. This radiosensitivity of oxygenated cells might be due to the generation of reactive oxygen species (ROS) that react with DNA free radicals generated by ionizing radiation. The hypoxic environment can be reverted if after destroying a part of the tumor, the inner parts are exposed and enter in contact with vessels carrying oxygen.

For this reason, the gaps between radiation doses are important to allow for reoxygenation of hypoxic tumor tissue[242].

One more factor that affects the efficacy of radiotherapy is the phase of the cell cycle in which tumor cells are at the time of treatment. Cells in mitosis are more sensitive to radiation, while cells in the late S-phase or a quiescent state are more resistant[243,244]. Due to this fact, fractioning of radiation dose is important to allow the progression of the cell cycle of those cells that are not dividing.

- DNA repair process after radiation therapy

Ionizing radiation works by producing double-strand breaks in the DNA, therefore DSBs repair mechanisms are essential in tumor cells to be able to overcome the effects of radiation.

One of the first responses to DSBs is the recruitment of the MNR complex (MRE11-RAD50-NBS1) to the site, which rapidly recruit ATM and causes its autophosphorylation (S1981), triggering the DNA damage response (DDR) signaling that starts with the phosphorylation of the adjacent histone H2AX in S139 (also known as γH2AX). This phosphorylation of H2AX is important for the recruitment of other relevant proteins in DDR like 53BP1, which serves many functions, including the process of DNA repair pathway choice[245]. ATM has a central organizing role in coordinating several cellular processes after DSBs recognition, not only controlling the DNA repair machinery, but also the cell cycle checkpoints, apoptosis, and senescence[246–248]. Although ATM is the main event in DDR, ataxia telangiectasia and Rad3-related protein (ATR) is also a player in initiating the DDR by responding to ssDNA produced in the DBSs. ATR acts as a kinase, much like ATM, controlling cell cycle checkpoints and phosphorylating many of the ATM substrates, helping ATM function[248].

After recognition of the DSB site, the damage can be repaired mainly through two mechanisms: non-homologous end-joining (NHEJ) and homologous recombination (HR).

  - Non-homologous end-joining

NHEJ is considered an error-prone and mutagenic mechanism because the DSB ends are ligated regardless of homology. This can generate modifications of the DNA sequence by deletions or insertions. This is the most common mechanism to repair DSB in eukaryotic cells because is a quick mechanism to repair the DNA breaks and minimize genetic instability in any phase of the cell cycle, but preferentially in G_0, G_1, and early S phase[249–252].

The first step in NHEJ is the recognition of the break by the helicase heterodimer KU70/KU80, which binds to the open strands and prevents degradation of the DNA molecule. DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is then recruited and activated, phosphorylating and recruiting X-ray repair cross-complementing protein 4 (XRCC4) that forms a complex with DNA ligase IV, responsible for the ligation of the DNA ends. Usually, DNA open ends are irregular and need processing before the ligation process can happen. In those cases, a variety of proteins are recruited to the DSB site after binding of the KU heterodimer. Some proteins that have been described to participate in this process are PNKP, responsible for adding and removing phosphates, Aprataxin, which catalyzes the removal of adenylate groups, or the exonuclease Artemis, responsible for degrading redundant ends in the DNA[252–257].

An alternative NHEJ has also been described, in which the DNA ends are excised by meiotic recombination 11 protein (MRE11) and the retinoblastoma binding protein 8 (RBBP8) exonucleases, allowing XRCC1 and ligase III to complete the end-joining process, facilitated by exposed microhomology regions[258–260].

- Homologous recombination (HR)

Homologous recombination requires a homologous DNA strand from the sister chromatid to repair the DSB, so, for this reason, HR is only active during the S and G_2 phases of the cell cycle[261]. After chromatin decondensation by H2AX phosphorylation and binding of 53BP1 to the DSB site, the complex RAP80-BRCA1 is recruited. BRCA1 is an HR mediator with multiple described functions, one of them being the removal of 53BP1 from the DNA ends making them accessible for the digestion of the 3' ends by the exonuclease activity of MRE11, part of the MRN complex[262–264]. Then the exonuclease I (EXO1) and the BLM/DNA2 complex elongate the DNA ends and the RPA protein binds to protect them[265,266]. Next, RAD51 replaces RPA, promoting homologous pairing and strand invasion of the sister chromatid with help of BRCA2 and an array of other proteins (RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, and RAD51AP1)[267–269]. Once the homologous DNA is aligned, RAD51 is liberated allowing for the DNA polymerase δ to sensitize the missing DNA and for ligation of the strands by the DNA ligase I[270,271].

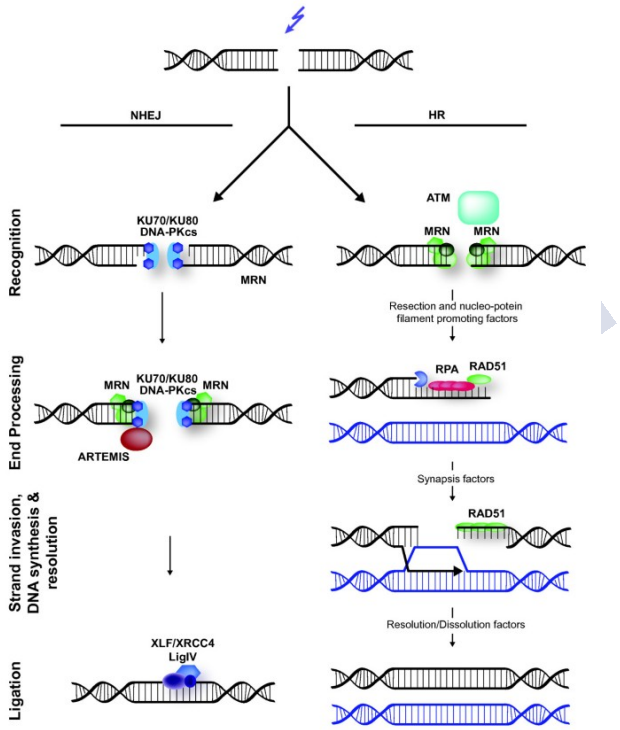

Figure 4. Mechanisms of DSBs repair. Schematic representation of NHEJ and HR process. (Reproduced with permission from Springer Nature)[272]

8.3. General mechanisms of therapeutic resistance

- Deregulation of apoptosis: Most cancer therapeutics ultimately induce the death of the cancer cell, and one of the major mechanisms of cell death is apoptosis. Therefore, evading the programmed cell death process is an efficient mechanism to survive the effects of cancer therapy. For this reason, Hanahan and Weinberg included the evasion of cell death as a hallmark of cancer in their famous review[273]. Evidence has accumulated over the years supporting altered mechanisms of apoptosis in cancer, showing that, in many cases cancer cells are dependent on anti-apoptotic proteins for their survival. Alterations in those proteins

(mutations, amplifications, overexpression, etc.) have been linked to resistance to chemotherapy and targeted therapies in different cancers[274].

- Autophagy is a biological process that degrades cellular components to maintain cell functions in conditions of stress. Even though the role of autophagy in cancer can be controversial, because it can work as a tumor suppressor and as a drug resistance mechanism enhancing cell survival[275], some studies have shown that blocking autophagy sensitizes cancer cells to cancer therapy[276,277].

- Activation of pro-survival signaling: in this mechanism of resistance, a secondary signaling pathway, inducing pro-survival signaling is activated to compensate for the inhibitory effects that anti-cancer treatment is producing. It is known that EGFR is activated as a resistant mechanism to chemotherapeutics[278,279].

In other cases what happens is an oncogenic bypass, in which the downstream signaling of a therapeutic target is activated by another protein. This is the case of ERBB3 activating PI3K/AKT signaling to produce resistance to EGFR inhibition[280].

This pro-survival signaling can also be activated by autocrine, paracrine, or endocrine signaling. Cytokines and growth factors can be released by tumor cells, or other cells in the tumor, like fibroblast, inducing signaling pathways leading to pro-survival signaling[281–283].

9. Heat shock protein 90 (HSP90) in cancer.

Heat shock proteins (HSPs) are a family of proteins that are an essential component of any cell, and they allow for the correct folding and stability of newly synthesized peptides [284–287]. For example, HSP90 has hundreds of client proteins, many of them kinases and nuclear receptors, participating in fundamental cell functions like cell cycle control, trafficking, and homeostasis [288,289].

Heat shock proteins are expressed in normal cells in response to stress (heat, oxidative stress, chemicals, etc.), allowing cells to survive under hostile conditions. Cancer cells exist in a constant state of cellular stress (hypoxia, starvation, protein misfolding, etc.) because of their rapid proliferation and the environment in which they develop. As a result of the stressful conditions, heat shock proteins are commonly found overexpressed in many cancers [290], helping neoplastic cells survive, and they are implicated in tumor progression processes such as: cell proliferation; differentiation; invasion; metastasis; and therapeutic resistance [273,290–292].

Cancer cells have overly active protein synthesis machinery due to the high proliferation rate they exhibit, and in most tumors, their proliferation is driven by mutated proteins. These mutations alter the primary structure of the protein and can produce misfolding. Therefore, enhanced chaperone machinery is essential to ensure the stabilization of the tumors. Hence, HSP90 is documented as an important component to oncogene addiction of cancer cells, facilitating their survival [293,294].

Many important roles of HSP90 in tumor cell biology have been described over years of research, some as crucial as the regulation of folding of AKT, an important component of PI3K/AKT/mTOR signaling pathway that participates in tumor progression [295,296]. Or the regulation of tumor suppressor proteins as important as p53, which is mutated in many cancers, and it has been demonstrated that the mutated p53 creates a regulation loop that stimulates the

transcription of HSP90, and thus helping stabilize the mutated protein [297]. Another important point of activity is the regulation of RB phosphorylation, which controls the progression of the cell cycle, by regulating the folding of CDK4, responsible for RB phosphorylation [298,299].

Given the relevance of the cellular processes in which HSP90 participates, increased interest has arisen in the treatment of cancers through HSP90 inhibition. Since the first HSP90 inhibitor, Geldanamycin, many compounds have been developed to inhibit the activity of HSP90 by blocking the ATP binding or disrupting the interaction with co-chaperones[298]. Most of the inhibitors developed have shown very promising results *in vitro* and pre-clinical *in vivo* experiments and many of those drugs have been evaluated in clinical trials for different types of cancers. However, the results have been disappointing and many of the trials had to be stopped due to toxicity or lack of antitumor activity [300]. Despite the lack of success with HSP90 inhibitors as a monotherapy, newly improved molecules and strategies are still being studied. In fact, the combination of HSP90 inhibitors with some drugs, like TKIs, have shown to be a better approach because the combination allows for a reduction in HSP90 inhibitor dose and still has an additive effect on kinase inhibition[300].

Aims

Lung cancer is responsible for over 20% of deaths caused by cancer, with non-small cell lung cancers accounting for nearly 85% of those deaths[1,4]. Approximately 40% of non-small cell lung cancers are unresectable, and despite the most recent advances in cancer treatment, radiotherapy remains the primary treatment for these types of tumors[13,301]. Unfortunately, in most cases, radiotherapy is largely a palliative treatment approach due to radioresistance which could be attributed to the heterogenicity in terms of cell types, etiology, pathology, and molecular characteristics[302]. The existence of cancer stem cells and cells that undergo an epithelial to mesenchymal transition within the tumor could provide an escape from the effects of conventional therapies[204,205,303], possibly by alterations of DNA double-strand breaks repair mechanisms[304]. Knowing this information, we hypothesize that non-small cell lung cancer cells which survive radiation can escape this treatment through the expression of cancer stem cell-like properties, epithelial to mesenchymal transition, and alteration of DNA repair mechanisms. Accordingly, we propose the following aims:

- **To determine the effect that radiation therapy exerts on surviving NSCLC cells.**

- Using well-studied non-small cell lung cancer cell lines we will generate cells that survive radiation treatment and study their phenotypic characteristics. We will analyze the stemness features that might appear in these surviving cells, as well as the epithelial to mesenchymal transition phenotype and their capability to invade new niches.

- **To study HSP90 inhibition as a treatment strategy in NSCLC.**

- Analyzing the effect of Ganetespib treatment as monotherapy in cancer progression, and looking at possible mechanisms of action by studying the effects of the treatment on cell cycle and apoptosis.
- We will study the validity of the combination treatment of Ganetespib with radiation in NSCLC cells and its effects on cell proliferation, pro-survival signaling, and DNA repair processes.

- **To establish and study a population of radioresistant NSCLC cell lines.**

- Using multiple doses of radiation we will generate radioresistant NSCLC cell lines and analyze their cancer stem cells and epithelial to mesenchymal transition characteristics.
- We will study the status of DNA repair pathways in the established radioresistant cell lines.
- We will assess the validity of HSP90 inhibition with Ganetespib in combination with radiation as a treatment strategy to eliminate radioresistant NSCLC cells *in vitro* and *in vivo*.

Results

10. Generation of Radiation-survived NSCLC cells and characterization.

10.1. Analysis of stemness

We first wanted to evaluate if the radiation treatment had any effect on the capacity of NSCLC cells to generate anoikis resistant clones. Seven days after IR treatment, cells were detached and seeded as single cells in suspension. In both cell lines, A549 and H460, cells that survived 5 Gy of radiation treatment produced a higher number of tumorspheres compared to the non-irradiated cells (Fig 5B).

Since the ability to grow detached is a trait of cells able to migrate and establish a new tumor[305], we set to analyze the expression of different proteins which have been recognized to be markers of stemness in NSCLC (Figure 5 C-F). We found that the pattern of expression of the analyzed CSCs markers differs with each cell type. Both A549 and H460 showed an upregulation in CD24 and CD44 expression in spheres that survived IR treatment. However, only A549 showed higher expression of CD166 in spheres generated from cells that survived the treatment

When we analyzed the expression of CSCs associated transcription factors (Figure 6), we observed that A549 and H460 showed higher expression of β-catenin in the nuclei of sphere cells that had been previously treated with IR. The expression of Oct-4 was, however, found to be greater only in A549 cells. On the contrary, H460, but not A549, showed increased expression of the Sox-2 transcription factor.

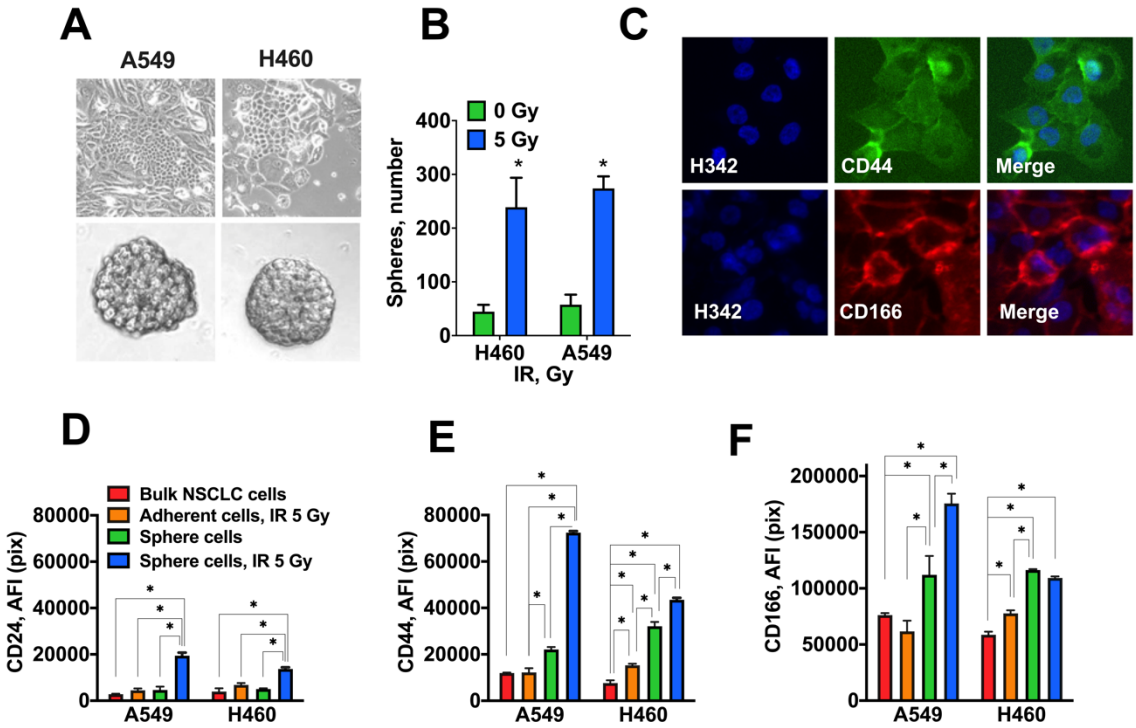

Figure 5. NSCLC cells that survive radiation have cancer stem cells phenotype. (A) Bright-field microscopy photography of A549 and H460 cell lines after 5Gy of radiation in adherent and attachment independent conditions. **(B)** Quantification of the number of tumorspheres in A549 and H460 cell lines when untreated and after irradiation. **(C-F)** Analysis of cancer stem cell markers by immunofluorescence. (C) Representative

immunofluorescence images for CD24 and CD166 in A549 spheres after radiation, and quantification of fluorescent intensity for CD24 (D), CD44 (E), and CD166 (F).

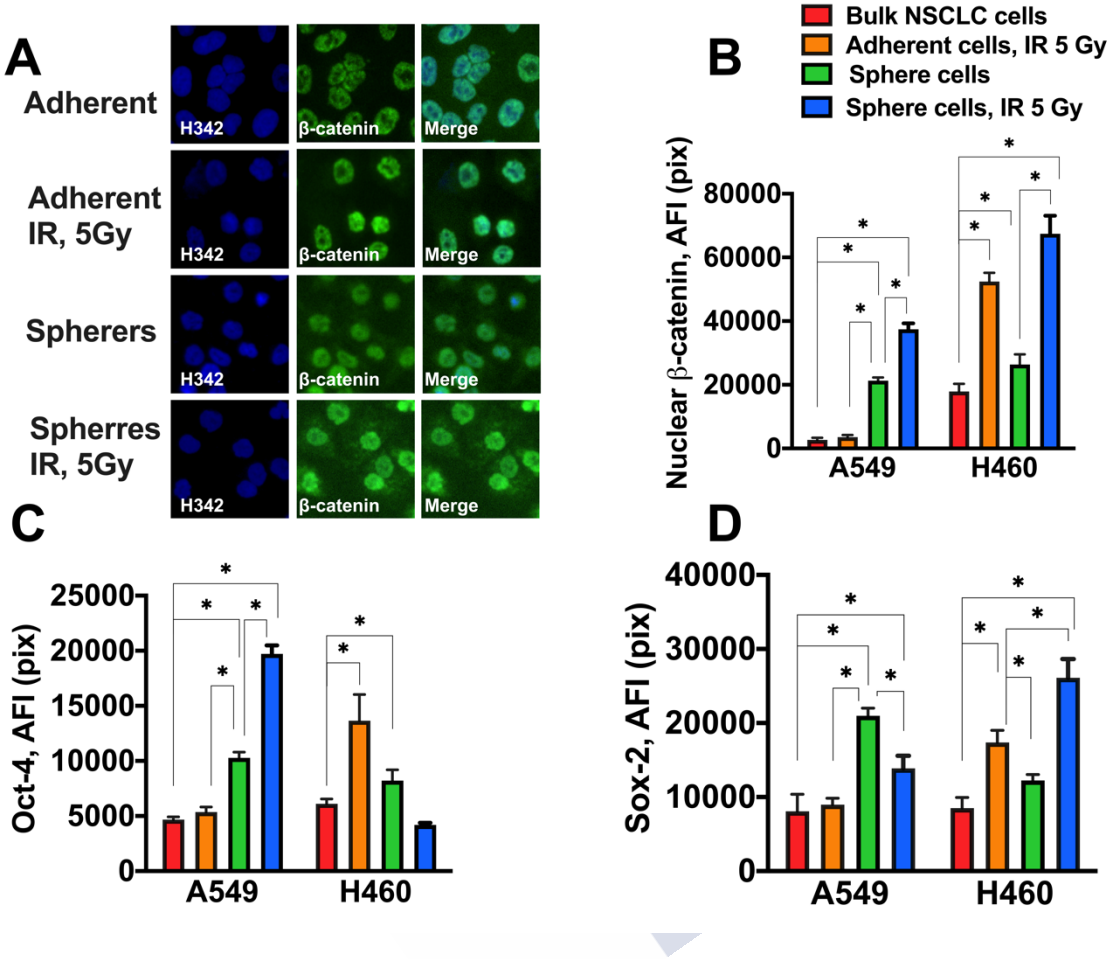

Figure 6. Radiation survived NSCLC cells show upregulation of transcription factors important in stemness. **(A)** Representative images of ß-catenin immunofluorescence staining in H460 cells growing as adherent and spheres, with and without radiation treatment. **(B-D)** Quantification of the staining for nuclear ß-catenin (B), OCT4 (C), and SOX-2 (D).

10.2. Analysis of Epithelial-Mesenchymal Transition characteristics

Since EMT is known to play an important role in treatment resistance[176,306], we planned to analyze the EMT status via the expression of SNAIL1 and TWIST by immunofluorescence (Figure 7). SNAIL1 signal was differentially higher in sphere cells that survived IR treatment and it was expressed ubiquitously in both cell lines. However, only A549 showed higher expression of TWIST in sphere cells after IR treatment and it was found to be located in the nuclear fraction of the cell.

