Mechanisms of Resistance to Targeted Therapy in Urothelial Carcinoma

Geoffrey Alasdair Pettitt

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract
Fibroblast growth factor receptor 3 (FGFR3) signalling is altered in ~80% of non-muscle-invasive and ~40% of muscle-invasive bladder cancers via activating mutations (point mutations or gene fusions), overexpression or both. FGFR inhibitors have entered clinical trials in advanced bladder cancer. As with other targeted therapies, resistance is expected to limit treatment efficacy. We have used in vitro models to explore resistance to FGFR inhibition. The urothelial cancer cell lines RT112 and RT4 express FGFR3-TACC3 fusion proteins and are sensitive to FGFR inhibition. Isogenic resistant cell lines, termed RT112 R1, R2, R3 and RT4 R1 were derived by long-term culture of parental cells in the FGFR inhibitor PD173074.

RT112 R1, R2 and RT4 R1 had an altered morphology and reduced proliferation rate compared to the parental lines. These changes were reversed when the resistant cells were cultured without PD173074 for four passages. Following this ‘drug holiday’ RT112 R1 and R2 retained PD173074 resistance, whereas RT4 R1 did not. The resistance mechanism in RT112 R1, R2 and RT4 R1 appears to be epigenetic. RT112 R3 retained an epithelial morphology and a proliferation rate similar to parental RT112. Exome sequencing uncovered a HRAS G12S mutation in RT112 R3. The retroviral transduction of a HRAS G12 mutation in RT112 parental induced PD173074 resistance. Microarray analysis was conducted to examine expression changes between parental and resistant lines. Metacore™ analysis identified the differential expression of pathways relating to cell cycle, epithelial-mesenchymal transition and Oncostatin M signalling. Microarray and immunoblot analysis identified a number of tyrosine kinases as potential resistance mediators. Viability assays showed that RT4 parental and R1 were sensitive to the EGFR inhibitor erlotinib and that RT112 parental and resistant lines were sensitive to the IGF1R inhibitor linsitinib.

Investigation of PD173074-resistant derivatives suggests that genetic and epigenetic mechanisms of resistance occur following prolonged FGFR inhibition.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>¹⁴C</td>
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</tr>
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<td>Micromolar</td>
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<td>ACER3</td>
<td>Alkaline ceramidase 3</td>
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<td>ALL</td>
<td>Acute lymphoblastic leukaemia</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>ELOVL5</td>
<td>Elongation of very long chain fatty acids protein 5</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ERBB</td>
<td>Erb-B2 Receptor Tyrosine Kinase (HER)</td>
</tr>
<tr>
<td>ETS</td>
<td>E-twenty-six</td>
</tr>
<tr>
<td>FABP4</td>
<td>Fatty acid binding protein 4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FGFRL1</td>
<td>Fibroblast growth factor receptor like-1</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence \textit{in situ} hybridization</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g</td>
<td>Gravity of Earth</td>
</tr>
<tr>
<td>GemCis</td>
<td>Gemcitabine plus cisplatin</td>
</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumour</td>
</tr>
<tr>
<td>gp130</td>
<td>Glycoprotein 130</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
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<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H3K27</td>
<td>Histone H3 lysine-27</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks' balanced salt solution</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HMGCR</td>
<td>3-hydroxy-3-methylglutaryl-CoA reductase</td>
</tr>
<tr>
<td>HMM</td>
<td>Hidden Markov Model</td>
</tr>
<tr>
<td>HRAS</td>
<td>Harvey Rat Sarcoma Viral Oncogene Homolog</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSD17B2</td>
<td>Hydroxysteroid 17-beta dehydrogenase 2</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF1R</td>
<td>Insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>IGV</td>
<td>Integrative genomics viewer</td>
</tr>
<tr>
<td>Indels</td>
<td>Insertions and deletions</td>
</tr>
<tr>
<td>InsR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>LIFR</td>
<td>Leukaemia inhibitory factor receptor</td>
</tr>
<tr>
<td>LRP8</td>
<td>Low-density lipoprotein receptor-related protein 8</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MAF</td>
<td>Mutation annotation format</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>Mb</td>
<td>Megabase</td>
</tr>
<tr>
<td>ME1</td>
<td>Malic enzyme 1</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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</tbody>
</table>
MgCl₂  Magnesium chloride
MIBC  Muscle-invasive bladder cancer
min  Minute
ml  Millilitre
mM  Millimolar
mRNA  Messenger RNA
MTT  3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium
MVAC  Methotrexate, vinblastine, doxorubicin plus cisplatin
ng  Nanogram
NGS  Next-generation sequencing
NLS  Nuclease localisation signal
NMIBC  Non-muscle-invasive bladder cancer
NRG  Neuregulin
OSM  Oncostatin M
OSMR  Oncostatin M receptor
PARP  Poly (ADP) ribose polymerase
PBS  Phosphate buffered saline
PBS-T  PBS containing 0.1% (v/v) Tween 20
PCA  Principal component analysis
PCR  Polymerase chain reaction
PCSK9  Proprotein convertase subtilisin/kexin Type 9
PD  PD173074
PD-1  Programmed cell death protein 1
PD-L1  Programmed death ligand 1
PDGFR  Platelet derived growth factor receptor
PDSS1  Decaprenyl diphosphate synthase subunit 1
PI3  Phosphatidylinositol-3
PIP₂  Phosphatidylinositol-4,5-bisphosphate
PIP₃  Phosphatidylinositol-3,4,5-triphosphate
PKC  Protein kinase C
PLCγ  Phospholipase C gamma
PolyPhen  Polymorphism Phenotyping v2
PPAR  Peroxisome proliferator activator receptor γ
OD  Optical density
qRT-PCR  Quantitative reverse transcription polymerase chain reaction
RIN  RNA integrity number
RIPA  Radioimmunoprecipitation assay
RNA  Ribonucleic acid
RNAi  RNA interference
RQ  Relative quantification
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SAP</td>
<td>Shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>SCD1</td>
<td>Stearoyl-CoA desaturase-1</td>
</tr>
<tr>
<td>SDHA</td>
<td>Succinate dehydrogenase complex flavoprotein subunit A</td>
</tr>
<tr>
<td>SDR16C5</td>
<td>Short chain dehydrogenase/reductase family 16C member 5</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SIFT</td>
<td>Sorting intolerant from tolerant</td>
</tr>
<tr>
<td>SNV</td>
<td>Single nucleotide variation</td>
</tr>
<tr>
<td>SST-RMA</td>
<td>Signal Space Transformation-Robust Multichip Average</td>
</tr>
<tr>
<td>STAG2</td>
<td>Stromal antigen 2</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TACC3</td>
<td>Transforming acid coiled coil 3</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>TGS</td>
<td>Tris/Glycine/SDS</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminoethane</td>
</tr>
<tr>
<td>TURBT</td>
<td>Transurethral resection of the bladder tumour</td>
</tr>
<tr>
<td>TV</td>
<td>Trypsin-versene</td>
</tr>
<tr>
<td>Tween</td>
<td>Polyxyethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEP</td>
<td>Variant Effect Predictor</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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</table>
Chapter 1
Introduction

1.1 Bladder cancer

1.1.1 Epidemiology and treatment of bladder cancer

It is estimated that there were 429,000 new cases and 165,000 deaths from bladder cancer worldwide in 2012 (Ferlay et al., 2015). The median age at diagnosis with bladder cancer is approximately 67 and in the western world approximately 95% of bladder cancers are urothelial carcinoma (Eble et al., 2004; Malats and Real, 2015). Bladder cancer occurs more frequently in males than females with a worldwide sex ratio of 3.5:1 (Ferlay et al., 2015). Smoking cigarettes, exposure to chemicals such as aromatic amines and consumption of phenacetin are major risk factors for developing bladder cancer (Miyazaki and Nishiyama, 2017). Bladder cancer patients commonly present with painless haematuria. Cystoscopy, transurethral resection of the bladder tumour (TURBT), computed tomography and magnetic resonance imaging are used in the diagnosis, staging and grading of bladder cancer (Witjes et al., 2014).

Bladder cancers are classified with the TNM staging system as follows: Tis: known as carcinoma in situ, the tumour is non-invasive; Ta: the tumour is non-invasive with a papillary structure protruding into the bladder lumen; T1: the tumour has invaded the subepithelial connective tissue; T2: the tumour has invaded the bladder’s muscle wall; T3: the tumour has invaded the perivesical fatty tissue surrounding the bladder, and T4: the tumour has invaded adjacent organs such as the uterus, vagina, prostate, pelvic wall or abdominal wall (Cheng et al., 2009). Non-invasive urothelial carcinomas are graded with the 2004 World Health Organisation (WHO) classification system according to cellular appearance as papillary urothelial malignancy of low malignant potential, non-invasive, low grade papillary urothelial carcinoma or non-invasive, high grade papillary urothelial carcinoma (Eble et al., 2004). Previously bladder cancers were graded with the 1973 WHO classification with the most differentiated tumours assigned grade 1,
tumours with an intermediate level of differentiation assigned grade 2 and the least differentiated tumours assigned grade 3 (World Health Organization, 1973).

Worldwide, 70-80% of bladder cancers present as non-muscle-invasive. These non-muscle-invasive bladder cancers (NMIBCs) recur in 50-70% of patients following treatment and progress to muscle-invasive disease in 10-15% of cases (Prout et al., 1992). The high rate of recurrence means that patients require lifetime surveillance and often multiple treatments during their lifetime. Therefore, bladder cancer is expensive to treat (Svatek et al., 2014). Sylvester et al. analysed the records of 2596 patients with superficial bladder cancer treated with TURBT and identified that the presence of multiple tumours, larger tumours and a higher prior recurrence rate were all prognostic of a greater risk of disease recurrence. Classification of the tumour as T1 rather than Ta, concomitant carcinoma in situ and a higher tumour grading were all prognostic of a greater risk of progression to muscle-invasive bladder cancer (MIBC) (Sylvester et al., 2006). Following the diagnosis of NMIBC, which is dependent upon the histological analysis of a tissue sample obtained with TURBT, patients with low risk of recurrence and progression receive treatment with a single immediate postoperative instillation of chemotherapy such as the DNA crosslinking agent mitomycin C. Patients with a high risk of recurrence and progression receive intravesical Bacillus Calmette-Guérin (BCG) vaccine instillations (Babjuk et al., 2016). BCG reduces recurrence and progression of NMIBC (Kamat et al., 2017). NMIBC patients with the highest risk of progression may receive radical cystectomy (Babjuk et al., 2016). MIBC is treated with the neoadjuvant chemotherapy combination of methotrexate, vinblastine, doxorubicin plus cisplatin (MVAC) or gemcitabine plus cisplatin (GemCis) and radical cystectomy. Neoadjuvant chemotherapy has been demonstrated to increase overall patient survival in randomized controlled trials (Hermans et al., 2017). Adjuvant chemotherapy, most commonly GemCis, is offered to patients who did not receive neoadjuvant chemotherapy or have stage T3 or T4 disease (Pradere et al., 2017; Witjes et al., 2016). Meta-analysis has shown that adjuvant therapy increases overall patient survival and disease-free survival in MIBC patients (Leow et al., 2014). There is a need to improve survival in MIBC patients: patients with locally advanced or metastatic MIBC have a 5-year survival rate of approximately 15% (von der Maase et al., 2005). Although more tumours are diagnosed in males, females have a higher mortality rate from their disease and are more likely to present with MIBC. This may be due to females experiencing a greater time from the onset of symptoms to GP referral and to treatment with TURBT (Bryan et al., 2015).
The immunotherapeutic monoclonal antibodies atezolizumab, pembrolizumab, nivolumab are approved by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for use as postplatinum single agents for the treatment of advanced urothelial carcinoma. Avelumab and durvalumab are also approved by the FDA but not the EMA for treatment of this condition. Atezolizumab and pembrolizumab are also approved by the FDA and EMA as single agent, first-line treatments of advanced urothelial carcinoma patients ineligible for treatment with cisplatin (Godwin et al., 2018). Pembrolizumab and nivolumab bind programmed cell death 1 (PD-1) and atezomomab, avelumab and durvalumab bind programmed death ligand 1 (PD-L1). Monoclonal antibodies targeting PD-1 and PD-L1 prevent the PD-1 and PD-L1 interaction and therefore prevent the inhibition of T-cell activation and enable T-cells to induce cancer cell death (Topalian et al., 2015). Atezolizumab was approved for use in urothelial carcinoma following a phase II clinical trial which assessed its efficacy in advanced urothelial carcinoma patients whose disease had progressed since, or who were ineligible for treatment with, platinum-based chemotherapy. The objective response rate of 15% was statistically significant compared to the historical objective response rate of 10% (Rosenberg et al., 2016). Since then a phase III clinical trial has been published assessing the efficacy of atezolizumab in advanced urothelial carcinoma patients whose disease had progressed following platinum-based chemotherapy. Overall survival was not significantly different between the atezolizumab and chemotherapy treatment groups (Powles et al., 2018). The efficacy of pembrolizumab was assessed in a phase III clinical trial with patients whose urothelial carcinoma had recurred or progressed following chemotherapy. Median overall survival was 10.3 months for the pembrolizumab group and 7.4 months for the chemotherapy group. This increase in overall survival was significant (Bellmunt et al., 2017). The efficacy of nivolumab was assessed in a phase II clinical trial in patients whose urothelial carcinoma had recurred or progressed following chemotherapy. An overall objective response was observed in 19.6% of patients. This was significantly greater than the historical control objective response rate of 10% (Sharma et al., 2017).

1.1.2 Molecular features of bladder cancer

NMIBC and MIBC have distinct molecular profiles. FGFR3 overexpression and mutations in FGFR3, KDM6A, STAG2 and RAS occur more frequently in NMIBC whilst alterations in genes which regulate TP53 and the cell cycle pathways occur
more frequently in MIBC. TERT mutations occur frequently in both NMIBC and MIBC. Mutation rates are higher in MIBC (Hurst et al., 2017; Robertson et al., 2017).

Genetic alterations in the TP53/cell cycle pathway occur frequently in bladder cancer (Lianes et al., 1994). The frequency of TP53 mutations increases with stage and grade and alterations in TP53 are weakly predictive of mortality, tumour progression and recurrence (Esrig et al., 1993; Malats et al., 2005). TP53 encodes the transcription factor TP53, which is a tumour suppressor. TP53 regulates expression of genes which control cell cycle, metabolism, DNA repair and apoptosis (Fischer, 2017). Robertson et al. conducted analysis including RNA-seq, whole exome sequencing and copy number analysis on a cohort of 412 MIBC tumours (Robertson et al., 2017). They reported that the TP53/cell cycle pathway was inactivated in 89% of MIBC tumours. 48% of the MIBC tumours had mutant TP53 (Robertson et al., 2017). Pietzak et al, who examined genomic changes in NMIBC from 105 patients and primary MIBC from 40 patients using targeted exon sequencing analysis, observed that TP53 was mutated in 21% of NMIBCs (Pietzak et al., 2017). MDM2 ubiquitinates the C-terminus of TP53 causing it to be degraded by the proteasome (Merkel et al., 2017). CDKN2A encodes p16INK4A which binds and inhibits CDK4 and CDK6 preventing the phosphorylation of RB1 and so inhibits cell cycle progression. CDKN2A also encodes p14ARF which inhibits MDM2 (Pomerantz et al., 1998; Zhao et al., 2016). Amplification and overexpression of MDM2 occurs in bladder cancer (López-Knowles et al., 2006; Maluf et al., 2006; Tuna et al., 2003). TP53 mutations occur mutually exclusively with amplification of MDM2 and deletion of CDKN2A (López-Knowles et al., 2006; Veerakumarasivam et al., 2008; Robertson et al., 2017). MDM2 amplification and overexpression were found in 6% and 19% of tumours, respectively in a cohort of 412 MIBC (Robertson et al., 2017). Additionally, it was observed that focal deletion of 9p21.3, which contains the tumour suppressor CDKN2A, occurred in 22% of MIBCs and CDKN2A mutations occurred in 7% of MIBCs. Alterations between CDKN2A were mutually exclusive with mutations observed in TP53 and RB1 (Robertson et al., 2017). Pietzak et al. observed genomic alteration, predominantly amplification, of MDM2 in 8% of NMIBCs and genomic alteration, predominantly deletion, of CDKN2A in 19% of NMIBCs (Pietzak et al., 2017). They observed that alterations in TP53 and MDM2 were associated with a higher stage and grade of bladder cancer (Pietzak et al., 2017). Homozygous deletion of CDKN2A occurs more frequently in bladder cancers with mutant FGFR3 than those with wildtype FGFR3 and homozygous deletion of CDKN2A associated with a higher stage in tumours with mutant FGFR3.
but not those with wildtype FGFR3 (Rebouissou et al., 2012). RB1 encodes the tumour suppressor RB1 which regulates cell cycle progression by modulating the activity of E2F transcription factors. Inactivating mutations in RB1 are a frequent event in bladder cancer and are associated with a higher stage and grade (Hong-Ji et al., 1993; Horowitz et al., 1990; Ishikawa et al., 1991). RB1 mutations, which were predominantly inactivating, were observed in 17% of MIBCs and deletion of RB1 was observed in 4% of MIBCs (Robertson et al., 2017). Pietzak et al. observed deletion and mutation of RB1 in 6% of NMIBCs. Alterations in cell cycle control genes were significantly associated with a higher stage and grade (Pietzak et al., 2017).

Fibroblast growth factor receptor 3 (FGFR3) is frequently overexpressed and FGFR3 is frequently mutated in bladder cancer. Alterations in FGFR3 occur more frequently in bladder cancers of a lower stage and grade (Kimura et al., 2001). Tomlinson et al. screened 158 urothelial carcinoma samples for mutations in FGFR3 exons 7, 10 and 15 with direct sequencing and examined FGFR3 protein expression in 149 samples with immunohistochemistry. Bladder cancer tumours were of a range of stages and grades. It was observed that 81% of non-invasive and 54% of invasive urothelial carcinomas had an activating FGFR3 mutation or overexpressed FGFR3 (Tomlinson et al., 2007). Robertson et al. observed FGFR3 mutations in 14% of MIBCs, FGFR3 fusion events in 2% of MIBC and focal copy number gain of the FGFR3 locus (4p16.3) in 2% of MIBCs (Robertson et al., 2017). Genomic alterations were observed in FGFR3 in 49% of 105 NMIBC patients, FGFR3 amplification was observed in 2 tumours (2% of the cohort), FGFR3 fusion events were observed in 4 tumours (4%) and missense mutations were observed in 47 tumours (45%) (Pietzak et al., 2017). FGFR3 mutations occurred in 79% of a cohort of 82 Ta urothelial tumours examined with either exome sequencing or selective target capture followed by next-generation sequencing (NGS) (Hurst et al., 2017). FGFR expression in urothelial carcinoma is further discussed in section 1.3.2.

Expression, mutation and copy number gain of members of the EGFR family of receptor tyrosine kinases (RTKs) are common in bladder cancer. Approximately 6% of MIBC exhibit gain of copy number of region 7p11.2 which contains EGFR (Robertson et al., 2017). Examination of membranous EGFR expression with immunohistochemistry showed that 61% of 74 bladder tumours of a variety of stages and grades expressed EGFR. This expression did not correlate with the stage or grade of the tumours (Ibrahim et al., 2009). 12% and 10% of MIBC tumours have mutations in ERBB2 and ERBB3 respectively. Gain of chromosome
7q12, which contains *ERBB2*, occurs in 5% of MIBC (Robertson et al., 2017). Examination of ERBB2 expression with immunohistochemistry in 1005 MIBC tumours showed that ERBB2 overexpression occurred in 9% of tumours (Laé et al., 2010). Fluorescence *in situ* hybridization (FISH) in the tumours in which immunohistochemistry had identified ERBB2 overexpression, revealed that 5% of the cohort of 1005 tumours exhibited *ERBB2* amplification (Laé et al., 2010). A single *ERBB2* missense mutation and no *ERBB2* amplifications were found in a panel of 23 low-grade NMIBCs and *ERBB2* amplifications or missense mutations were observed in 15 out of 82 (18%) high grade NMIBCs. *ERBB2* alterations were associated with a higher stage and grade and *FGFR3* and *ERBB2* alterations were observed to be mutually exclusive (Pietzak et al., 2017).

Alterations in the PI3 kinase pathway are frequently found in bladder cancers (Askham et al., 2010; Aveyard et al., 1999; Cairns et al., 1998; Knowles et al., 2003; Platt et al., 2009; Ross et al., 2013; Wang et al., 2000). *PIK3CA* encodes the phosphatidylinositol-3 (PI3) kinase p110α subunit. PI3 kinase phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to produce phosphatidylinositol-3,4,5-triphosphate (PIP3). This leads to AKT activation which promotes cell growth and survival (Fruman et al., 2017). TSC1 forms part of the TSC complex which inhibits the activity of the mTORC1 complex which in turn regulates cell growth via the promotion of translation. TSC1 is inhibited by AKT (Mieulet and Lamb, 2010). Of the 412 MIBCs examined with whole exome sequencing, 22% harboured *PIK3CA* mutations, which were predominantly activating, and 8% had *TSC1* mutations, which were often truncating (Robertson et al., 2017). *PIK3CA* and *TSC1* mutations were observed in 28% and 11% of a cohort of 105 NMIBC respectively. *PIK3CA* and *TSC1* mutations were found not to be significantly associated with bladder cancer stage and grade (Pietzak et al., 2017).

Mutations in members of the RAS family of GTPases, which activate the mitogen activated protein (MAP) kinase and PI3 kinase pathways, are observed in bladder cancer. Robertson *et al.* observed *HRAS*, *KRAS* and *NRAS* mutations in 5, 4 and 2% of MIBCs respectively (Robertson *et al.*, 2017). RAS mutations are usually found mutually exclusively with *FGFR3* mutations in bladder cancer suggesting the mutations have an overlapping function (Jebar *et al.*, 2005). RAS mutations occur more frequently in NIBCs than MIBCs (Hurst *et al.*, 2017).

Genes encoding chromatin modifiers, such as *KDM6A*, *CREBBP*, *EP300* and *ARID1A* are frequently mutated in bladder cancer (Gui *et al.*, 2011; van Haafent *et al.*, 2009). *KDM6A* encodes a histone H3 lysine-27 (H3K27)
One consequence of the histone demethylase activity of KDM6A in fibroblasts is an increase in expression of retinoblastoma binding proteins required for the function of RB1 as a tumour suppressor (Wang et al., 2010). Its role in epithelial cells, including the urothelium, is unknown. CREBBP and EP300 encode CREB-binding protein and p300 respectively which can act as both tumour suppressors or oncogenes. These proteins have a similar structure, with 63% amino acid sequence identity, and are largely functionally redundant. CREB-binding protein and p300 function as histone acetyltransferases, interact with transcription factors and recruit transcription machinery and transcriptional coactivators to the promoter. The tumour suppressive role of these proteins may be due to their interaction with TP53, BRCA1, and FOXO3a (Wang et al., 2013a). ARID1A is a subunit of the BAF chromatin remodelling complex. BAF has been found to bind RB1, aiding RB1 repression of E2F1 activity: this could be a key tumour suppressive function of the BAF complexes (Hodges et al., 2016; Trouche et al., 1997).