To further confirm the EMT phenotype, we analyzed the expression of Fibronectin, E-cadherin, Vimentin, and N-cadherin (Figure 8). In both cell lines, Vimentin and N-cadherin expression increased in sphere cells that were irradiated when compared to cells growing in adherent conditions. In the case of A549, Vimentin expression was not higher in sphere cells after IR treatment compared with the non-irradiated cells. This cell line also showed differences

in the presence of Fibronectin in sphere cells compared to the adherent cells, and increased presence in sphere cells that had been previously irradiated. In contrast, Vimentin expression in H460 was substantially larger in sphere cells that had been treated with IR. Further confirmation of the EMT phenotype of these cells is the fact that E-cadherin expression is repressed in both cell lines when treated with radiation and grown in suspension.

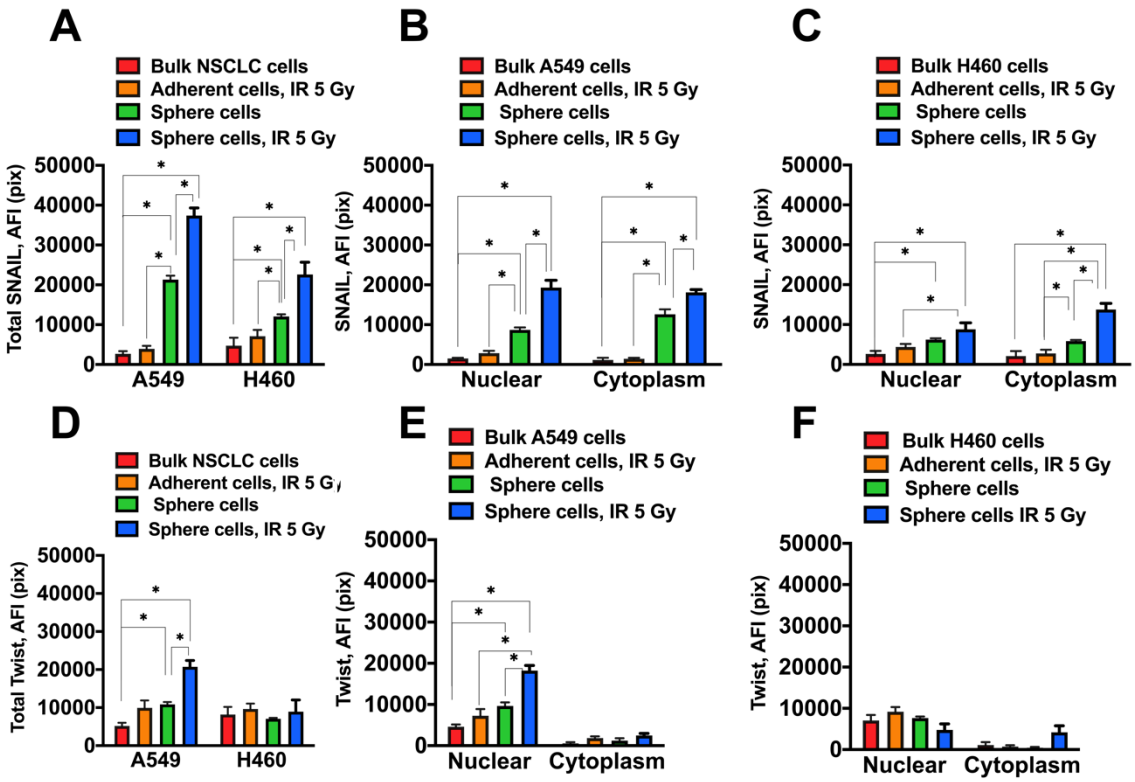

Figure 7. NSCLC sphere cells have increased expression of EMT transcription factors. (A) Analysis of SNAIL1 expression in A549 and H460 growing in adherent and attachment independent conditions, with and without radiation. **(B-C)** Distribution of SNAIL1 in the same cell populations. **(D)** Total average fluorescence intensities for TWIST1 immunofluorescence staining and cellular distribution **(E-F)** in adherent cultures and spheres, untreated and post-radiation.

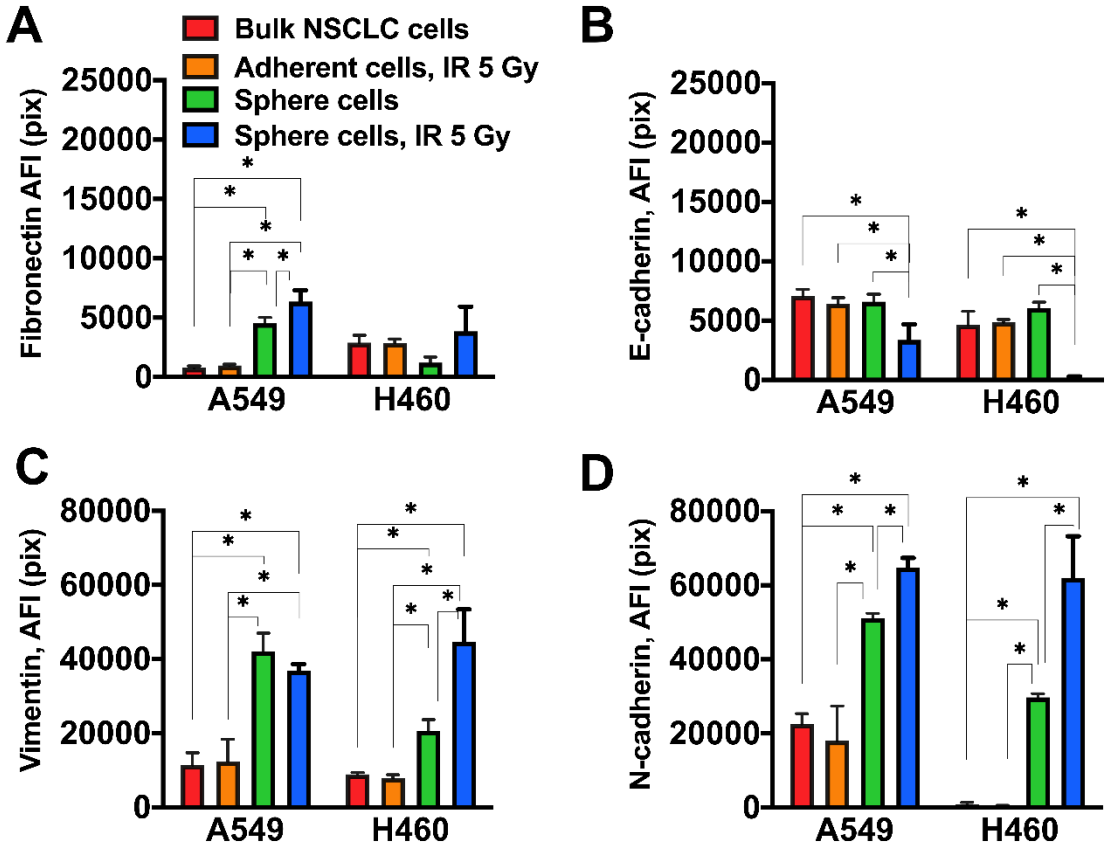

Figure 8. NSCLC cells that survived radiation have upregulation of EMT markers. Average fluorescence intensity for Fibronectin **(A)**, E-Cadherin **(B)**, Vimentin **(C)**, and N-Cadherin **(D)** are shown in A549 and H460 cells growing as adherent culture and tumorspheres, untreated and treated with 5 Gy of radiation.

10.3. Analysis of receptors involved in stemness and EMT

CXCR4 expression is important for the maintenance of stemness in drug-resistant NSCLC[307,308], and PDGFR is relevant in the signaling cascade leading to EMT[309]. Knowing this, we chose to analyze the expression status of these two receptors. We found that irradiated sphere cells showed more expression of CXCR4 in their membrane surface. Similarly, PDGFR-β showed higher expression after cells were irradiated, and in a much more dramatic way in sphere cells after radiation treatment (Figure 9).

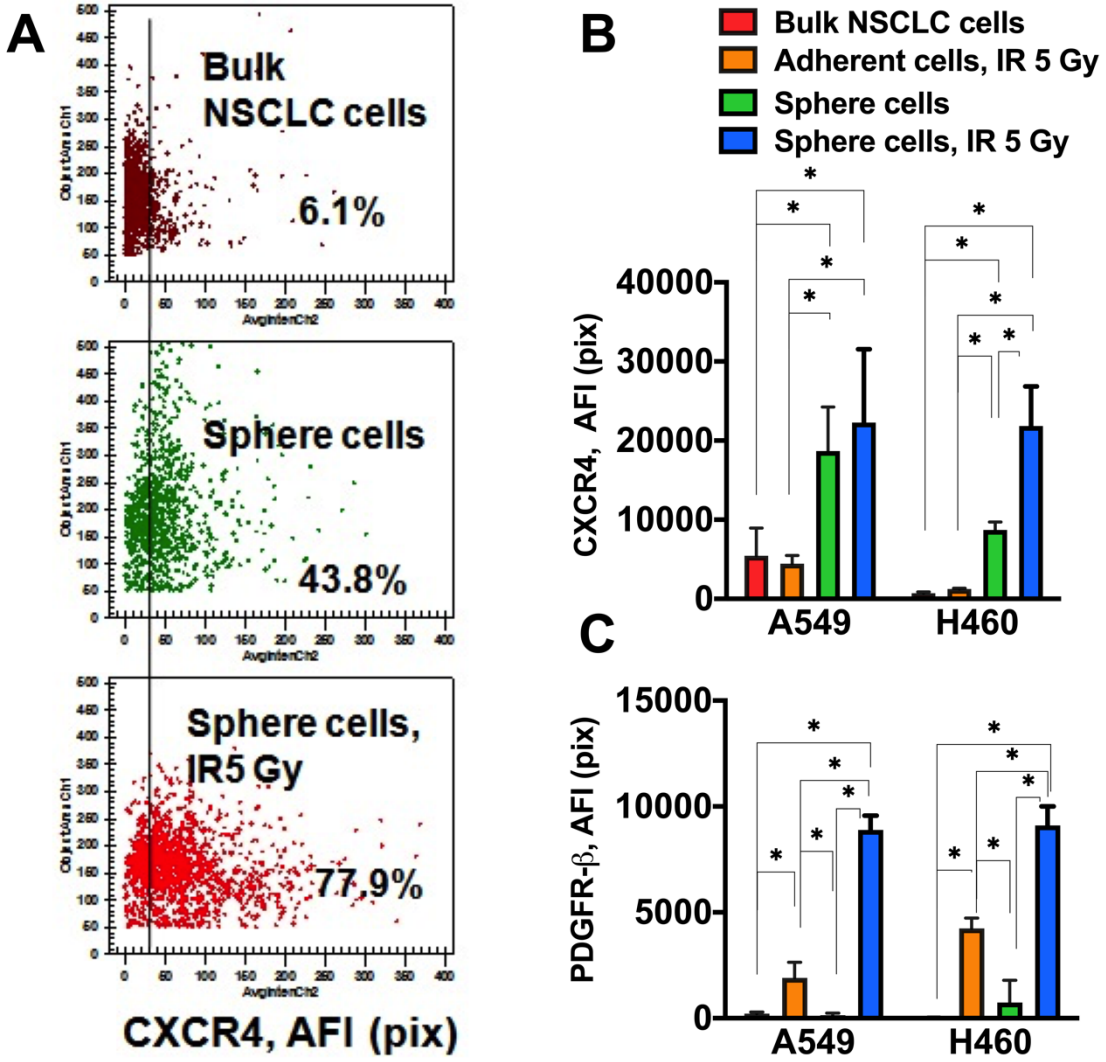

Figure 9. Radiation survived NSCLC sphere cells have upregulation of CXCR4 and PDGFR-β. (A) Fluorescence intensity of CXCR4 in A549 adherent non-irradiated cells (Brown), non-irradiated sphere cells (Green), and irradiated sphere cells (Red) plotted against object area. Each dot represents one cell. **(B-C)** Quantification of the fluorescence intensity in adherent and sphere cells, radiated and non-irradiated in A549 and H460 for CXCR4 (B) and PDGFR-β (C).

10.4. Migration ability analysis

After observing the expression of EMT markers in these cell lines, we tested whether this phenotype had an impact on the motility of the cells. As shown in figure 10, sphere cells, irradiated and non-irradiated, could re-establish the monolayer of cells at a quicker pace than those cells growing in adherent conditions. When treated with IR, the cell motility is hampered, both in adherent cells and cells growing as tumorspheres.

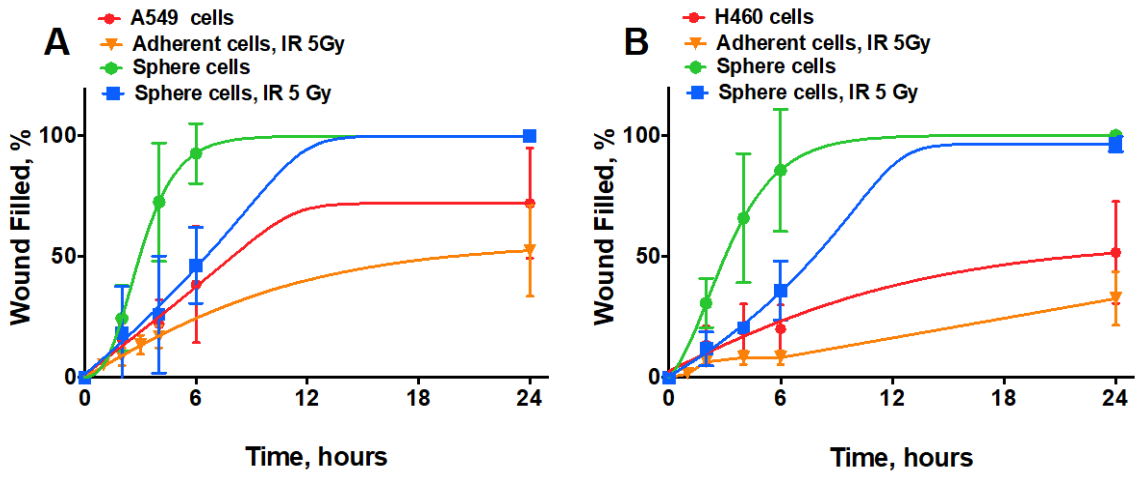

Figure 10. Radiation survived NSCLC sphere cells show a high migratory capacity. Comparison of migratory capacity in wound healing assay of untreated cells and cells that survived radiation for A549 **(A)** and H460 **(B).**

10.5. Treatment strategy for the elimination of tumor initiating cells

To test the influence of the PDGFR-β signaling in the survival of the spheres cells that survived radiation, we used tyrosine kinase inhibitors with known activity anti PDGFR, Axitinib and Dasatinib, in colony formation assay. We observed that both inhibitors, were able to potentiate the antiproliferative effects of irradiation in A549 and H460 (Figure 11).

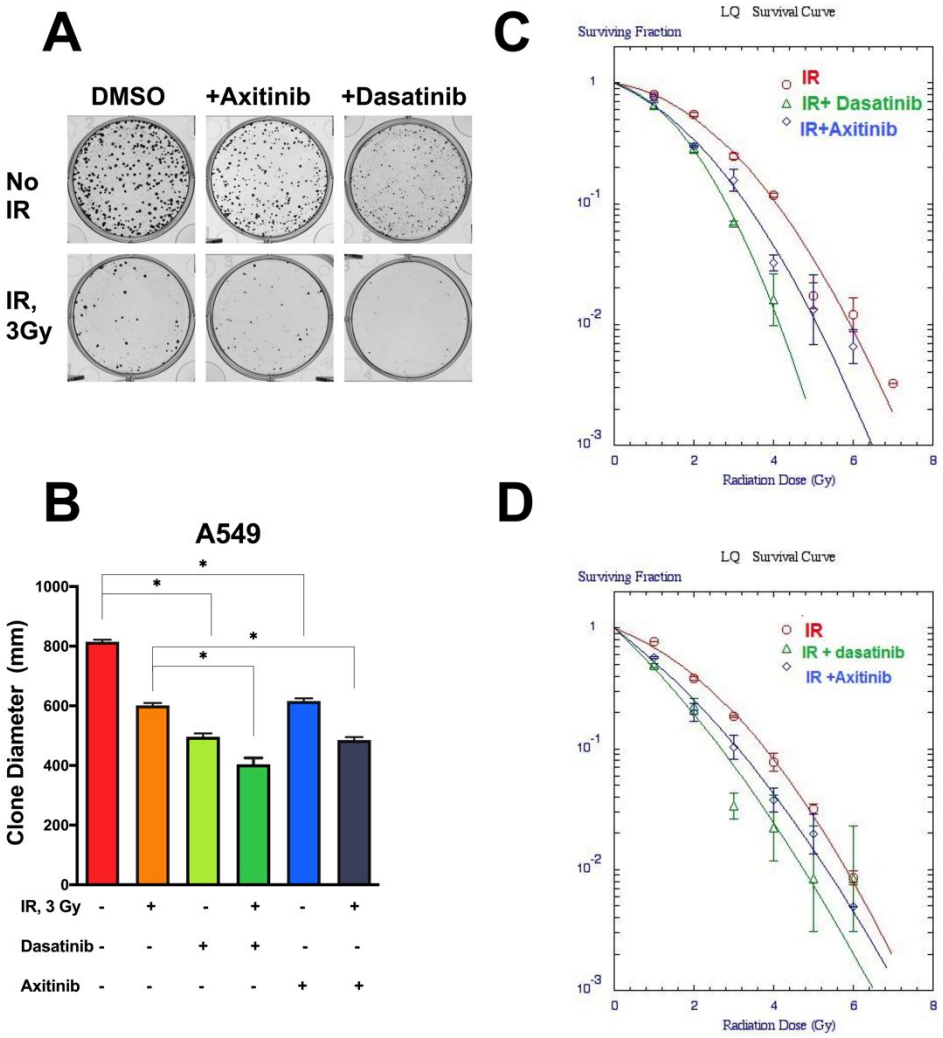

Figure 11. Tyrosine kinase inhibition potentiates the effects of irradiation. (A) Representative images of A549 colony formation assay treated with 3 Gy of radiation, and in combination with Axitinib or Dasatinib. **(B)** Graph representing the averages diameters of cell clones for each treatment. **(C-D)** Radiation survival curves for A549 (C) and H460 (D) growing with and without RTK inhibitors.

11. HSP90 inhibition as a treatment strategy

The cells that survived radiation therapy seem to have characteristics that predispose them to higher resistance of further treatments, and the ability to survive in an attachment independent environment. Despite the promising data shown with the use of RTK inhibitors, it is well known that targeted treatments aimed at inhibiting the tyrosine kinase activity of receptors, often lead to acquired resistances in the tumor[310,311]. For this reason we found useful to explore other novel approaches to target tumor cells that are able to resist and reemerge after a radiation treatment. A possible effective treatment option to eradicate this surviving population could be targeting HSP90, a chaperone that is involved in the maturity and folding of many known oncogenic drivers[312,313]. To further explore this, we decided to test the viability of HSP90 inhibition in NSCLC cells *in vitro*.

11.1. Effects of HSP90 inhibition with Ganetespib in NSCLC

11.1.1. Proliferation and motility

To begin, we evaluated whether Ganetespib had an antitumor effect on A549, T2821, and T2851 cell lines. All three cell lines showed sensitivity to Ganetespib treatment after 72h of incubation. T2821 was the most sensitive out of the three (IC50, 21.2 ± 0.9 nM), with lower sensitivities for T2851 (IC50, 43.4 ± 1.5 nM) and A549 (IC50, 49.9 ± 1.9 nM) (Figure 11A).

Similar results were obtained when we assessed the sensitivity in non-adherent conditions with the same cell lines (Figure 12B-C). T2821 cells again showed the greatest sensitivity to treatment (IC50 ~0.9 nM/IC100 ~4 nM), followed by A549 (IC50 ~1.4 nM/IC100 ~4 nM) and T2851 being the least sensitive (IC50 ~1.2 nM/IC100 ~10 nM).

Ganetesbip also exhibited the capacity to reduce cell motility in wound healing assays. A549 displayed the highest rate of motility of the three cell lines, but in all cases, Hsp90 inhibition with Ganetespib showed reduced motility of the cells (Figure 12D-F).

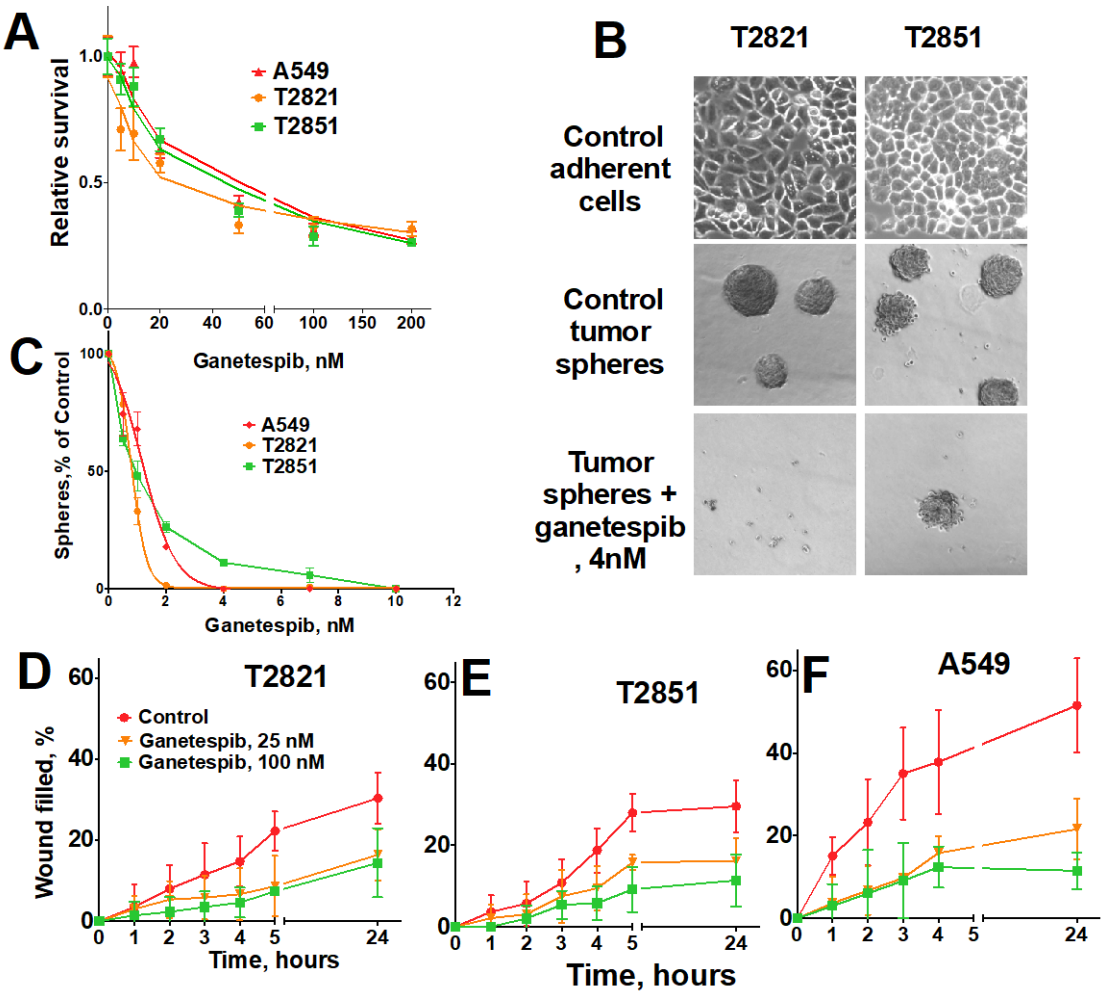

Figure 12. HSP90 inhibition with Ganetespib affects NSCLC cells survival and motility. (A) Viability of NSCLC cells after 72h in the presence of varying doses of Ganetespib. **(B)** Images of T2821 and T2851 growing in adherent conditions, as untreated tumorspheres and spheres in presence of 4 nM Ganetespib. **(C)** Ability of NSCLC cells to form tumorspheres in presence of Ganetespib at different concentrations. **(D-F)** Analysis of migratory capacity of NSCLC cells in presence of Ganetespib.

11.1.2. Apoptosis and cell cycle

To determine if the effect on migration was due to apoptosis, cells were treated with increasing concentrations of Ganetespib at different time points and analyzed with Annexin V-PI staining by flow cytometry (Figure 13A-C). Apoptosis was not detected in cells treated with low concentrations of Ganetespib (3 nM). At higher concentrations of the drug (30 and 300 nM) apoptosis was induced in the cells and it was time and concentration-dependent.

The cell cycle profile of the treated cell lines was also analyzed in comparison to the same non-treated cells. An arrest of the G2 phase of the cell cycle was observed and it was paired with descent in G1 and S phases in all three cell lines (Figure 13D-F).

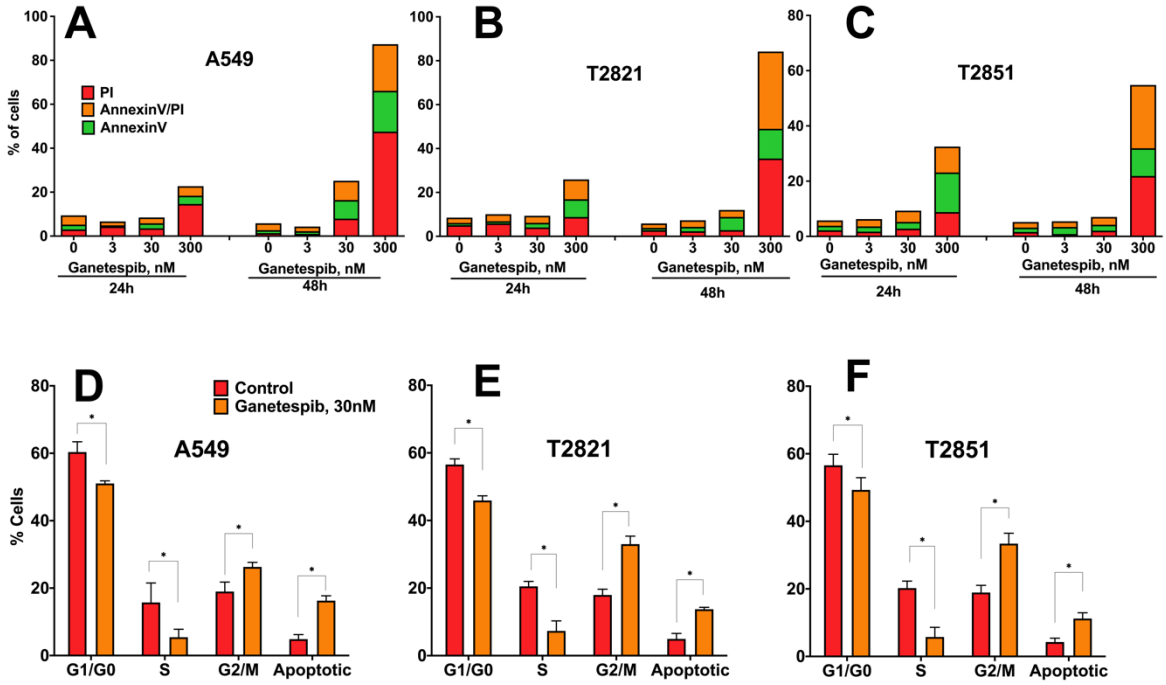

Figure 13. Ganetespib induces apoptosis and cell cycle arrest in NSCLC cells. (A-C) Quantification of cells in early apoptosis (Green), late apoptosis (Orange), and dead (Red) of NSCLC cells at 24 and 48h with different doses of Ganetespib. **(D-F)** Analysis of the effect of Ganetespib on the cell cycle of NSCLC cells after 48h of treatment.

11.1.3. Effects of Ganetespib on proteins involved in autophagy and senescence

The data suggests that Ganestespib treatment could induce a process of autophagy or senescence in these cells. To study this, we examined LC3 and HMGB1 as markers for autophagy processes (Figure 14A-B). We found both proteins to be increased in expression after exposure to 300 nM of Ganetespib for 48h. However, HMGB1 was mainly localized in the nuclei, suggesting an apoptotic event rather than a major autophagy process. Since this change in autophagy markers was not observed in lower dose treatments, we have analyzed the role of senescence in the growth inhibition process. We analyzed the expression levels of CDK inhibitors p21 and p27 along with p53 (Figure 14C-E). At the same time, we measured the morphological change by looking at the nuclei size (Figure 14F-G), which is another characteristic of senescent cells. p21, p27, and p53 showed to accumulate when cells were

treated with high doses of Ganetespib. Similarly, at 48h, only the treatment with high doses of HSP90 inhibitor produced an increase in the size of the nuclei. When the nuclei area of the treated cells was analyzed after 5 days of treatment we could observe a significant increase in nuclei size compared to the non-treated cells at doses of 3 nM (Figurer 14G).

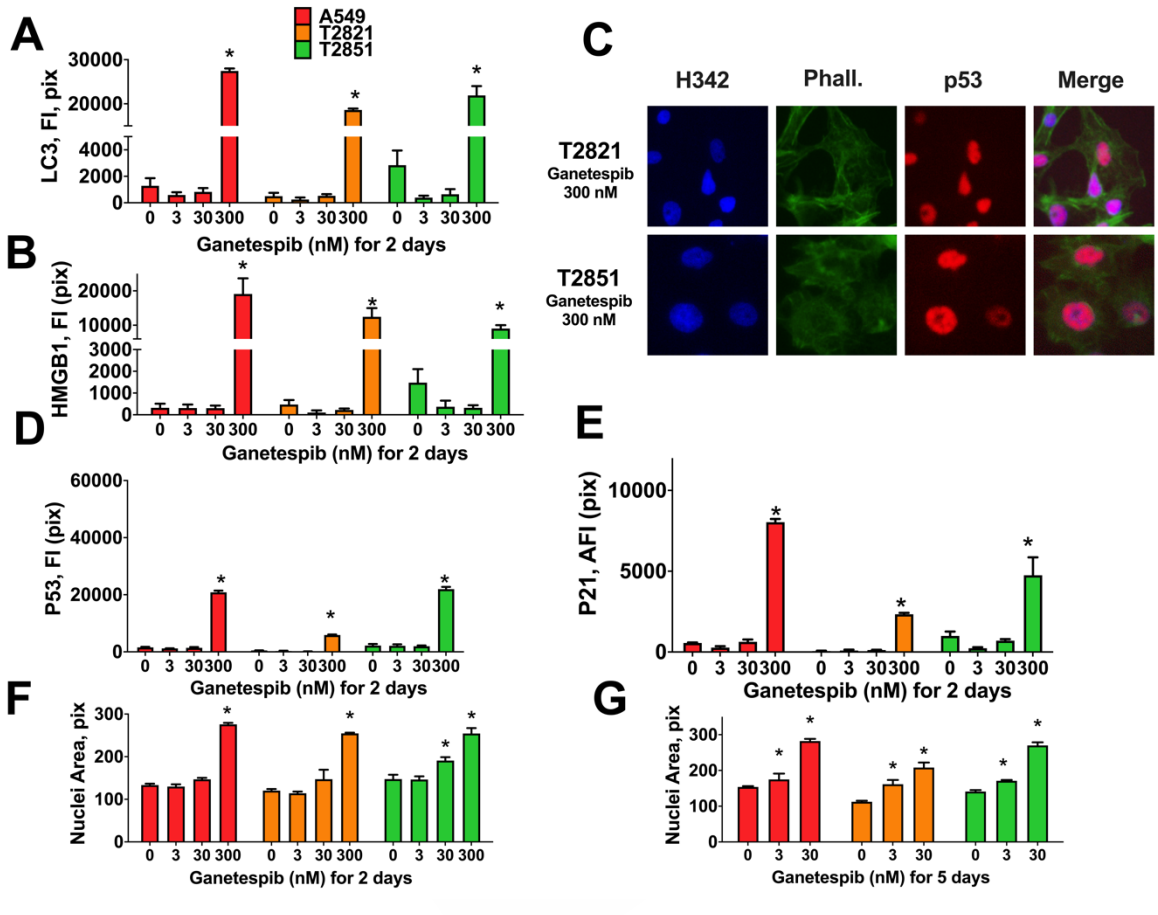

Figure 14. NSCLC cells treated with Ganetespib show characteristics of senescent phenotype. (A-B) Analysis of autophagy associated proteins LC3 and HMGB1 after 48h of Ganetespib treatment by immunofluorescence. **(C-D)** Representative images for p53 immunostaining are shown (C), and quantification of fluorescent intensity of p53 after 48h of treatment with Ganetespib (D). **(E)** Analysis of expression of p21 after exposure of the cells to Ganetespib for 48h. **(F-G)** Measurement of nuclei size of NSCLC cells after 48h of Ganetespib treatment, immediately after treatment (F) and five days after treatment(G).

11.1.4. Analysis of senescence after Ganetespib treatment

To confirm the senescent phenotype of the treated cells, we performed the β-galactosidase staining assay after exposing the cells to Ganetespib at different concentrations for 72h. We observed that not only was the number of cells per field lower as the concentration of drug increased, but the number of β-galactosidase staining positive cells had a direct relationship with the concentration (Figure 15).

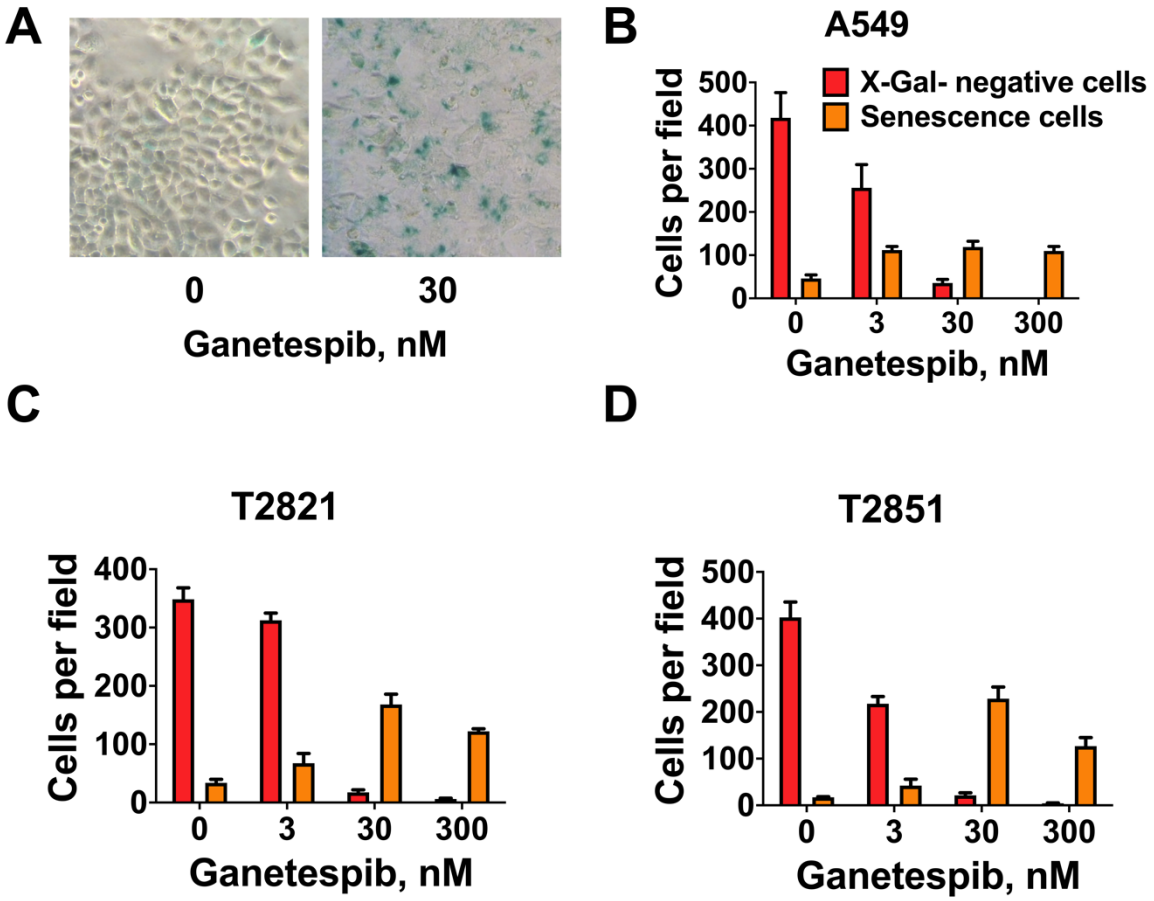

Figure 15. Ganetespib induces senescence in NSCLC cells. (A) Representative images of β-galactosidase staining of A549 cells. **(B-D)** Quantification of β-galactosidase stained cells with varying concentrations of Ganetespib at 72h in A549 (B), T2821 (C), and T2851 (D).

11.2. Combination of Ganetespib with radiation in NSCLC cells

11.2.1. Effect on survival of the combinatorial treatment

After seeing the potential of Ganetespib treatment in NSCLC cells, we evaluated the possibility of combining the first line of treatment, radiotherapy, with Hsp90 inhibition. To make sure these cells still expressed HSP90 after IR treatment, we checked the levels of Hsp90 expression in cells that have been irradiated by western blot (Figure 16A). Once we confirmed that the chaperone is also expressing in cells that survived radiation, we tested different concentrations of Ganetespib (3, 30 and 300 nM) in combination with a single dose of radiation of 5 Gy and counted the number of cells after 48h and 72h to assess the effect on proliferation. We observed that the number of cells per well decreased when cells were incubated with 300 nM of Ganetespib at both time points. More promisingly, after 72h, a dose 10 times lower, showed a significant reduction in the number of cells (Figure 16B-C).

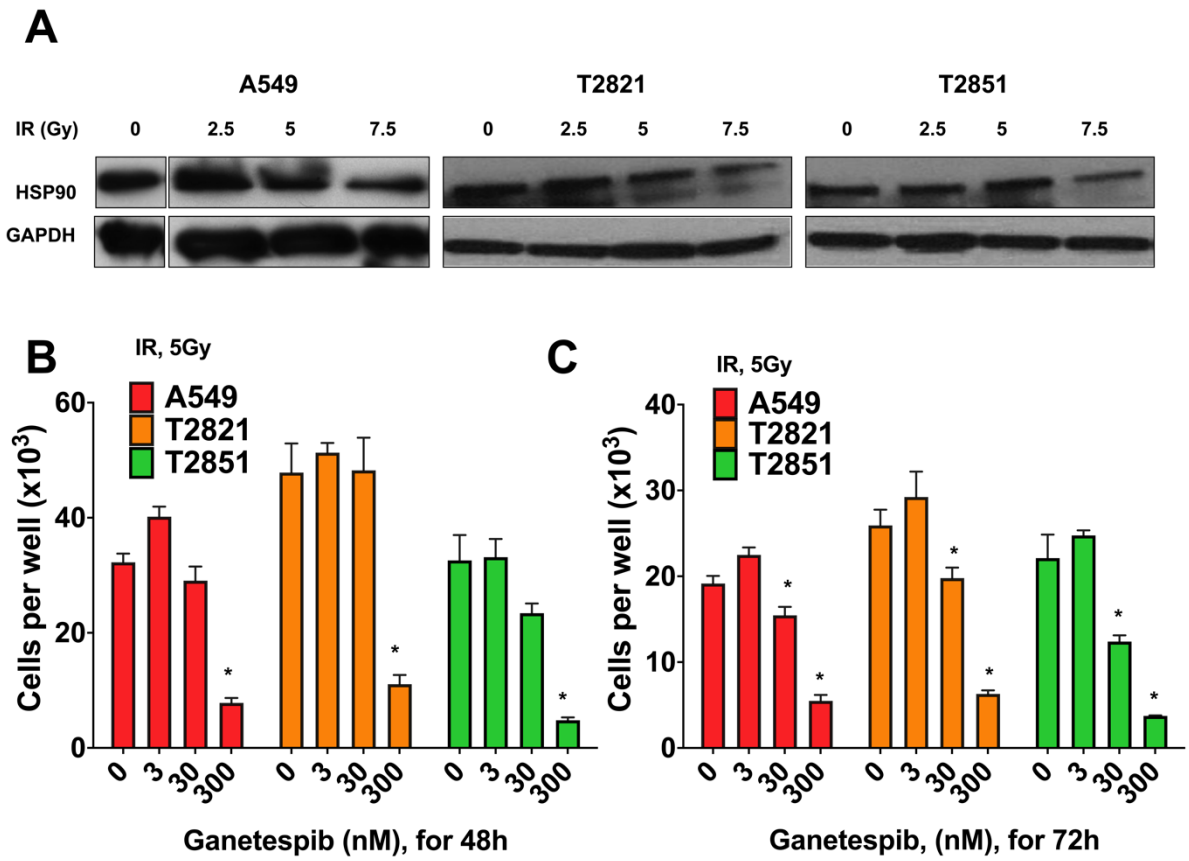

Figure 16. Ganetespib has an additive effect on radiation in NSCLC cells proliferation. (A) Western blot analysis of HSP90 expression levels in A549, T2821, and T2851 72h after irradiation. **(B-C)** Quantification of the number of cells per well in NSCLC cells treated with 5 Gy of radiation and Ganetespib at 48h (B) and 72h (C).

11.2.2. Comparison of the effects on cell survival with Ganetespib as pre- and post-radiation

In the next experiment, we compared the effect of pre-treating the cells with ganetespib before radiation treatment, as opposed to treating the cells with the inhibitor after the IR treatment was performed. We observed that pre-treating the cells with Ganetespib 24h before exposing them to radiation dramatically increased the effect of radiation therapy (Figure 17A-C). When cells were exposed to the drug after they were irradiated, even though the difference in survival was significant, it was not as drastic as the pre-treatment schedule (Figure 17D-F).