Gui et al. identified mutations in 9 MIBC with whole exome sequencing. Genes identified as mutated in this initial cohort were then examined with targeted exon sequencing in a further 51 MIBC and 37 NMIBC (Gui et al., 2011). They observed that mutations in chromatin remodelling genes occurred in 59% of samples, the most common of which were in KDM6A, which was mutated in 21% of tumours, and CREBBP, EP300 and ARID1A which were each mutated in 13% of tumours (Gui et al., 2011). KDM6A mutations were frequent in tumours of a low stage and grade with 32% of Ta and T1 tumours and 13% of tumours stage T2 or greater exhibiting mutated KDM6A. ARID1A, CREBBP and EP300 mutations were not significantly associated with tumour stage and grade (Gui et al., 2011). In the Robertson et al. cohort of 412 MIBCs, mutations, predominantly inactivating, were observed in greater than 5% of tumours in each of the following chromatin remodelling genes: KDM6A (26%), KMT2A (11%), KMT2C (18%), KMT2D (28%), CREBBP (12%), EP300 (15%), KANSL1 (6%), ARID1A (25%), ASXL1 (6%) and ASXL2 (9%) (Robertson et al., 2017). In a panel of 105 NMIBC, KDM6A, ARID1A, CREBBP and EP300 were mutated in 38%, 21%, 21% and 15% of NMIBCs respectively (Pietzak et al., 2017). Hurst et al. observed predominantly inactivating mutations in KDM6A (52%), KDMT2D (30%), EP300 (18%), ARID1A (18%), KDMT2C (15%) and CREBBP (15%) in a cohort of 82 Ta urothelial tumours (Hurst et al., 2017).

Reduced expression of stromal antigen 2 (STAG2) occurs in bladder cancer due to truncating and missense mutations (Gui et al., 2011; Taylor et al.,
STAG2 functions as a subunit of the cohesin complex to adhere to the centromeric regions of sister chromatids following DNA replication in the cell cycle. This maintains the cohesion of sister chromatids until they are required to separate during metaphase (Aquila et al., 2018; Peters et al., 2008). STAG2 mutations were observed in 22% of a cohort of 307 bladder tumours and STAG2 mutations were associated with a low stage and grade of bladder cancer (Taylor et al., 2014). Examination of STAG2 with immunohistochemistry showed that 18% of 295 urothelial carcinomas, of a range of stages and grades, did not express STAG2. Additionally, Sanger sequencing on a cohort of 47 NMIBC and 64 MIBC found that 32% of the NMIBC and 13% of the MIBC exhibited STAG2 mutations. (Solomon et al., 2013). It has been suggested that the loss of STAG2 expression may promote aneuploidy and tumour evolution due to its role in regulating chromosomal segregation. However, examination of STAG2 expression, with immunohistochemistry, and chromosomal alterations, with high resolution SNP or BAC arrays, in a panel of 23 TaG1/TaG2 urothelial carcinomas showed that 9 tumours lacked STAG2 expression and loss of STAG2 expression often occurred in chromosomally stable tumours (Balbás-Martínez et al., 2013). Additionally knockdown of STAG2 in the bladder cancer cell lines RT112, UM-UC-11 and 639V did not increase the development of aneuploidy (Balbás-Martínez et al., 2013). Furthermore, Taylor et al. observed that STAG2 mutation was not associated with an increase in copy number alterations in bladder cancer (Taylor et al., 2014). Therefore, loss of STAG2 may exert its function as a tumour suppressor in urothelial carcinoma through a mechanism other than preventing the development of aneuploidy.

*TERT* promoter mutations are found in approximately 80% of bladder cancer patients, most commonly G>A base changes -124 bp and -146 bp upstream of the ATG start site. *TERT* promoter mutations are not associated with stage and grade in bladder cancer and therefore *TERT* mutations may occur early in tumour development (Allory et al., 2014; Hurst et al., 2014; Killela et al., 2013; Kinde et al., 2013; Liu et al., 2013). *TERT* encodes the enzymatic subunit of telomerase (TERT) which prevents shortening of telomeres during DNA replication. Cells which do not express TERT are able to undergo only 50-90 rounds of replication prior to the induction of cell senescence (Heidenreich and Kumar, 2017). *TERT* promoter mutations create binding sites for the E-twenty-six (ETS) transcription factors (Bell et al., 2015; Horn et al., 2013). Borah et al. showed that TERT promoter mutations were associated with increased expression of *TERT* mRNA and protein and an increase in telomere length in urothelial carcinoma cell lines (Borah et al., 2015).
Figure 1.1 Molecular pathogenesis of urothelial carcinoma. The two major pathways of urothelial carcinoma pathogenesis are shown in green and blue. Key molecular alterations and percentages at diagnosis are shown. Image adapted from Knowles and Hurst (2015).

Gene expression profiling of bladder tumours has been used to identify molecular subtypes with differing clinical outcomes. Sjödahl et al. performed mRNA microarray expression analysis and unsupervised hierarchical cluster analysis on a cohort of 308 NMIBCs and MIBCs. Additionally, tumours were examined for the presence of FGFR3, PIK3CA and TP53 mutations and the expression of key genes was determined with immunohistochemistry. This identified 5 subtypes of bladder cancer: urobasal A, urobasal B, genomically unstable, infiltrated and squamous cell carcinoma (SCC)-like. The tumour subtype with the best prognosis was urobasal A whilst urobasal B and SCC-like had the worst prognosis. Urobasal A and B subtypes both exhibited high expression of FGFR3, CCND1 and TP63 and frequently harboured FGFR3 mutations. However, whilst urobasal A had a similar pattern of cytokeratin expression to normal urothelium, urobasal B exhibited high expression of KRT6A, KRT6C and KRT14. Urobasal B also had a high frequency of TP53 mutations. The SCC-like subtype also exhibited high expression of basal markers but did not exhibit the FGFR3 gene expression signature observed in urobasal A and B. The genomically unstable subtype had a high frequency of TP53 mutations and grossly rearranged genomes. The infiltrated subtype exhibited high expression of immunologic and extracellular matrix genes. Immunohistochemistry revealed a high expression of CD3 and ACTA2 in the infiltrating subtype tumours, confirming the presence of T cells and myofibroblasts (Sjodahl et al., 2012).

Damrauer et al. performed consensus clustering on gene expression data, curated from publically available datasets, from 262 high grade NMIBCs. This
analysis identified two subtypes. In one subtype tumours exhibited higher expression of urothelial basal markers (KRT14, KRT5, KRT6B and CD44) and had a higher frequency of alterations in the RB1 pathway. In the other subtype tumours exhibited higher expression of markers associated with urothelial umbrella cells (UPK1B, UPK2, UPK3A and KRT20) and this subtype had a higher frequency of FGFR3 and TSC1 mutations. It was observed that the basal-like subtype of tumours had a significantly shorter disease specific and overall survival (Damrauer et al., 2014).

Choi et al. performed mRNA microarray expression profiling and unsupervised hierarchical cluster analysis with 73 MIBCs. The MIBCs clustered into 3 molecular subtypes: basal, luminal and p53-like (Choi et al., 2014). Basal tumours exhibited high expression of basal markers (CD44, CDH3, KRT14, KRT16, KRT5, KRT6A, KRT6B and KRT6C), mesenchymal markers (TWIST1/2, SNAI2, ZEB2 and VIM) and p63 pathway genes. This subtype had a significantly shorter disease specific survival. Luminal tumours expressed luminal markers (FGFR3, FOXA1, GPX2, ERBB2, ERBB3, CYP2J2, GATA3, PPARG, KRT19, KRT7, KRT6, FABP4, KRT20, CD24, KRT18 and XBP1), exhibited PPARγ pathway activation and this subtype was enriched with activating FGFR3 mutations. The p53-like subtype also expressed luminal markers but possessed the determining feature of an activated wild-type p53 gene expression signature. p53-like subtype tumours were typically resistant to chemotherapy. Expression profiling was conducted with a cohort of 43 MIBC prior to and following neoadjuvant chemotherapy. It was observed that post-treatment, resistant tumours were enriched with the p53-like subtype (Choi et al., 2014).

Robertson et al. conducted consensus clustering with the RNA-seq data generated from 408 MIBCs. This split the MIBCs into 5 subtypes: luminal, luminal papillarly, luminal infiltrated, basal-squamous and neuronal (Robertson et al., 2017). The luminal, luminal papillarly and luminal infiltrated subtypes had high expression of UPK2, UPK1A, PPARG, FOXA1 and GATA3. The luminal papillarly subtype was enriched with tumours with a papillary structure whilst the luminal infiltrated subtype exhibited a myofibroblast and smooth muscle expression signature and had increased expression of immune markers. The basal-squamous subtype exhibited high expression of the basal and stem-like markers CD44, KRT5, KRT6A and KRT14 and high expression of TGM1, DCS3 and PI3 which are markers of squamous differentiation. This subtype was enriched with TP53 mutations. The neuronal subtype exhibited high expression of markers of neuronal differentiation and neuroendocrine markers. Three of the tumours in this subtype
had a neuroendocrine histology whilst the other 17 tumours in this subtype did not display histological features suggestive of a neuroendocrine origin. Overall survival after 5 years was highest for the luminal papilliary subtype, intermediate for the luminal and basal squamous subtypes and lowest for the luminal infiltrated and neuronal subtype (Robertson et al., 2017).

Sjodahl et al. conducted hierarchical cluster analysis with microarray expression data and examined the expression of 28 proteins with immunohistochemistry in 307 MIBCs. This categorised tumours into the following subtypes: urothelial-like, genomically unstable, basal/SCC-like, mesenchymal-like, and small-cell/neuroendocrine-like. The urothelial-like and genomically unstable subtypes both exhibited expression of luminal markers. However, the urothelial-like subtype was distinct from the genomically unstable subtype as it exhibited higher expression of FGFR3, CCND1 and RB1 and lower expression of E2F3. The basal/SCC-like, mesenchymal-like, and small-cell/neuroendocrine-like subtypes exhibited lower expression of luminal markers. The basal/SCC-like subtype exhibited high expression of KRT5 and KRT14 and low expression of GATA3 and FOXA1 whereas the mesenchymal-like subtype exhibited high expression of the mesenchymal markers vimentin and ZEB2 and the small-cell/neuroendocrine-like subtype exhibited high expression of the neuroendocrine marker TUBB2B and the epithelial marker EpCAM (Sjödahl et al., 2017).

Seiler et al. conducted expression profiling with 343 MIBC prior to these tumours being treated with neoadjuvant chemotherapy and developed a single-sample genomic subtyping classifier to categorize tumours as claudin-low, basal, luminal-infiltrated or luminal. 476 MIBC, which were not treated with neoadjuvant chemotherapy, from previously published datasets were assigned subtypes. This study found that patients with basal tumours had a poor prognosis when not given neoadjuvant chemotherapy but that the overall survival of patients in this subtype was improved when treated with neoadjuvant chemotherapy. Patients with luminal subtype tumours had a good prognosis whilst the luminal-infiltrated and claudin-low subtypes had a poor overall survival. Treatment with neoadjuvant chemotherapy had little effect on the prognosis of the luminal, luminal-infiltrated or claudin-low subtypes (Seiler et al., 2017).

Hedegaard et al. conducted RNA-seq analysis on a cohort of 460 NMIBCs and conducted consensus clustering with this expression data. This identified three subtypes which were named class 1-3 and a 117 gene classifier was developed to assign independent samples to a subtype/class. Hedegaard et al. suggested that
the luminal-like class 2 may represent a Tis pathway of progression whilst the basal-like class 3 tumours may represent a Ta pathway of progression. Class 1 and 2 tumours both exhibited high expression of uroplakins compared to class 3 tumours. However, class 1 tumours had high expression of genes associated with early cell cycle in contrast to class 2 tumours which exhibited high expression of genes associated with late cell cycle. Additionally, class 2 tumours exhibited higher expression of KRT20 which is a luminal marker and associated with carcinoma in situ. The class 3 subtype did not exhibit high expression of early or late cell cycle genes. Low expression of uroplakins and high expression of the basal markers KRT5 and KRT15 was observed in class 3 tumours and some class 3 tumours exhibited high expression of CD44. High expression of KRT14, was observed in both class 2 and 3 tumours. Class 1 and 2 tumours exhibited a higher frequency of FGFR3 mutations whilst class 3 tumours exhibited a higher frequency of TP53 mutations. Tumours of a high stage and grade or with concomitant carcinoma in situ were more frequent in classes 2 and 3. Class 1 tumours had the highest rate progression-free survival followed by class 3 whilst class 2 tumours had the lowest. (Hedegaard et al., 2016).

The different attempts to use gene expression profiling to classify bladder cancer have differed in the subtypes identified. However, there is a consensus that there are basal and luminal subtypes of bladder cancer and that tumours expressing basal markers have a worse prognosis than those expressing luminal markers.

1.2 Targeted therapy for cancer

1.2.1 Targeted therapies

Targeted therapies act against molecules specific to, or aberrantly expressed in, cancerous cells to induce cell death and reduce cell division. In contrast, traditional chemotherapy acts less specifically by targeting rapidly dividing cells whether or not they are cancerous. Targeted therapies can take the form of either monoclonal antibodies, small molecule inhibitors or gene therapy. Gene therapy, including oncolytic viral therapy, involves the delivery of DNA or RNA to target cells. The aim is to induce gene expression of an absent or defective gene in target cells, to increase expression of a gene which will induce cell death, by for example
stimulating an immune response, or to cause cell death due to viral infection. Delivery of the DNA is usually achieved with a viral vector (Fukuhara et al., 2016; Naldini, 2015). This Introduction will focus on monoclonal antibody and small molecule inhibitor treatments as these therapies are most relevant to this project.

Many targeted therapies have been developed against RTKs, a class of proteins which are frequently activated in cancer. Upon ligand binding RTKs undergo a conformational change which results in oligomerisation. The oligomers then use ATP to autophosphorylate tyrosine residues in the RTK tyrosine kinase domain and to phosphorylate intracellular signalling molecules (Ullrich and Schlessinger, 1990). RTKs activate intracellular signalling pathways such as the MAP kinase and the PI3 kinase pathways. Small molecule inhibitors commonly have specificity for the ATP binding site of tyrosine kinases which enables them to competitively or non-competitively inhibit RTK activity and downstream signalling (Klein et al., 2005). Monoclonal antibodies have been developed with specificity for RTKs. These monoclonal antibodies can induce cell death by a number of mechanisms including cell surface receptor agonist or antagonist activity, induction of phagocytosis and antibody-dependent cell-mediated cytotoxicity. Monoclonal antibodies and small molecule inhibitors specific for tyrosine kinases are now established treatments for cancer (Fabbro, 2015; Scott et al., 2012). Examples of targeted agents which have been found to increase patient survival are detailed below.

The monoclonal antibody trastuzumab is specific for the human epidermal receptor 2 (ERBB2/HER2). Binding of trastuzumab to ERBB2 reduces downstream signalling through the PI3 and MAP kinase pathways and induces antibody-dependent cellular cytotoxicity (Vu and Claret, 2012). Trastuzumab increased the ten-year overall survival rate from 75.2 to 84% in women with primary, operable node positive or high-risk node negative, ERBB2-positive breast cancer without metastasis when used in conjunction with adjuvant chemotherapy (Perez et al., 2014).

Hussain et al. examined the efficacy of treatment with trastuzumab, paclitaxel, carboplatin, and gemcitabine in patients with ERBB2-positive advanced urothelial carcinoma. Out of the cohort of 44 patients, there were 5 complete responses and 26 partial responses to treatment (Hussain et al., 2007). Powles et al. conducted a phase III clinical trial in patients with EGFR- or ERBB2-positive metastatic urothelial carcinoma. The efficacy of maintenance treatment with lapatinib, an EGFR and ERBB2 small molecule inhibitor, following first-line
chemotherapy, was compared to treatment with a placebo. Lapatinib did not significantly increase progression-free survival or overall survival (Powles et al., 2017).

The small molecule inhibitor erlotinib is a first-generation EGFR tyrosine kinase inhibitor (TKI). Erlotinib reversibly binds the adenosine triphosphate (ATP) binding site on the epidermal growth factor receptor (EGFR) intracellular domain. Erlotinib was the first EGFR TKI to be approved by the FDA, following a phase III clinical trial in patients with non-small cell lung cancer (NSCLC) which accounts for 80-85% of lung cancers. This trial found that erlotinib could prolong survival in these patients following the failure of one or two chemotherapy regimens (Gridelli et al., 2010; Zhang, 2016).

Seront et al. assessed the efficacy of the mTOR inhibitor everolimus in patients whose urothelial carcinoma had progressed following treatment with platinum-based chemotherapy. A disease control rate of 27% was observed at 8 weeks (Seront et al., 2012). Milowsky et al. assessed the efficacy of everolimus in patients with progressive metastatic urothelial carcinoma previously treated with chemotherapy in a phase II clinical trial. The primary endpoint of 2-month progression-free survival was not met (Milowsky et al., 2013). However, a patient from this clinical trial, who achieved a durable and complete response to everolimus was examine by Iyer et al. with whole exome sequencing. This identified a E636fs mutation in TSC1 (Iyer et al., 2012). TSC1 acts in complex with TSC2 to regulate the GTPase activity of Rheb and therefore inhibit activation of the mTORC1 complex which contains mTOR (Huang and Manning, 2008). Iyer et al. examined the tumours of a further 13 patients who were enrolled in the same clinical trial with a targeted deep sequencing assay and identified that three other tumours had TSC1 nonsense mutations. Two of the three patients whose tumours had a TSC1 mutation had a minor response to everolimus, as did a patient who had a somatic TSC1 missense mutation of unknown consequence. Of the 9 patients whose tumours were identified to have wildtype TSC1, only one patient's disease did not progress (Iyer et al., 2012).

FGFR TKIs have been assessed in clinical trials with bladder cancer patients. These are discussed in section 1.4.1.
1.2.2 Mechanisms of resistance to targeted therapies

Resistance to therapy can be classified as intrinsic, if the cancer does not show any response to treatment, or acquired where the cancer initially responds to treatment before resistance develops. Targeted agents typically have specificity for RTKs or intracellular signalling molecules which induce cell proliferation and survival by activating intracellular signalling pathways such as the PI3 kinase and MAP kinase pathways. Resistance to targeted agents frequently occurs via maintaining activation or re-activation of the intracellular signalling pathways vital for cell proliferation and survival. This can occur via the activation of an RTK not inhibited by the targeted agent or maintenance of signalling via the targeted RTK due to upregulation or mutation of the receptor. Alternatively, resistance to targeted agents can occur due to alterations in intracellular signalling molecules which induce intracellular pathway activation independent of upstream signalling. Examples of how resistance has arisen against a wide variety of targeted agents are detailed below.

The first generation EGFR small molecule TKIs erlotinib, gefitinib and icotinib, which are reversible ATP-competitive inhibitors, were developed to treat NSCLC (Zhang, 2016). The most common NSCLC EGFR mutations are exon 19 in-frame deletions and L858R point mutations. In Europe, mutant EGFR is found in approximately 15% of NSCLC. This subtype of NSCLC responds to first generation EGFR TKIs in more than 60% of cases (Midha et al., 2015q Russo et al., 2015). The T790M EGFR mutation was first detected in a patient with NSCLC. The mutation was absent in the patient’s original biopsy specimen but present after the patient relapsed after treatment with gefitinib. This mutation introduces a methionine side chain, which impedes EGFR TKI binding to the ATP binding site of EGFR (Kobayashi et al., 2005). Activation of the T790M mutation accounts for approximately 60% of cases of acquired resistance to first generation EGFR TKIs (Zhang, 2016). Second generation EGFR TKIs were developed to overcome resistance arising from the T790M mutation. These TKIs irreversibly inhibit EGFR by covalent bonding to the ATP binding site in the EGFR tyrosine kinase domain. However, patients treated with second generation EGFR TKIs suffer epithelium-based toxicities due to inhibition of wildtype EGFR, limiting the tolerated dose and clinical usage of these molecules (Huang and Fu, 2015q Zhang, 2016). Third generation TKIs, like second generation inhibitors, covalently bind EGFR. However, these have greater affinity for mutant EGFR receptors and therefore can be given to patients at an effective dose without inducing intolerable side effects (Hossam et al., 2016). Clinical trials with a number of third generation EGFR TKIs have been
conducted or are in progress (Wang et al., 2016). The third generation EGFR TKI osimertinib has been approved by the FDA and conditionally approved by the EMA for the treatment of advanced or metastatic NSCLC with the T790M EGFR mutation (Santarpia et al., 2017). Other EGFR mutations that induce resistance to EGFR TKIs include D761Y, L747S and T854A, which are located in the tyrosine kinase domain. The mechanisms by which these mutations induce resistance are unknown (Balak et al., 2006; Bean et al., 2008; Costa et al., 2007).

Overexpression or mutation of an alternative RTK, or overexpression of a receptor ligand, are mechanisms by which resistance to EGFR-targeted agents can arise. These mechanisms enable the activation of growth and survival pathways such as MAP kinase and PI3 kinase. Signalling via other EGFR family members, MET, insulin-like growth factor 1 receptor (IGF1R), vascular endothelial growth factor receptors (VEGFRs), FGFRs and platelet derived growth factor receptor (PDGFR) have all been implicated in resistance to inhibition of EGFR (Akhavan et al., 2013; Azuma et al., 2014; Bianco et al., 2008; Engelman et al., 2007; Nakata et al., 2014; Peled et al., 2013). For example, amplification of ERBB2 and downstream re-activation of the MAP kinase pathway has been identified as a mechanism of resistance to cetuximab in derivatives of colorectal cancer and NSCLC cell lines which had undergone long-term culture in cetuximab (Yonesaka et al., 2011). ERBB2 amplification has been identified as a mechanism of both intrinsic and acquired resistance to treatment with anti-EGFR monoclonal antibodies in colorectal cancer patients (Martin et al., 2013; Yonesaka et al., 2011). Sartore-Bianchi et al. conducted a phase II clinical trial testing the combination of trastuzumab and lapatinib in ERBB2-positive metastatic colorectal cancer previously treated with cetuximab or panitumumab. An objective response was observed in 8 out of 27 patients (Sartore-Bianchi et al., 2016).

Mutant KRAS is found in lung adenocarcinomas with wildtype EGFR, most commonly mutated at G12, G13 or Q61, which reduce GTPase activity and permanently activate RAS. Constitutively active KRAS induces MAP kinase pathway signalling irrespective of RTK activation. Patients with mutant KRAS have a worse response rate to EGFR TKIs (Pan et al., 2016; Pao et al., 2005). Colorectal cancer is treated with the anti-EGFR monoclonal antibodies cetuximab and panitumumab. Approximately 50% of metastatic colorectal tumours have mutations in KRAS or NRAS, most commonly at residues G12, G13, Q61 or A146 (Boleij et al., 2016). These patients do not benefit from treatment with anti-EGFR monoclonal antibodies (Amado et al., 2008; Karapetis et al., 2008). This result suggests that the anti-tumour effect of anti-EGFR monoclonal antibodies is mediated by inhibition of
the MAP kinase pathway downstream of EGFR and that the efficacy of EGFR-targeted agents can be limited by MAP kinase reactivation.