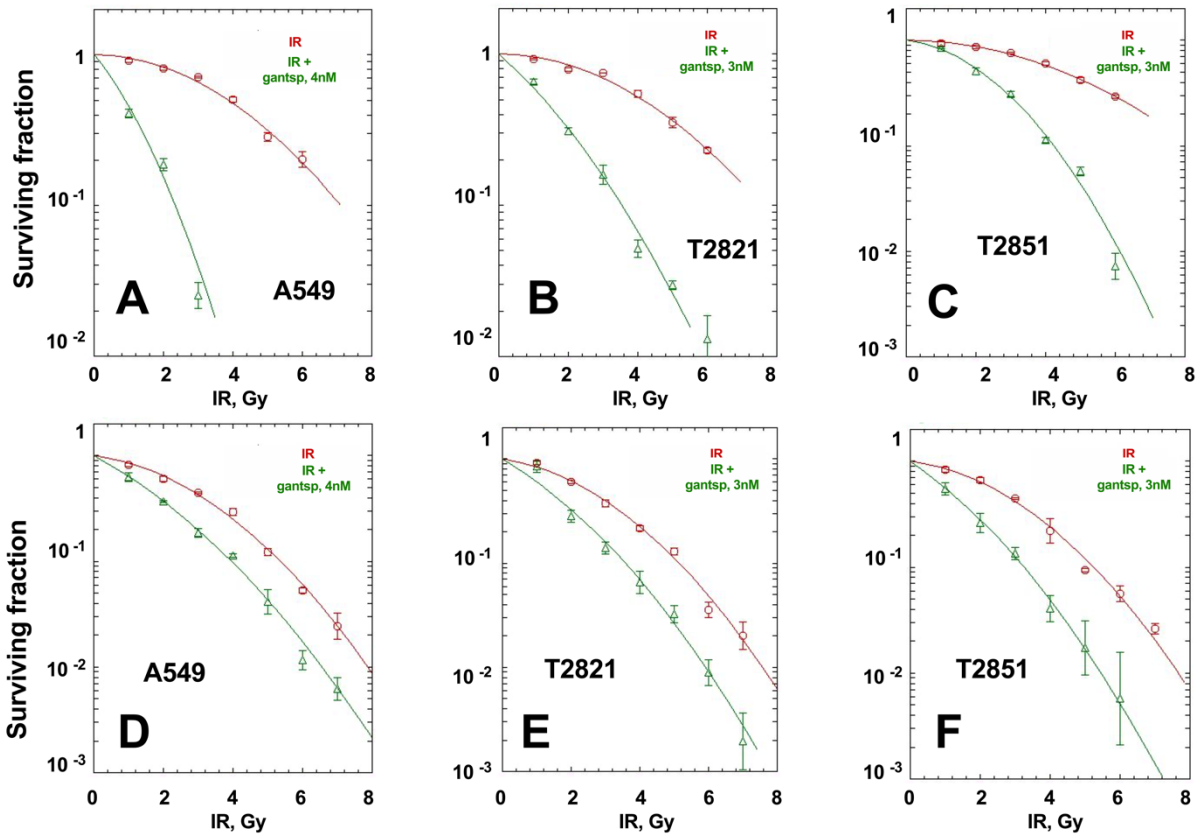

Figure 17. Ganetespib improves the effects of radiation over NSCLC cell survival. (A-C) Colony-forming ability of NSCLC cells treated with Ganetespib 24h before irradiation. **(D-F)** Colony-forming assay in NSCLC cells treated with Ganetespib after radiation treatment.

11.2.3. Influence of Ganetespib treatment on the DNA repair process

11.2.3.1. γH2AX foci formation

Next, we assessed the phosphorylated histone H2AX foci formation, as a measurement of DNA double-strand breaks repair. When cells were incubated with Ganetespib as a monotherapy, only 300 nM dose produced a little increase in the number of γH2AX foci at 12h (Figure 18B-D), suggesting that high concentrations of the drug could influence the DNA repair process. When both treatments, IR and Ganetespib, were tested in combination we observed

consistent differences in the three cell lines at 6h and 12h post-radiation treatment. Ganetespib at 3nM dose influences the resolution of the DNA repair foci (Figure 18E-G). The effect was more dramatic as the dose increased (Figure 18H).

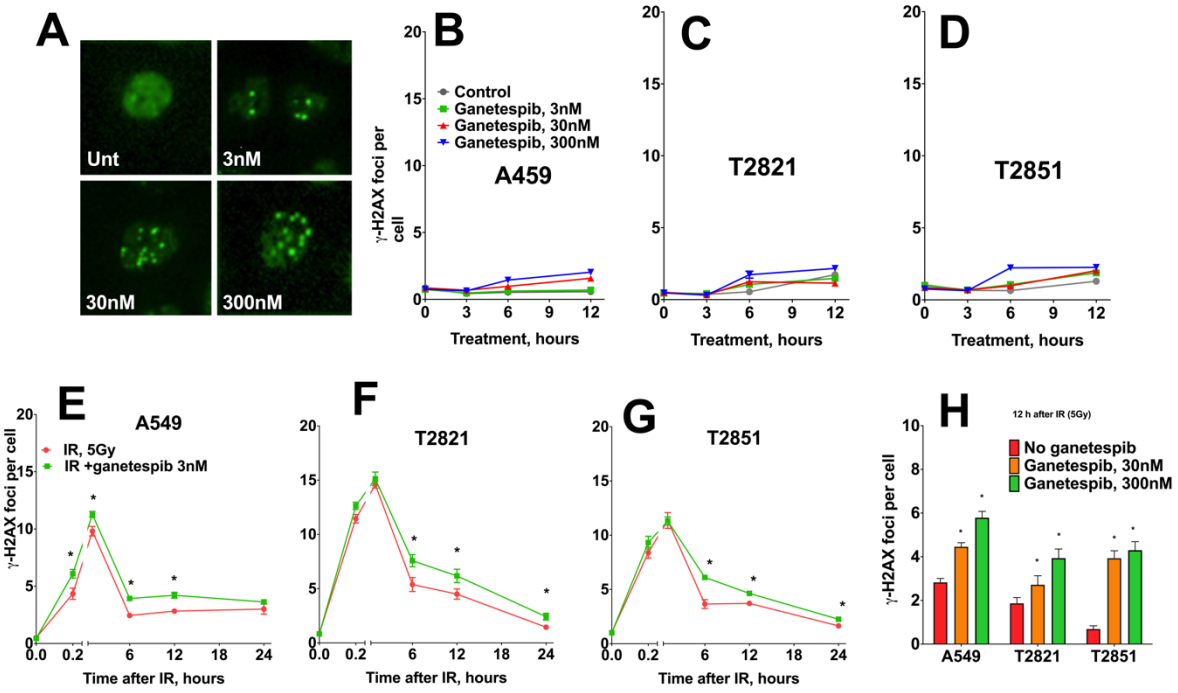

Figure 18. Ganetespib impairs DNA repair after radiation treatment. (A) Representative images of γH2AX foci in A549 cells untreated and treated with radiation and Ganetespib at 3 nM, 30 nM, and 300 nM. **(B-D)** Analysis of the number of γH2AX foci in NSCLC cells with Ganetespib as monotherapy. **(E-G)** Quantification of the number of γH2AX foci per cell in response to radiation with and without Ganetespib treatment in NSCLC cells. **(H)** Representation of the number of γH2AX foci per cell 12h post-treatment with radiation and radiation plus Ganetespib at 30nM and 300nM.

11.2.3.2. Effect on ATM, ATR, and HSP90 phosphorylation, and 53BP1 expression

Considering the prior findings, we studied the influence of HSP90 inhibition on key DNA repair proteins such as pATM, pATR, and 53BP1. As Expected, IR treatment produced an increase in ATM phosphorylation, but pretreatment of cells with Ganetespib reduced the levels of pATM in the three cell lines (Figure 19). Similarly, pATR and 53BP1 expression were affected by the treatment with Ganetespib (Figure 20A-B). For the three proteins, the inhibitory effect of Ganetespib was dose-dependent.

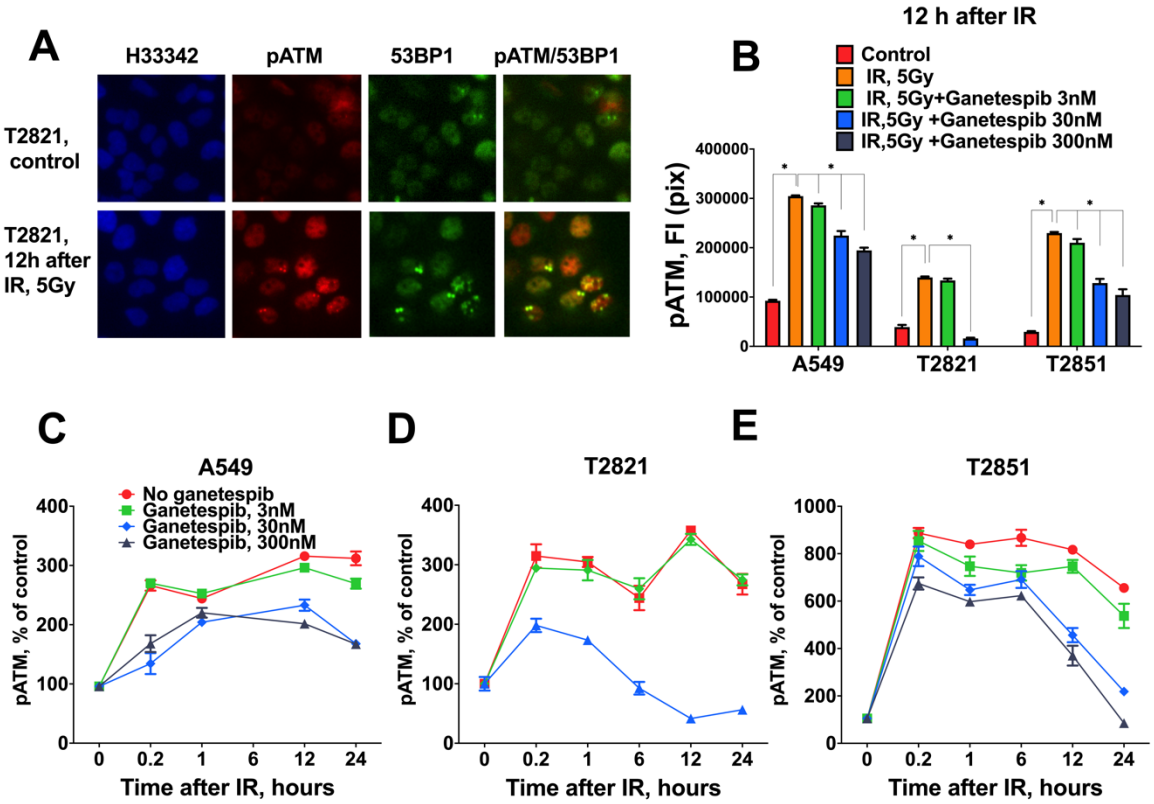

Figure 19. Ganetespib reduces the phosphorylation of ATM after IR treatment in NSCLC cells. (A-B) Analysis of ATM phosphorylation (S1981) 12h after radiation. Representative images of pATM showing co-localization with 53BP1 foci are shown (A) and fluorescence intensity quantification of pATM staining in untreated cells, treated with 5 Gy of radiation, and radiation combined with Ganetespib at different doses (B). **(C-E)** Evolution of ATM phosphorylation after radiation with and without Ganetespib at different doses in A549 (C), T2821 (D), and T2851 (E).

Furthermore, we have analyzed the status of HSP90 phosphorylation. The involvement of HSP90 in DNA repair has previously been established. It has been shown that HSP90 phosphorylates on residue Th7 after DNA damage and it localizes at sites of double-strand breaks[314]. Like the other DNA repair proteins analyzed, the phosphorylation of HSP90 was affected by the treatment with Ganetespib, producing a decrease in the phosphorylated protein signal (Figure 20C).

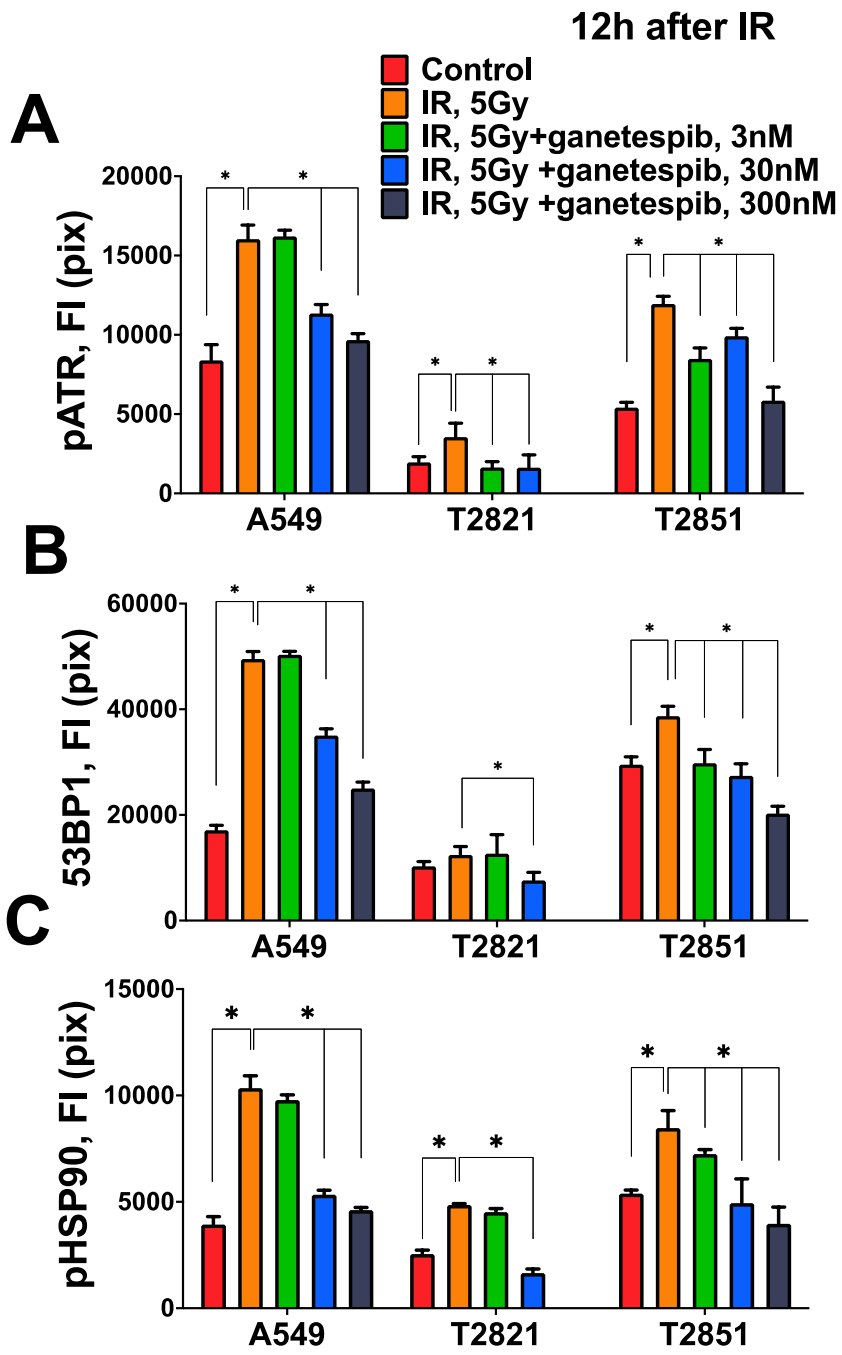

Figure 20. Ganetespib reduces ATR and HSP90 phosphorylation and 53BP1 expression after radiation. Fluorescent intensity of pATR (S428)(**A**), 53BP1 **(B)**, and pHSP90 (T7)**(C)** 12h after irradiation with and without Ganetespib treatment in A549, T2821 and T2851 cell lines.

11.2.4. Analysis of the status of relevant signaling pathways

For the next step, we wanted to investigate whether Ganetespib at low doses could influence the response of important biological processes by measuring key proteins (Figure 21). RAD51 is an important protein involved in homologous recombination occurring during DNA

double-strand break repair[315,316]. We observed that RAD51 was affected by Ganetespib treatment at 3 nM in T2821 and T2851, but not in A549 cells. AKT phosphorylation was used to study the status of activation of the PI3K/AKT pathway, which has been demonstrated to be important in cell proliferation in NSCLC cells[317]. We observed that phosphorylation of AKT in S473 was activated after IR treatment, however, treatment of the cells with Ganetespib reduced the amount of phosphorylated AKT in T2821 and T2851, but not in A549. Lastly, we analyzed the expression of the CDK inhibitor p21, which is expressed in low amounts in untreated cells. We observed that the combination treatment of IR and Ganetespib at 3 nM induced an important accumulation of p21 in all 3 cell lines, suggesting that Ganetespib could potentiate the effects of IR damage leading to growth arrest and senescence.

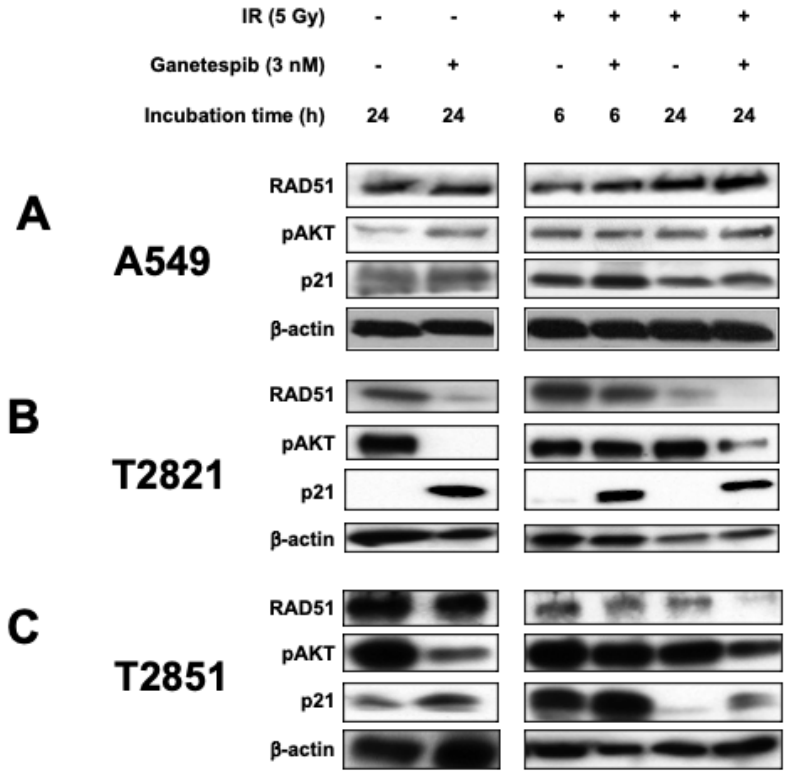

Figure 21. Ganetespib influences proteins relevant in different signaling pathways. (A-C) Western blot image showing levels of expression for RAD51, pAKT (S473), and p21 in A549 **(A)**, T2821 **(B)**, and T2851 **(C)** cell lines untreated, treated with radiation, treated with Ganetespib, and combination of both treatments.

11.2.5. Analysis of senescence in combinatorial treatment

Our data suggest that the combination of Ganetespib with IR therapy may induce DNA-SCARs, which don´t need the participation of RAD51, leading to a senescent phenotype as revealed by the increase in p21 expression. To confirm this, we investigated the effect of Ganetespib treatment when combined with radiation over three major proteins in DNA-SCARs: pATM, pH2AX, and 53BP1.

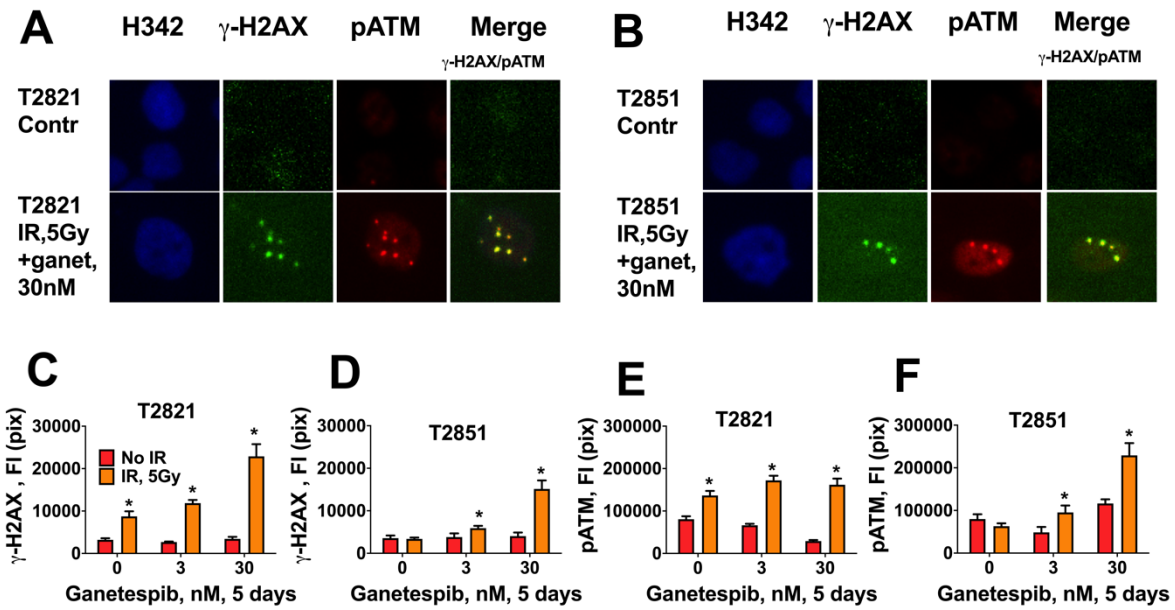

Figure 22. Ganetespib in combination with radiation prevents DNA repair resolution. (A-B) Representative images showing colocalization of pATM and γH2AX in T2821 and T2851 five days post-radiation with and without Ganetespib. **(C-D)** Fluorescence intensity of γH2AX immunostaining five days after irradiation and when combined with Ganetespib at 3 nM and 30 nM in T2821 and T2851 cell lines. **(E-F)** Quantification of pATM (S1981) fluorescence in irradiated cells combined with Ganetespib after five days.

We observed that treating the cells with Ganetespib for 5 days after IR treatment significantly increases the amount of γH2AX and pATM in all three cell lines (Figure 22). Similarly, 53BP1 presence in the cells was increased when cells were treated with Ganetespib after IR (Figure 23). Moreover, cells treated with radiation and Ganetespib for 5 days showed a higher number of cells stained with X-gal when compared with cells that have been treated with radiation only, supporting the hypothesis that Ganetespib might induce the formation of DNA-SCARs leading to senescent phenotypes.

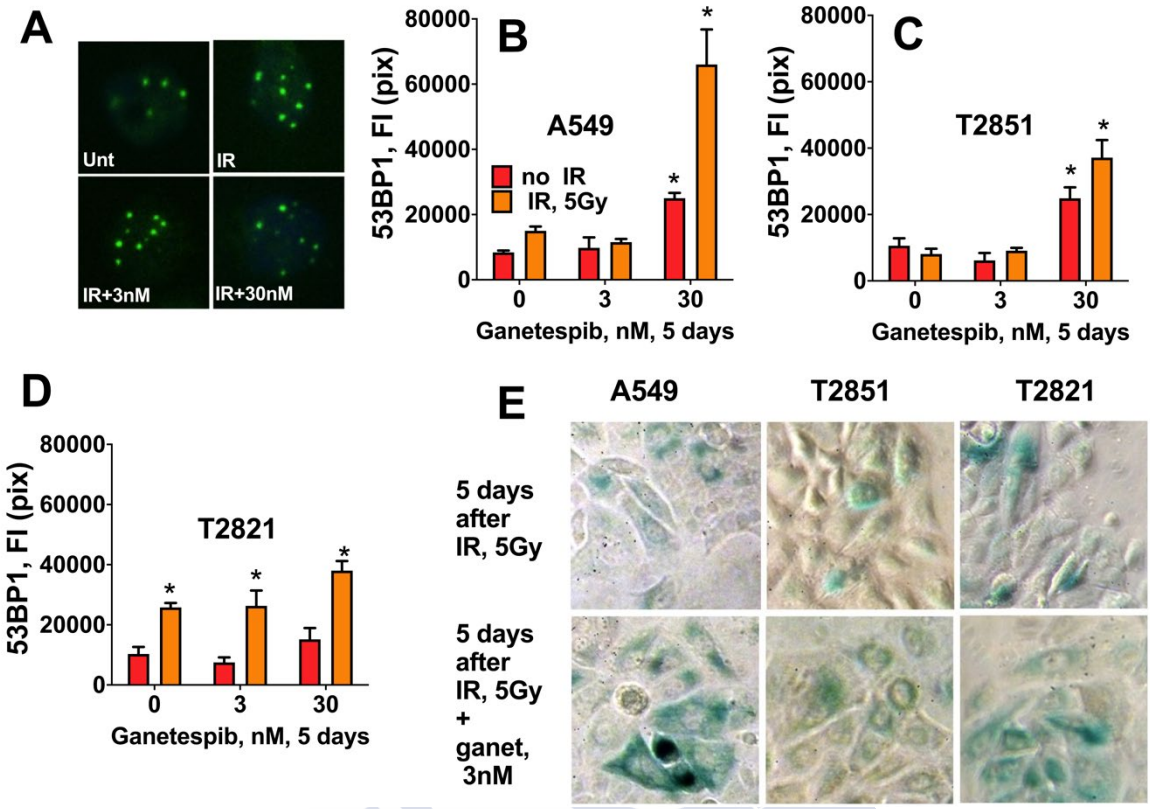

Figure 23. Ganetespib makes radiation-induced DNA damage persistent leading to senescence. (A) Representative images of 53BP1 radiation-induced foci staining in T2821 cells untreated (top left), treated with radiation (top right), and radiation plus Ganetespib at 3nM (lower left) and 30 nM (lower right). **(B-D)** Quantification of the fluorescence intensity for 53BP1 in A549, T2821, and T2851 cells treated with Ganetespib in combination with radiation after five days. **(E)** Representative images of ß-galactosidase staining five days after irradiation with and without Ganetespib in A549, T2821, and T2851.

12. Study of radioresistant NSCLC cell lines

12.1. Establishment of NSCLC radioresistant cell lines

Considering the previous results, we decided to study a population of truly radioresistant cells. For this, we used the tumor patient-derived cell lines T2821 and T2851. The parental cell lines were treated with repeated doses of radiation, twenty times with 2 Gy, four times with 5 Gy, and three times with 10 Gy. The derived radioresistant cell lines were named T2821/R and T2851/R respectively. After the cell lines were established we determined the plating efficiency of the newly generated cells. We observed that the radio-resistant cells had significantly lower plating efficiency than the parental cell lines from which they were generated (Table 3). Then, we confirmed the radioresistance of these cell lines by colony-forming assay. We observed that the derived cell lines effectively could generate more clones after they were exposed to radiation (Figure 24B-C).

Next, we tested whether the resistant cell lines generated had increased resistance to other treatments that induced DNA damage. To accomplish this, we performed a survival assay with cisplatin, in which we observed that the resistant cells showed a higher IC50 than the parental cells (Table 3).

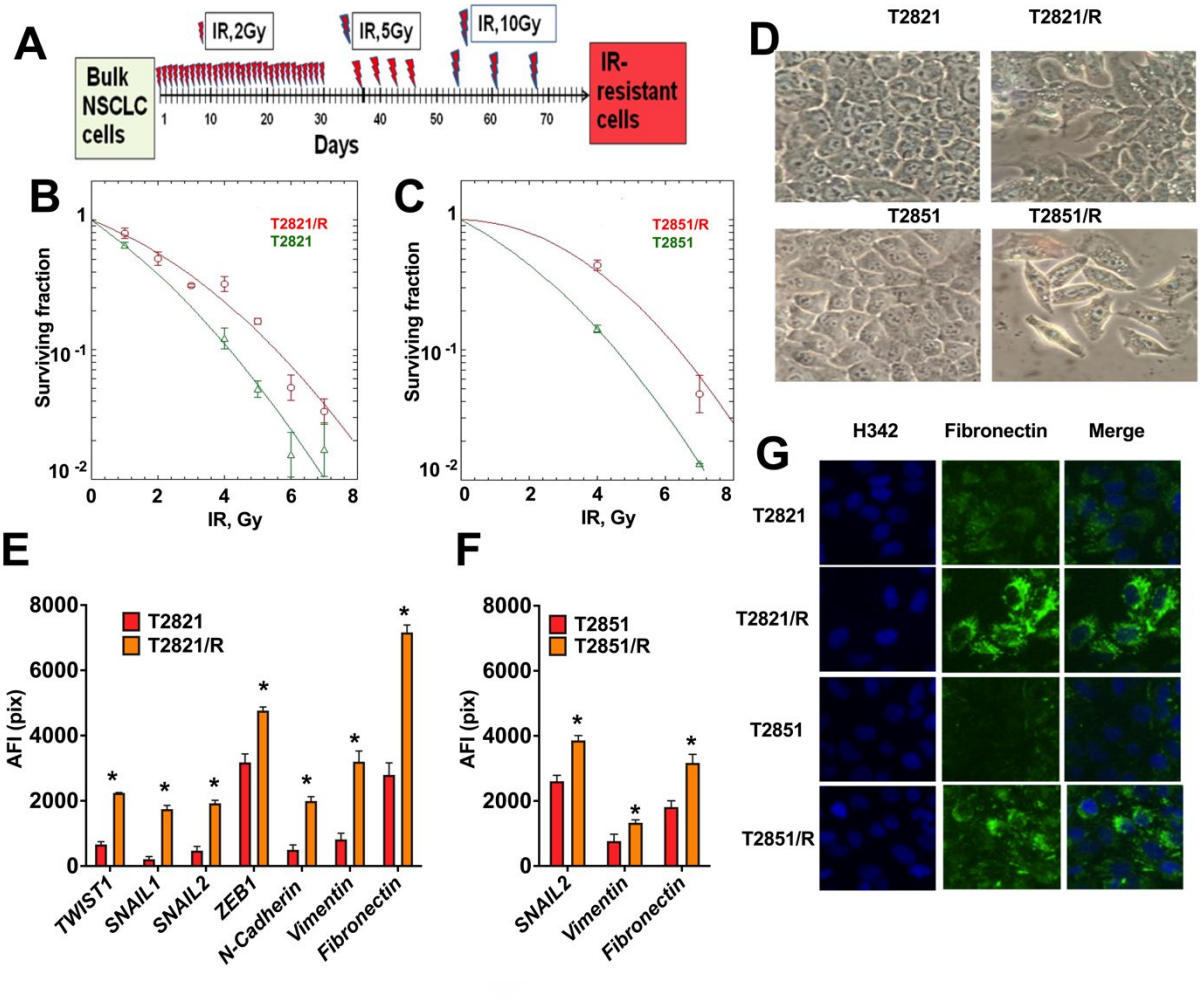

Figure 24. Generation of radiation-resistant NSCLC cells. (A) Diagram representation of the strategy used to generate the radioresistant cells. **(B-C)** Clonogenic survival assay showing higher resistance to radiation treatment in the new cell lines T2821/R and T2851/R. **(D)** Images of parental cell lines T2821 and T2851 and the derived radioresistant cell lines T2821/R and T2851/R in culture. **(E-F)** Quantification of fluorescent intensity for different EMT markers in T2821 versus T2821/R (E) and T2851 versus T2851/R (F). **(G)** Representative images of the immunofluorescence staining for Fibronectin in the four cell lines.

	T2821	T2821/R	T2851	T2851/R
Plating efficiency	66.98 ± 1.95	*50.82 ± 1.57	58.07 ± 3.99	53.76 ± 2.58
IR sensitivity, D_0	1.279 ± 0.12	*1.546 ± 0.06	1.351 ± 0.07	*1.659 ± 0.04
Cisplatin IC_{50}, μM	3.67 ± 0.31	*8.31 ± 0.66	7.29 ± 0.69	*9.26 ± 0.92

Table 3. Characterization of radioresistant cell lines. Values of plating efficiency (%), sensitivity to radiation (Gy), and sensitivity to cisplatin treatment (μM) in T2821, T2821/R, T2851, and T2851/R. All values expressed with standard deviation. Asterisks represent statistical significance compared to the respective parental cell line with $\rho < 0.05$.

12.2. Characterization of NSCLC radioresistant cell lines

12.2.1. Analysis of EMT, stemness and other pathways relevant for NSCLC progression

In culture, the resistant cell lines showed morphological changes with respect to the cell lines from which they derived. While the parental cell lines showed tight cell-cell junctions, as is expected of epithelial cell cultures, the resistant cell lines had a more scattered pattern and the cells showed a more spindle-like morphology (Figure 24D). To verify if the morphology changes were due to an EMT process, we analyzed, by immunofluorescence (Figure 24E-G), several EMT markers. We observed an increase in expression on several proteins important for the EMT events. On T2821/R we observed upregulation of Twist1, Snail1, Snail2, Zeb1, N-cadherin, Vimentin, and Fibronectin. The phenotype was different in T2851/R, presenting upregulation in only Snail2, Vimentin, and Fibronectin.

In the next step, we set to study some of the pathways known to be involved in cancer progression and therapy resistance. Activation of the PI3K/AKT pathway is known to be involved in radiotherapy resistance in NSCLC, and when we analyzed the expression of total AKT and its phosphorylated form, we observed that the radioresistant cell lines have a significantly higher amount of AKT and an increase in pAKT (S743) compared to the parental cell lines (Figure 25A-B). We also found that total STAT3 expression was higher in resistant cell lines. Other important factors in pro-survival signaling, like SDF1 and its receptor (CXCR4), are expressed in bigger amounts in the resistant cell lines. Similarly, IL-6, STAT3, and PDGF-BB have increased expression in radioresistant cells (Figure 25). We did not find a difference in the expression of IL-6R, PDGFR-β, or GRP130 between radioresistant and parental cell lines. The fact that resistant cells have higher production of the aforementioned growth factors suggests that there might be autocrine and/or paracrine mechanisms helping these cells to survive.

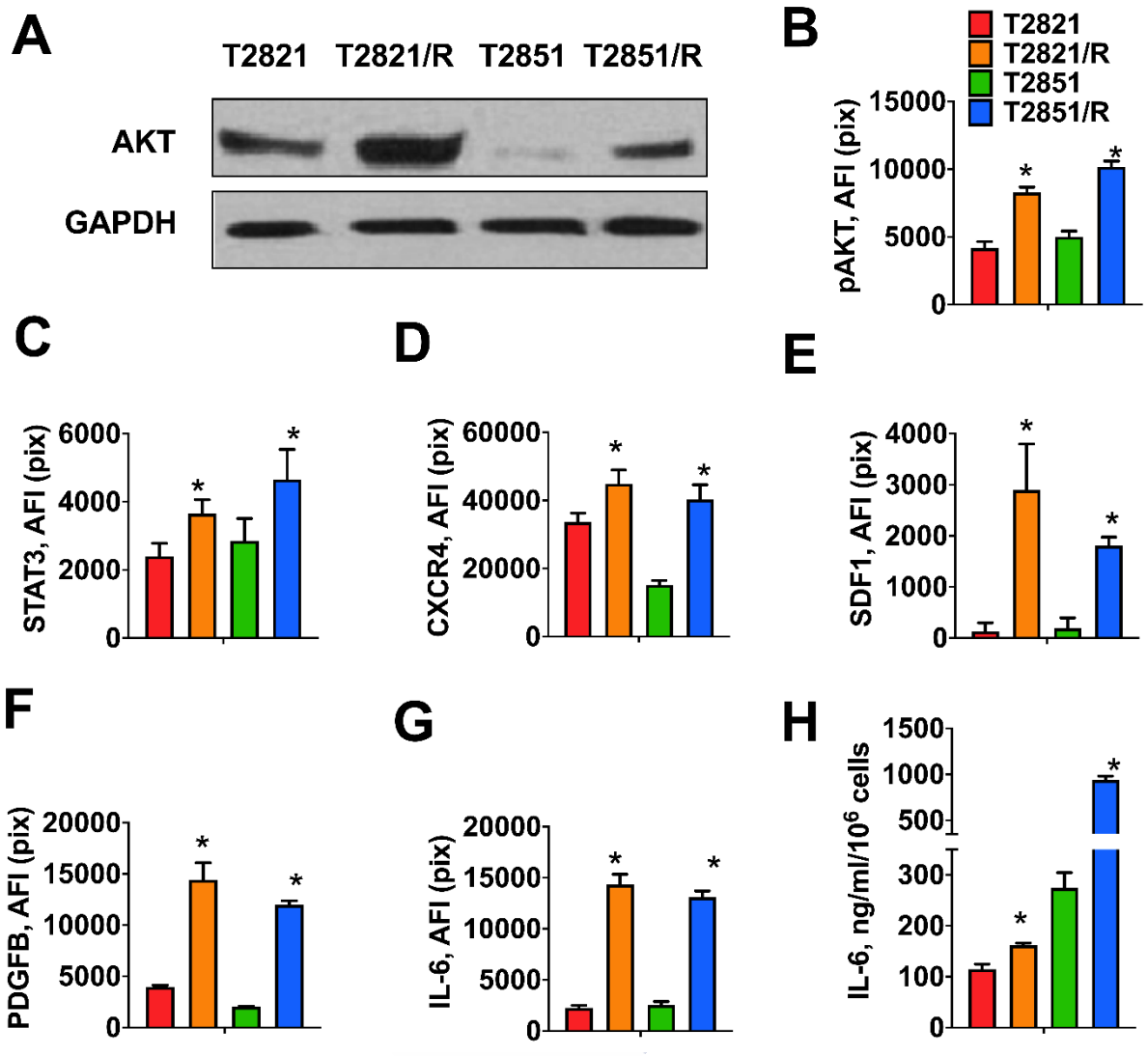

Figure 25. The radioresistant cell lines have upregulation of important proteins for cell survival and proliferation. (A) Western blot analysis of AKT expression levels. **(B-D)** Immunofluorescent staining quantification for the expression of pAKT (S473), STAT3, and CXCR4. **(E-G)** Average fluorescence intensity for the staining of growth factors SDF1, PDGFBB, and IL-6 produced in T2821/R, T2851/R, and the parental cell lines. **(H)** Measurement of IL-6 production by ELISA in parental and radioresistant T2821 and T2851 cell lines.

12.2.2. Analysis of proliferation rates and cell cycle

12.2.2.1. Doubling time

Observing that T2821/R and T2851/R seem to produce growth factors in a higher amount than the parental cell lines, we compared the doubling times of the four cell lines, and we observed that resistant cell lines have a longer doubling time when compared with the parental cell lines (Table 4).

Cell line	Time (h)					Doubling time (h)	*p*-value
	0	8	24	48	72		
T2821	0.26 (0.07)	0.48 (0.22)	1.70 (0.29)	5.88 (0.45)	11.55 (1.84)	12.80 (0.79)	
T2821/R	0.27 (0.11)	0.51 (0.18)	1.56 (0.17)	5.18 (0.42)	9.05 (0.40)	13.77 (0.92)	p = 0.4237 compared to T2821
T2851	0.31 (0.10)	0.49 (0.28)	2.20 (0.41)	6.22 (0.55)	12.10 (0.29)	13.00 (0.95)	p = 0.8704 compared to T2821 p = 0.5623 compared to T2821/R
T2851/R	0.28 (0.06)	0.88 (0.44)	2.13 (0.19)	4.51 (0.36)	5.45 (0.40)	17.84 (1.99)	p = 0.0184 compared to T2821 p = 0.0635 compared to T2821/R p = 0.0282 compared to T2851

Table 4. Doubling times and significance values for the statistical comparisons of doubling times. The table shows the mean of the number of cells (x105) estimated at each time point and the standard deviations between parentheses in the columns on the left, and cell line doubling times and statistical significance in the two columns on the right.

12.2.2.2. Analysis of cell cycle profile in response to radiation

When we analyzed the cell cycle in response to radiation, we observed that there was no major difference between parental and radioresistant cell lines. As expected, we observed an accumulation on the G2 phase eight hours after they were exposed to IR. The cycle progresses back to the untreated profile after 24 hours, except in T2821/R, in which we observe an accumulation on the G1 phase at 24 and 30 hours post-radiation. We did not observe differences in the untreated cells at different time points, therefore the figure only shows the profile for cells at time zero hours (Figure 26).

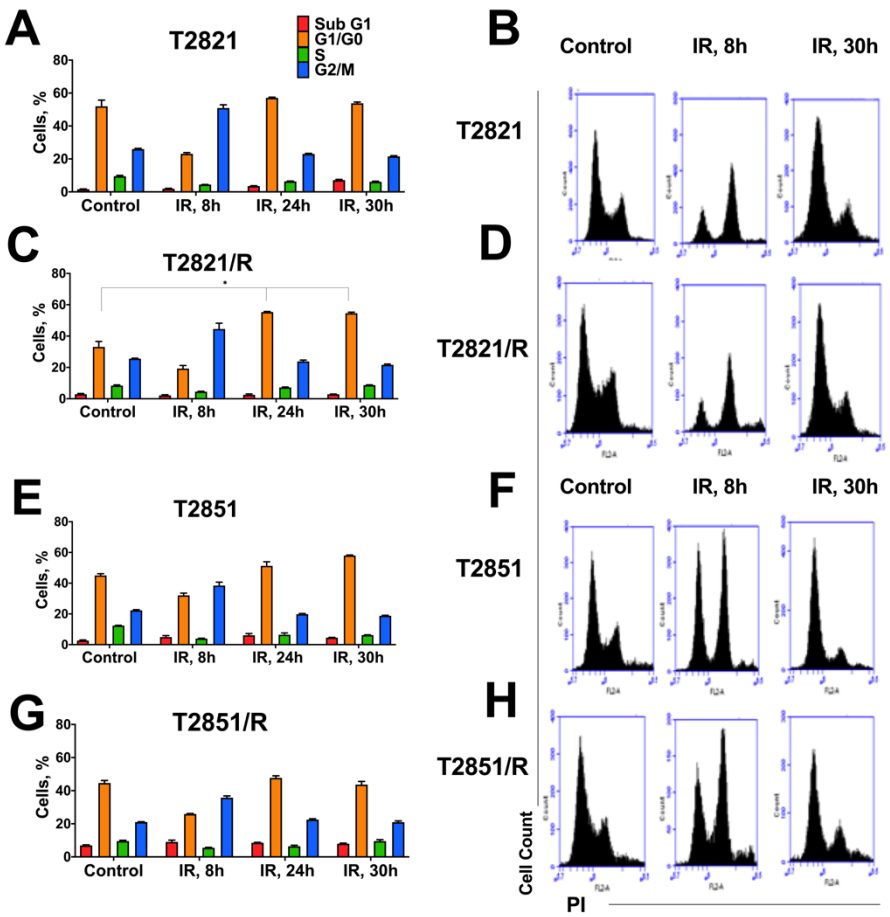

Figure 26. Radioresistant cells do not show important changes in cell cycle distribution compared to the parental cell lines. (A, C, E, G) Analysis of the percentage of cells in each phase of the cell cycle for T2821, T2821/R, T2851, and T2851/R at different time points post-radiation. **(B, D, F, H)** Representative flow cytometry histograms of cell cycle distribution for the same cell lines in the same conditions as shown on the bar graphs.