Another member of the MAP kinase pathway which is frequently mutated in cancer is BRAF, a kinase activated by RAS. Activating BRAF mutations are found in cancers including melanoma, papillary thyroid carcinoma, serous micropapillary ovarian carcinoma, colorectal and NSCLC (Niault and Baccarini, 2010). The most common activating BRAF mutation is V600E which accounts for 90% of BRAF mutations in cancer (Obaid et al., 2017). Approximately 6% of colorectal tumours have mutant BRAF, most commonly V600E which is located in the kinase domain (Mao et al., 2015). V600E mutant BRAF is not dependent on RAS-mediated RAF dimerization for activation (Haling et al.). Metastatic colorectal cancers treated with cetuximab and chemotherapy that have mutant BRAF have a significantly worse prognosis than those with wildtype BRAF (De Roock et al., 2010). Additionally, colorectal cancers exhibit intrinsic resistance to BRAF inhibition due to activation of EGFR (Corcoran et al., 2012; Prahallad et al., 2012). Examination of the efficacy of the combination of BRAF and EGFR inhibition or BRAF, MEK and EGFR inhibition in patients with BRAF-mutant advanced colorectal cancer has been examined in phase I/II clinical trials and found to have acceptable tolerability and clinical activity (Atreya et al., 2015; Schellens et al., 2015; Yaeger et al., 2015). BRAF activating mutations are also reported in NSCLC patients with acquired resistance to EGFR TKIs (Ohashi et al., 2012).

Clinical trials have found the BRAF TKIs vemurafenib and dabrafenib to be effective in the treatment of metastatic melanoma (Chapman et al., 2011; Hauschild et al.). Melanomas have been found to activate the MAP kinase pathway to overcome treatment with BRAF TKIs. Mutations in NRAS, KRAS and MEK1 as well as amplification and alternative splicing of BRAF are found in melanoma patients who developed resistance to BRAF TKI monotherapy (Shi et al., 2014). The RTKs EGFR, IGF1R and PDGFRβ have been reported to be activated as mechanisms of acquired resistance to BRAF inhibition (Girotti et al., 2013; Nazarian et al., 2010; Villanueva et al., 2010). Treatment of melanomas with the MEK inhibitors cobimetinib and trametinib used in combination with BRAF TKIs has been found to be more effective than BRAF TKI monotherapy. The combinatorial treatment hinders the emergence of resistance via re-activation of the MAP kinase pathway and is less toxic (Grimaldi et al., 2017). The combination of BRAF and MEK inhibition is also effective in some BRAF-mutant metastatic colorectal cancer patients (Corcoran et al., 2015).
Activation of the PI3 kinase pathway can also lead to resistance to BRAF inhibition. Mutations in the PI3 kinase pathway genes AKT1, AKT3, PIK3CA, PIK3CG, PIK3R2, PTEN, PHLPP1 have been reported in BRAF TKI-resistant melanoma (Shi et al., 2014). Activation of ERBB3 and AKT has been reported as a mechanism of resistance to BRAF or MEK inhibition in melanoma cell lines (Fattore et al., 2013).

Monoclonal antibodies and TKIs have been developed that are specific to ERBB2. Aberrant activation of ERBB2 signalling, via overexpression, amplification or, less frequently, mutation is found in cancers including breast, gastric, lung, bladder, colon, ovary, endometrium and head and neck. The monoclonal antibody trastuzumab is used in the treatment of ERBB2-overexpressing breast, gastric and gastroesophageal cancer (Iqbal and Iqbal, 2014). In order to determine the suitability of patients for treatment with trastuzumab, ERBB2 overexpression or amplification is determined by immunohistochemistry or in situ hybridisation (Bartley et al., 2016; Wolff et al., 2013).

One mechanism of resistance to trastuzumab is the synthesis of truncated ERBB2, termed p95HER2, which lacks the extracellular portion of the protein due to cleavage of the extracellular domain of full length ERBB2 by matrix metalloproteases (Codony-Servat et al., 1999). Alternatively, initiation of translation can occur from methionine residues located close to the ERBB2 transmembrane domain at codons 611 and 678 of full-length ERBB2, generating a C-terminal ERBB2 fragment (Anido et al., 2006). p95HER2 proteins can be classified by their size: either 100 to 115kDa or 95 to 100kDa. 100-115kDa fragments are constitutively active homodimers due to intermolecular disulphide bonds formed between cysteine residues in the short extracellular portion of this protein. 95-100kDa sized proteins lack these cysteine residues and most likely form dimers due to homotypic affinity between the ERBB2 transmembrane and intracellular domains (Arribas et al., 2011). The trastuzumab binding site is in the extracellular domain of ERBB2 and therefore this antibody does not bind p95HER2. It has been demonstrated that inducing expression of p95HER2 in breast cancer cell lines and xenograft models results in resistance to trastuzumab. However, p95HER2 expression did not induce resistance to lapatinib as lapatinib binds the ATP-binding site in the kinase domain of EGFR and ERBB2. (Scaltriti et al., 2007). Lapatinib has since been shown to inhibit tumour growth and ERBB2 signalling in patients whose tumours express p95HER2 (Han et al., 2012; Scaltriti et al., 2010).
Heterodimerisation of IGF1R with ERBB2 has been described as a mechanism of resistance in the breast cancer cell line SK-BR-3 which became trastuzumab resistant following long-term culture in trastuzumab. Inhibition of IGF1R increased sensitivity to trastuzumab by reducing IGF1R and ERBB2 heterodimerization (Nahta et al., 2005). Patients whose breast tumours express IGF1R at the cell membrane, as opposed to those with tumours with mainly cytoplasmic IGF1R expression, have been shown to be significantly less likely to respond to trastuzumab and vinorelbine (Harris et al., 2007).

Whilst ERBB2 mutations are rare in cancer, they have been reported in the ERBB2 tyrosine kinase domain in lung, breast, colorectal, head and neck, bladder and gastric cancer (Bekaii-Saab et al., 2006; Bellmunt et al., 2015; Cohen et al., 2005; Kubo et al., 2009; Lee et al., 2006; Stephens et al., 2004; Zito et al., 2008). ERBB2 mutations are most commonly located in the extracellular domain or kinase domain. ERBB2 mutations have been reported in breast cancers including T798M which was observed to induce resistance to lapatinib, an EGFR- and ERBB2-targeted TKI, when expressed in breast cancer cell lines. The T798M ERBB2 mutation is analogous to the EGFR T790M mutation in the EGFR kinase domain ATP binding site (Bose et al., 2013; Rexer et al., 2013). Lapatinib resistance could be overcome by treatment with the irreversible EGFR and ERBB2 TKI neratinib. In July 2017, neratinib was approved by the FDA for early stage breast cancer with amplification or overexpression of ERBB2 previously treated with trastuzumab (Deeks, 2017).

Studies of breast cancer cell lines have found low expression of PTEN and presence of PIK3CA mutations to correlate with resistance to trastuzumab (Kataoka et al., 2010; O’Brien et al., 2010). Rexer et al. observed that the breast cancer cell line BT474 acquired a PI3KCA helical domain mutation, E542K, following long-term culture in lapatinib. This mutation enabled the resistant derivative to maintain activation of the PI3 kinase pathway in the presence of lapatinib, as PIK3CA is downstream of ERBB2. In contrast, lapatinib inhibited the PI3 kinase pathway in parental BT474 (Rexer et al., 2014). Rexer et al. also observed that treatment with the PI3K inhibitor BKM120 in addition to trastuzumab and lapatinib induced tumour regression in an ERBB2 amplified, mutant PIK3CA mouse xenograft model of breast cancer whereas, treatment with trastuzumab and lapatinib without BKM120 did not (Rexer et al., 2014). The link between PI3 kinase pathway alterations and trastuzumab resistance has been examined in breast cancer patients. Further research is required to elucidate the effect of PIK3CA mutations and PTEN loss and the response to trastuzumab (Razis et al., 2011; Wang et al., 2013b). In vitro
work established that the combination of an mTOR or AKT small molecule inhibitor and trastuzumab could overcome resistance to trastuzumab in breast cancer cell line and xenograft models (Lu et al., 2007). The efficacy and safety of adding everolimus, an mTOR inhibitor, to the combination of trastuzumab and the chemotherapeutic agent vinorelbine was tested in a phase III clinical trial in patients with ERBB2-positive, trastuzumab-resistant breast cancer. It was found that everolimus increased the progression-free survival of patients although this additional treatment also increased adverse effects (André et al., 2014).

The Philadelphia chromosome is found in greater than 90% of cases of chronic myeloid leukaemia (CML) and 10-15% of cases of adult acute lymphoblastic leukaemia (ALL). This genetic alteration is formed by the Philadelphia chromosomal translocation: t(9;22)(q34;q11), and results in the production of BCR-ABL fusion protein which has constitutive activity, unlike ABL kinase (Salesse and Verfaillie, 2002; Wong and Witte, 2004). Treatment of Philadelphia chromosome-positive CML with imatinib, an inhibitor of BCR-ABL, improves patient survival (Hochhaus et al., 2017; O'Brien et al., 2003). However, resistance to imatinib is common, with 60-95% of chronic stage CML patients becoming unresponsive to the TKI within 12 to 24 months. Mutations in BCR-ABL are a mechanism of intrinsic and acquired resistance; the kinase domain of the protein is a mutation hotspot. The most common BCR-ABL mutations to arise following treatment with imatinib are T315I, G250E, M351T, and E255K/V (Soverini et al., 2014). The second generation ABL TKIs nilotinib, dasatinib and bosutinib have greater potency than imatinib and were designed to overcome imatinib resistance mediated by point mutation in BCR-ABL (Chen and Chen, 2015; Weisberg et al., 2006). Following clinical trials which demonstrated the superiority of nilotinib and dasatinib over imatinib, these TKIs were approved by the FDA and EMA as first line treatments for use in Philadelphia chromosome-positive CML and for treatment of Philadelphia chromosome-positive CML resistant to or intolerant to prior therapy (Jabbour et al., 2015). However, treatment with second generation ABL TKIs does not overcome the T315I BCR-ABL mutation (Hochhaus et al., 2007; Kantarjian et al., 2007). This mutation prevents the formation of a hydrogen bond between the oxygen atom in threonine 315 and an imatinib amino group, hindering the binding of imatinib to BCR-ABL (Gorre et al., 2001; Schindler et al., 2000). The TKI ponatinib, which inhibits a number of kinases including ABL, VEGFRs and FGFRs, was developed to avoid the steric clash with the bulky isoleucine residue and therefore inhibits T315I BCR-ABL in addition to wildtype ABL. Ponatinib has been approved by the EMA and FDA for Philadelphia chromosome positive CML
harbouring the T315I mutation and for the treatment of Philadelphia positive CML resistant to or intolerant to other TKIs (Jabbour et al., 2015). However, resistance to ponatinib has been found to arise via further mutation of residue 315: I351M, or via compound BCR-ABL mutations including E255V/T315I (Zabriskie et al., 2014). A less common mechanism of resistance to imatinib is an increase in BCR-ABL copy number via amplification of BCR-ABL or gain of an additional Philadelphia chromosome. The additional copies of BCR-ABL induce increased protein expression and activation of BCR-ABL (Hochhaus et al., 2002; le Coutre et al., 2000).

Studies using cancer cell lines have shown that cells can enter a drug-tolerant or resistant state mediated by gene expression changes and epigenetic resistance mechanisms with an apparent lack of genetic alteration. Sharma et al. generated derivatives of the NSCLC line PC9 tolerant to EGFR TKIs by culturing parental PC9 in gefitinib for 9 days. These cells mediated their resistance by activation of IGF1R and this drug tolerant state was dependent upon the activation of the histone demethylase KDM5A (Sharma et al., 2010). Hou et al. reported that KDM5A protein and RNA expression was increased in the breast cancer lines SUM149 and SUM102 following 6 and 9 days culture in erlotinib. They conducted stable knockdown of KDM5A in the breast cancer cell lines SUM149 and HCC1937. It was observed that stable KDM5A knockdown reduced the number of drug tolerant SUM149 and HCC1937 cells following 30-day culture in erlotinib (Hou et al., 2012). Gale et al. developed the KDM5A inhibitor YUKA1. It was observed that treatment with YUKA1 reduced the formation of gefitinib-resistant colonies during the long-term culture of PC-9 with gefitinib. YUKA1 also reduced the formation of trastuzumab-resistant colonies during the long-term culture of the breast cancer cell line BT474 with trastuzumab (Gale et al., 2016).

Hata et al. cultured greater than 1200 pools of approximately 5000 parental PC9 cells in gefitinib for 2 weeks. Some pools of PC9 cells produced resistant derivatives with the EGFR T790M mutation and some PC9 pools produced drug tolerant derivatives which did not have this gatekeeper mutation, similar to the PC9 derivatives described by Sharma et al. (Hata et al., 2016; Sharma et al., 2010). Repetition of this procedure with the third generation EGFR TKI WZ4002, which effectively inhibits EGFR T790M, yielded only the drug tolerant derivatives which lacked the T790M mutation. Further culture of the drug tolerant derivatives in gefitinib led to acquisition of the EGFR T790M mutation. However, these cells retained resistance to WZ4002 induced apoptosis. This study indicates that further
resistance mechanisms can arise from cancer cells persisting in a drug tolerant state, highlighting the importance of targeting drug tolerant cells (Hata et al., 2016).

Ramires et al. expanded a single clone of PC9 for approximately 20 doublings. This population of cells was then treated with erlotinib for approximately 2 months at which point the emergent colonies of drug tolerant cells were isolated and cultured in erlotinib for a further 6-8 months. In total, this process yielded 17 different resistant colonies. These colonies all retained greater resistance to erlotinib than PC9 following culture without erlotinib for 40 weeks. They then conducted a drug screen, testing the sensitivity of the resistant cells to 560 compounds in combination with erlotinib. Exome sequencing data was then used to attempt to identify a genetic basis for the drug sensitivities identified in the drug screen. Despite the fact that the resistant cells were derived from a single clone, the cells exhibited differential sensitivity to drugs in the screen. Some colonies exhibited broad resistance to the drugs tested. One resistant clone exhibited sensitivity to MET inhibitors and was found to contain a MET amplification. Some exhibited sensitivity to EGFR inhibitors capable of inhibiting EGFR T790M, and were observed to have acquired this mutation. Other colonies were sensitive to MEK inhibitors and were observed to have acquired NRAS or RAF1 mutations (Ramirez et al., 2016). This study demonstrates that several mechanisms of resistance can emerge from a single clone in a drug tolerant state.

Gastrointestinal stromal tumours (GISTs) often have activating mutations in the RTK encoding genes KIT and PDGFRA (Heinrich et al., 2003; Hirota et al., 1998). Mühlenberg et al. observed that the combination of the non-selective histone deacetylase inhibitor vorinostat and imatinib was additive in KIT-positive GIST cell lines. Vorinostat was shown to reduce KIT mRNA expression and increase acetylation of HSP90, a KIT chaperone, inducing KIT degradation (Muhlenberg et al., 2009). Bauer et al. conducted a phase I clinical trial assessing the efficacy of the combination of imatinib and the non-selective histone deacetylase inhibitor panobinostat in overcoming resistance in patients with GISTs refractory to the combination of imatinib and the multitargeted TKI sunitinib. One out of the 11 patients showed a partial response, 7 had stable disease and 3 patients had progressive disease (Bauer et al., 2014).

15-20% of colorectal cancer patients have global high CpG island methylation phenotype which induces inactivation of tumour suppressors such as the mismatch repair gene MLH1 (Mojarad et al., 2013). Garrido-Laguna et al. conducted a phase I/II clinical in treated metastatic colorectal cancer patients with
wildtype KRAS and a minimum of 2 prior therapies. All but one patient had previously been treated with cetuximab. 20 patients were treated with combination of decitabine, a hypomethylating agent, and panitumumab. A partial response was observed in 2 patients, stable disease was observed in 11 patients and progressive disease was observed in 7 patients (Garrido-Laguna et al., 2013).

Non-cancerous cells in the tumour microenvironment have been reported to affect tumour drug resistance. Paulsson et al. used immunohistochemistry to examine the stromal expression of PDGFRβ, a regulator of fibroblasts, in 360 tumours from premenopausal, and 528 tumours from postmenopausal, breast cancer patients treated with and without tamoxifen, a selective oestrogen receptor modulator. Kaplan-Meier analysis showed that pre- and postmenopausal patients with low expression of PDGFRβ had a significant increase in recurrence-free survival when treated with tamoxifen. In contrast, a significant treatment benefit was not observed in patients with high stromal PDGFRβ expression. This study did not identify a causal link between high stromal PDGFRβ expression and tamoxifen resistance (Paulsson et al., 2017). Park et al. reported that depletion of CD8+ cytotoxic lymphocytes with an anti-CD8α- antibody induced rapid tumour relapse in BALB/c mice bearing tumours established from the neu-positive mammary carcinoma cell line TUBO treated with an anti-neu antibody. This suggests a T-cell dependent resistance mechanism (Park et al., 2010).

1.3 Altered FGFR signalling in cancer

1.3.1 Structure of FGFRs

The FGFR family of transmembrane glycoproteins (FGFRs1-4) bind to and are activated by 18 FGFs and 4 FGF homologous factors that have differing affinities for each of the FGFRs and their splice variants. The different FGFs signal through FGFR in a paracrine or endocrine manner by forming a complex of 2 FGFs, 2 FGFRs and 2 heparan sulphate molecules (Ornitz et al., 1996; Zhang et al., 2006). Fibroblast growth factor receptor like-1 (FGFRL1), also known as FGFR5, is the most recently identified member of the FGFR family (Sleeman et al., 2001). It has been suggested that FGFRL1 is a decoy receptor (Steinberg et al., 2010; Trueb et al., 2003) although more recent evidence indicates that it may activate the MAP kinase pathway independently of ligand (Silva et al., 2013). Upon ligand binding,
FGFRs 1-4 form homodimers, and intracellular autophosphorylation can lead to signalling through the MAP kinase, PI3 kinase, PLC\(\gamma\) and JAK/STAT pathways (Böttcher and Niehrs, 2005; Klint and Claesson-Welsh, 1999). From the extracellular N-terminus through to the C-terminus FGFR1-4 contain the following: a signal peptide, immunoglobulin (Ig)-like domain I, an acid box consisting of a sequence of glutamic and aspartic acids, the heparan sulphate binding domain, Ig-like domain II, Ig-like domain III, a transmembrane domain, a juxtamembrane linker, a split tyrosine kinase domain and an inhibitory carboxy-terminal tail (Fig. 1.1)(Belov and Mohammadi, 2013). The Ig-like domains I-III are named D1-3 with D1 at the N-terminus and D3 proximal to the membrane. D1 and the acid box are autoinhibitory whilst D2 and D3 bind FGFs and therefore are responsible for specificity of each receptor. FGFR1 can be alternatively spliced to produce FGFR1\(\alpha\), which contains Ig-like domains I-III or FGFR1\(\beta\) which contains only Ig-like domains II and III (Johnson et al., 1991). FGFR1\(\alpha\) exhibits greater affinity for FGF1 and heparan than FGFR1\(\beta\) (Shi et al., 1993; Wang et al., 1995). FGFR1-3 can undergo alternative splicing expressing either exon 8 or 9, producing IIIb or IIIc isoforms respectively. Exons 8 and 9 encode the C-terminus of D3 and therefore this alternative splicing event affects ligand specificity. For example, FGF1 activates all FGFRs whereas FGF2 preferentially activates the FGFR IIIc isoforms. Expression of FGFR IIIb isoforms is found in epithelial tissue, in contrast to IIIc isoforms which are expressed in mesenchymal tissue (di Martino et al., 2012; Olsen et al., 2004; Ornitz et al., 1996; Zhang et al., 2006). FGFR3 IIIb contains the following tyrosine phosphorylation sites: Y579, Y649/650, Y726, Y762 and Y772 (Fig. 1.1). Phosphorylation of residue Y726 is important in the activation of the MAP kinase, PI3 kinase, STAT1 and STAT3 pathways. Phosphorylation of Y649/650, which are situated in the protein’s activation loop, is required for kinase activity. Phosphorylation of Y762 allows binding of PLC\(\gamma\) and the PI3 kinase p85 subunit and may induce greater phosphorylation of STAT1 and STAT3. Y772 inhibits activation of the PI3 kinase pathway (Hart et al., 2001). Pathway activation downstream of FGFR3 is summarised in Fig. 1.2.
Figure 1.2 Protein structure of FGFR3 IIIb (green) and the FGFR3-TACC3 (blue) fusion proteins found in RT112 and RT4. Yellow spots highlight the location of FGFR3 phosphorylation sites. The red arrow indicates the breakpoint in RT112 and RT4 FGFR3-TACC3. Blue arrows indicate the most common sites of FGFR3 mutation in bladder cancer. SP: signal peptide, IgI-III immunoglobulin like domains, AB: acid box, TM: transmembrane domain, TK1 and TK2: tyrosine kinase domain, TACC: Transforming acid coiled-coil. Image adapted from di Martino et al., 2016.

Figure 1.3 Signalling pathways activated by FGFR3. Orange denotes the MAP kinase pathway, magenta: the JAK/STAT pathway, grey: the PLCγ pathway and green: the PI3 kinase pathway. Blue denotes the FGFR3 homodimer, red: heparan sulphate and yellow: FGFs. P indicates the FGFR3 phosphorylation sites. Image adapted from di Martino et al., 2016.
1.3.2 FGFR mutation and overexpression

FGFR signalling is activated in many different cancers: some examples are summarised below.

Moelans et al. observed amplification of FGFR1 in 17% of 104 invasive breast cancers examined with multiplex ligation-dependent probe amplification (Moelans et al., 2010). Turner et al. interrogated whole genome gene expression data from 295 invasive breast cancers, examining overexpression of genes located in the 8p11-12 amplicon, which contains FGFR1. It was observed that amplification of FGFR1 was associated with the luminal B breast cancer subtype which has a poor prognosis. 16-27% of luminal B subtype breast cancers had FGFR1 amplification (Turner et al., 2010). Hiest et al. determined that 16% of a cohort of squamous NSCLCs had amplification of FGFR1 using FISH (Heist et al.). Theelen et al. conducted immunohistochemistry on 653 NSCLC tumours and observed high protein expression of FGFR1 in 10.6% of samples (Theelen et al., 2016).

Amplification and mutation of FGFR1 are uncommon events in bladder cancer. Fischbach et al. identified FGFR1 amplification in 2 out of 122 bladder cancers of a range of stages and grades with FISH (Fischbach et al., 2015). Ross et al. conducted NGS targeting the exons of 182 cancer related genes and the introns of 14 genes often rearranged in cancer on a panel of 35 stage IV, predominantly high grade urothelial carcinomas. Three tumours had FGFR1 gene amplification, one patient had a T141R FGFR1 mutation and one tumour had a FGFR1-NTM fusion (Ross et al., 2014). Tomlinson et al. examined expression of FGFR1 in 27 bladder cell lines and 73 bladder tumours with quantitative real time PCR (qRT-PCR) and observed that FGFR1 mRNA expression was increased in the majority of bladder cancer cell lines and tumours compared to normal human urothelial cells. Knockdown of FGFR1 and treatment with the FGFR TKI PD173074 (PD) identified the bladder cancer cell line JMSU1 as dependent upon FGFR1 expression for survival (Tomlinson et al., 2009). Tomlinson et al. examined expression of FGFR1α and FGFR1β with isoform specific qRT-PCR in 37 bladder cancer cell lines and 50 bladder tumours and observed that the majority of bladder cancer cell lines and tumours had undergone a FGFR1α to FGFR1β isoform switch. A higher ratio of FGFR1β to FGFR1α expression was associated with a higher stage and grade in tumours. Low concentrations of FGF1 were found to activate the MAP kinase pathway and cell proliferation to a greater extent in immortalized normal human urothelial cells transduced to express FGFR1α compared to cells transduced to express FGFR1β (Tomlinson and Knowles, 2010). FGFR1
expression has been associated with a mesenchymal phenotype in bladder cancer cell lines in contrast to expression of FGFR3 which has been associated with an epithelial phenotype (Cheng et al., 2013).