12.2.3. Analysis of DNA repair pathways

12.2.3.1. Expression of genes involved in DNA repair

To further analyze the profile of the resistant cells, we analyzed the mRNA expression of several genes involved in DNA repair processes. For this, we used an RT-qPCR array that included 84 different genes involved in base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and double-strand break repair (DSBR). The gene profile was altered in T2821/R and T2851/R in comparison to the respective parental cell lines. A bigger number of genes was altered in T2821/R than in T2851/R. We found ten genes with higher expression in T2821/R compared to T2821. Such genes were involved in BER (NEIL1 and LIG3), NER (ATXN3, ERCC1, and PNKP) and DBSR (ATM, RAD50, RAD51C, RAD51D, and RAD52). On the contrary, three genes were found to be downregulated in T2821/R: UNG (involved in BER), RPA3 (involved in NER), and RAD51 (part of the DSBR machinery). Interestingly, the gene expression profile changes were different between the two resistant cell lines. T2851/R showed only upregulation changes in DSBR genes: EXO1; FEN1; RAD18; RAD51 RAD54L; and XRCC2. Some of the genes found to have changes in expression were

double-checked by conventional RT-qPCR. Levels of mRNA expression for ATM, RAD50, RAD51, ERCC1, and XRCC2 were corroborated.

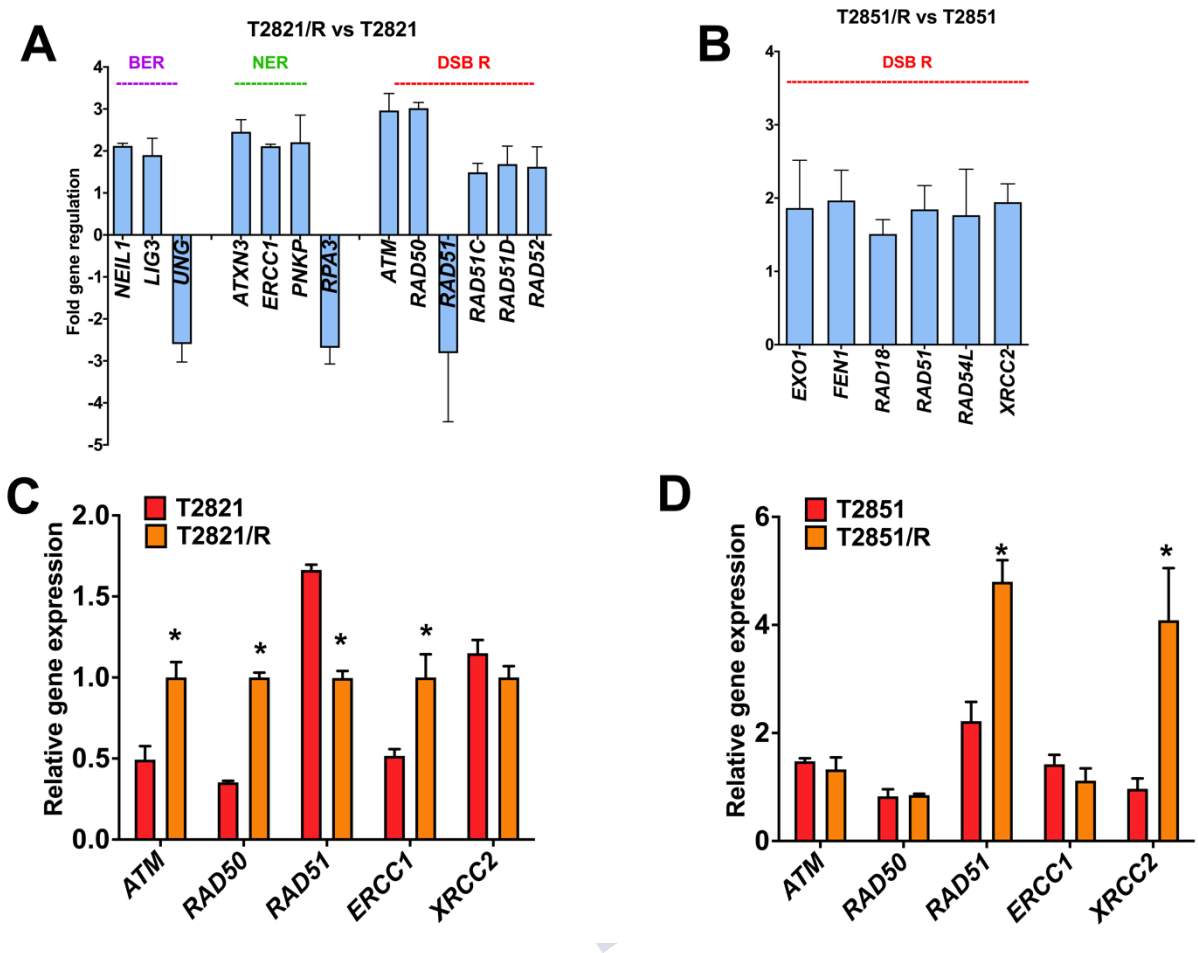

Figure 27. Radioresistant NSCLC cells exhibit altered expression of genes associated with DNA repair. (A-B) Fold change regulation of gene expression in radioresistant versus parental cell lines obtained by RT-qPCR array. **(C-D)** Gene expression relative to GAPDH mRNA expression of each sample by RT-qPCR in T2821 and T2851/R (C), and T2851 and T2851/R (D).

12.2.3.2. Analysis of the DNA machinery response to radiation

12.2.3.2.1. γH2AX and 53BP1 foci formation

Next we wanted to analyze whether the radioresistant phenotype was due to an increased capacity of DNA DSB repair (Figure 28). To determine this, we used γH2AX and 53BP1 foci as a measurement of DSB repair after IR treatment. We found T2821/R and T2851/R controls to have a similar number of γH2AX and 53BP1 foci per cell compared to the respective parental cell lines. Radiation treatment-induced foci formation in the four cell lines at 0.5 hours and one hour after treatment, however, the number of foci in T2821 and T2851 were significantly higher than the respective resistant cell lines for γH2AX and 53BP1. Six hours after radiation, the number of γH2AX foci per cell was reduced to one-third of those present at one hour post-treatment, suggesting that most of the DNA repair process had already occurred by that point. T2821 and T2821/R showed the same number of γH2AX and 53BP1 foci per cell at 6 and 24 hours post-radiation. On the contrary, a significantly higher number of foci was still observable in T2851/R compared to T2851 at those time points.

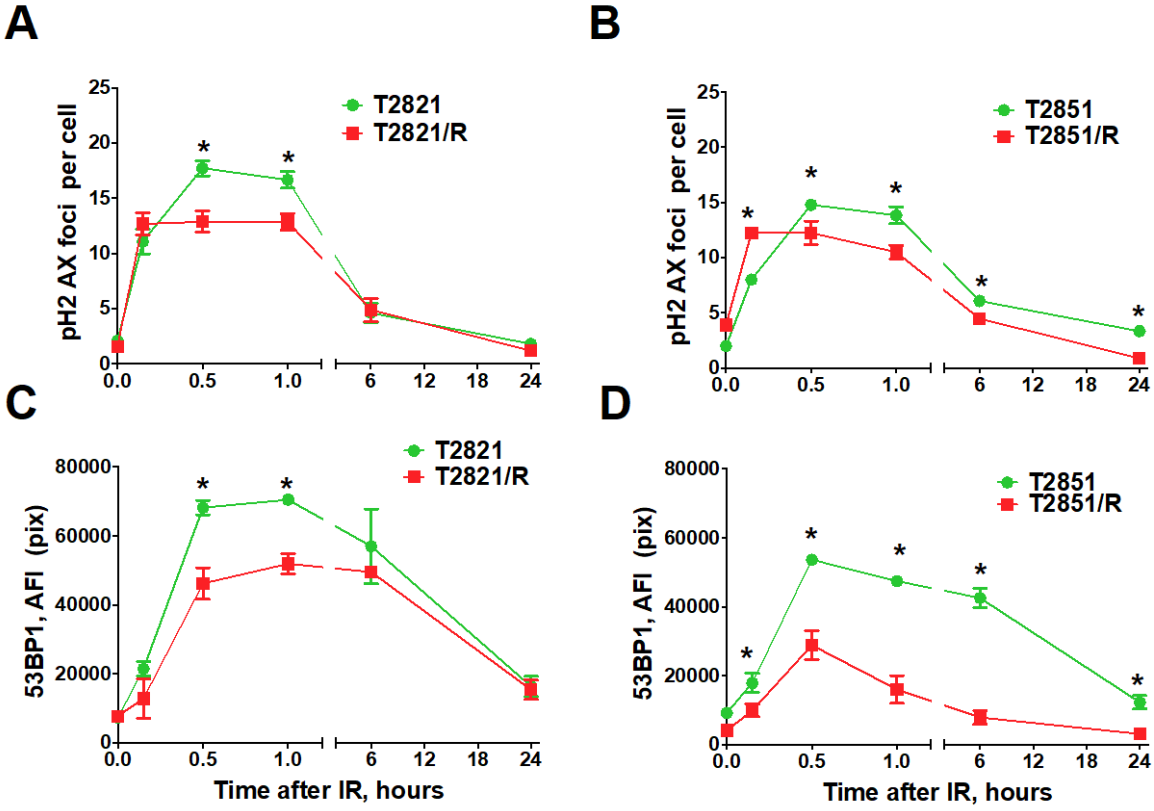

Figure 28. Radiation resistant NSCLC cells exhibit a decreased response to radiation-induced DNA damage. **(A-B)** Number of γH2AX foci formation in response to radiation throughout 24h in T2821 compared to T2821/R (A) and T2851 compared to T2851/R (B). **(C-D)** Average fluorescence intensity of 53BP1 after radiation treatment for 24h in T2821, T2821/R, T2851, and T2851/R cell lines.

12.2.3.2.2. Activation of ATM and ATR and expression of RAD51 after radiation

To further characterize the DNA repair response, we analyzed the activation of ATM and ATR, and the expression of RAD51 over time in cells that were exposed to 5 Gy of radiation. The treatment triggered phosphorylation of ATM in all cell lines, but, interestingly, the parental cells had a higher amount of phosphorylated protein compared to the radioresistant cells derived from them (Figure 29A-B). Similarly, pATR (S428) was lower in T2821/R and T2851/R compared to their respective parental cell lines(Figure 29C-D). Nevertheless, RAD51 expression was remarkably different in the two pairs of cell lines. T2821/R had significantly lower expression of the protein compared to T2821 (Figure 29E). On the other hand T2851 and T2851/R showed similar levels of expression of RAD51 during six hours post-treatment (Figure 29F).

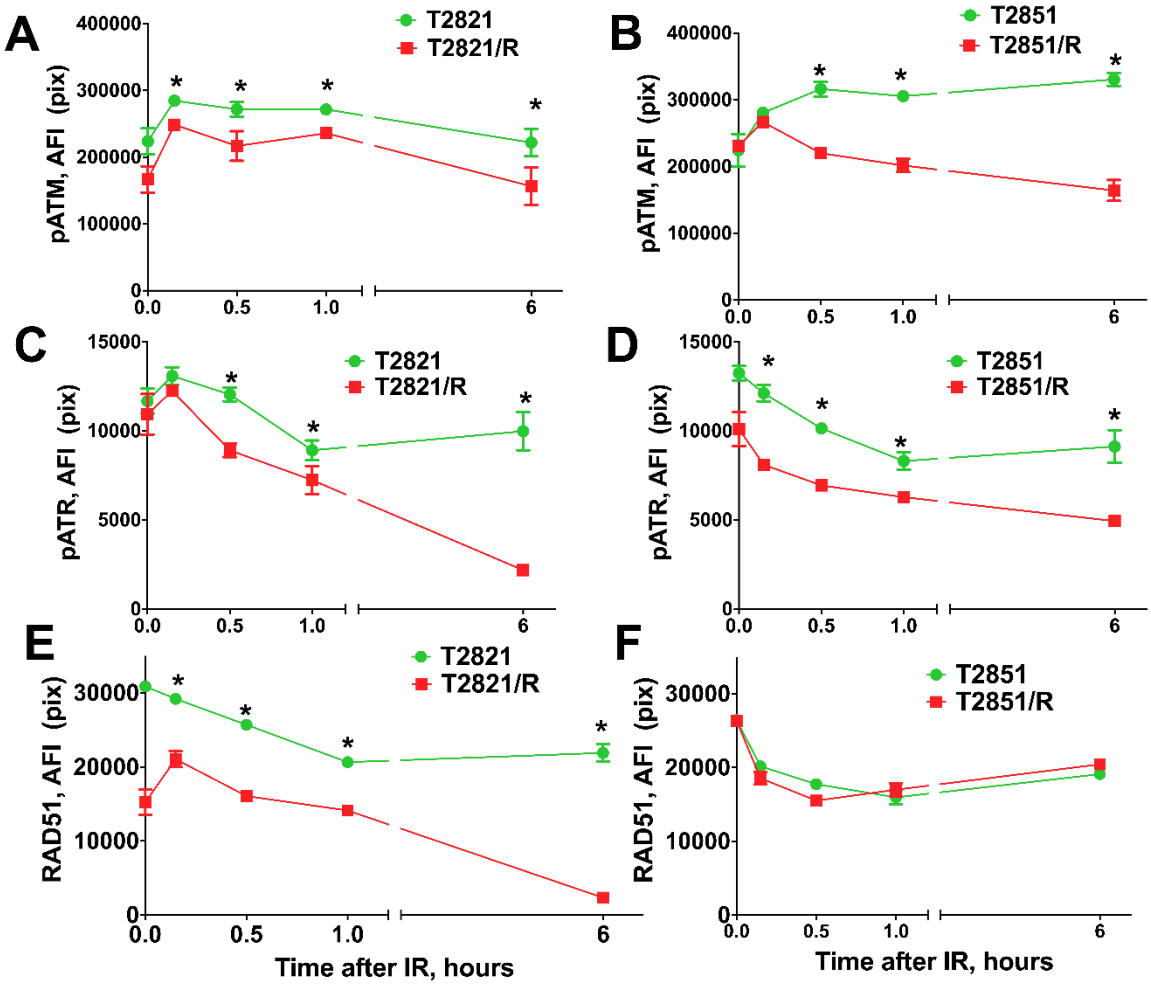

Figure 29. Activation of ATM and ATR in response to radiation is lower in radioresistant NSCLC cell lines, and RAD51 expression differs between each cell line. (A-B) Quantification of pATM (S1981) in response to 5 Gy of radiation during 6 hours in T2821/R and T2851/R compared to the parental cell lines. **(C-D)** Fluorescence intensity of pATR (S428) staining after treating T2821, T2821/R, T2851, and T2851/R with 5Gy of radiation. **(E-F)** Expression levels of RAD51 as measured by immunofluorescence after radiation treatment in T2821/R, T2851/R, and the respective parental cell lines.

12.2.4. Treatment strategy for radioresistant cells

12.2.4.1. *In vitro* evaluation of HSP90 inhibition as a treatment for radioresistant NSCLC cells

The data shown previously suggests that the treatment of NSCLC cells with repeated doses of radiation induces the generation of radioresistant cells with varying expression of genes and proteins involved in DNA repair, EMT, and proliferation regulation. For this reason, we proposed to test a treatment that would target several of these proteins that we found changed in the radioresistant cell lines. To assess this approach, we chose to target the HSP90 chaperone using Ganetespib as its inhibitor.

First, we analyzed the expression of HSP90 by western blot (Figure 30A) and immunofluorescence (Figure 30B), observing that T2821/R and T2851/R and their respective parental cell lines were expressing HSP90 in sufficient amounts to be able to test the inhibitory treatment. Knowing this, we analyzed the growth inhibitory effect and safety of Ganetespib *in vitro*. As shown in Figure 26C, T2821, T2851, and their respective radioresistant derived cells showed inhibition of proliferation with the treatment of Ganetespib at 72 hours. However, the human fibroblast cell line, IMR-90, did not show any effect on proliferation in doses up to 200 nM of Ganetespib.

We showed before that T2821 and T2851 motility is inhibited by Ganetespib (Figure 11). In this case, we demonstrated that Ganetespib has a similar effect on T2821/R and T2851/R with doses of 25 nM of Ganetespib being able to reduce the motility of the cells (Figure 30D-E).

We have also shown previously that radioresistant cells have an increased amount of pAKT (S473) and a higher production of cytokines like IL-6 (Figure 25). Treatment of T2821/R and T2851/R can reduce the amount of phosphorylated AKT and also decrease the production of IL-6 in both cell lines (Figure 30F-G). At the same time, Ganetespib at a concentration of 3 nM has a similar effect on proliferation in T2821/R and T2851/R as it does in the respective parental cell lines (Figure 30H-I). When the HSP90 inhibition is combined with radiation treatment, we observe that Ganetespib has an additive toxic effect, improving the effects on survival, as shown by clonogenic assay (Figure 30J-M).

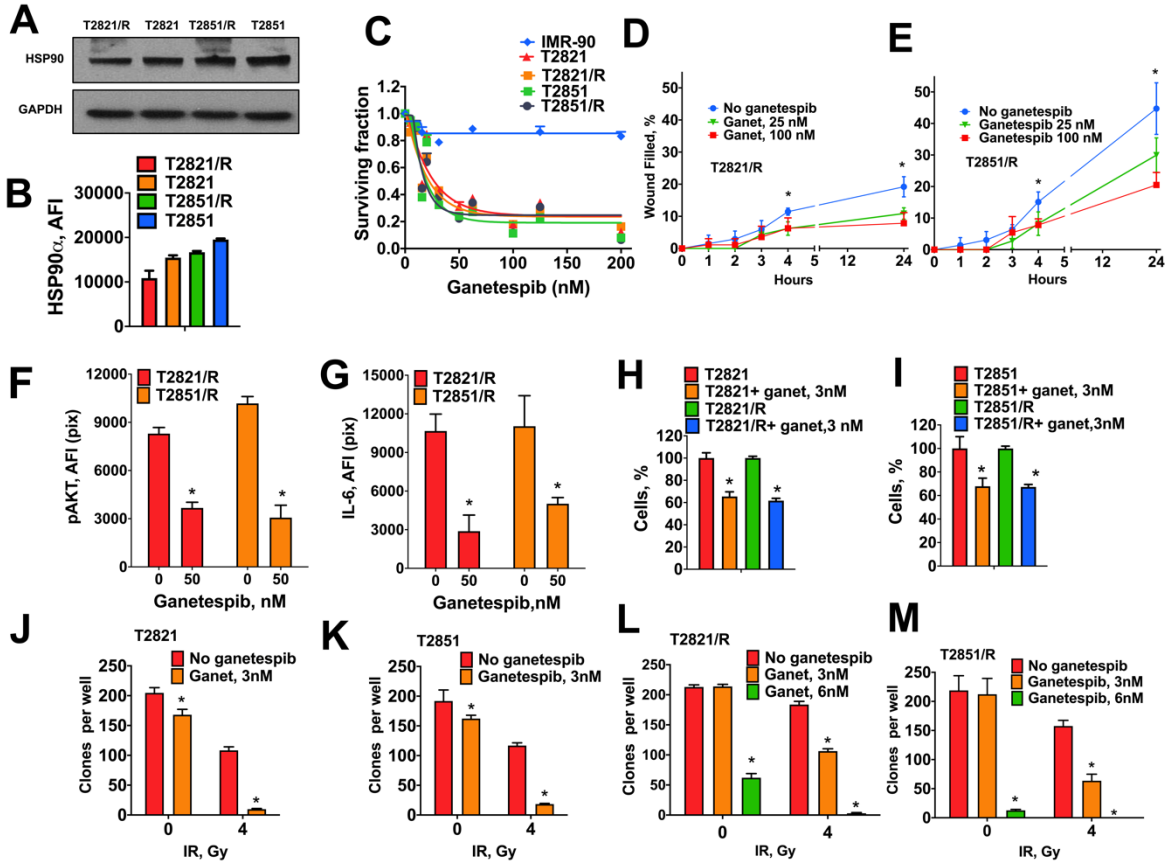

Figure 30. HSP90 inhibition with Ganetespib impairs cell proliferation and improves the effects of radiation. **(A-B)** HSP90 expression levels in T2821, T2821/R, T2851, and T2851/R analyzed by western blot (A) and immunofluorescence staining (B). **(C)** Effects on the survival of Ganetespib at different doses after 72 hours of treatment in T2821, T2821/R, T2851, T2851/R, and the fibroblast cell line IMR-90. **(D-E)** Analysis of Ganetespib effect on migration capacity in radioresistant cell lines. **(F-G)** Quantification of the levels of phosphorylated AKT (F) and production of IL-6 (G) in T2821/R and T2851/R when treated with Ganetespib for 48 hours. **(H-I)** Analysis of effect on proliferation of Ganetespib at low doses in T2821/R (H) and T2851/R (I). **(J-M)** Effect on survival when radiation therapy is combined with Ganetespib treatment as measured in a colony-forming assay for T2821 (J), T2851 (K), T2821/R (L), and T2851/R (M).

12.2.4.2. *In vivo* evaluation of combinatorial treatment in NSCLC cells

Finally, we tested whether the observed results obtained *in vitro* with Ganetespib would translate into an effective and viable treatment in an *in vivo* model. We established xenograft tumors of the T2821 cell line in SCID mice and separated them into four groups of five mice each: control, treated with 5Gy of radiation, treated with Ganetespib, and treated with radiation combined with Ganetespib. We observed that monotherapy treatments had a similar effect on tumor growth, however, when both treatments were used in combination, the tumor volume increase was significantly slower than those treated by monotherapy (Table 5 and Figure 31A).

Comparison	*p*-value (*F*-test)	Adjusted *p*-value (Bonferroni Correction)
IR vs. Untreated	0.0062	0.0372
Ganetespib vs. Untreated	<0.0001	<0.0001
IR + Ganetespib vs. Untreated	<0.0001	<0.0001
Ganetespib vs. IR	0.0814	0.4884
IR + Ganetespib vs. IR	<0.0001	<0.0001
IR + Ganetespib vs. Ganetespib	<0.0001	<0.0001

Table 5. Statistical significance values for the comparison of individual tumor growth rates among the different treatment groups.

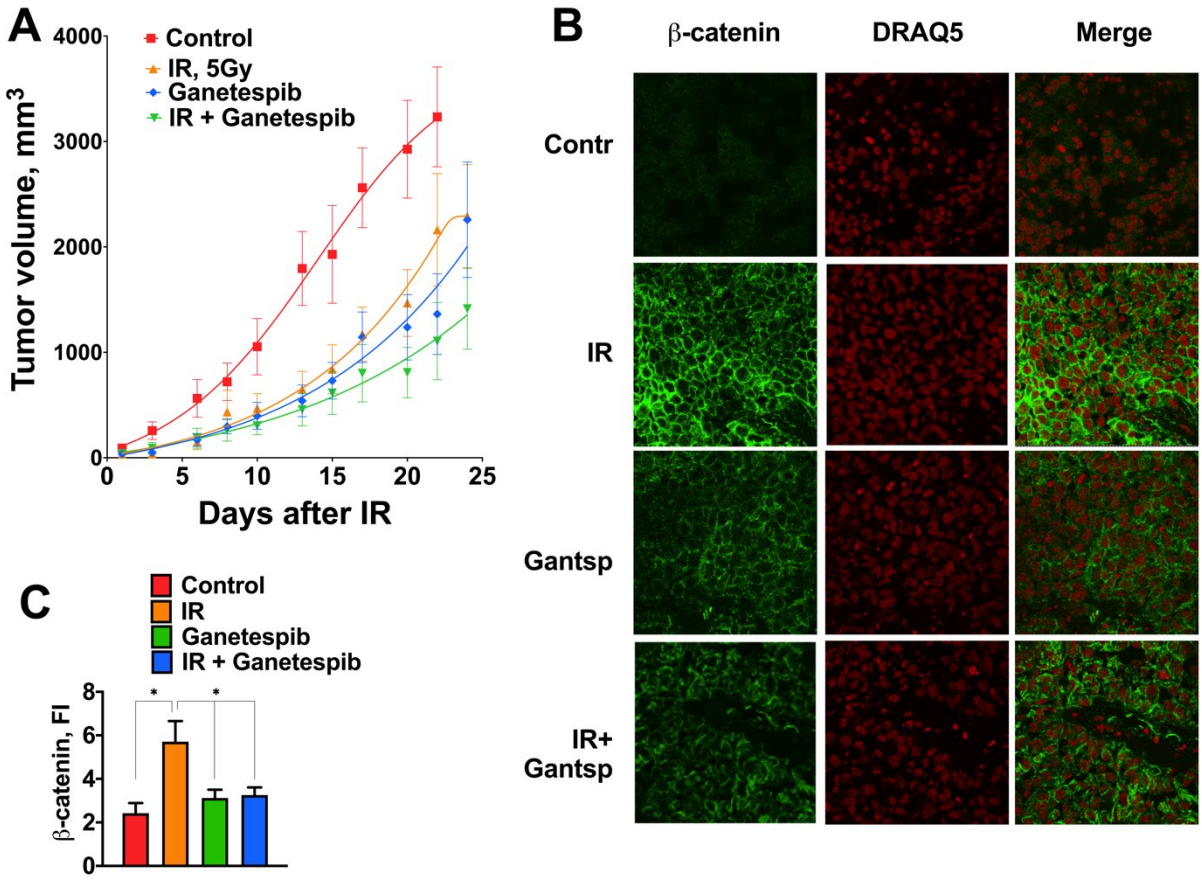

Figure 31. The combination of radiation and Ganetespib treatment inhibits tumor growth in NSCLC xenografts. (A) Graph showing the evolution of tumor growth in T2821 xenografts with different treatments. **(B-C)** ß-catenin immunostaining of the T2821 xenograft tumors: (B) Representative images of the staining and (C) quantification of fluorescence intensity in the different treatment groups.

To evaluate the status of the tumors at the end of the *in vivo* experiment, we chose immunofluorescence analysis of the expression of β-catenin, since it is involved in the regulation of stemness, EMT and metastasis in NSCLC[308,318,319]. We observed a radiation-induced upregulation of β-catenin expression, whereas Ganetespib as monotherapy did not influence the expression of β-catenin. However, Ganetespib was able to abrogate the

upregulation of β-catenin expression in T2821 tumors after induction with the radiation treatment (Figure 27B-C).

Discussion

One of the first descriptions of lung cancer was done in 1912 by Isaac Alder[320] when this affection represented approximately 1% of the cancer diagnoses. Since then, lung cancer prevalence worldwide has been on the rise, now accounting for an estimated one quarter of all deaths caused by cancer[4,321]. Early researchers identified tobacco use as the major exposure risk for developing lung cancer and other respiratory system malignancies, with close to 80% of the cases attributable to this factor[322]. Other factors that aid in the development of lung malignancies have been identified over decades of research, such as genetic susceptibility and poor diet habits. Despite all the advances in cancer diagnosis and treatment, lung cancer still has a 5-year survival rate of around 10%. This number is even lower when we look at the 52% of patients that present with distant metastasis, as they have a 5-year survival rate lower than 4%[4,321].

Lung cancer diseases can be classified into two broad categories based on their histological characteristics, small cell lung cancer (SCLC) representing 15% of the diagnoses, and non-small cell lung cancer (NSCLC), which accounts for the remaining 85%. The latter can be further divided into three major subtypes: adenocarcinoma (the most abundant with close to 40% of the cases); squamous cell carcinoma; and large cell carcinoma[1,321].

In most cases, NSCLC is diagnosed when the disease is in its advanced stages. Early diagnosed cases are treated with surgical resection, but when this type of intervention is not possible because of advanced tumor stage or other clinical problems, radiation alone, or in combination with chemo- or targeted- therapy, is the treatment utilized[301]. Radiation is usually applied in low repetitive doses to achieve destruction of the tumor. However, continuous treatment might provide selective pressure on tumor cells to escape the antiproliferative effects of radiation treatment. This radioresistance can be attributable to different characteristics of the tumor, like the amount of cancer stem cells (which are resistant to chemo- and radiotherapies), the DNA repair capability, and the proliferation rates of the tumor cells[202,304,323,324].

Development of treatment resistances in tumors often leads to a more difficult treatment of the recurrences, therefore it is of high relevance to minimize the survival of cells that can escape the treatment.

In this scenario, we set to study the effects of radiation therapy on NSCLC cells to better understand the characteristics of radioresistance. Our first objective was to study whether radiation therapy-induced cancer stem cell phenotypes in the tumor cell population. We have observed that NSCLC cells that have been treated with radiation have an increased capacity to grow in attachment independent conditions and express higher amounts of previously defined cancer stem cell markers. The increased expression of CD44, a receptor for hyaluronic acid with an important role in cell adhesion and migration, has been well described in previous research as a cancer stem cell marker in many epithelial cancers[152,154]. CD44 has known interactions with proteins important in cancer progression and invasion, like EGFR, PDGFR, and matrix metalloproteinases, and its level of expression correlates with the response to radiotherapy in larynx cancers[151,325]. Also, we observed that the transmembrane glycoprotein CD166 was upregulated in cells growing in attachment independent conditions. CD166 is a member of the immunoglobulin family implicated in cell adhesion and migration which has been described as a cancer stem cell marker in NSCLC[326].

Cancer stem cell markers have been a controversial subject because there are no known universal markers that can identify the population of cells with self-renewal capabilities and increased treatment resistance. Therefore, we found it more compelling to analyze the expression of a set of described markers. Apart from the surface markers CD44 and CD166, we studied the status of cancer stem cell transcription factors OCT4 and SOX2 in cells that survived radiation treatment. Their transcription is regulated via the NOTCH pathway, one of the main

signaling pathways involved in the maintenance of stemness in cancer cells[327,328]. We found that OCT4 was highly upregulated in A549 sphere cells that survived radiation. In contrast, SOX2 was expressed in higher quantities in H460 radiation survived sphere cells than in adherent cells or untreated spheres, but in both cases we encountered higher expression of nuclear β-catenin, part of the WNT signaling cascade involved in activation and maintenance of cell proliferation and metastasis. These differences in expression of different effectors involved in cancer maintenance and proliferation lead us to speculate that each type of tumor can find a different strategy to ensure cancer proliferation and progression.

The boundaries between stemness and EMT transition phenotypes have become more diffuse as years of research have passed, and nowadays the difference between these two described phenotypes is not clear[191,194,198,306]. In fact, we found EMT markers such as SNAIL1, TWIST, Fibronectin, Vimentin, and N-cadherin to be overexpressed in the same populations where the CSC markers were seen to appear, all while the expression of E-cadherin was repressed. In 2008, Mani et. Al. showed that EMT produces stem characteristics in epithelial cells[173]. It is plausible to think that these two phenomena, once thought of as separate processes, are part of the same strategy tumor cells use to escape cancer treatments, migrate, and produce metastasis. In support of this idea, we saw increased expression of CXCR4, which is important for the maintenance of stemness [167,307], and PDGFR-β, which plays a significant role in commitment to mesenchymal lineages[309].

We showed that it is possible to impair the growth of radiation survived cells by inhibiting the signaling transduction of the tyrosine kinase receptor PDGFR-β. However, it is well known that tumor cells can generate resistance to these inhibitors by spontaneously producing mutations in such receptors, or their signaling effectors, or even compensating the signaling by overexpressing other receptors and/or ligands that drive the downstream signaling. These two mechanisms have been shown in NSCLC tumors with a gain of function in EGFR, that, after an initial response to treatments with erlotinib or gefitinib, became resistant to the treatment due to secondary mutations in ABL kinase[329]. An alternative way that they became resistant to the treatment was by the overexpression of the MET receptor which can compensate for the loss of EGFR signaling[330]. Another well-identified resistance mechanism is that of imatinib, an ABL kinase inhibitor used to treat chronic myeloid leukemia. For patients treated with imatinib, the cause for relapse is the reactivation of the BCR-ABL kinase. A mutation in the kinase domain avoids the binding of the inhibitor without altering the kinase function of the protein[331,332].

Even though using tyrosine kinase inhibitors as a treatment approach showed to be useful in eliminating radioresistant cells *in vitro*, we found it more interesting to explore other possible strategies to circumvent the resistances that have been arising when RTK inhibitors are used in the clinic. On this note, targeting a nodal point in protein maturation, the chaperone machinery, could overcome the challenges that arise when targeted therapies are used. Thus, HSP90, an evolutionarily conserved chaperone, arises as an attractive therapeutic target since it takes part in the stabilization and activation of hundreds of proteins, many of which are key players in constitutive cell processes, such as tyrosine kinases, transcription factors, and nuclear receptors. Among the HSP90 clients, there are known oncogenic drivers in lung cancer, including: EGFR; ERBB2; BRAF; or the fusion proteins EML4-ALK and BCR-ABL[313,333,334]. It is now known that the chaperone family is often upregulated in many types of cancer, and it has been correlated with poor prognosis in cancer patients. It could be that the increased levels of heat shock proteins in cancer cells confer protection to the physiological stress in which the tumors develop (hypoxia, nutrient deprivation), or simply allow the neoplastic cells to escape the apoptotic processes that should arise from imbalanced signaling[291,335]. Kamal et. al showed

years ago that HSP90 has a higher ATPase activity in cancer cells than in normal cells, which confer those proteins a much higher affinity to 17-AAG, an ATP competitive HSP90 inhibitor[336], making them in this way more susceptible to HSP90 inhibition treatment than normal cells.

We have shown in this research that using Ganetespib as a tool for HSP90 inhibition, we were able to abrogate the proliferation of NSCLC cell lines and eliminate anoikic resistant cells. Our data confirm that Ganetespib produces cell cycle arrest in the G2/M phase, as shown in other studies[337–339], and potentiates the senescent phenotype induced by radiation. The blockade of HSP90 activity directly impacts the DNA repair machinery, as seen by the accumulation of important effectors like pATM, pATR, pH2AX, and 53BP1 after treatment with Ganetespib. Moreover, we observed that Ganetespib treatment leads to a degradation of the DNA repair protein RAD51, implicated in the homologous recombination used in the double-strand breaks repair that follows radiation exposure. We observed accumulation of the proteins p21, p53, p27, and those cells also present positive staining for β-galactosidase, all of them established markers for senescent cells. All these events are consistent with the description of DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence) made by Rodier et. al in 2011[340]. Thus, in response to radiation-induced DNA damage, cells activate ATM and ATR signaling pathways to stop the cell cycle and initiate DNA repair[341]. If the activity of HSP90 is blocked, in this case with Ganetespib, the activation and recruitment of DNA repair proteins can be impacted, producing a delay in the repair machinery leaving the cells with unrepaired γH2Ax foci. Other studies have demonstrated that HSP90 inhibition prolongs the duration of DNA repair foci after radiation in prostate, breast, and cervical cancer cell lines[342,343]. Also, others have suggested that HSP90 inhibitors radio-sensitize tumor cells through downregulation of RAD51 protein, therefore abrogating homologous recombination[344,345]. However, our data suggest that many more DNA repair players can be affected by HSP90 inhibition.

Currently, the development of new pharmacotherapies for NSCLC are addressing tumor-specific molecular aberrations, however, radiation is still used without stratification of the diseases[346–349]. Although advances in the use of high-dose radiation have been highly effective in the control of lung cancer, patients often suffer from recurrences and distant metastasis[349,350].

Repetitive exposure to radiation treatments induces an adaptive response in the tumor, resulting in the generation of radiation-resistant cells that negatively affect the patient prognosis[349,351]. To explore these events, we have generated two radioresistant NSCLC cell lines derived from patient tumors with different genetic backgrounds. The radioresistant cell lines were generated by mimicking the repeated radiation treatment fallowed in patients.

Both 2821/R and 2851/R showed not only resistance to radiation, but also cisplatin. This is important because platinum-based chemotherapies are often used in combination with radiotherapy in NSCLC patients[13,301] This cross-resistance can be associated with the EMT phenotype exhibited by these cell lines, a phenotype that we already observed in the first experiments when NSCLC cells were exposed to a single dose of radiation. Also, these radioresistant cells show an increase in AKT phosphorylation, corroborating previous publications where they showed that the signaling pathway PI3K/AKT is activated in response to radiation treatment in NSCLC cells[352]. PI3K/AKT is a pathway that plays a major role in suppressing apoptosis by promoting cell proliferation, cell cycle progression, and protein translation.

Elevated production of IL-6 and PDGFB were observed in 2821/R and 2851/R compared to the parental cell lines. IL-6 is a proinflammatory cytokine that was found to be expressed in more than half of NSCLC and was associated with reduced survival in lung cancer patients[353].

IL-6 production could help cancer proliferation and migration by being secreted in the tumor environment and producing paracrine signaling to activate the JAK/STAT pathway. A similar process can be hypothesized for the increased production of PDGFB, which is involved in the process of cell migration and tumoral angiogenesis[354,355]. Expression of SDF-1 (CXCL12) and its receptor CXCR4 was also higher in the radioresistant compared to the parental cell lines. The activation of the SDF-1/CXCR4 signaling axis is a characteristic of metastatic and drug-resistant tumor cells[307,356,357]. This information confirms what we have already observed in cells that have survived a single dose of radiation. In those experiments, we observed upregulation of CXCR4 and PDGFRβ receptors that could be playing an important role in the survival of NSCLC cells after radiation treatment.

Even though both 2821/R and 2851/R show EMT phenotype, and changes in important pro-survival signaling, the acquired radio-resistance is the result of multiple changes in genes and protein expression, which we found by analyzing the expression of genes related to different DNA repair mechanisms. Both cell lines exhibited changes in gene expression related to double-strand break repair (DSBR), but none of the genes found to be altered were coincident for both cell lines. 2821/R had changes in genes related to other DNA repair processes like Base excision repair (BER) and Nucleotide excision repair (NER). This data is consistent with what other studies have found on compared analysis of DNA repair gene expression between parental and isolated radioresistant subpopulations in other cancer cell lines[358–363].

Our data demonstrate that repeated exposure of tumor cells to radiation selects for radioresistant cells that can survive the treatment via changes in multiple survival pathways and DNA repair mechanisms. These components that we have found to be altered in the radioresistant cell lines are known clients of the chaperone HSP90.

It is known that the AKT/mTOR signaling drives the translation of HIF-1α in irradiated tumors[364], and that HIF1α regulates the expression of CXCR4 and SDF-1[357]. In addition, an autocrine signaling loop has been proposed between HIF1α and STAT3, where HIF1α induces IL-6 production leading to activation of STAT3[365]. Interestingly, AKT, HIF1α, and STAT3 are known clients of HSP90 chaperone, therefore when we treated the radioresistant cell lines with Ganetespib, we saw a reduction in AKT phosphorylation and lower production of IL-6. In this way, by blocking HSP90 activity, we were able to disrupt these signaling loops and other likely players in the resistance mechanism of these cells to potentiate the effect of radiotherapy. This was true not only *in vitro*, but we showed that Ganetespib potentiates the effect of radiation therapy in a xenograft model of the T2821 cell line.

Analysis of xenografted tumors treated with Ganetespib showed that HSP90 inhibition was able to abrogate the upregulation of β-catenin, demonstrating that important signaling pathways, like WNT/β-catenin, important for cancer progression and metastasis can be disrupted, making a positive impact on tumor shrinkage. Dispite this promising results, they have to be further evaluated due to the limitations of the xenograft model, because of potential interactions that are factored out when using immunocompromised animals.

Further studies need to be done to establish whether the radiation survived cells are a subset population already existing in the tumor, or, on the contrary, the pressure of treatment induces biological changes in the tumor cells adapting them to survive and re-establish the tumor. Nevertheless, our *in vitro* and *in vivo* data supports our initial premises that treating NSCLC tumors with repeated doses of radiation promotes the appearance of radioresistant cells with stem and EMT characteristics that arise from changes in multiple biological processes, and that these advantages can be eliminated by targeting an internodal molecule, as it is HSP90, that affects many biological processes important for the survival of NSCLC cells after radiotherapy.

Conclusions

1. **NSCLC cells that survive radiation therapy, express cancer stem cell as well as epithelial to mesenchymal transition markers.**

   As we showed in Title 10, lung cancer cells that have been exposed to radiation have and increased capacity of growing in attachment independent conditions, and express CSCs markers such as CD44, OCT4 or SOX2. Moreover, they also show expression of markers characteristic of mesenchymanl lineages like SNAIL or N-cadherin.

2. **HSP90 inhibition with Ganetespib induces cell cycle arrest and senescent features in NSCLC cells.**

   We observed, in experiments explained in Title 11, that Ganetespib treatment induces cell cycle arrest in phase G2/M. This arrest drives the cells to enter a senescent state as corroborated with the analysis of nuclear size of the cells and the β-galactosidase staining.

3. **The combination of HSP90 inhibition and radiation impairs cell proliferation by interrupting important pro-survival signaling and disrupting the DNA repair process in NSCLC cells.**

   The clonogenic assay shown in figure 17 demonstrates that the combination of radiation and HSP90 inhibition improves the antiproliferative effects of radiation alone. Moreover, we have shown in Title 11.2.3 that Ganetespib affects the resolution of DNA repair foci by preventing the phosphorylation of important players of the DNA repair process such as ATM and ATR, as well as the inhibition of AKT phosphorylation, important in the prosurvival PI3K/AKT/mTOR signaling pathway.

4. **Radioresistant NSCLC cells exhibit epithelial to mesenchymal features.**

   The data explained in figure 24 shows the increased expression of different EMT markers in radioresistant NSCLC cells compared to their parental cell lines.

5. **The DNA repair machinery in radioresistant NSCLC cell lines is altered.**

   The analysis of expression of genes related to the DNA repair process by qPCR showed and alteration in the expression pattern of several genes in the radioresistant cell lines. Furthermore, the data presented in figures 28 and 29 show how the resolution of DNA repair foci and phosphorylation of key players of the process is different from the parental cell lines.

6. **HSP90 inhibition with Ganetespib affects proliferation of NSCLC radioresistant cell lines by blocking important pro-survival signaling pathways.**

   In figure 30, we show data revealing the antiproliferative effects of HSP90 inhibition with Ganetespib in radioresistant cells. In addition, we show data that proves that Ganetespib treatment dramatically reduces the phosphorylation of AKT.

7. **The treatment combination of Ganetespib and radiation *in vivo,* abrogates tumor growth and downregulates the expression of cancer stem cell markers induced by radiation.**

   As shown in figure 31, the treatment combination of radiation and Ganetespib significantly impairs tumor growth in a xenograft tumor model. Additionally, immunofluorescent analysis of the tumors showed a reduction of β-catenin expression in tumors treated with Ganetespib.

Materials and methods

13. Cell lines

Five cell lines were used for the experimentation explained in this document.

Cell line name	Source	Cell type	Culture Medium
A549	ATCC (CCL-185)	Lung, Epithelial carcinoma	F-12K (ATCC)
H460	ATCC (HTB-177)	Lung, Epithelial carcinoma	RPMI-1640 (Corning Life sciences)
T2821	Primary from lung biopsy	Lung, Epithelial carcinoma	RPMI-1640 (Corning Life sciences)
T2851	Primary from lung biopsy	Lung, Epithelial carcinoma	RPMI-1640 (Corning Life sciences)
IMR-90	ATCC (CCL-186)	Lung, Normal fibroblasts	EMEM (ATCC)

Table 6. Cell lines

To generate the T2821 and T2851 cell lines, surgical samples were obtained from the University of Pittsburgh Cancer Institute Lung Specialized Program of Research Excellence (SPORE) with written consent from patients following the guidelines of the Institution Review Board protocol approved by the University of Pittsburgh Scientific Review Committee (IRB# 01-09-27-07). The tumor samples were evaluated by a pathologist and genotyped.