Activating FGFR2 mutations are found in approximately 10% of endometrial cancers. The most common is S252W, which increases FGFR2 binding affinity for several FGFs (Helsten et al., 2016; Pollock et al., 2007). Theelen et al. observed high protein expression of FGFR2 in 12.9% of samples of the 653 NSCLC tumours examined with immunohistochemistry. Diez de Medina et al. examined FGFR2 mRNA expression in 54 urothelial carcinomas of a variety of stages and grades and observed FGFR2 expression to be increased in 2 tumours and 18 tumours expressed FGFR2 below 30% of the level observed in normal urothelium. In MIBCs loss of FGFR2 expression correlated with a poor prognosis. Therefore, FGFR2 may be tumour suppressive in urothelial carcinoma (Diez de Medina et al., 1997).

In multiple myeloma, about 40% of patients have a t(4:14) translocation and approximately 70% of tumours with this translocation overexpress wildtype FGFR3 due to the juxtaposition of FGFR3 with IGH gene regulatory sequences (Kalfff and Spencer, 2012). FGFR3 mutations are found at a low frequency in multiple myeloma, colorectal cancer, and cervical carcinoma (Cappellen et al., 1999; Chesi et al., 1997; Jang et al., 2001). FGFR3 mutations are found at a high frequency in seborrheic keratoses (Hafner et al., 2010; Hafner et al., 2006)

The prevalence of FGFR3 mutation and overexpression in NMIBC and MIBC is discussed in section 1.1.2. The most common FGFR3 mutations in bladder cancer are S249C, Y375C, R248C and G372C (Fig. 1.1). All these mutations introduce a cysteine in or close to D2 or the transmembrane domain of the receptor, inducing the formation of intermolecular disulphide bonds and constitutive phosphorylation and activation of FGFR3 (Bernard-Pierrot et al., 2006). K650E/M/Q/T FGFR3 kinase domain mutations are found in less than 1% of urothelial carcinomas and stabilise the kinase in its active state (Huang et al., 2013). FGFR3 mutations are normally mutually exclusive with RAS mutations in bladder cancer, suggesting that mutations in these genes perform an overlapping function (Jebar et al., 2005).

Altered expression of FGFR4 has not been described in urothelial carcinoma (di Martino et al., 2016). FGFR4 kinase domain mutations are found in approximately 8% of rhabdomyosarcomas (Taylor et al., 2009).
1.3.3 FGFR Fusion Proteins

The first FGFR fusion protein to be identified was FGFR3-TACC3 in glioblastoma multiforme (Singh et al., 2012). FGFR1-TACC1 or FGFR3-TACC3 fusions are found in approximately 3% of glioblastoma multiforme (Singh et al., 2012). FGFR fusion protein transcripts have been detected in bladder, prostate, oral and breast cancer as well as others (Wu et al., 2013).

FGFR3 fusion proteins were first observed in bladder cancer by Williams et al. It was observed that the bladder cancer cell lines RT112, RT4 and SW780 express FGFR3 fusion proteins of a higher molecular weight than wildtype FGFR3 in addition to wildtype FGFR3 (Williams et al., 2013). RT112 expresses an FGFR3-transforming acid coiled coil 3 (TACC3) fusion containing residues 1-760 (exons 1-18 of IIIb isoform) of FGFR3 and TACC3 residues 649-838. RT4 also expresses an FGFR3-TACC3 fusion containing residues 1-760 (IIIb isoform) of FGFR3, but this fusion contains TACC3 residues 433-638 (Fig 1.1) (Williams et al., 2013). TACC3 forms a complex with ch-TOG and clathrin which aids the formation of inter-microtubule bridges, stabilising kinetochore fibres during mitosis (Booth et al., 2011; Peset and Vernos, 2008). These FGFR3-TACC3 fusions are due to a rearrangement of the 4p16.3 region containing both FGFR3 and TACC3. SW780 expresses an FGFR3-BAI1-associated protein 2-like 1 (BAIAP2L1) fusion with residues 1-760 (IIIb isoform) of FGFR3 and BAIAP2L1 residues 18-522 as a result of a reciprocal translocation between chromosome 4 and 7 (Williams et al., 2013). BAIAP2L1, also called IRTKS (insulin receptor tyrosine kinase substrate), regulates the actin cytoskeleton (Millard et al., 2007).

FGFR3 exon 19 is not encoded in the FGFR3-TACC3 fusion. FGFR3 exon 19 contains a PLCγ binding site, Y762, and a site involved in the negative regulation of the PI3 kinase pathway, Y772 (Hart et al., 2001). As anticipated, when expressed in immortalized normal human urothelial cells, the FGFR3 fusion proteins did not induce phosphorylation of PLCγ but did induce ERK phosphorylation (Williams et al., 2013). Williams et al. considered the possibility that the FGFR3-TACC3 fusion proteins may possess aberrant activation compared to wildtype FGFR3 due to the loss of FGFR3 exon 19. The fusion proteins induced anchorage-independent growth and a more spindle-like morphology when ectopically expressed in the mouse embryonic fibroblast cell line NIH3T3. This is in contrast to the result of ectopic expression of a truncated FGFR3 with a stop codon at the end of exon 18 to prevent expression of C-terminal exon 19. This did not induce a morphological change and induced anchorage-independent growth to a lesser extent. This suggests that the fusion partner rather than simply loss of the
FGFR3 terminal exon is important for the oncogenic potential of the fusion proteins (Williams et al., 2013). TACC3 is thought to regulate microtubule stability via the formation of a complex with ch-TOG and clathrin (Ding et al., 2017). The FGFR3-TACC3 fusion contains the TACC3 TACC domain which mediates dimerization (Guo et al., 2015; Simpson et al., 2004). The inclusion of the TACC domain in FGFR3-TACC3 may mediate homodimerization of the fusion protein, inducing activation of the tyrosine kinase domain. Compared to wildtype FGFR3, FGFR3-TACC3 is reported to have increased total kinase activity, additional phosphorylation sites and an increase in FGF independent activation of the MAPK pathway (Nelson et al., 2016).

Sequencing of 126 urothelial tumours identified the following gene fusions: one FGFR1-NTM, four FGFR3-TACC3, one FGFR3-JAKMIP1 and one FGFR3-TNIP2. Information on the stage and grade of tumours was not provided in this study (Helsten et al., 2016). Guo et al. identified two FGFR3-TACC3 fusion events in a panel of 42 urothelial carcinomas of a variety of stages and grades with RNA-Seq (Guo et al., 2013). One FGFR3-TACC3 fusion event was present in a superficial tumour and one was present in an invasive tumour (Guo et al., 2013). Robertson et al. examined 412 MIBCs with RNA-Seq and identified that FGFR3-TACC3 fusions were present in 10 (2%). Pietzak et al. identified that of the 105 NMIBCs examined with targeted exon sequencing, FGFR3 fusion events occurred in 4 (4%) (Pietzak et al., 2017). These studies show that FGFR3 fusion events are present at a low frequency in both NMIBC and MIBC.

1.4 Previous Research into FGFR-targeted therapy

1.4.1 Current FGFR-targeted therapies in development

Many FGFR TKIs have been assessed in clinical trials. Dovitinib is a multitargeted TKI. Trudel et al. observed that dovitinib inhibited FGFR1, FGFR3, KIT, vascular endothelial growth factor receptor 1 (VEGFR1), VEGFR2, VEGFR3, FLK3, platelet-derived growth factor receptor α (PDGFRα) and PDGFRβ with IC50s less than 28nM in an in vitro kinase assay (Trudel et al., 2005). It has been trialled in patients with NSCLC, bladder cancer, endometrial cancer and multiple myeloma, (Das et al., 2015; Keam et al., 2015; Konecny et al., 2015; Milowsky et al., 2014; Scheid et al., 2015). It was assessed in a phase II clinical trial in advanced urothelial cancer
patients. Although it was well tolerated it had limited single agent activity in urothelial cancer patients with either mutant or wildtype FGFR3 (Milowsky et al., 2014).

The FGFR1-2, VEGFR1-3, PDGFR α and PDGFRβ TKI lucitanib was evaluated in a phase I/IIa clinical trial in 76 patients with advanced metastatic solid tumours including breast, colon, rectal, lung and thyroid cancer. Lucitanib was assessed in patients with FGFR1 or FGF3/4/19 amplification or whose disease was of a histological type potentially sensitive to anti-angiogenic therapy patients and whose disease was progressing following previously stable disease in response to anti-angiogenic-based treatment. The objective response rate was 30.4% in the FGF aberrant tumours and 25.9% in the angiogenesis-sensitive tumours (Soria et al., 2014).

The FGFR1-3 TKI AZD4547 has been tested in phase I and II clinical trials in FGFR1-amplified NSCLC, FGFR1-amplified breast cancer and FGFR2-amplified gastroesophageal cancer. In these trials AZD4547 was well tolerated but the efficacy of AZD4547 was often disappointing; however, Smyth et al. did report a confirmed response in 3 out of 9 patients with FGFR2-amplified gastroesophageal cancer (Arkenau et al., 2014; Paik et al., 2017; Smyth et al., 2015). Other trials with AZD4547 are in progress or yet to commence, including a phase I trial, ID NCT02546661, in metastatic MIBC patients who have progressed on prior treatment (Powles et al., 2016).

The FGFR1-3 TKI BGJ398 has been assessed in a phase I clinical trial in patients with advanced solid tumours with FGFR alterations. BGJ398 was found to have a tolerable safety profile and a partial response was observed in some patients with FGFR1-amplified squamous NSCLC and FGFR3-mutant urothelial cancer (Nogova et al., 2017). Pal et al. evaluated the efficacy of single agent treatment with BGJ398 in 67 patients with metastatic urothelial carcinoma who had progressed on or were intolerant to platinum-based chemotherapy and whose tumours had alterations in FGFR3. There was an overall response rate of 25% and disease stabilisation was observed in an additional 39% of patients. FGFR3 gatekeeper mutations (V443L, V443M, and L496V) were detected in the cell-free DNA (cfDNA) of 4 patients during treatment (Pal et al., 2018).

The FGFR1-4 TKI erdafitinib has been assessed in a phase I trial in a number of advanced solid tumours. Of the 8 urothelial carcinoma patients in this trial, a partial response was reported in 2 patients with FGFR3-TACC3 translocations and a patient with a FGFR2-BICC1/FGFR2-CASP7 translocation
(Tabernero et al., 2015a). Erdafitinib was assessed in another phase I clinical trial in patients with advanced solid tumours and found to have a manageable safety profile. No patients had a partial or complete response. This trial had only one bladder cancer patient (Tabernero et al., 2015b). Completed, ongoing or planned clinical trials examining treatment with FGFR targeted agents in bladder cancer patients are detailed in table 1.1.

<table>
<thead>
<tr>
<th>FGFR targeted agent</th>
<th>Manufacturer</th>
<th>Molecular targets</th>
<th>Clinical trials (NCT number, phase)</th>
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<td>FLT3, KIT, FGFR1-3, VEGFR1-4</td>
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<td>NCT02546661, phase I&lt;br&gt;NCT02465060, phase II</td>
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<tr>
<td>BGJ398</td>
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<td>FGFR1-4</td>
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<td>Janssen Pharmaceuticals</td>
<td>FGFR1-4</td>
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<td>BioCln Therapeutics</td>
<td>FGFR3</td>
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<td>NCT02529553, phase I</td>
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</table>

Table 1.1 Completed, ongoing or planned clinical trials examining treatment with FGFR targeted agents in bladder cancer patients. Clinical trials identified using clinicaltrials.gov.

1.4.2 Previous work on resistance to FGFR inhibitors

The V561 residue is the FGFR1 ‘gatekeeper’ residue, responsible for FGFR1 ATP binding site nucleotide and small molecule inhibitor specificity. The FGFR1 V561M mutation introduces a large methionine side chain mutation that causes steric hindrance with the FGFR inhibitors PD and E3810 but not others such as AZD4547. AZD4547 retains binding affinity due to flexibility caused by an ethyl linker in its structure. This mutation also increases the stability of the hydrophobic
spine of residues indicative of the active form of the catalytic domain (Sohl et al., 2015; Yoza et al., 2016).

Long-term culture of the myeloma cell line KMS-11 with the FGFR inhibitor AZ12908010, which was discovered during the development of, and is closely related to, AZD4547, produced a resistant derivative with an FGFR3 V555M mutation, analogous to the FGFR1 V561M gatekeeper mutation. The KMS-11 AZ12908010-resistant derivative had also acquired resistance to AZD4547 and PD. The methionine side chain introduced by this mutation, similarly to the FGFR1 V561M mutation, appears to disrupt binding of TKIs to the ATP-binding site (Chell et al., 2013).

The murine IL-3-dependent, pro-B cell line, Ba/F3, was stably transduced to overexpress FGFR2. The stable FGFR2-expressing Ba/F3 cells were seeded into 96 well plates and cultured in the presence of dovitinib and colonies which grew out were expanded. This screening procedure was repeated with Ba/F3 cells stably expressing the FGFR2-activating mutations, S252W or N550K. In total 14 FGFR2 mutations which induced resistance to dovitinib were identified, 13 of which occurred in the FGFR2 tyrosine kinase domain (M536I, M538I, I548V, N550H/K/S/T, V565I, E566A/G, L618M, K642N and E719G). One mutation, Y770fsX14, was in the FGFR2 c-terminal tail. One of these mutations was N550K, which accounts for 25% of FGFR2-mutant endometrial cancers. This mutation induced the greatest cross-resistance to PD. N550 is part of an autoinhibitory network of residues, and mutations affecting this site allow the kinase to adopt an active state. The only mutation identified that caused resistance to ponatinib, a TKI that inhibits BCR-ABL and FGFR1-4, was V565I. The gatekeeper residue V565I stabilizes the hydrophobic spine, in a similar way to some of the other reported mutations, but also sterically hinders FGFR TKI binding. This steric hindrance is a possible cause of the ponatinib resistance (Byron et al., 2013).

A siRNA screen identified EGFR as limiting the sensitivity of three bladder cancer cell lines with mutant FGFR3, RT4, RT112 and MGH-U3, to PD. RT112 and 639V, a bladder cancer cell line with an FGFR3 point mutation, showed increased phosphorylation of EGFR upon acute treatment with PD. A RT112 xenograft mouse model showed that treatment with PD and gefitinib reduced tumour volume to a greater extent than either of the drugs given separately (Herrera-Abreu et al., 2013).
Long term treatment of RT112 with the pan-FGFR TKI BGJ398 yielded resistant derivatives with phosphorylated ERBB2 and ERBB3 and sensitivity to a combination of inhibitors of the EGFR family (Wang et al., 2014).

A screen of a shRNA library that targeted kinases and kinase-related genes in RT112 identified that inhibition of the PI3 kinase pathway and EGFR enhanced the effect of AZD4547. Phosphorylation of EGFR and ERBB3 was observed in RT112 following AZD4547 treatment. The pan-PI3 kinase inhibitor BKM120 plus AZD4547 synergistically reduced proliferation in RT112 and JMSU-1, a urothelial carcinoma cell line with FGFR1 amplification (Wang et al., 2017b).

A secreted protein cDNA library was screened in RT112 cultured in BGJ398. The EGF family ligands neuregulin 1 (NRG1), NRG2 and transforming growth factor alpha (TGF-α) and the MET ligand HGF were identified as able to rescue RT112 from this FGFR TKI (Harbinski et al., 2012).

1.4.3 RT112 and RT4
In order to examine how urothelial carcinomas may acquire resistance to FGFR TKIs this study has made use of the cell lines RT112 and RT4. RT112 was derived in 1973 from a female of unknown age with untreated urothelial carcinoma, histological grade 2. Clinical stage of the disease was not recorded (Marshall et al., 1977; Masters et al., 1986). RT4 was derived in 1968 from a 63 year old male with urothelial carcinoma treated with gold beads, clinical stage T2, histological grade 1 (Rigby and Franks, 1970). RT112 is mainly diploid (Williams et al., 2005). This cell line has a BRCA2 Q2731R missense mutation, a CDH6 S2380X nonsense mutation, a TERT promoter mutation and homozygous deletion of CDKN2A (Aveyard and Knowles, 2004; Earl et al., 2015; Nickerson et al., 2017). BRCA2 is crucial in the repair of DNA double strand breaks by homologous recombination maintaining genomic stability (Fradet-Turcotte et al., 2016). CDH6 encodes cadherin 6 which is a type II classical cadherin (Shimoyama et al., 1999).

RT4 is mainly tetraploid, has a DNMT3A S129L missense mutation, an ELF3 217_219del frameshift deletion, ESPL1 P1937A missense mutation, FAT4 R3716H missense mutation, a PALB2 L337S missense mutation and a TSC1 D505fs frameshift deletion. RT4 also has TP53 loss of heterozygosity and homozygous deletion of CDKN2A (Aveyard and Knowles, 2004; Earl et al., 2015; Nickerson et al., 2017; Williams et al., 2005). DNMT3A encodes DNA (cytosine-5)-methyltransferase 3A. ELF3 is a transcriptional regulator in the urothelium (Bock et
ELF3 frameshift deletions and insertions, splice site and missense mutations are found in bladder cancer (Dadhania et al., 2016). Guo et al. found ESPL1 mutations in 6% of urothelial carcinomas (Guo et al., 2013). ESPL1 encodes separase which enables the segregation of sister chromatids at the metaphase to anaphase transition (Solomon et al., 2014). FAT4, a member of the cadherin superfamily, inactivates the oncogenic transcription co-activators YAP and TAZ which promote cell proliferation (Katoh, 2012). PALB2 is a tumour suppressor which binds BRCA2 and recruits it to the site of DNA damage (Park et al., 2014). RT112 and RT4 express wildtype FGFR3 and FGFR3-TACC3 fusion proteins (Fig. 1.1) (Williams et al., 2013).

RT112 and RT4 were chosen for this study as they are reported to be sensitive to PD (Lamont et al., 2011). PD acts by binding the ATP-binding pocket in the tyrosine kinase domain of FGFR1 and FGFR3 (Mohammadi et al., 1998). Lamont et al. reported RT112 and RT4 as having IC50s of 15nM and 5nM respectively for PD (Lamont et al., 2011). Herrera-Abreu et al. found that treatment of RT112 with 1mM PD for 3 days reduced cell viability by over 50% (Herrera-Abreu et al., 2013). In order to produce a model of acquired resistance to FGFR inhibition, RT112 and RT4 were cultured in PD for 20 passages. Resistant derivatives that arose from this treatment were examined in this project and are denoted RT112 R1, RT112 R2, RT112 R3 and RT4 R1.

## 1.5 Aims

There is a need to improve the survival of patients with MIBC. FGFR inhibitors are now entering clinical trials to assess their suitability for the treatment of urothelial carcinoma. Whilst intrinsic and acquired resistance has reduced the efficacy of targeted agents specific for tyrosine kinases in other types of cancer, research into these resistance mechanisms has enabled further improvements in the treatment of cancer patients. This project has been conducted in anticipation that resistance to FGFR inhibitors will develop in patients with urothelial carcinoma. The aims of this research are the following:

1. Examine the differences in morphology, cell proliferation and sensitivity to PD between RT112 parental and RT112 R1, R2 and R3 and between RT4 parental and RT4 R1.
2. Determine the mechanisms by which the RT112 and RT4 resistant derivatives continue to grow in the presence of PD.

3. Suggest drug combinations which may overcome resistance based upon the identified resistance mechanisms and test the efficacy of these drug combinations.
Chapter 2
Materials and Methods

Manufacturer names are detailed after the first mention of each product. See Appendix A for full name and address of all suppliers. See Appendix B for buffers and solutions.

2.1 Tissue culture

2.1.1 Tyrosine kinase inhibitors (TKIs) used in this study

The TKIs used in this study were the FGFR inhibitor PD173074 (Sigma-Aldrich), the EGFR inhibitor Erlotinib (Cayman Chemical), the EGFR, ERBB2 and ERBB3 inhibitor Sapitinib (ApexBio Technology), the IGF1R inhibitor Linsitinib (Biovision) and the MET inhibitor Capmatinib (Adooq Bioscience). All TKIs were dissolved in DMSO and stored at -20°C: PD173074 and Sapitinib were stored as 10mM solutions, Erlotinib was stored as a 40mM solution, Linsitinib was stored as a 20mM solution and Capmatinib was stored as a 4.85mM solution.

2.1.2 Cell lines used in this study

The epithelial cell lines RT112 and RT4 were used (Marshall et al., 1977; Masters et al., 1986). The cell line identity of parental RT112 and parental RT4 was confirmed with short tandem repeat profiling in 2012. The RT112 and RT4 profiles were compared with reference profiles from the ATCC and DSMZ databases. All resistant derivatives were previously generated in the Knowles lab (described in Chapter 3). RT112 R1, RT112 R3 and RT4 R1 were cultured in 1μM PD173074 (Sigma-Aldrich) whilst RT112 R2 was cultured in 2μM PD173074. During production of the resistant derivatives RT112 R1, R2 and RT4 R1 were split 1 in 3 and R3 was split 1 in 5.
2.1.3 Cell culture

All cell culture was performed under aseptic conditions in a Biomat class II laminar flow hood (MAT). Cells were removed from liquid nitrogen storage, rapidly thawed at 37°C, recovered into 10ml of growth medium, pelleted by centrifugation at 1000 \( \times \) g for 4 min then resuspended in an appropriate volume of growth medium and transferred to sterile 25cm\(^2\) or 75cm\(^2\) vented canted-neck culture flasks (Corning). Cells were maintained at 37°C in a humidified incubator (Sanyo) in an atmosphere of 5% CO\(_2\) in air. RT112 cells were cultured in RPMI-1640 growth medium (Sigma-Aldrich) supplemented with 10% foetal calf serum (FCS; Biosera) and 2mM GlutaMAX (Life Technologies). RT4 was cultured in McCoy’s 5A growth medium (Sigma-Aldrich) supplemented with 10% FCS and 2mM GlutaMAX. Resistant derivatives were cultured in the same media as their parental cell line supplemented with PD173074. The resistant derivatives RT112 R1, RT112 R3 and RT4 R1 were routinely cultured in 1μM PD173074 whilst RT112 R2 was cultured in 2μM PD173074. Cells were cultured for no more than 10 passages from frozen stocks.