Tumor ID	Tumor stage	Genetic alterations			
		KRAS	**EGFR**	**BRAF**	**EML4-ALK**
T2821	IV (T4Nx)	No	No	No	No
T2851	IB (T2N0)	No	Exon 21	No	No

Table 7. Characterization of tumor samples used to generate cell lines.

All cell lines were grown in a humidified atmosphere at 37ºC with 5% of CO_2. All cell culture media were supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% Penicillin and streptomycin solution (Gibco).

13.1. Establishment of cell lines from tumor samples

Tumor samples were disaggregated manually, with a surgical scalpel, in complete culture medium. The small pieces of tissue, and the media, were placed in a 35 mm petri dish with just enough media to cover the bottom, so that the tissue would be in touch with the plate and would not float when moved. Media was refreshed every 24 hours. Once colonies were established, they were subcultured repeatedly into clean plates to eliminate fibroblast growth until a clean culture was achieved.

13.2. Culture of irradiated adherent cells

To obtain radiation survived adherent cells, cell lines were seeded in 12-well plates at a density of 20000 cells/well. The next day, the cell culture plates were irradiated at a single dose of 5 Gy and then subcultured for 14 days. These cells were then trypsinized and used for further experimentation.

13.3. Culture of lung tumorspheres

Attachment independent culture was done in serum-free 0.8% methylcellulose-based medium MammoCult™ (Stem Cell Technologies) supplemented with 20 ng/ml EGF and bFGF (BD Biosciences), and 4 μg/ml of insulin from bovine pancreas (Sigma-Aldrich).

NSCLC cells growing as adherent culture irradiated or untreated were trypsinized (after seven days of subculture) and seeded in triplicate at a density of 500 cells/well in ultra-low attachment 24-well plates (Corning) and cultured for 12 days. The medium was supplemented with fresh growth factors twice a week. Tumor spheres were counted under a contrast-phase microscope using a 10X objective lens.

To assess the self-renewing potential, tumorspheres of the first generation were collected by gentle centrifugation, mechanically dissociated into a single-cell suspension, and replated into ultra-low attachment plates in the aforementioned media with supplements.

14. Cell doubling time

Approximately $3x10^5$ cells were seeded in 100 mm plates in complete media in triplicates for each time point. The next day, cells were trypsinized, resuspended into a single cell suspension, and manually counted using a Neubauer chamber under a light microscope, to establish the baseline number of cells. In the same way, other plates were trypsinized and cells were counted after 8h, 24h, 48h, and 72h of incubation.

15. Drugs

The tyrosine kinase inhibitors Axitinib and Dasatinib were purchased from LC Laboratories. And the HSP90 inhibitor Ganetespib [3-(2,4-dihydroxy-5-isopropylphenyl)-4-(1-methyl-1H-indol-5-yl)-1H-1,2,4-triazol-5(4H)-one] was provided free of charge by Synta Pharmaceuticals Corporation.

16. Irradiation

Cell lines were irradiated in suspension or as a monolayer in culture plates in complete culture medium using a Shepherd Mark 168 irradiator (^{137}CS irradiator) (JL Shepherd) at a dose rate of 70.6 rad/min at room temperature.

For *in vivo* experiments, mice were irradiated using a Varian linear accelerator (Varian Medical Systems Inc.) at 6MeV photons with a dose rate of 300 monitor units per minute. Mice were immobilized and shielded by a 10/2 mm value layer block to ensure that only the tumor area in the right hind limb was irradiated.

17. Colony-forming assay

Exponentially growing cells were trypsinized, counted, and resuspended at a concentration of 1000 cells/ml. The cell suspension was then irradiated at different doses (0-10 Gy) and 500 cells /well were plated in 6-well plates immediately after, in triplicates for each treatment condition. The next day, medium was changed and the appropriate drug concentration or DMSO was added. The media and drugs were refreshed every other day.

After 7 days of culture, the colonies were methanol fixed, stained with Crystal Violet, and colonies bigger than 50 cells were counted using a GelCount Colony Counter (Oxford Optronix).

For assays where cells were pretreated with the drug, the cells were seeded in the same way described above, allowed to attach to the plate overnight and the next day the drugs were added for 24h. Then, the 6-well plates were irradiated at the appropriate doses.

18. Wound healing assay

Cell cultures growing exponentially were trypsinized, resuspended as a single cell suspension, and seeded in complete media at 80% confluency in 6-well plates, previously marked at the bottom with a horizontal line. Collagen IV coated 6-well plates (BD Biosciences) were used to seed cells growing as tumorspheres to facilitate their attachment. Cell cultures were left to attach overnight, and the next day a wound on the monolayer was done, using a 200 µl pipet tip, perpendicular to the mark on the plate, and the monolayer was washed four times with PBS (Corning) to eliminate cell debris. Fresh complete media was added to the wells. The closing of the wound was monitored for 24h and pictures were taken at 0, 2, 4, 6, and 24h using a ZEISS Axiovert 40C light microscope. The distances between the monolayer borders were measured using the marking on the plate as a reference by analysis with ImageJ (https://imagej.nih.gov)[366].

19. Cellomics Arrayscan HCS reader

The Cellomics Arrayscan HCS reader (Cellomics/ThermoFisher) is an imaging system used for the detection of fluorescently labeled components of the cell. The system uses a set of lasers with excitation and emission filters to selectively capture fluorescent signals. The system acquires images of multiple fields for each plate well or slide, which are analyzed according to defined algorithms using the Arrayscan II Data Acquisition and Viewer version 3.0 (Cellomics).

19.1. Immunofluorescence staining procedure for Cellomics Arrayscan imaging

Cell cultures growing exponentially were trypsinized, resuspended as a single cell suspension in fresh medium, and seeded in 96-well plates (Collagen IV coated for tumorspheres) at 80% confluency. The next day, the cells were fixed with 2% paraformaldehyde (PFA) solution and washed using FACS buffer (1% bovine serum albumin in PBS). Cells were incubated in FACS buffer for 1h at room temperature to block unspecific binding of the antibodies and then the appropriate diluted primary antibody was added to the well. Primary antibodies were incubated for 1h at room temperature. After, wells were washed three times for five minutes, and a secondary antibody fluorescently labeled AlexaFluor©, with 488, 546, or 680 dyes (Invitrogen), was added for one hour. Next, wells were washed three times for 5 minutes and incubated with a 2 μg/ml Hoechst33342 solution (ThermoFisher) for 20 min to stain cell nuclei and help with single-cell identification and optimize focusing. All washes and antibody dilutions were made in FACS buffer, and incubations were carried at room temperature.

For the labeling of intracellular proteins, after fixation, cells were incubated with a 0.1% Triton X-100 solution, for 10 min, for permeabilization and then washed three times for 5 min with FACS buffer before continuing with the staining procedure as described above.

For each protein analyzed, wells stained only with the secondary antibody were used for background detection and subtraction during the analysis.

19.1.1. Immunofluorescence detection of DNA repair markers

Cell cultures growing exponentially were trypsinized, resuspended as single-cell suspensions, and seeded in 96-well plates at 80% confluency in complete medium. The next day cells were irradiated at a dose of 5Gy and incubated at 37ºC for the appropriate time (0-24h). Cells were fixed with 2% PFA and kept in PBS until the staining procedure, which was followed as described above, with permeabilization.

For experiments with Ganetespib pretreatment, the next day after seeding the cells, the appropriate concentration of Ganetespib or DMSO was added to the media (0-300 nM) for 2h before irradiation.

Antibody	Source
53BP1	Millipore
β-catenin	Cell Signaling Technology
CD166	R&D Systems
CD24	Abcam Inc
CD44	Beckman Coulter
CXCR4	R&D Systems
E-cadherin	Cell Signaling Technology
Fibronectin	Abcam Inc
HMGB1	Abcam Inc
HSP90	Cell Signaling Technology
IL-6	Abcam Inc
LC3	Cell Signaling Technology
N-cadherin	Abcam Inc
OCT4	Cell Signaling Technology
p21	Cell Signaling Technology
p53	Novus Biologicals
pAKT (S473)	Cell Signaling Technology
pATM (S1981)	Abcam Inc
pATR (S428)	Cell Signaling Technology
PDGFRβ	R&D Systems
pHSP90 (T7)	Cell Signaling Technology
RAD51	Abcam Inc
SDF-1	Sigma-Aldrich
SNAIL	Abcam Inc
SOX2	R&D Systems
STAT3	Cell Signaling Technology
TWIST1	Abcam Inc
Vimentin	Abcam Inc
γH2AX	Millipore
Secondary AlexaFluor	Invitrogen

Table 8. Antibodies used for immunofluorescence detection.

20. Cell proliferation and viability assays

Cell cultures growing exponentially were trypsinized, resuspended as single-cell suspensions, and 5000 cells/well were seeded in 96-well plates. The next day, Ganetespib (0-300 nM) or DMSO was added to the wells and incubated for 72h at 37ºC. The number of viable cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT, Sigma-Aldrich). 10 µl of 5 mg/ml MTT solution was added to each well and incubated at 37ºC for 2h. Then, the formazan precipitate was dissolved using 100 ml of solubilizing solution (4 mM HCl, 0.1% NP40 in isopropanol). Absorbance was read at 590 nm.

For some experiments, cell number was measured by counting the nuclei, after Hoechst 33342 staining, using the Cellomics ArrayScan HCS Reader.

21. Cell cycle analysis

Cells exponentially growing in 6-well plates were treated either with Ganetespib (0-30 nM) for 48 hours, or irradiated at 5 Gy, and cultured for 0-30h. After this time, the cells were trypsinized and resuspended as a single cell suspension in ice-cold PBS. Immediately, ice-cold 70% ethanol was added dropwise to fix the cells while in agitation to reduce cell clumping. The samples were incubated overnight at 4ºC to ensure fixation. The next day, cells were washed twice with PBS and incubated, in the dark, in 500 µl of PI staining solution containing 50 µg/ml Propidium iodide (Sigma-Aldrich), 100 µg/ml RNase (ThermoFisher), and 0.1% Triton X-100 in PBS. The cell cycle profiles were analyzed by flow cytometry using a BD Accuri™ C6 cytometer (BD Biosciences).

22. Apoptosis assay

Cells growing exponentially as monolayers in 6-well plates were incubated with Ganetespib (0, 3, 30, 300 nM) and incubated for 24 and 48 hours. Then cells were trypsinized and resuspended as single cells, washed with PBS, resuspended in binding buffer, and stained using AlexaFluor®488-conjugated Annexin V and propidium iodide (PI), following the protocol provided by the manufacturer of the kit used: Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (ThermoFisher). After 15 min of staining in the dark, cells were diluted with 400 µl of binding buffer and analyzed by flow cytometry using a BD Accuri™ C6 cytometer (BD Biosciences). Untreated cells were used to define the gating strategy of the analysis.

23. β-galactosidase staining

Senescence-associated β-galactosidase activation was detected using the Senescence β-Galactosidase Staining Kit from Cell Signaling Technology. Cells were seeded in 24-well plates and treated with 5 Gy of radiation, Ganetespib (3 or 30 nM), or both and incubated for five days.

Staining was done according to the manufacturer protocol as follows. Cells were washed with PBS and fixed. Next, the β-Galactosidase staining solution was added to the wells and incubated at 37ºC overnight. Pictures were taken of multiple fields of each well using a ZEISS Axiovert 40C light microscope and the number of blue-stained cells were counted using ImageJ (https://imagej.nih.gov)[366].

24. Western blotting

Cell cultures growing exponentially in 100 mm plates were treated with radiation (0-7.5 Gy) with and without Ganetespib (3 nM) and grown for up to 72h, depending on the experiment. After treatment incubation, the cells were washed with ice-cold PBS and lysed using Triton based lysis buffer (Cell Signaling Technology). The lysates were clarified by centrifugation and the supernatant containing the protein extract was recovered. Equal amounts of protein were mixed with Laemmli buffer (Sigma-Aldrich) and boiled at 95ºC for 5min. Those processed lysates were resolved by SDS-PAGE using a precast 4-20% gradient acrylamide gel (Bio-Rad). After the electrophoresis, the proteins were transferred into a PVDF membrane (Bio-Rad) via the wet transfer method. The membranes were blocked with 5% non-fat milk in TBS for 1h. Next, the membranes were incubated with the appropriate dilutions of antibodies overnight at 4ºC. The next day, the membranes were washed three times for 10 min with TBS-Tween20 and incubated with HRP conjugated secondary antibody for 1 hour at room temperature. After this incubation, the membranes were washed three times for 10 minutes and chemiluminescent detection reagent (ThermoFisher) was added. Signals were detected by exposure using X-ray film.

Antibody	Source
HSP90	Cell Signaling Technology
RAD51	Santa Cruz Biotechnology
p21	Cell Signaling Technology
pAKT S473	Cell Signaling Technology
GAPDH	Sigma-Aldrich
b-actin	Sigma-Aldrich

Table 9. Antibodies used for western blotting.

25. Tumor xenografts

Twenty immunocompromised NOG-F female mice (NOD.Cg-Prkdcscid Il2rgtm1Sug/JicTac), purchased from Taconic Biosciences Inc and, ranging from six to eight weeks of age, were used for the experiment. Mice were housed according to the IACUC approved protocol number 15025156, at the Hillman Cancer Center animal facility at the University of Pittsburgh (U.S.A.), in cages of 5 animals in a specific pathogen free area, where they received appropriate veterinary supervision.

To establish the xenografted tumor, two million T2821 cells were inoculated subcutaneously into the right hind limb of twenty NOG-F mice. Cells were injected as a single cell suspension in 100 µl of PBS. Tumors were allowed to form for 7 days. At this point mice bearing established tumors of 100-200 mm^3 were randomized into four treatment groups: Control, Ganetespib, Irradiation, and Irradiation+Ganetespib. According to published methods, mice received an intraperitoneal injection of either Ganetespib (25 mg/Kg), formulated in 10/18 DRD (10% DMSO, 18% cremophor RH40, 3.6% dextrose, 68.4% water) or vehicle only, 48h before the irradiation. The tumor area was irradiated with 5 Gy as previously described and Ganetespib or vehicle treatments were continued twice a week for the duration of the experiment.

Mice were weighed twice a week for health monitoring and tumor measurements were taken three times a week. The tumor volume was defined as $[(W_1 \cdot W_1 \cdot W_2) \cdot (\pi/6)]$, where W_1 is the smallest tumor diameter and W_2 the largest.

The number of mice was decided taking the minimum subjects to perform a statistical analysis (3 per treatment group) and allowing for 2 extra for the possibility of casualties during the course of the experiment.

25.1. Tumor analysis

After the animal experiment concluded, mice were euthanized and the xenografted tumors were harvested and snap-frozen in OCT compound (Fisher HealthCare) then stored at -80ºC. 8 mm cryostat sections were cut from the center of the tumor and air-dried on slides for staining. Tissue was fixed with 4% PFA for 10 mins and then washed with PBS. The tissue was incubated with a solution of 1% bovine serum albumin and 0.1% TritonX (Blocking buffer), to block unspecific binding of the antibodies during 1 hour. Afterward, the slides were incubated with anti- β-catenin antibody FITC conjugated (BD Biosciences) overnight at 4ºC in the dark. Excess antibody was washed with blocking buffer. Nuclei of the cells were stained with Draq5™ (BD Biosciences). Images of the stained tissue were acquired with a Leica Laser Confocal Microscope, model DMRE TCS SL, and analyzed using ImageJ (https://imagej.nih.gov)[366].

26. Analysis of cytokines

26.1. Analysis of intracellular cytokines

Cells growing exponentially were seeded in 96-well plates at 10^4 cell/well. The next day, 2 μM monensin (Sigma-Aldrich), a transport inhibitor that blocks the release of cytokines, was added to the wells for 48 hours. After this time, the cells were fixed and permeabilized. The staining procedure and analysis continued, as described in previous sections, for the Cellomics ArrayScan Reader.

26.2. Analysis of secreted IL-6

Cells growing exponentially were seeded in 96-well plates at 3000 cells/well and cultured for 72 hours. At this time, the culture media was collected and analyzed using an ELISA kit (R&D systems) performed by the Hillman Cancer Center Luminex Facility.

The 96-well plates containing the cells were fixed with 4% PFA and the cell nuclei were stained with Hoechst 33342. Then, the number of cells per well was estimated using the Cellomics ArrayScan Reader.

27. Real-time PCR

Cells growing exponentially were used to extract RNA using the RNeasy kit (Qiagen). RNA purity and concentration were assessed using a Nanodrop 2000c spectrophotometer (ThermoFisher) and cDNA was sensitized with the qScript cDNA Supermix from QuantaBio.

Fast SYBR Green Master Mix, from Life Technologies, was used to perform the real-time PCR for the genes of interest in the StepOnePlus Real-Time PCR System (Applied Biosystems). The analysis was done by comparative quantification of gene expression using the ΔΔCt method.

A Human DNA repair RT^2 Profiler PCR array kit from Qiagen was also used. The protocol was followed as stated by the manufacturer. cDNA isolated from the cell lines was mixed with the SYBR Green/ROX qPCR Master Mix and aliquoted in the 96-well preloaded with gene-specific primer sets. The real-time PCR was performed in a StepOnePlus Real-Time PCR System. Analysis of the raw data was done using the GeneGlobe Data Analysis Center Web (Qiagen).

Gene name	Primer sequence
RAD51	GGGAATTAGTGAAGCCAAAGC
	TATGATCTCTGACCGCCTTTG
ATM	ATTCCGACTTTGTTCCCTCTG
	CATCTTGGTCCCCATTCTAGC
RAD50	CTGTTTGATGTTTGTGGTAGCC
	TGGTTTTCGTCTGTTAGCTGAG
ERCC1	AATTTGTGATACCCCTCGACG
	TGTGAGATGGCATATTCGGC
XRCC2	CAGTTGGTGAATGGCGTTG
	CTACCTTCAAGTCGGGCAAG

Table 10. Sequences of the primers used in RT-PCR.

28. Statistical analysis

Statistical analysis was done using the software GraphPad Prism vs.7 (GraphPad Software Inc.).

Comparison between groups was done with the two-sided two-sample *t*-test for data normally distributed, or the Wilcoxon rank test for data that is not normally distributed.

One-way or two-way ANOVA were used to compare multiple groups, followed by a two-sample *t*-test for pair-wise comparisons.

For the cell doubling time experiment, a simple linear regression model was built for the logarithm of the cell number per well for each cell line, using the time as a continuous explanatory variable. For each cell line, the estimated time for cell doubling was calculated based on the slope of the regression line.

Irradiation survival curves of *in vitro* experiments were analyzed using the linear-quadratic and single-hit multitarget models. Statistical comparison used the final slope of the survival curves, representing multi-event killing (D_0), and an extrapolation of the width of the shoulders on the survival curve (ñ). D_0 and ñ from several experiments were compared with a two-sided two-sample *t*-test.

Statistical analysis of the tumor growth was done using a linear mixed model built on the log-transformed volume data, in which treatment and time were used as fixed effects, and assuming a random intercept and random slope for each mouse. The comparison of the slopes

between groups was done using an *F*-test and the *p*-values were adjusted with the Bonferroni correction for multiple comparisons.

For all the statistical analyses a *p*-value of 0.05 or lower was considered significant.

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Publications where the research data explained in this thesis has been published:

- Data explained in Title 10 (Generation of radiation survived NSCLC cells and characterization) was published in:

Gomez-Casal, R., Bhattacharya, C., Ganesh, N., Bailey, L., Basse, P., Gibson, M., Epperly, M., & Levina, V. Non-small cell lung cancer cells survived ionizing radiation treatment display cancer stem cell and epithelial-mesenchymal transition phenotypes. *Molecular Cancer*, *12*(1), 94. https://doi.org/10.1186/1476-4598-12-94

Molecular Cancer Journal Impact Factor: 34.3(SCOPUS, 2020), 27.401 (JCR, 2020).

- Data explained in Title 11 (HSP90 inhibition as a treatment strategy) and subtitle 12.2.4.2 (In vivo evaluation of combinatorial treatment in NSCLC cells) was published in:

Gomez-Casal, R., Bhattacharya, C., Epperly, M. W., Basse, P. H., Wang, H., Wang, X., Proia, D. A., Greenberger, J. S., Socinski, M. A., & Levina, V. The HSP90 Inhibitor Ganetespib Radiosensitizes Human Lung Adenocarcinoma Cells. *Cancers*, *7*(2), 876–907. https://doi.org/10.3390/cancers7020814

Cancers Journal Impact Factor: 4.4 (SCOPUS, 2020), 6.639 (JCR, 2020).

- Data explained in Title 12 (Study of radioresistant NSCLC cell lines) was published in:

Gomez-Casal, R., Epperly, M. W., Wang, H., Proia, D. A., Greenberger, J. S., & Levina, V. Radioresistant human lung adenocarcinoma cells that survived multiple fractions of ionizing radiation are sensitive to HSP90 inhibition. *Oncotarget*, *6*(42), 44306–44322. https://doi.org/10.18632/oncotarget.6248

Oncotarget Journal Impact Factor: 10.4 (SCOPUS, 2020), 5.168 (JCR, 2016).

NOTES:

-Roberto Gómez Casal has participated in the design and performance of the experiments, as well as the analysis of the data published in the three research articles cited above.

-All three journals where the data of this thesis has been published follow the “Open Access” guidelines, therefore no explicit authorization was required for the reproduction of the data contained in the publications.

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