2.1.4 Cell passage

Cells were routinely passaged at, or just prior to, confluence. Medium was aspirated, cells were rinsed in calcium- and magnesium-free phosphate buffered saline (PBS) solution then incubated in PBS containing 0.1% (w/v) EDTA for 2 min at 37°C. The PBS-EDTA was aspirated and cells were incubated with 1ml 0.05% trypsin-0.02% EDTA (TV; Sigma-Aldrich) for a 75cm\(^2\) flask, for approximately 5 min until the cells detached. TV was inhibited by re-suspension of cells in fresh medium containing 10% FCS. A fraction of this single cell suspension was transferred to a new flask containing fresh growth medium.

2.1.5 Cell counting

Cells were counted to seed plates with the correct number of cells per well for growth curves and cell viability assays, and to calculate cell numbers during growth curves. A single cell suspension was produced as described above. Cells were counted with a Beckman Coulter Z2 cell and particle counter (Beckman Coulter). Counts were performed twice per sample and a mean value was calculated.
2.1.6 Growth curves

A single cell suspension was produced from cells at 70-80% confluence as described above. Cells were seeded in 6 well plates (Corning) at a density of 1 x 10^4 cells/well in 2ml of medium. Medium was changed every 3 to 4 days. Triplicate wells were counted for each time point and a mean value calculated.

2.1.7 Cell Viability Assay - CellTiter-Blue™

For each cell line a 70-80% confluent 75cm^2 flask was selected and a single cell suspension produced as described above. A flat-bottomed 96 well plate (Corning) was seeded with 100μl of cells per well in medium without any TKI or vehicle present. Cells were seeded at a density that would give 80% confluence at the end of the assay in the untreated control. After 24 h incubation medium was replaced with 100μl of medium containing the appropriate concentration of TKI(s) or vehicle. Cells were then incubated for 72 h after which medium was replaced and cells were incubated for a further 48 h. Throughout this assay cells were incubated at 37°C in 5% CO₂. After 120 h of incubation with TKI(s), 20μl of CellTiter-Blue pre-warmed to 37°C was added to each well. The plates were kept dark and incubated for 2 h at 37°C in 5% CO₂. Fluorescence was measured on a Berthold Mithras LB 940 Multimode microplate reader. Excitation was at 540nM and emission was at 590nM respectively. Results were blanked with a medium no cell control and normalized to the vehicle (DMSO) control. Dose response curves and IC50 values were calculated using GraphPad Prism® 7. Cell viability assays in Figure 3.8, Figure 7.7 and Figure 7.8 were conducted by Matt Dunning.

2.1.8 Retroviral transduction of RT112 parental

Retroviral transduction was performed by Dr Julie Burns:

Amphotropic retrovirus was produced as follows: 50% confluent Phoenix Amphi cells were transfected overnight with virus backbone plasmids: pBABEpuro, pBABEpuro-RAS V12, pLXSNeo or pLXSNeo-RAS V12 using transfection reagent TransIT-293 (Mirus) according to the manufacturer’s protocol. Medium was changed daily and virus-containing medium was harvested after 72 and 96 h. The 72 and 96 h virus supernatants were pooled, passed through a 0.45μm filter and stored at -80°C in 1.5ml aliquots. RT112 parental cells were transduced as follows: 50% confluent cells in a 25cm² flask were transduced overnight with retroviral
supernatant (1.5 ml virus supernatant + 1.5 ml normal medium + 8µg/ml polybrene (Sigma-Aldrich 107689)). The following morning the transduction mix was replaced with normal growth medium and selection with 1µg/ml puromycin or 600µg/ml G418 was started after 48 h. Selection was maintained until stocks were frozen after 14 days (LXSN) or 19 days (BABE). When thawed the cells were maintained in half of the selection drug concentration.

2.2 Examination of protein expression

2.2.1 Protein extraction

70-80% confluent cells in 75cm² cell culture flasks were selected and washed twice with phosphate buffered saline (PBS). Cells were lysed on ice in 250µl of RIPA buffer (Appendix A) containing 6.25µl protease inhibitor cocktail (P8340) and 2.5µl phosphatase inhibitor cocktail 2 (P5726) (Sigma-Aldrich). The flasks were incubated on ice for 5 min then cells were detached using a cell scraper (Sarstedt). Cell lysates were transferred to 1.5ml micro-centrifuge tubes and cell debris pelleted by centrifugation at 12850 x g for 10 min at 4°C. The supernatants were transferred to new 1.5ml micro-centrifuge tubes and stored at -80°C.

2.2.2 Bradford assay (protein quantification)

Proteins used for immunoblotting and immunoprecipitation were quantified by Bradford assay. Protein concentrations were measured using the Bio-Rad protein assay, which is based on the Bradford dye-binding method, according to the manufacturer’s protocol. The absorbance of a series of bovine serum albumin standards was measured on a Bio-Rad SmartSpec Plus spectrophotometer and used to generate a standard curve. The absorbance of the samples was then measured and the standard curve used to calculate protein concentrations.

2.2.3 Immunoblotting

Protein samples were diluted with RIPA buffer to contain 30µg of protein in 20µl. 5µl of 5x SDS loading buffer (Appendix A) was added and samples were denatured for 3 min at 100°C then placed on ice for 3 min. 10µl of Precision Plus Protein
Prestained Standard (Bio-Rad) and 20μl of samples were loaded onto 7.5% polyacrylamide Bio-Rad Mini-PROTEAN TGX precast gels. Gels were electrophoresed in 1x TGS buffer (Bio-Rad) (Appendix A) at 3W per gel, until the blue dye front reached the bottom of the gel. Proteins were transferred to nitrocellulose membranes using the high molecular weight program on a Trans-Blot Turbo Transfer System (Bio-Rad) with a Trans-Blot Turbo transfer pack (Bio-Rad) of either mini or midi format. Membranes were blocked in 4% (w/v) non-fat milk (Panreac AppliChem) in PBS containing 0.1% (v/v) Tween 20 (Fisher Scientific) (PBS-T) for 30 min. Membranes were then incubated with primary antibody in 2% (w/v) non-fat milk in PBS-T for 1 h at room temperature (RT) or overnight at 4°C on a shaking platform. Hybridised membranes were washed 4 x 10 min in PBS-T and incubated with HRP-conjugated secondary antibody in 2% (w/v) non-fat milk in PBS-T for 1 h at RT or overnight at 4°C on a shaking platform. Membranes were then washed for 4 x 10 min in PBS-T. HRP-labelled antibody was detected by chemiluminescence using Luminata Forte Western HRP Substrate (Merk Millipore) and imaged with a ChemiDoc MP System and Image Lab software (Bio-Rad). Membranes were stripped at 55°C for 40 min in stripping buffer (Appendix A), washed 3 x 10 min in PBS-T and re-hybridised to examine β-actin expression as a loading control. See Table 2.1 for a list of antibodies and their concentrations.

Table 2.1 Antibodies used in immunoblotting.

<table>
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<th>Dilution</th>
</tr>
</thead>
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<td></td>
<td></td>
</tr>
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<td>Santa Cruz sc-81178</td>
<td>Mouse monoclonal</td>
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</tr>
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<td>R &amp; D Systems BBA10</td>
<td>Mouse monoclonal</td>
<td>1/1000</td>
</tr>
<tr>
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<td>Dako M7237</td>
<td>Mouse monoclonal</td>
<td>1/1000</td>
</tr>
<tr>
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<td>Abcam ab1416</td>
<td>Mouse monoclonal</td>
<td>1/500</td>
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<tr>
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<td>1/1000</td>
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<td>Rabbit</td>
<td>1/500</td>
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</tr>
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<td>Anti rabbit-HRP</td>
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<td>Goat</td>
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</tr>
</tbody>
</table>

### 2.3 Examination of RNA expression

### 2.3.1 RNA extraction and quantification

RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol and the concentration determined by measuring the absorbance at 260nm using a NanoDrop 8000 UV-Vis Spectrophotometer (LabTech). RNA was stored at -80°C.

### 2.3.2 DNase treatment and examination of RNA concentration, purity and integrity prior to DNA microarray analysis

RNA samples were DNase-treated prior to examination with the Affymetrix GeneChip® Human Transcriptome Array 2.0. 10μg of RNA was treated with 3U of DNase I in DNase reaction buffer containing 40U RNase OUT™ in a total volume of
34μl for 15 minutes at RT. The reaction was inhibited by the addition of 4μl of 25mM EDTA (all reagents from Life Technologies). The sample volume was adjusted to 100μl with RNase-free water and RNA was purified with an RNeasy Mini Kit (Qiagen) using the RNA Clean-up protocol according to the manufacturer’s protocol. A NanoDrop 8000 UV-Vis Spectrophotometer was used to establish the concentration and purity of RNA samples by measuring the absorbance at 260nm and the 260/280nm absorbance ratio respectively. The integrity of the RNA was examined on a 2200 TapeStation (Agilent) using the RNA ScreenTape System (Agilent) according to the manufacturer’s instructions, and RIN values recorded. An example RNA profile of one sample is shown in Fig. 2.1 RNA was stored at -80°C.

2.3.3 Wet lab microarray procedure - conducted by Hologic/Tepnel

Total RNA was amplified using the Affymetrix GeneChip® WT PLUS Reagent Kit according to manufacturer’s instructions. The resulting cDNA was quantified using optical density (OD) (NanoDrop). The cDNA was normalised and hybridised onto Affymetrix Human Transcriptome 2.0 microarrays for 16 hours at 45°C. Microarrays were washed and stained using the Affymetrix GeneChip® Hybridization, Wash, and Stain Kit according to manufacturer’s instructions using the Affymetrix GeneChip® Fluidics Station 450. Microarrays were scanned using an Affymetrix GeneChip® 7G microarray scanner. Data quality was analysed using Affymetrix® Expression Console™ Software.

2.3.4 Microarray data analysis

Quality control of individual sample hybridisations and normalisation of data was conducted with Affymetrix Expression Console. To adjust for the variability in probe cell intensity data between microarray samples, Signal Space Transformation - Robust Multichip Average (SST-RMA) normalisation was conducted with CEL files generating CHP files. SST removes significant fold change compression and RMA minimises probe variance (Irizarry et al., 2003). Affymetrix Transcriptome Analysis Console was used to determine which genes had undergone a significant change in gene expression between experimental conditions. The criteria for a significant change in intensity were a) a linear fold change greater than or equal to 2 and b) an unpaired one-way Analysis of Variance (ANOVA) p-value less than 0.05. MetaCore™ was used to examine which pathways were significantly altered
between experimental conditions. Partek Genomic Suite® was used to conduct hierarchical clustering with Euclidean distance and complete linkage.

![Figure 2.1 Profile of a RNA sample from RT4 R1 No PD generated on the 2200 TapeStation.](image)

The 18S and 28S ribosomal subunits are quantified from the electropherogram and the software uses the ratio of these subunits to produce a RNA Integrity Number (RIN). RINs range from 1 to 10 with a value of 1 indicating total RNA degradation and a value of 10 indicating no degradation (Schroeder et al., 2006). All RNA samples used in the microarray analysis had RINs greater than or equal to 8.7.

### 2.3.5 cDNA synthesis

RNA was reverse transcribed into first strand cDNA using SuperScript II Reverse Transcriptase (Life Technologies) according to the manufacturer's protocol. 1μg of RNA, 250ng random primers and 1μl dNTP mix (10mM each) were added to a 0.5ml micro-centrifuge tube and the volume was made up to 12μl with molecular biology grade water (Sigma Aldrich). Samples were heated at 65°C for 5min and quick chilled on ice. 4μl 5X First Strand Buffer [250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂], 2μl 0.1M Dithiothreitol (DTT) and 1μl RNaseOUT™ was added to each tube. Samples were incubated at room temperature for 2min then 200 units of SuperScript II reverse transcriptase were added to each tube. Samples were incubated at 42°C for 50min and the reactions were stopped by heating at 70°C for 15min. Finally, 80μl of molecular biology grade water was added. cDNA was stored as 25μl aliquots at -80°C.
2.3.6 PCR of cDNA

PCR was performed using first strand cDNAs to examine the expression of total FGFR2, FGFR2 IIIb, FGFR2 IIIc and HPRT1. Each reaction contained 1x AmpliTaq Gold® 360 buffer, 1.5mM MgCl₂, 0.2μM of forward and reverse primers (see Table 2.2 for primer sequences), 200μM dNTPs, 1U AmpliTaq Gold® 360 DNA Polymerase and 5μl cDNA in a total reaction volume of 25μl. Reactions were performed in non-skirted 96 well PCR plates (Thermo Scientific) in a Veriti thermal cycler (Applied Biosystems). Cycling conditions were 95°C for 5 min, 35 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 60 s followed by a final extension step of 72°C for 5 min.

2.3.7 Agarose gel electrophoresis of PCR products

5μl of DNA loading dye (Appendix A) was added to PCR products. 500ng of 100bp DNA ladder (New England Biolabs, N3231S) and 10μl of PCR products were loaded onto 2% agarose (w/v) gels cast using 1x TBE (Severn Biotech)(Appendix A) and containing 0.6μg/ml ethidium bromide (Severn Biotech). Gels were electrophoresed in 1x TBE buffer at 90 V for 1 h and imaged using a Bio-Rad ChemiDoc MP.

Table 2.2 Primers used in FGFR2 standard PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FGFR2 exons 7-11 (all isoforms)</strong></td>
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</tr>
<tr>
<td>Forward</td>
<td>GTGGTCGGAGGAGACGTAGA</td>
</tr>
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<td>Reverse</td>
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<tr>
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<tr>
<td>Reverse</td>
<td>CTGAAGTACTCATTATAGTCAAGG</td>
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</table>
2.3.8 Quantitative real time PCR (qRT-PCR)

Quantitative real-time PCR was performed using first strand cDNAs and TaqMan or SYBR Green assays. For TaqMan assays, pre-made and optimised gene expression assays (Applied Biosystems) were used (Table 2.3). Each reaction contained 10μl 2x TaqMan Gene Expression Master Mix (Applied Biosystems), 1μl TaqMan gene expression assay, 7μl dH₂O and 2μl cDNA. Previously optimised SYBR Green assays were used for analysis of KDM6A mRNA levels. Each reaction contained 12.5μl 2x SYBR Green mix, 1.5μl of each forward and reverse primer (10μM stock) (see Table 2.4 for primer sequences), 9μl dH₂O and 2μl cDNA. Reactions were performed in triplicate in 96-well optical plates (Applied Biosystems) in a 7500 Real-Time PCR System (Applied Biosystems). Cycling conditions consisted of an initial hold at 50°C for 2 min, followed by 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Melt curve analysis was performed for SYBR Green assays after the PCR amplification step. Expression levels were normalised to the expression of the housekeeping gene succinate dehydrogenase complex flavoprotein subunit A (SDHA) (Ohl et al., 2006) and measured relative to the parental cell lines value.

Table 2.3 TaqMan assays used in qRT-PCR.

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<th>Assay ID</th>
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</thead>
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<td>UPK2</td>
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</tr>
</tbody>
</table>

Table 2.4 Primers used in *KDM6A* SYBR Green qRT-PCR

### 2.4 DNA sequencing

#### 2.4.1 DNA extraction

DNA used for exome sequencing and copy number analysis was purified from cells using a Gentra Puregene Kit (Qiagen) following the cultured cells protocol according to the manufacturer's instructions. DNA used for Sanger sequencing and SNaPshot assay was purified using a QIAamp DNA Mini kit (Qiagen) following the blood or body fluids spin protocol. For each protocol, DNA was purified from one 80-100% confluent 75cm² culture flask

#### 2.4.2 DNA quantification

DNA used for exome sequencing and copy number analysis was quantified using the PicoGreen dsDNA quantitation assay (Thermo Scientific) as follows: Lambda DNA standards were prepared to final concentrations of 1000, 100, 10, 1 and 0 ng/ml and samples were diluted 1 in 500 in 1 x TE buffer. 100μl of these standards and diluted samples were pipetted into a black 96 well optical plate (BMG Labtech Ltd). Then 100μl of PicoGreen reagent (diluted 200 fold in 1x TE) was added to each well. Standards and samples were mixed by pipetting and incubated at RT for 2 minutes before being quantified by measurement of fluorescence (excitation
480nm, emission 520nm) with a BMG Fluostar Galaxy plate reader. DNA used for Sanger sequencing and SNaPshot assay was quantified and tested for purity with a NanoDrop 8000 UV-Vis spectrophotometer by measuring the absorbance at 260nm and the calculation of a 260/280nm absorbance ratio respectively.

2.4.3 Exome sequencing

Exome sequencing was conducted with RT112 R1, RT112 R3 and parental RT112 samples. 3μg of DNA was made up to a final volume of 250μl with TE buffer (pH 8 0.1mM EDTA). DNA was sonicated with a Covaris S2 sonicator with the following settings:

38 cycles of:

1. 500 cycles per burst, intensity 8, duty cycle 15%.
2. 1000 cycles per burst, intensity 9.9, duty cycle 19.9%.

Libraries were generated and enriched for exomic regions using the SureSelect® Human All Exon V6 capture library (Agilent) according to the manufacturer’s protocols. An overview of the production of libraries for NGS is shown in Fig. 2.2. The concentration and size of DNA fragments was assessed using a 2200 TapeStation (Agilent) with High Sensitivity D1000 ScreenTapes (Agilent) according to the manufacturer’s instructions. The points at which the 2200 TapeStation (Agilent) was used to determine the concentration and size of DNA fragments are detailed in Fig 2.2. RT112 parental, RT112 R1, RT112 R3 and 2 samples not relevant to this project were indexed, pooled and run in a single lane on an Illumina HiSeq 3000 in paired-end mode. DNA sequencing was performed by the Leeds Institute of Molecular Medicine Next Generation Sequencing Facility.

2.4.4 Exome sequencing data analysis

Procedure conducted by Dr Olivia Alder: Base calling and quality control was performed using Illumina’s Real Time Analysis software with standard settings. Sequence files were QC checked using FastQC (v0.10.0) before preprocessing. Adapter contamination and low-quality read ends (< 20) were trimmed using cutadapt 1.3 and fastq-tools 0.8. Any read in which either pair had a length less than 19 was removed from subsequent analysis. Read Mapping and Genotype Calling Alignment was performed using BWA 0.7.10 mem GRch38 reference. Duplicate reads were removed using the Picard v1.56 MarkDuplicates program. Local realignment around indels was performed using the GATK v1.3
RealignerTargetCreator and IndelRealigner in Smith-Waterman mode with reference to dbSNP v132. The Picard v1.56 FixMateInformation program was used to ensure that all mate-pair information was in sync between each read and its mate following local realignment. Base quality scores were recalibrated using GATK v1.3 CountCovariates and TableRecalibration with reference to dbSNP v132. BAM files were sorted and then indexed using SAMtools index. Somatic Variant Analysis Pileup files (created using SAMtools mpileup, with parameters – d 5000 and q -20) were used as input to VarScan2 v2.3.5. This was used in somatic mode, with a strand filter, to identify somatic single nucleotide variations (SNVs) and small insertions and deletions (indels). Results were then processed using processSomatic to extract the somatic mutations with the specification that zero reads were to support the variant in the normal sample. SNVs and indels were identified in RT112 R1 and RT112 R3 with parental RT112 as the normal sample. To be called, a mutation needed to satisfy the following two criteria:

1) a variant allele frequency of greater than 20%
2) an absence of the mutation in RT112 parental.

High confidence somatic variant and indel calls were converted to mutation annotation format (MAF) using Variant Effect Predictor (VEP) version 81 via the Ensembl Virtual Machine.

2.4.5 Copy number analysis

Copy number alterations were examined by low pass genomic sequencing. Emma Black constructed NGS libraries with NEBNext® reagents according to the manufacturer’s instructions. Data was processed by Fiona Platt. BAM files were generated as described in the exome sequencing section (2.5.4). Following the generation of BAM files, ngCGH was used to compare number of read counts between the cell line and blood or parental samples using a window size of 1000. The Nexus Copy Number software package was used to conduct GC correction and copy number calling using the FASST2 Segmentation Algorithm which is a Hidden Markov Model (HMM) based approach. Segmentation was conducted with a significance threshold of 1.0E-5, with a requirement of at least 3 probes per segment and a maximum probe spacing of 1000 between adjacent probes before breaking a segment.

The log₂ ratio thresholds were set as follows: single copy gain; 0.25, single copy loss; -0.25, two or more copy gain; 1.25 and homozygous loss; -1.25.
Figure 2.2 Overview of production of next-generation sequencing libraries. Adapted from SureSelectXT Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library protocol.
2.4.6 PCR and Sanger sequencing of HRAS exon 1

Standard PCR was performed with each reaction containing 1x AmpliTaq Gold® 360 buffer, 2.5mM MgCl₂, 0.2μM of forward and reverse primers (see Table 2.5 for primer sequences), 1mM dNTPs, 1U AmpliTaq Gold® 360 DNA Polymerase and 20ng of DNA in a total reaction volume of 25μl. Reactions were performed in non-skirted 96 well PCR plates (Thermo Scientific) in a Veriti thermal cycler (Applied Biosystems). Cycling conditions were 95°C for 5 min, 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30s followed by a final extension step of 72°C for 10 min. Agarose gel electrophoresis was used to check that PCR was successful.

1μl ExoProStar (Illumina) was added to 2.5μl PCR products. This was mixed by pipetting, incubated at 37°C for 15 min then incubated at 80°C for 15 min. Sequencing was conducted with the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions (see Table 2.5 for primer sequences). Products were purified by adding 1μl of 3M sodium acetate (pH5.3) and 25μl 95% (v/v) ethanol to each sample, incubating at RT for 30 min and centrifugation at 2000 x g for 30 min at 4°C. The PCR plate was inverted on absorbent paper to remove the supernatant. 75μl 95% (v/v) ethanol was added to each sample and samples were spun at 2000 x g for 15 min at 4°C. The PCR plate was spun inverted on tissue paper at 730 x g for 1 min. Pellets were dried for 5 min at 95°C and resuspended in 15μl Hi-Di formamide (Applied Biosystems). Samples were heated at 95°C for 2 min and then kept on ice. Samples were then run on a ABI 3130xl Genetic Analyzer (Applied Biosystems).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
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</tr>
<tr>
<td>Reverse</td>
<td>TCGTCCACAAAATGGTTCTG</td>
</tr>
</tbody>
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2.4.7 SNaPshot analysis of HRAS c.DNA 34

Standard PCR was performed following the same methodology as for Sanger sequencing. 3μl of PCR product was run on a 2% agarose gel to check the PCR was successful. 3μl of shrimp alkaline phosphatase (Sigma-Aldrich) and 2μl Exonuclease I (Affymetrix) were added to the remaining 12 μl PCR product. This was then spun down for 10 s at 190 x g, incubated for 60 min at 37°C and 15 min at
72°C. Each SNaPshot reaction contained 1μl shrimp alkaline phosphatase (SAP)/exonuclease-treated PCR product, 2.5μl SNaPshot Multiplex Ready Reaction Mix, 1 x BigDye Terminator sequencing buffer, 5μM HRAS c.DNA 34 probe, and molecular biology grade water to a final volume of 9μl (all reagents Applied Biosystems). The sequence (5’->3’) of the HRAS c.DNA 34 probe was T₁₁₇CTGGTGTTGGTGCGGCC. SNaPshot analysis was conducted with the following cycling conditions: 35 cycles of 95°C for 10 s, 58.5°C for 40 s, hold at 4°C. 1μl shrimp alkaline phosphatase was added to the labelled SNaPshot extension products which were then incubated for 60 min at 37°C followed by 15 min at 72°C and spun down for 10 s at 190 x g. SNaPshot products and GeneScan™ 120 LIZ™ dye size standard (Applied Biosystems) were diluted 1 in 10. 1μl diluted SNaPshot product, 0.2 μl 120 LIZ™ dye size standard and 9.8μl Hi-Di formamide were mixed, spun down for 10 s at 190 x g, denatured for 5 min at 100°C, cooled on ice and run on a ABI 3130xl Genetic Analyzer.

**Figure 2.3 Overview of the SNaPshot analysis of HRAS c.DNA 34.** Abbreviations: SAP; shrimp alkaline phosphatase, ddNTP; dideoxynucleoside triphosphate. Adapted from the SNaPshot® Multiplex System for SNP genotyping product bulletin.
Chapter 3
Characterisation of Parental and PD173074 Resistant cells

3.1 Introduction

The FGFR TKI PD173074 (PD) is a potent selective inhibitor of the FGFR family of receptor tyrosine kinases. Mohammadi et al. reported that in an in vitro kinase inhibition assay FGFR1 was inhibited with an IC50 of 21.5nM. FGFR1 autophosphorylation was inhibited with a half maximal inhibitory concentration (IC50) of 1-5nM in the fibroblast cell line NIH3T3 which endogenously expresses FGFR1 (Mohammadi et al., 1998). Pardo et al. demonstrated that 100nM of PD inhibited autophosphorylation of FGFR2 in NCI-H510A, a small cell lung cancer cell line (Pardo et al., 2009). In another study PD inhibited FGFR3 autophosphorylation in NIH3T3 transfected with FGFR3 with an IC50 of approximately 5nM (Trudel et al., 2004). Mohammadi et al. demonstrated that, in an in vitro kinase inhibition assay, PD inhibited the cytoplasmic domain of PDGFR with an IC50 of 17.6µM and Src with an IC50 of 19.8µM. EGFR, insulin receptor (InsR), MEK and protein kinase C (PKC) were inhibited with IC50s greater than 50µM. VEGFR2 was inhibited with an IC50 of 100-200nm in the fibroblast cell line NIH3T3. PD binds the ATP-binding site of FGFRs via the formation of hydrogen bonds and van der Waal forces, competitively inhibiting ATP-binding (Mohammadi et al., 1998).

PD has been found to reduce cell viability in bladder cancer cell lines which express point mutated FGFR3 or FGFR3-TACC3 fusion proteins (Herrera-Abreu et al., 2013; Lamont et al., 2011; Miyake et al., 2010). Lamont et al. reported RT112 and RT4 to have IC50s of 15nM and 5nM in PD respectively. Herrera-Abreu et al. reported a half maximal effective concentration (EC50) of 27nM in RT112. FGFR TKIs including PD have been used previously to study resistance in FGFR-expressing cell lines including RT112 and RT4. The findings of these previous studies are summarised in section 1.4.2 of the Introduction. This chapter will describe how isogenic resistant lines were derived from parental RT112 and RT4 and compare the morphology, cell proliferation and sensitivity to PD of parental and resistant lines. Expression of FGFR3 and, due to a morphological change in some resistant lines, markers of epithelial-mesenchymal transition (EMT) were examined.
3.2 Results

3.2.1 Derivation of resistant lines

RT112 and RT4 resistant derivatives were derived by long-term culture in PD by Helen McPherson. Characterisation of resistant derivatives was not conducted prior to this project. 1µM was selected as the initial concentration of PD to culture parental RT112 and RT4 in to generate resistant derivatives. This was because PD exerted maximal effect on the growth of RT112 and RT4 at these concentrations but some RT112 and RT4 cells could survive in these PD concentrations. RT112 was cultured in PD for a total of 21 passages in order to generate isogenic resistant derivatives (Fig. 3.1). Cells were passaged when 90% confluent throughout the generation of resistant derivatives. Parental RT112 was cultured in 1µM PD and split 1 in 2 between passages 40 and 43. This took 74 days. At passage 43 two sublines were created, one which was split 1 in 3 and one split 1 in 5 for the rest of the resistant line derivation. The subline split 1 in 3 was cultured until passage 45, 15 days later, when this subline was further split to produce two sublines. One continued to be cultured in 1µM PD and the other was cultured in 2µM PD. The subline cultured 1 in 3 in 1µM PD was cultured for a further 117 days when it was frozen down at passage 61 and termed R1. The subline cultured 1 in 3 in 2µM PD was cultured for a further 120 days from passage 45 when it was frozen down at passage 61 and called R2. The subline split 1 in 5 was cultured in 1µM PD for a further 135 days from passage 43. Stocks of this resistant derivative were frozen at passage 61. This resistant derivative was termed R3.

Initially during the derivation of RT112 R1, R2 and R3 the media contained many floating dead cells. The number of floating cells in all sublines had reduced by passage 52. RT112 parental cells grow as a tightly packed layer of small epithelial cells when cultured without PD. Treatment with PD induced RT112 to have a spindle-shaped mesenchymal morphology with a loss of intercellular adhesion. (Fig. 3.2). The cells gradually began to proliferate at a faster rate, based upon the time taken to reach confluence between passages.

RT4 R1 was produced by long-term culture of RT4 in 1µM PD for 211 days from passage 12 to passage 28, splitting at 90% confluence (Fig. 3.1). At passage 28 the resistant derivative was frozen down and termed RT4 R1. With the initial PD treatment, as with RT112, there were many dead floating cells. The number of floating cells had reduced by passage 23. During the treatment with PD the RT4 cells became flatter and had many peripheral cytoplasmic extensions in contrast to
the morphology of RT4 when not cultured in PD (Fig. 3.3). Parental RT4 cells, cultured without PD, grow in tightly packed clusters. During the derivation of the RT4 resistant line, the RT4 cells continued to grow in clusters but did not grow on top of each other (Fig. 3.3). When initially treated with PD the RT4 cells stopped proliferating or proliferated very slowly. With continued PD treatment, the RT4 cells began proliferating at a faster rate. However, the proliferation rate continued to be slower than the rate observed in parental RT4. With both RT112 and RT4 there were no obvious pre-existing resistant cells or a specific time when resistance appeared to develop. The development of resistance was gradual. The morphology and proliferation rate of the final resistant derivatives is examined in sections 3.2.2 and 3.2.3. RT112 and RT4 resistant lines were maintained in their respective concentrations of PD at all times unless otherwise stated.

Figure 3.1 Derivation of resistant derivatives from parental RT112 and RT4. Passage number is denoted with a ‘p’ prior to the passage number.
3.2.2 Morphology of resistant lines cultured with and without PD173074

RT112 parental cells have an epithelial morphology when cultured with no PD. This is in contrast to the mesenchymal morphology observed during the derivation of resistant lines by long-term culture of RT112 parental in PD (Fig. 3.2). RT112 R1 and R2 cultured in PD exhibited a mesenchymal morphology whilst R3 had a more epithelial morphology (Fig. 3.4). Wang et al. reported that RT112 cells cultured long-term in the FGFR TKIs BJG398 and ponatinib to produce RT112 resistant derivatives exhibited a mesenchymal morphology. It was found that removal of the
FGFR TKIs resulted in the derivatives regaining an epithelial morphology within 2-4 weeks (Wang et al., 2014). In order to determine if resistance to PD and the morphological change observed in R1 and R2 were dependent on continual culture in PD, RT112 resistant derivatives were cultured without PD. Following culture for 2 passages without PD, approximately 2 weeks, the cells reverted to an epithelial morphology (Fig. 3.5).

RT4 parental cells, RT4 R1 cells cultured in PD and R1 cells cultured without PD for 4 passages are pictured in Fig. 3.6. RT4 has an epithelial morphology and RT4 grow as a tall palisade of tightly clustered cells rather than spreading out across the culture surface. RT4 R1 also grows in clusters but the cells have many cytoplasmic extensions. These cytoplasmic extensions are not observed in RT4 parental. RT4 R1 regained a morphology similar to RT4 parental after being cultured without PD for 2 passages.

![Figure 3.4 Morphology of RT112 resistant derivatives.](image)

Images were taken with phase contrast microscopy. The white bar indicates 100µm.
3.2.3 Proliferation of parental and resistant lines

To determine if the cell density at confluence and growth rate of the RT112 resistant lines differed from parental RT112, proliferation assays were conducted with RT112 parental, R1, R2 and R3 and growth curves constructed (Fig. 3.7). RT112 parental was cultured in medium not supplemented with PD. R1 and R3 were cultured in 1μM PD and R2 was cultured in 2μM PD. Parental RT112 reached a final density of 6.65 x 10^6 cells/well whereas R1 and R2 reached a final density of 8.38 x 10^5 cells/well and 1.06 x 10^6 cells/well respectively. R3 reached a final density of 1.64 x 10^6 cells/well. It seemed likely that PD was limiting the proliferation of RT112 resistant derivatives, rather than the resistant cells having an intrinsically slower growth rate than RT112 parental. To test this, RT112 resistant derivatives were cultured out of PD for 4 passages, 3-5 weeks, and proliferation assays were conducted with these cells without PD. Population doubling times were calculated for RT112 parental and resistant derivatives and are summarised in Table 3.1. The doubling time was shortest for RT112 parental not cultured with PD at 25.6 h. R2 and R3 cultured in PD both had doubling times of 38.2 h and R1 + PD had the longest doubling time of 51.1 h. The doubling times of R1 and R2 were reduced, 31.3 h and 34.5 h respectively, when cultured without PD (Table 3.1). The doubling time of parental + PD and R3 no PD was not examined. These results indicate that PD treatment hampered proliferation and cell density at confluence of the RT112 resistant lines despite these lines exhibiting PD resistance. The reduced rate of cell

Figure 3.5 Morphology of RT112 R1 and R2 cultured without PD for 4 passages. Images were taken with phase contrast microscopy. The white bar indicates 100μm.
proliferation in RT112 R1 and R2 was partially reversed when the cells were cultured in the absence of PD for 4 passages.

Proliferation assays were not conducted in RT4 parental and RT4 R1. However, from cell culture it was apparent that RT4 R1 cultured in PD proliferated at a slower rate than RT4 parental cultured without PD. It was also clear that, despite the long-term culture in PD during the production of RT4 R1, the proliferation rate of RT4 R1 increased upon culture without PD.

Figure 3.6 Morphology of RT4 parental cultured without PD, RT4 R1 cultured with PD and R1 cultured without PD for 4 passages. Images were taken with phase contrast microscopy.
Figure 3.7 Growth curves for RT112 parental and resistant derivatives. Cells were seeded in 6 well plates, with a growth area of approximately 9.5 cm² per well, at a density of $1 \times 10^4$ cells/well in 2ml of medium. RT112 parental was cultured in media not supplemented with PD. R1 and R3 were cultured in 1µM PD and R2 was cultured in 2µM PD. Error bars show standard error of the mean. GraphPad Prism software was unable to plot some error bars due to small standard error of the mean.

Table 3.1 RT112 parental and resistant derivative doubling times. Cells were seeded in 6 well plates, with a cell growth area of approximately 9.5 cm² per well, at a density of $1 \times 10^4$ cells/well in 2ml of medium. RT112 R1 and R2 were cultured without PD for 4 passages prior to seeding for doubling time experiments. Doubling times were calculated from cell counts at days 3 and 7 or 4 and 8 post seeding. ND stands for no data.

<table>
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<th>Doubling time (h) Cells cultured without PD173074</th>
<th>Doubling time (h) Cells cultured with PD173074</th>
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</thead>
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<tr>
<td>Parental</td>
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<td>ND</td>
</tr>
<tr>
<td>R1</td>
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<td>51.1</td>
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</tr>
<tr>
<td>R3</td>
<td>ND</td>
<td>38.2</td>
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</tbody>
</table>

3.2.4 Sensitivity to PD173074

To examine the sensitivity of RT112 parental and RT112 resistant lines to PD, cell viability assays were conducted (Fig. 3.8). RT112 parental had 33% viability, R1 had 53% viability, R2 had 67% viability and R3 had 90% viability in 1µM PD relative to the vehicle control. Therefore, sensitivity to PD increased in the order R3 < R2 < R1 < parental. This confirmed that the derivatives had gained resistance to PD, with
R3 being most resistant and showing no sensitivity up to 1µM PD. Viability was approximately 30% in parental cells treated with between 0.1 and 1µM PD indicating that some parental cells survived this PD treatment. Parental RT112 had an IC50 of 36nM PD estimated from sigmoidal dose response curve plotted in GraphPad Prism®.

![Graph showing cell viability of parental RT112 and resistant derivatives in PD173074.](image)

**Figure 3.8 Cell viability of parental RT112 and resistant derivatives in PD173074.** Viability of cells was assayed following 120 h treatment with PD173074 and normalised to the vehicle control. Error bars show standard error of the mean. This assay was repeated 3 times and a representative example is shown. Sigmoidal dose response curve was plotted in GraphPad Prism®. GraphPad Prism® software was unable to plot some error bars due to small standard error of the mean.

We had observed that RT112 R1 and R2 cells regained an epithelial morphology similar to parental RT112 following culture without PD for 2 passages (approximately 2 weeks). To determine if sensitivity to PD was retained following culture without PD and a return to an epithelial morphology, resistant derivatives were cultured without PD for 4 passages, 3-5 weeks, and viability in 1µM PD was examined (Fig. 3.9). Despite R1 and R2 reverting to an epithelial morphology the cells remained resistant to PD compared to RT112 parental. However, the difference in sensitivity to PD between RT112 R1 cultured without PD and the parental line was not striking. R3 also retained PD resistance compared to RT112 parental. In this aspect RT112 R1, R2 and R3 differed from the mesenchymal-like BGJ398 and ponatinib resistant RT112 derivatives described by Wang et al. Upon culture without PD for 2-4 weeks and morphological reversion, these RT112
resistant cells were almost as sensitive to FGFR inhibition as parental RT112 (Wang et al., 2014).

Figure 3.9 Cell viability of parental RT112 and resistant derivatives in PD173074 following a drug holiday. Resistant derivatives were cultured without PD173074 for 4 passages (3-5 weeks). Viability of cells was assayed following 120 h treatment with PD173074 and normalised to the vehicle control. Error bars show standard error of the mean. This assay was repeated twice and a representative example is shown.

To determine if RT4 R1 was less sensitive to PD than RT4 parental and if RT4 R1 retained its resistance following culture without PD, viability assays were conducted. Sensitivity to PD was assessed in RT4 parental, R1 which had been cultured with PD and R1 cultured without PD for 4 passages (3-5 weeks) (Fig. 3.10). RT4 parental was sensitive to PD with an IC50 of 19nM. RT4 R1 + PD had some resistance to PD and did not reach IC50. R1 no PD was sensitive to PD with an IC50 of 17nM. IC50s were estimated from sigmoidal dose response curve plotted in GraphPad Prism®. RT4 parental had 11% viability, R1 cultured in PD had 55% viability and R1 cultured out of PD for 4 passages had 22% viability in 1μM PD relative to the vehicle control. These results indicate that RT4 R1 had gained resistance to PD but that this resistance was not maintained following culture.
without PD. The sensitivity of RT4 R1 cultured without PD was in contrast to the RT112 PD resistant derivatives cultured without PD for the same number of passages, as these retained PD resistance.

![Figure 3.10 Cell viability of RT4 parental and R1 in PD173074.](image)

**Figure 3.10 Cell viability of RT4 parental and R1 in PD173074.** Viability of cells was assayed following 120 h treatment with PD173074 and normalised to the vehicle control. Error bars show standard error of the mean. This assay was repeated twice and a representative example is shown. Sigmoidal dose response curve was plotted in GraphPad Prism®.

### 3.2.5 Expression of FGFR3 and EMT markers

Due to the mesenchymal-like morphology observed in RT112 R1 and R2, expression of mesenchymal and epithelial markers was examined to determine if these cells had undergone an EMT. Cadherins are a class of membrane proteins which form part of the adherens junctions. Adherens junctions facilitate the attachment of neighbouring cells to one another (Singh et al., 2017). FGFR3 expression is of clear importance due to the dependency of RT112 and RT4 on FGFR3 signalling and the specificity of PD for the FGFR family. In addition to this, FGFR3 expression has been associated with an epithelial phenotype in bladder cancer cell lines (Cheng et al., 2009). Expression of FGFR3 and the mesenchymal marker N-cadherin were examined in RT112 parental and resistant derivatives (Fig. 3.11). Expression of FGFR3 was highest in parental cells cultured without PD and treated with PD for 24 h, lower in the resistant derivatives cultured without PD and lowest in the resistant derivatives cultured in PD. Herrera-Abreu et al. and Wang et
al. both reported that total FGFR3 expression is maintained in RT112 during 24 h treatment with an FGFR TKI (Herrera-Abreu et al., 2013; Wang et al., 2014). This is concordant with our results. N-cadherin was increased in RT112 R1 + PD and R2 + PD compared to the parental line cultured without PD. Like RT112 R1 and R2, the FGFR TKI resistant cells produced by Wang et al. had increased protein expression of N-cadherin.

![Image of immunoblot analysis](image-url)

**Figure 3.11** Immunoblot of FGFR3 and N-Cadherin protein expression in parental RT112 and resistant derivatives. A) Immunoblot analysis. B) Protein expression relative to parental – PD. Expression was examined in parental, parental treated with PD173074 for 24 h, R1, R2 and R3 cultured with PD173074 and R1, R2 and R3 cultured without PD173074 for 4 passages. β-actin was used as a loading control. Immunoblots were conducted three times and a representative example is shown. The immunoblot shown was used to normalise FGFR3 and N-cadherin relative to β-actin using Image Lab software.
Vimentin, a mesenchymal marker, provides mechanical strength to intermediate filaments. Intermediate filaments are a cytoskeleton component and vimentin makes intermediate filaments less likely be damaged during cell migration (Singh et al., 2017). As expression of N-cadherin was increased in RT112 R1 and R2 cultured in PD, expression of the epithelial marker E-cadherin and vimentin was also examined in these resistant derivatives (Fig. 3.12). E-cadherin expression was not reduced in RT112 R1 and R2. Vimentin expression was increased in RT112 R1 and R2, though only by a small magnitude. RT112 R1 and R2 are therefore distinct from the Wang et al. resistant derivatives which had reduced expression of E-cadherin protein (Wang et al., 2014). The results so far suggest that RT112 R1 and R2 have undergone a partial EMT. RT112 R3 appears to have not undergone an EMT.

Figure 3.12 Immunoblot of E-Cadherin and vimentin protein expression in RT112 parental, R1 and R2. A) Immunoblot analysis. B) Relative protein expression. T24 was included as a negative control for E-cadherin and JMSU1 was included as a positive control for vimentin. β-actin was used as a loading control. Immunoblots were conducted three times and a representative example is shown. The expression of E-cadherin and vimentin relative to β-actin was calculated using Image Lab software. E-cadherin expression was quantified relative to RT112 parental and Vimentin was quantified relative to JMSU1.
To determine if EMT-like changes were present in RT4 R1 as observed in RT112 R1 and R2, expression of N-cadherin, vimentin, E-cadherin and FGFR3 were examined in RT4 parental and R1 (Fig. 3.13). The increase in expression of N-cadherin and decrease in expression of FGFR3 observed in RT112 R1 and R2 was not observed in RT4 R1. Vimentin expression was low in RT4 parental and RT4 R1. E-cadherin expression was not reduced in RT4 R1 relative to RT4 parental. These results indicate that RT4 R1 did not undergo an EMT during its derivation.

**Figure 3.13 Immunoblot of N-cadherin, vimentin FGFR3 and E-Cadherin protein expression in RT4 parental and R1.** A) Immunoblot analysis. B) Protein expression relative to parental RT112. T24 was included as a positive control for N-cadherin and vimentin. RT112 parental and JMSU1 were included as positive and negative controls for FGFR3 and E-cadherin respectively. β-actin was used as a loading control. Immunoblots were conducted three times and a representative example is shown. The expression of FGFR3 and E-cadherin relative to β-actin was calculated using Image Lab software. FGFR3 and E-cadherin expression were quantified relative to RT112 parental.
3.3 Discussion

The sensitivity of RT112 to PD has previously been examined. Lamont et al. found RT112 to have an IC50 of 15nM and 10-20% viability in 1µM PD in a 120 h assay (Lamont et al., 2011). Herrera-Abreu et al. found RT112 to have an EC50 of 27nM and a cell viability of 36% in 1µM PD in a 72 h assay (Herrera-Abreu et al., 2013). In this study, it was found that the viability of RT112 in PD was not entirely abolished with viability approximately 1/3 of the vehicle control in parental cells treated with between 0.1 and 1µM PD. The viability of RT4 parental was not abolished with PD with approximately 12% viability relative to the vehicle control being maintained with treatment with between 0.1 and 1µM PD. This indicates that some RT112 and RT4 parental cells were able to survive PD treatment. Lamont et al. reported RT4 to have an IC50 of 5nM in a 120 h assay, whereas we found RT4 to have an IC50 of 19nM in an assay of the same time length (Lamont et al., 2011). The differences in the viability results between Herrera-Abreu et al. and Lamont et al. and our viability assays could be due to differences in methodology. Herrera-Abreu et al. treated with PD for 72 h and assayed viability with CellTiter-Glo whereas for our assay, PD treatment was for 120 h and viability was assessed with Cell-Titer Blue. Herrera-Abreu et al. calculated an EC50 whereas we calculated an IC50. Lamont et al. treated with PD for 120 h, similar to our assay but changed media 48 h post PD treatment whereas in our assay medium was replaced after 72 h. Lamont et al. assessed viability with a 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium (MTT) assay. The RT112 used by Herrera-Abreu et al. was obtained from the same laboratory in which this project was conducted. The Lamont et al. paper was produced in the same laboratory as this project. It is therefore unlikely that the observed differences in viability are due to differences in the RT112 and RT4 cells used. There was large variability between individual cell viability assays, for example, in figure 3.8 the cell viability of parental RT112 treated with 1µM PD was reduced to 33% whereas in figure 3.9 the viability of RT112 parental treated with 1µM PD was reduced to approximately 50%. Therefore, it is not possible to draw conclusions by comparing the results of different viability assays. It would have been beneficial to have included the resistant lines cultured in PD as a control when the resistant lines cultured out of PD were assayed for their PD resistance. This would have determined whether the RT112 resistant lines maintained the same level of PD resistance when cultured with and without PD rather than simply demonstrating that the resistant derivatives cultured out of PD were more resistant than parental RT112. Additionally, for each cell viability experiment it would have
been more reliable to have presented the average of the cell viability assay repeats, rather than presenting one representative example. This would have enabled a statistical test to have been conducted to determine whether observed differences in viability were significant.

RT112 R1, RT112 R2 and RT4 R1 cells had a different morphology from their parental lines. These resistant lines returned to the parental morphology upon culture without PD. RT112 R1 and R2 retained their resistance to PD following culture without PD whereas RT4 R1 did not. RT112 R3 was distinct from RT112 R1, RT112 R2 and RT4 R1 in that it retained the epithelial morphology of its parental line. Despite the mesenchymal-like morphology of RT112 R1 and R2 cultured in PD these cells did not appear to have undergone a complete EMT. Expression of E-cadherin was not decreased in these resistant derivatives. Expression of N-cadherin and vimentin was increased suggesting that RT112 R1 and R2 had undergone a partial EMT. No evidence was found to suggest that RT4 R1 had undergone an EMT. However, the analysis of epithelial and mesenchymal markers was limited to only a few proteins and therefore it is possible that RT4 R1 or RT112 R3 may have also undergone some expression changes associated with an EMT. In bladder cancer cell lines, expression of FGFR3 has been associated with an epithelial phenotype, in contrast to FGFR1 expression which has been associated with a mesenchymal phenotype (Cheng et al., 2013). Hanze et al. found that phenotypically mesenchymal bladder cancer cell lines were more resistant to the FGFR TKI dovitinib than epithelial bladder cancer cell lines (Hanze et al., 2013). This could be related to the activation of FGFR3 versus FGFR1 signalling in these lines, as there was some overlap between the panel of bladder cancer cell lines examined by Cheng et al. and Hanze et al. Therefore, a shift from an epithelial phenotype to a more mesenchymal phenotype as a result of FGFR inhibition is logical in the FGFR3-dependent lines used in this project. This switch to a more mesenchymal phenotype is of interest as a mesenchymal phenotype is associated with greater cell migration and invasion. Therefore, if FGFR inhibitors induce an EMT in patients this could lead to tumour metastasis (Singh et al., 2017).

Some studies examining the response of RT112 to FGFR inhibition, which have focused on the short-term mechanisms of survival, have not detailed EMT-like changes occurring in RT112 (Harbinski et al., 2012; Herrera-Abreu et al., 2013; Wang et al., 2017b). This would be expected as this study found that the EMT-like morphological changes in RT112 became apparent only after long-term culture in PD rather than acute exposure. Wang et al. reported a mesenchymal morphology, increased cell migration, increased protein expression of the mesenchymal markers
fibronectin and N-cadherin and reduced expression of E-cadherin with long-term culture of RT112 in the FGFR TKIs BGJ398 and ponatinib. They derived resistant cells via gradual increase in exposure to the TKI and treatment with an initial high dose of TKI. These changes were reversed upon culturing the cells out of drug for 2-4 weeks. In contrast to our RT112 resistant derivatives which maintained their resistance following culture out of PD for 4 passages, the Wang et al. resistant cells did not maintain their FGFR TKI resistance following culture without drug for 2-4 weeks. Wang et al. do not detail the time taken to derive their resistant cells (Wang et al., 2014). Our RT4 resistant derivative, RT4 R1, did re-acquire PD sensitivity when cultured out of PD for 4 passages. If resistance to FGFR inhibition could be reversed in patients simply by pausing treatment with the FGFR TKI, then this would be an ideal way to overcome the resistance as it avoids the use of potentially toxic drug combinations. Our results suggest that pausing treatment would not induce the cancer cells to revert to sensitivity to an FGFR TKI in all cases. However, our RT112 resistant derivatives may have re-acquired sensitivity to PD if cultured out of PD for a greater length of time.

The rapid reversibility of the phenotypes of RT112 R1, RT112 R2 and RT4 R1 indicates that the phenotypic changes are induced by alterations in transcription rather than via stable genomic changes. The altered transcription is likely to be mediated by epigenetic modifications. Sharma et al., reported that a treatment of the NSCLC cell line PC-9 with the EGFR TKIs gefitinib or erlotinib for 9 days yielded a population of drug tolerant cells with increased protein expression of the histone demethylase KDM5A. KDM5A demethylates lysine 4 of histone 3 (H3K4me2 and H3K4me3) thereby repressing transcription (Santos-Rosa et al., 2002). KDM5A promotes cell proliferation and inhibits cell differentiation, this activity is modulated by the tumour suppressor RB1 (Benevolenskaya et al., 2005; Lin et al., 2011). Knockdown of KDM5A with RNA interference significantly reduced the number of drug tolerant PC-9 cells generated by culture in an EGFR TKI (Sharma et al., 2010). Hypermethylation of PDLIM4 which encodes PDZ and LIM domain protein 4 has been associated with shortened survival in CML patients treated with imatinib. Gene hypermethylation normally reduces gene transcription, therefore this epigenetic alteration may induce imatinib resistance by reducing PDLIM4 inactivation of the tyrosine kinase Src (Jelinek et al., 2011). Roh et al. reported that, in the urothelial carcinoma cell lines KU-7 and 5637, overexpression and knockdown of FOXM1 significantly increased and decreased cell viability in the presence of the chemotherapeutic agent doxorubicin respectively. FOXM1 is a global regulator of DNA methylation. FOXM1 was shown to regulate expression of
ABCG2 and drug efflux. ABCG2 is a member of the ATP-binding cassette (ABC) transporter family which induce drug resistance via drug efflux (Roh et al., 2018; Teh et al., 2012). As epigenetic alterations are heritable, it is possible that an epigenetic alteration maintains expression changes in RT112 R1 and R2 following the culture of these cells without PD for 4 passages. An epigenetic alteration could therefore enable the persistence of the PD resistant in RT112 R1 and R2.

The reduction in FGFR3 expression in the RT112 PD resistant derivatives suggests that signalling via FGFR3 in these cells is reduced and indicates that FGFR3 overexpression or mutation or drug efflux is not the cause of resistance in these cells. Resistance could be mediated by activation of an alternative RTK, as has been described previously in response to FGFR TKIs in RT112. Harbinski et al. showed that inducing expression of HGF, NRG1, NRG2 or TGF-α in RT112 could mediate short-term survival to exposure to PD in RT112 (Harbinski et al., 2012). Herrera-Abreu et al. identified EGFR signalling as mediating short-term survival to PD in RT112 (Herrera-Abreu et al., 2013). Wang et al. produced RT112 resistant derivatives via long-term culture in FGFR inhibitors which owed their resistant phenotype to the activation of ERBB2 and ERBB3 (Wang et al., 2014). As RT112 R3 is able to maintain a phenotype more similar to its parental line than RT112 R1, RT112 R2 or RT4 R1, this resistant derivative may have developed resistance via a mutation. A V555M mutation in the FGFR3 kinase domain was reported in KMS-11, a myeloma cell line, following long-term treatment with a FGFR TKI, AZ12908010. This mutation prevents the binding of TKIs to the ATP-binding site (Chell et al., 2013). However, as FGFR3 expression was downregulated in R3, it is unlikely that resistance has arisen in this line via a mutation in FGFR3. Alternative genetic mechanisms by which resistance may have arisen include other mutations in FGFR3 which induce activity by a different mechanism, an activating mutation in a different RTK, or mutation in a signalling molecule downstream of FGFR3. Chapter 5 will examine if there is a genetic mechanism of resistance in the RT112 and RT4 resistant derivatives.
Chapter 4
Receptor Tyrosine Kinase Signalling

4.1 Introduction

FGFR3 expression is associated with an epithelial morphology and FGFR1 expression is associated with a mesenchymal morphology in bladder cancer cell lines (Cheng et al., 2013; Tomlinson et al., 2012). It was therefore thought that RT112 R1 and R2, which have a more mesenchymal morphology, could have increased activation of FGFR1, possibly by altered FGF expression. FGFR1, FGF1 and FGF2 expression was examined in this Chapter for this reason. However, any mechanism which induced FGFR1 activation in the resistant lines would have to overcome the inhibition of FGFR1 by PD, which inhibits FGFR1-3 (Mohammadi et al., 1998; Pardo et al., 2009; Trudel et al., 2004). Activation of an alternative RTK is a commonly reported mechanism of resistance to targeted agents. Such activation of a replacement RTK would re-activate intracellular signalling pathways inhibited by FGFR3 inhibition. Wang et al. found RT112 activated ERBB2 and ERBB3 signalling as a mechanism of resistance to the FGFR TKIs BGJ398 and ponatinib upon long-term culture in these inhibitors (Wang et al., 2014). Herrera-Abreu et al. identified EGFR activation as a mechanism of short-term survival in RT112 treated with PD (Herrera-Abreu et al., 2013). Harbinski et al. identified MET activation by HGF as a mechanism of short-term survival in response to BGJ398 (Harbinski et al., 2012). This previously conducted research is examined in section 1.4.2 of the Introduction. As these RTKs had been previously implicated in FGFR TKI resistance it was imperative to examine total expression and phosphorylation of EGFR family members and MET. Examination of RTK expression was conducted predominantly in RT112 R1, RT112 R2 and RT4 R1. This was because it was thought that RT112 R3 was the most likely resistant derivative to have a genetic mechanism of resistance because, compared to RT112 R1 and R2, it maintained a maximal cell density and morphology were more similar to RT112 parental. Exome sequencing will be conducted in Chapter 5 with the aim of determining whether RT112 R3 has a genetic mechanism of resistance. Expression and phosphorylation of AKT, a serine/threonine kinase which is part of the PI3 kinase pathway, and ERK, a serine/threonine kinase in the MAP kinase signalling cascade, will be examined in RT112 parental and resistant lines in this Chapter. The PI3 kinase and
MAP kinase pathways, amongst others, are activated by FGFR3 and are key determinants of cell proliferation and survival (di Martino et al., 2016). Intracellular pathways activated in resistant cells could potentially be targeted as a mechanism to overcome resistance. For example, the PI3 kinase inhibitor BKM120 and FGFR TKI AZD4547 have been found to be synergistic in reducing cell proliferation in RT112 and urothelial carcinoma cell line JMSU1, which has amplification of FGFR1 (Wang et al., 2017b).

4.2 Results

4.2.1 FGFR and FGF expression

FGFR1 mutations are not found in bladder cancer but FGFR1 DNA amplifications are reported albeit infrequently (Fischbach et al., 2015). Cheng et al. examined a panel of bladder cancer cell lines and found FGFR3 dependence to be associated with an epithelial phenotype and FGFR1 dependence to be associated with a mesenchymal phenotype (Cheng et al., 2013). Increased activation of FGFR1 could be a cause of resistance. However, PD is a pan-FGFR TKI, which inhibits FGFR1 with an IC50 in the nanomolar range (Mohammadi et al., 1998). Therefore, a significant increase in protein expression or an FGFR1 gatekeeper mutation would be required to activate FGFR1 signalling. Exome sequencing, which is described in Chapter 5, will uncover any genetic alterations involving FGFR1 or other FGFRs. FGFR1 and FGFR3 expression was examined by qRT-PCR in the epithelial RT112 parental line as well as R1 and R2, which had undergone phenotypic changes associated with a mesenchymal phenotype (Fig. 4.1). FGFR1 expression was significantly increased and FGFR3 expression was significantly reduced in R1 and R2. The reduction in FGFR3 mRNA expression is concordant with immunoblot analysis conducted in Chapter 3 which found reduced expression of FGFR3 protein in RT112 R1 and R2 (Fig. 3.11). Protein expression of FGFR1 was examined in RT112 parental, RT112 R1, RT112 R2, RT4 parental and RT4 R1 by immunoblot (Fig. 4.2). There was no increase in FGFR1 expression in the resistant derivatives compared to their parental lines. Expression of FGFR1 was low in all RT112 samples compared to the JMSU1 positive control. Therefore, the increased FGFR1 mRNA expression in RT112 R1 and R2 did not result in a detectable increase in FGFR1 protein expression. Phosphorylation of FGFR1 was not examined.
However, the low expression of this receptor suggests that it is not inducing resistance in the resistant lines or inducing the EMT-associated changes observed in RT112 R1 and R2.

The FGFR family undergoes alternative splicing, mutually exclusively including exons 8 and 9 to produce isoforms with different specificities for FGFs. If exon 8 is included the IIIb isoform is produced whereas if exon 9 is included the IIIc isoform is produced. FGF1 activates FGFR1-3 IIIb and IIIc isoforms. FGF2 has a greater specificity for the IIIc isoforms of FGFR1-3 which are associated with a mesenchymal phenotype, rather than the IIIb isoforms associated with an epithelial morphology (Chellaiah et al., 1994; Ornitz et al., 1996; Zhang et al., 2006). It was thought that differential expression of these FGFs could be inducing greater activation of IIIc rather than IIIb isoforms in RT112 R1 and R2. FGF1 and FGF2 expression was examined in RT112 parental, R1 and R2 by qRT-PCR (Fig. 4.3). Expression of FGF1 was not consistently higher or lower in R1 and R2 compared to parental in the 4 repeats of the qRT-PCR analysis. FGF2 levels were increased in R1 and R2 compared to parental by over 200-fold. However parental RT112 had a high cycle threshold and therefore the parental FGF2 mRNA level was low.

![FGFR1 and FGFR3 expression in R1 and R2](image)

**Figure 4.1 qRT-PCR of FGFR1 and FGFR3 in RT112 parental R1 and R2.** Expression was normalised to parental to produce relative quantification values (RQ). Error bars indicate RQmin and RQmax which indicate the 95% confidence interval calculated by the 7500 Real-Time PCR System software. qRT-PCR was repeated 3 times and one representative result is shown. Resistant derivatives were tested against parental for significantly different gene expression with the Mann Whitney U test, two-tailed, p<0.05. Asterisks indicate which genes were significantly differentially expressed between resistant derivatives and parental cells.
Figure 4.2 Immunoblot analysis of FGFR1 protein expression in RT112 and RT4. Expression was examined in RT112 parental, RT112 R1, RT112 R2, RT4 parental and RT4 R1. JMSU1 and T24 were included as positive and negative controls for FGFR1 respectively. β-actin was used as a loading control. Immunoblots were conducted three times and a representative example is shown.

As with other FGFRs, expression of FGFR2IIIb is associated with an epithelial phenotype whereas FGFR2IIIc expression is associated with a mesenchymal morphology (Ranieri et al., 2016). Expression of FGFR2 isoforms was examined in RT112 R1 and R2 to determine if isoform switching had occurred (Fig. 4.4). PCR of
exons FGFR2 7-11 produces a 623-626 bp product and detects both FGFR2 IIIb and IIIc. PCR of exons 7-8 produces a 278 bp product and detects FGFR2 IIIb expression. PCR of exons 7-9 produces 267 bp product and detects FGFR2 IIIc. The FGFR2 IIIb isoform but not the FGFR2 IIIc isoform was detected in all of the RT112 samples. Therefore, R1 and R2 had not switched expression of FGFR2 isoforms and this had not induced the mesenchymal change in RT112 R1 and R2. It is possible that isoform switching has occurred in other FGFRs, although expression of FGFR3 is decreased and FGFR1 expression is low in the RT112 resistant derivatives (Fig. 3.11 and Fig. 4.2).

4.2.2 EGFR and ERBB2 activation

The EGFR family of RTKs homodimerize or heterodimerize resulting in phosphorylation of the intracellular kinase domain and activation of the MAP kinase, PI3 kinase and PLCγ pathways. (Roskoski, 2014). EGFR amplification, ERBB2 amplification and mutations and ERBB3 mutations are found in MIBC (Robertson et al., 2017). Increased signalling via EGFR has been reported as a method of survival in RT112 following short term treatment with PD (Herrera-Abreu et al., 2013). For this reason, immunoblot analysis of total and phospho-EGFR was examined in RT112 parental, R1 and R2 (Fig. 4.5). Expression of phospho-EGFR and total EGFR was low in RT112 parental, R1 and R2. Phospho-EGFR expression
was not increased in R1 and R2 compared with parental cells indicating that activation of EGFR is not mediating resistance in these resistant lines.

![Immunoblot analysis of phospho-EGFR and total EGFR protein expression in RT112. A) Immunoblot analysis. B) Protein expression relative to 97-7. Expression was examined in parental, R1 and R2. 97-7 was included as a positive control for phospho-EGFR and total EGFR. UMUC3 and T24 were included as negative controls for phospho-EGFR and total EGFR. β-actin was used as a loading control. Immunoblots were conducted three times and a representative example is shown. The immunoblot shown was used to normalise phospho-EGFR relative to β-actin using Image Lab software.](image)

Expression of phospho-EGFR (Y1068) and total EGFR was also examined in RT4 parental and RT4 R1 (Fig. 4.6). Growth factor receptor-bound protein 2 (Grb2) binds to the phosphorylated Y1068 of EGFR. Therefore, autophosphorylation of this residue is required for EGFR-mediated activation of the MAP kinase pathway (Rojas et al., 1996). Expression of both phospho-EGFR and total EGFR was increased in RT4 R1 compared to parental R1. Therefore, signalling via EGFR is a possible mechanism of resistance in RT4 R1.

No ligand with specificity for ERBB2 has been identified, so it is suspected that ERBB2 only signals via heterodimerization with other EGFR family members.
The other EGFR family members preferentially dimerize with ERBB2 (Roskoski, 2014). The ERBB3 kinase domain is impaired in its ability to undergo autophosphorylation and so ERBB3 homodimers are unable to elicit activation of intracellular signalling pathways (Shi et al., 2010). Activation of ERBB2 and ERBB3 signalling has been reported as a mechanism of resistance in RT112 to BGJ398 and ponatinib (Wang et al., 2014). For this reason, protein expression of phospho-ERBB2, total ERBB2 and phospho-ERBB3 was examined in RT112 parental, R1 and R2. Examination of total ERBB3 was attempted but was unsuccessful. No increase in phospho-ERBB2 or total ERBB2 was observed in RT112 R1 or R2 (Fig. 4.7). Therefore, activation of ERBB2 does not appear to contribute to resistance in these cells.

Figure 4.6 Immunoblot analysis of phospho-EGFR and total EGFR protein expression in RT4. A) Immunoblot analysis. B) Protein expression relative to 647V. Expression was examined in parental and R1. 647V was included as a positive control for phospho-EGFR (Tyrosine 1068) and total EGFR. JMSU1 was included as a negative control for phospho-EGFR and total EGFR. β-actin was used as a loading control. Immunoblots were conducted three times and a representative example is shown. Protein expression from the immunoblots shown was normalised compared to the total protein per well using Image Lab software and quantified relative to the positive control.
Figure 4.7 Immunoblot analysis of phospho-ERBB2 and total ERBB2 protein expression in RT112. Expression was examined in parental, R1 and R2. SKBR3 and JMSU1 were included as positive and negative controls for phospho-ERBB2 and total ERBB2 respectively. β-actin was used as a loading control. Immunoblots were conducted three times and a representative example is shown.

4.2.3 ERBB3 activation

Phosphorylation of ERBB3 in RT112 parental, R1 and R2 was examined with immunoblotting (Fig. 4.8). An increase in ERBB3 phosphorylation was observed in RT112 R1 and R2 compared to parental. It is possible that this activation of ERBB3 signalling contributes to resistance in these derivatives. As immunoblotting for total ERBB3 was unsuccessful, it is not possible to tell if the increase in ERBB3 phosphorylation is accompanied by an increase in ERBB3.

Expression of phospho-ERBB2, total ERBB2 and phospho-ERBB3 was examined in RT4 parental and R1 (Fig. 4.9). Expression of phospho-ERBB2 and total ERBB2 was low in RT4 parental and R1 compared to the positive control SKBR3. Expression of phospho-ERBB3 was increased in R1, although it remained much lower than in the positive control, the breast cancer cell line SKBR3. The increase in phospho-ERBB3 could also contribute to PD resistance in this line.
Figure 4.8 Immunoblot analysis of phospho-ERBB3 protein expression in RT112. A) Immunoblot analysis. B) Protein expression relative to SKBR3. Expression was examined in parental, R1 and R2. SKBR3 and JMSU1 were included as positive and negative controls for phospho-ERBB3 respectively. β-actin was used as a loading control. Immunoblots were conducted three times and a representative example is shown. The immunoblot shown was used to normalise phospho-ERBB3 relative to β-actin using Image Lab software.
Figure 4.9 Immunoblot analysis of phospho-ERBB2, total ERBB2, phospho-ERBB3 and total ERBB3 protein expression in RT4. A) Immunoblot analysis. B) Protein expression relative to parental RT4. Expression was examined in parental and R1. SKBR3 and BFTC909 were included as positive and negative controls for phospho-ERBB2, total ERBB2, phospho-ERBB3 and total ERBB3. β-actin was used as a loading control. Immunoblots were conducted three times and a representative example is shown. The immunoblot shown was used to normalise phospho-ERBB3 relative to β-actin using Image Lab software.

Expression of the ERBB3 and ERBB4 ligand neuregulin 1 was reported to be upregulated in BGJ398 resistant derivatives. Treatment with neuregulin 1 was also found to rescue RT112 and RT4 from BGJ398-induced reduction in cell viability (Wang et al., 2014). It was thought that neuregulin 1 could be inducing phosphorylation of ERBB3 in RT112 R1, RT112 R2 and RT4 R1. Expression of NRG1, which encodes neuregulin 1, was examined in RT112 parental, R1 and R2 by qRT-PCR. Expression of NRG1 was increased by approximately 3 and 2-fold in RT112 R1 and R2 compared to parental respectively (Fig. 4.10). This increase in NRG1 expression, although of a low magnitude, could be the cause of the increased phosphorylation of ERBB3 in RT112 R1 and R2.
4.2.4 MET activation

Activation of MET with hepatocyte growth factor (HGF) has been reported to rescue RT112 treated with BGJ398 (Harbinski et al., 2012). Cheng et al., using immunohistochemistry, identified high MET expression in 7 out of 142 urothelial carcinoma patients (Cheng et al., 2002). MET, in addition to other EGFR family members, is able to heterodimerize with ERBB3 to induce intracellular signalling (Pérez-Ramírez et al., 2015; Tanizaki et al., 2011). Engelman et al. reported that MET amplification induced resistance to EGFR inhibition in the NSCLC cell line HCC827 cultured in gefitinib. MET activation mediated phosphorylation of ERBB3 which induced activation of the PI3 kinase pathway. This resistance could be overcome by combined EGFR and MET inhibition. Engelman et al. also reported MET amplification in NSCLC patient samples with acquired resistance to gefitinib (Engelman et al., 2007).

It was thought that activation of MET could be mediating the increase in ERBB3 phosphorylation observed in RT112 R1 and R2. Expression of phospho-MET and total MET was measured in RT112 parental cultured in PD for 24 h, parental cultured without PD and R1, R2 and R3 each cultured with PD or without PD for 4 passages. Expression of phospho-MET and total MET was not examined.
in RT4 parental and R1 due to time limitations and because it was thought that the increased phosphorylation of EGFR was the likely mediator of PD resistance in RT4 R1. An increase in phospho-MET was observed in R3 cultured with and without PD and a smaller increase was observed in R1 and R2 cultured with PD. However, phospho-MET expression remained low in R1 and R2 compared to the positive control 5637. (Fig. 4.11). It is possible that MET activation could be inducing survival in the RT112 resistant derivatives.

**Figure 4.11 Immunoblot analysis of phospho-MET and total MET protein expression in RT112.** A) Immunoblot analysis. B) Protein expression relative to 5637. Expression was examined in parental, parental treated with PD173074 for 24 h, R1, R2 and R3 cultured with PD173074 and R1, R2 and R3 cultured without PD173074 for 4 passages. 5637 was included as a positive control for phospho-MET and total MET and β-actin was used as a loading control. Immunoblots were conducted twice and a representative example is shown. The immunoblots shown were used to normalise phospho-MET and total MET relative to β-actin using Image Lab software.
4.2.5 AKT and ERK activation

The MAP kinase and PI3 kinase pathways regulate cell proliferation and survival and are activated downstream of FGFR3 (Hart et al., 2001). Therefore, the activation of these pathways could be the determinant of the differences in cell proliferation between parental and resistant derivatives observed in Chapter 3. To determine whether the PI3 kinase and MAP kinase pathways were activated, expression of phospho-AKT, total AKT, phospho-ERK and total ERK was examined in RT112 parental acutely treated with PD, parental cultured without PD, and resistant derivatives cultured with and without PD (Fig. 4.12). Expression of phospho-AKT was increased in R1 and R2 + PD and reduced in parental + PD. Expression of phospho-ERK was also decreased in parental + PD. Expression of total ERK remained fairly constant between parental and resistant derivatives. The reduction of phospho-ERK and phospho-AKT in RT112 parental acutely treated with PD is likely to contribute to the reduced proliferation in these cells. All resistant derivatives cultured in PD maintained ERK phosphorylation. These results suggest that finding an alternative mechanism of activating the MAP kinase and PI3 kinase pathways may be key to overcoming FGFR inhibition with PD. The increase in phosphorylation of AKT observed in R1 and R2 cultured with PD but not R3 cultured with PD highlights a difference in the resistance mechanism between these lines. The mechanism of resistance employed in R1 and R2 appears to strongly activate the AKT pathway; whereas the resistance mechanism in R3 activates the PI3 kinase pathway only to a similar level to parental RT112 not treated with PD.
Figure 4.12 Immunoblot analysis of phospho-AKT, AKT, phospho-ERK and ERK protein expression in RT112. A) Immunoblot analysis. B) Protein expression relative to parental - PD. Expression was examined in parental, parental treated with PD73074 for 24 h, R1, R2 and R3 cultured with PD173074 and R1, R2 and R3 cultured without PD173074 for 4 passages. β-actin was used as a loading control. Immunoblots were conducted twice and a representative example is shown. Phospho-AKT expression from the immunoblot shown was normalised compared to the total protein per well using Image Lab software and quantified relative to parental no PD. The immunoblot shown was used to normalise phospho-ERK relative to β-actin using Image Lab software. Phospho-ERK expression was quantified relative to parental no PD.
4.3 Discussion

FGFR1 expression has been associated with a mesenchymal phenotype and FGFR3 has been associated with an epithelial phenotype (Cheng et al., 2013). FGFR1 activation is known to induce EMT in urothelial carcinoma cell lines (Tomlinson et al., 2012). It was thought that the mesenchymal phenotypic changes observed in RT112 R1 and R2 could be caused by a switch in dependency from FGFR3 to FGFR1. FGFR1 protein expression remained low in the resistant derivatives and therefore the partial EMT observed in RT112 R1 and R2 and PD resistance is not induced by increased expression of this RTK. It was also considered that activation of the FGFR IIIc rather than IIIb isoform could be inducing the mesenchymal phenotypic changes observed in RT112 R1 and R2. An increase in FGF2 expression was observed in RT112 R1 and R2. This FGF preferentially activates FGFR IIIc isoforms. However, no switching to the IIIc isoform was observed in FGFR2 in RT112 R1 and R2. Other FGFRs were not examined for isoform switching. However, as FGFR1 and FGFR3 expression is low in RT112 R1 and R2 cultured in PD isoform switching of these receptors is unlikely to be a cause of the partial EMT in these lines. It is crucial to note that, as PD is a pan-FGFR inhibitor, signalling via FGFRs including IIIc isoforms should not be possible in the presence of PD unless a gatekeeper mutation has occurred. The V555M FGFR3 mutation has been identified as a mechanism of acquired resistance to FGFR inhibition (Chell et al., 2013). Unless an activating FGFR mutation or FGFR amplification is identified in the resistant derivatives the hypothesis that FGFR isoform switching is inducing phenotypic changes in the PD resistant derivatives can be discounted. Genetic analysis of resistant derivatives will be described in Chapter 5.

Increased phosphorylation of ERBB3 and MET was observed in RT112 R1 and R2. An increase in phospho-MET was observed in RT112 R3 but phospho-ERBB3 was not examined in this line. The phosphorylation of these receptors could be contributing to the resistant phenotype in these lines. ERBB3 is not able to signal via homodimerization due to low kinase function. However, MET, in addition to other EGFR family members, is able to heterodimerize with ERBB3 to induce intracellular signalling (Pérez-Ramírez et al., 2015; Tanizaki et al., 2011). It is therefore possible that MET is heterodimerising with ERBB3 in the resistant derivatives and contributing to the resistance to PD. The activation of RTKs in RT112 resistant lines was examined with a phospho-RTK array (PathScan® RTK signaling antibody array Kit #7982). However, as the results of this array were
disappointing with a low signal detected from RTK specific spots, the results of this analysis were not presented in this Chapter. An alternative phospho-RTK array from a different supplier was identified but analysis was not conducted with this array due time limitations.

Phosphorylation of EGFR was markedly increased in RT4 R1 and was considered a strong candidate resistance mechanism. EGFR is able to signal via homodimerization or heterodimerization with other EGFR family members (Roskoski, 2014). A slight increase in phospho-ERBB3 was observed in RT4 R1 but phospho-ERBB2 was not detected in RT4 R1. This analysis of expression and phosphorylation is limited to a few RTKs: other signalling via other RTKs could be inducing resistance. The genetic analysis in Chapter 5 and transcriptomic analysis in Chapter 6 may uncover further signalling pathways implicated in resistance to PD. RTKs can be targeted by TKIs used clinically and the dependence of resistant lines on these RTKs will be tested in Chapter 7 of this thesis.

The increased phosphorylation of AKT in RT112 R1 and R2 compared to RT112 parental and R3 highlights that there is a difference in the resistance mechanism employed in R1 and R2 compared to the mechanism employed in R3. However, the cause of this increased phosphorylation is unknown. The maintenance of ERK and AKT activation in RT112 R3 to a similar level as observed in RT112 parental was expected as R3 has the most similar phenotype to the parental line of all the resistant derivatives. Upon culture without PD, phospho-AKT expression was reduced in RT112 R1 and R2, FGFR3 expression was increased in all RT112 resistant derivatives and cell proliferation was increased in all resistant derivatives. This suggests that the resistant derivatives resume signalling via FGFR3 upon drug removal.
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<th>Protein</th>
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</tr>
</thead>
<tbody>
<tr>
<td>N-cadherin</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Constant</td>
</tr>
<tr>
<td>FGFR3</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Constant</td>
</tr>
<tr>
<td>Phospho-EGFR</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Not assayed</td>
<td>Increased</td>
</tr>
<tr>
<td>EGFR</td>
<td>Constant</td>
<td>Constant</td>
<td>Not assayed</td>
<td>Increased</td>
</tr>
<tr>
<td>Phospho-ERBB2</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not assayed</td>
<td>Not detected</td>
</tr>
<tr>
<td>ERBB2</td>
<td>Constant</td>
<td>Constant</td>
<td>Not assayed</td>
<td>Not detected</td>
</tr>
<tr>
<td>Phospho-ERBB3</td>
<td>Increased</td>
<td>Increased</td>
<td>Not assayed</td>
<td>Increased</td>
</tr>
<tr>
<td>Phospho-MET</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Not assayed</td>
</tr>
<tr>
<td>MET</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Increased</td>
<td>Not assayed</td>
</tr>
</tbody>
</table>

Figure 4.13 Key protein expression changes identified in RT112 and RT4 resistant derivatives by immunoblot in Chapter 3 and 4. Protein expression of each resistant derivative cultured in PD is given relative to the parental line cultured without PD.
Chapter 5
Genetic Differences Between Parental and Resistant Cells

5.1 Introduction

This Chapter will examine whether there is a genetic mechanism of resistance in any of the RT112 or RT4 derivatives. Genetic mechanisms of resistance to targeted agents discussed in Chapter 1 included the FGFR3 V555M mutation which was previously described as a mechanism of acquired resistance to FGFR inhibition in the myeloma cell line KMS-11 (Chell et al., 2013). One possible mechanism by which resistance could have arisen in the RT112 and RT4 resistant lines is via the acquisition of a mutation in an RTK. It is probable that any genetic change identified as a cause of resistance will induce activation of the MAP kinase and PI3 kinase pathways as these have been identified as inhibited in parental RT112 upon acute treatment but active in RT112 resistant derivatives. In addition to mutations in the receptor itself, mutations may be identified in these pathways, in genes such as the RAS genes, \( \text{RAF}, \text{PIK3CA}, \text{PTEN} \) and \( \text{AKT} \) (discussed in Chapter 1, section 1.2.2).

Increased copy number of \( \text{ERBB2} \) has been associated with resistance to EGFR monoclonal antibodies in colorectal cancer patients (Martin et al., 2013). Le Coutre et al. cultured the CML cell line LMA84 with imatinib for 6 months. The line became resistant to imatinib due to amplification of the \( \text{BCR-ABL} \) fusion gene which induced an increase in BCR-ABL protein expression (le Coutre et al., 2000). Short-term survival and resistance to FGFR TKIs in RT112 has been reported to arise due to activation of alternative RTKs, but this has not been found to be due to gene amplification (Harbinski et al., 2012; Herrera-Abreu et al., 2013; Wang et al., 2014).

Two studies examined the mechanism by which RT112 cells survive short-term treatment with PD and focused on identifying non-genetic changes in the FGFR TKI-treated cells rather than genetic differences (Harbinski et al., 2012; Herrera-Abreu et al., 2013). They identified MET and EGFR activation respectively as inducing short term survival. During the course of this study, Wang et al., conducted long-term culture of RT112 in FGFR TKIs and conducted mRNA sequencing and reported activation of \( \text{ERBB2} \) and \( \text{ERBB3} \) as a non-genetic mechanism of resistance in their resistant derivatives (Wang et al., 2014).
In Chapter 4, a small increase in ERBB3 phosphorylation was identified in RT112 R1 and R2 (Fig. 4.8) and an increase in phosphorylation of MET was observed in RT112 R1, R2 and R3 (Fig. 4.11). An increase in total and phosphorylated EGFR was observed in RT4 R1 (Fig. 4.6). It is possible that the phosphorylation of these RTKs could be mediated by an activating mutation in or an increase in the copy number of these RTKs. It was hypothesised that RT112 R3 was the most likely RT112 resistant derivative to have a genetic mechanism of resistance as it maintained a maximal cell density and morphology more similar to RT112 parental than RT112 R1 or R2. It was also thought that RT112 R1 and RT112 R2 may have the same resistance mechanism due to these resistant derivatives having a similar phenotype. Therefore, RT112 R2 was not examined with whole exome sequencing or copy number analysis. If a genetic alteration of interest was identified in RT112 R1, analysis would be conducted to determine if the same mutation was present in RT112 R2. RT4 parental and R1 were not examined by whole exome sequencing as it was considered likely that EGFR was mediating resistance in RT4. Therefore, genetic analysis of the RT4 parental and resistant line focused on determining if EGFR was amplified or mutated in RT4 R1.

5.2 Results

5.2.1 Copy number analysis of RT112 Parental, R1 and R3

Copy number analysis was conducted in RT112 parental, R1, R2 and R3 with low pass whole genome sequencing. An unmatched blood sample was used as a reference sample in this study because we do not have a matched reference sample for RT112 or RT4. The sequence of centromeres contains long arrays of near identical tandem repeats. The repetitive nature of these regions prevents accurate sequence alignment (Hayden, 2012). This can result in copy number gains and losses being falsely identified in centromeric regions and therefore apparent copy number alterations identified in these regions were ignored. The genome-wide copy number profiles of chromosomes 1-22 for RT112 parental, R1 and R3 normalised to a blood sample are presented in Fig. 5.1. The copy number profiles of chromosome 8 and 9 for RT112 parental, R1 and R3 are shown in Appendix C, Fig. C.1, to enable the visualization of the smaller copy number alterations present on these chromosomes. Copy number alterations shared
between RT112 parental, R1 and R3 are summarised in Table 5.1. The karyotype of parental RT112 maintained in this laboratory has been analysed previously by M-FISH and copy number changes have been examined with array comparative genomic hybridisation at passage 33 (Hurst et al., 2004; Williams et al., 2005). In this study RT112 parental was passage 37 when DNA was harvested for copy number analysis. The copy number data fits well with the genomic alterations described by Williams et al. and Hurst et al. (Hurst et al., 2004; Williams et al., 2005). For example, a gain of chromosome 20 was observed by Williams et al., Hurst et al. and in this study. Williams et al. observed a gain of 4p and Hurst et al. reported that RT112 had a gain of 4p11–pter. This was consistent with the copy number gain of 4p16.3-p11 observed in RT112 parental in this study. Williams et al. describe RT112 as having a del(1)(p3?4) and Hurst et al. observed loss of 1p35.3–pter. In our copy number data, a loss of 1p36.33-p36.21 in RT112 parental, R1 and R3 was observed. No loss of 2q was reported by Williams et al. whilst loss of 2q22.1–q23.1 was reported by Hurst et al. and a loss of 2q21.3-q23.2 was observed in RT112 parental, R1 and R3. This may be due to the more limited resolution of M-FISH (Hurst et al., 2004).

To examine the differences between the three lines, the copy number data of RT112 R1 and R3 were normalised to parental. Results are presented in Figure 5.2. Copy number differences between RT112 parental and R1 and R3 are summarised in Table 5.2 and Table 5.3, respectively. As RT112 parental is an uncloned line, copy number differences between RT112 parental and the RT112 resistant derivatives may be due to selection of a subclone rather than due to a genomic alteration that has occurred during the derivation of the resistant derivatives. In addition to the 2q21.3-q23.2 copy number loss in RT112 parental, R1 and R3, copy number loss of 2q37.2-q37.3 was observed in RT112 R1. The copy number analysis conducted with RT112 parental as the reference sample indicated that RT112 R1 had gain of 19p13.3-q11. However, RT112 parental and R3 have loss of 19p (Fig. 5.1) and therefore it is likely that RT112 R1 is a subclone with normal chromosome 19.
Figure 5.1 Copy number profiles for all autosomes for RT112 parental, R1 and R3 normalised to a patient blood sample. Copy number plots were generated in Nexus Copy Number 8.0 and show log$_2$ ratio of the read counts of RT112 samples normalised to a patient blood sample for chromosomes 1-22 (GRCh38 reference). Each point represents the log$_2$ ratio for a section of DNA of window size 1000 bp. Copy number gains (above the zero line) and losses (below the zero line) shared by RT112 parental, R1 and R3 are highlighted with arrows and are also detailed in Table 5.1.
Table 5.1 Copy number alterations shared by RT112 parental, R1 and R3. Copy number data was generated using low-pass whole genome sequencing and normalised to data from an unrelated patient’s blood sample.

<table>
<thead>
<tr>
<th>Chromosome:Position (Mb; hg38)</th>
<th>Cytoband</th>
<th>Copy Number Loss/Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:744,421-29,096,605</td>
<td>p36.33-p36.21</td>
<td>loss</td>
</tr>
<tr>
<td>2:135,548,251-149,171,997</td>
<td>q21.3-q23.2</td>
<td>loss</td>
</tr>
<tr>
<td>5:0-46,508,621</td>
<td>p15.33-p11</td>
<td>gain</td>
</tr>
<tr>
<td>8:7,295,847-38,098,889</td>
<td>p23.1-p11.23</td>
<td>loss</td>
</tr>
<tr>
<td>8:39,000,213-41,275,121</td>
<td>p11.22-p11.21</td>
<td>gain</td>
</tr>
<tr>
<td>8:43,801,423-45,928,436</td>
<td>p11.1-q11.1</td>
<td>loss</td>
</tr>
<tr>
<td>8:45,928,436-145,138,636</td>
<td>q11.1-q24.3</td>
<td>gain</td>
</tr>
<tr>
<td>9:21,409,526-23,540,699</td>
<td>p21.3</td>
<td>loss</td>
</tr>
<tr>
<td>11:0-8,295,312</td>
<td>p15.5-p15.4</td>
<td>loss</td>
</tr>
<tr>
<td>16:78,500,343-90,338,345</td>
<td>q23.1-q24.3</td>
<td>gain</td>
</tr>
<tr>
<td>20:0-64,444,167</td>
<td>p13-q13.33</td>
<td>gain</td>
</tr>
</tbody>
</table>

RT112 R1 exhibited copy number loss of 4p16.3-q22.1. There was a possible loss of 4p16.3-p11 in RT112 R3, however this did not reach the required threshold of -0.25 to be classified as a loss. FGFR3 is located within the chromosome 4 cytoband p16.3. RT112 parental exhibited a gain of 4p16.3-p11 which was not observed in RT112 R1 or R3 (Fig. 5.1). Therefore, the loss of 4p16.3-q22.1 in RT112 R1 appears to be due to this resistant line lacking the 4p gain observed in RT112 parental. Both RT112 R1 and R3 exhibited gain of 8p11.23-p11.1. This region contains FGFR1. RT112 R1 exhibited copy number gain of 5p15.33-q11.1 which contains the genes LIFR and OSMR which encode the oncostatin M (OSM) pathway receptors leukaemia inhibitory factor receptor (LIFR) and oncostatin M receptor (OSMR), respectively. RT112 R1 also exhibited copy number gain of 5q11.1-q11.2 which contains the gene IL6ST which encodes the OSM pathway receptor glycoprotein 130 (gp130) (Fig. 5.2, Table 5.2).
Figure 5.2 Copy number profiles of RT112 R1 and R3 normalised to RT112 parental. Copy number plots were generated in Nexus Copy Number 8.0 and show log$_2$ ratio of the read counts of RT112 R1 and R3 normalised to parental for chromosomes 1-22 (GRCh38 reference). Each point represents the log$_2$ ratio for a section of DNA of window size 1000 bp. Copy number gains (above the zero line) and losses (below the zero line) are highlighted with arrows and are also detailed in Table 5.2 and Table 5.3.
Table 5.2 Copy number differences between RT112 parental and R1. Copy number data was generated using low-pass whole genome sequencing and copy number alterations identified using RT112 parental as the reference sample and RT112 R1 as the test sample.

<table>
<thead>
<tr>
<th>Chromosome:Position (Mb; hg38)</th>
<th>Cytoband</th>
<th>Copy Number Loss/Gain</th>
<th>Potential Candidate Genes</th>
<th>Number of Genes in Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:235,053,661-238,107,785</td>
<td>q37.2 – q37.3</td>
<td>loss</td>
<td>SH3BP4</td>
<td>17</td>
</tr>
<tr>
<td>4:90,569,813-190,026,739</td>
<td>q22.1 – q35.2</td>
<td>gain</td>
<td>FABP2, SMAD1</td>
<td>311</td>
</tr>
<tr>
<td>5:0-48,800,000</td>
<td>p15.33 – q11.1</td>
<td>gain</td>
<td>LIFR, OSMR</td>
<td>136</td>
</tr>
<tr>
<td>5:49,712,701-57,604,516</td>
<td>q11.1 – q11.2</td>
<td>gain</td>
<td>IL6ST, MAP3K1</td>
<td>34</td>
</tr>
<tr>
<td>8:38,057,101-45,200,000</td>
<td>p11.23 – q11.1</td>
<td>gain</td>
<td>FGFR1</td>
<td>47</td>
</tr>
<tr>
<td>19:209,898-26,200,000</td>
<td>p13.3 – q11</td>
<td>gain</td>
<td>FGF22, INSR, JUNB, JUND</td>
<td>590</td>
</tr>
</tbody>
</table>
Table 5.3 Copy number differences between RT112 parental and R3. Copy number data was generated using low-pass whole genome sequencing and copy number alterations identified using RT112 parental as the reference sample and RT112 R3 as the test sample.

<table>
<thead>
<tr>
<th>Chromosome:Position (Mb; hg38)</th>
<th>Cytoband</th>
<th>Copy Number Loss/Gain</th>
<th>Potential Candidate Genes</th>
<th>Number of Genes in Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:85,661-49,061,875</td>
<td>p16.3 – p11</td>
<td>loss</td>
<td>FGFR1, FGFR3, FGFRBP1, FGFRBP2</td>
<td>301</td>
</tr>
<tr>
<td>4:51,843,881-190,026,739</td>
<td>q12 – q35.2</td>
<td>gain</td>
<td>KIT, SMAD1</td>
<td>507</td>
</tr>
<tr>
<td>8:38,057,101-43,883,945</td>
<td>p11.23 – p11.1</td>
<td>gain</td>
<td>FGFR1</td>
<td>47</td>
</tr>
</tbody>
</table>
5.2.2 Copy number analysis of RT4 parental and RT4 R1

Copy number analysis was conducted with low pass whole genome sequencing in RT4 parental and R1. The copy number profiles of RT4 parental and RT4 R1 normalised to a blood sample are presented in Figure 5.3 and copy number changes shared between RT4 parental and R1 are summarised in Table 5.4. The karyotype of parental RT4 has been analysed previously by M-FISH and copy number changes have been examined by with array comparative genomic hybridisation (Hurst et al., 2004; Williams et al., 2005). The reported genomic alterations in RT4 parental were concordant with the copy number changes observed in our copy number data. For example, Williams et al. reported RT4 parental, which is tetraploid, to have three copies of chromosome 9 and five copies of chromosome 12 and Hurst et al. reported loss of chromosome 9 and gain of chromosome 12 (Hurst et al., 2004; Williams et al., 2005). This is in agreement with our copy number data in which there was copy number loss of chromosome 9 and gain of chromosome 12 in both RT4 parental and RT4 R1.

The copy number profile of RT4 R1 normalised to RT4 parental is presented in Figure 5.4 and copy number differences between RT4 parental and RT4 R1 are summarised in Table 5.5. As our RT4 parental is a clone, any changes in copy number differences observed in RT4 R1 are likely to be due to alterations acquired during culture in PD, rather than selection of existing subclones, although changes could still be passenger events. The alterations that differed between RT4 parental and RT4 R1 were gain of 5p15.33-q11.1 and the loss of 5q11.1- q35.3. A gain of the region from 5p15.33-q11.1, which contains the LIFR and OSMR genes was also observed in RT112 R1. These genes encode receptors in the OSM signalling pathway (Fig. 6.15). Transcriptome analysis conducted in Chapter 6 identified differential expression of OSM pathway signalling genes in RT112 resistant derivatives compared to parental RT112 (Fig. 6.9, 6.10, D.6 and D.7). Tables of the genes in 5p15.33-q11.1 which were differentially expressed between RT4 parental and RT4 R1 and between RT112 parental and RT112 R1, determined by transcriptome analysis, are included as supplementary data. It was possible that the increase in EGFR expression in RT4 R1 could be due to EGFR amplification. EGFR is located on chromosome 7, cytoband p11.2. No change in copy number in this region was observed between RT4 parental and RT4 R1, therefore the increased EGFR expression observed is not due to EGFR amplification.
Figure 5.3 Copy number profiles of RT4 parental and R1 normalised to a patient blood sample. Copy number plots were generated in Nexus Copy Number 8.0 and show log$_2$ ratio of the read counts of RT4 samples normalised to a patient blood sample for chromosomes 1-22 (GRch38 reference). Each point represents the log$_2$ ratio for a section of DNA of window size 1000 bp. Copy number gains (above the zero line) and losses (below the zero line) shared by RT4 parental and R1 are highlighted with arrows and are also detailed in Table 5.4.
Table 5.4 Copy number alterations shared by RT4 parental and RT4 R1. Copy number data was generated using low-pass whole genome sequencing and normalised to data from an unrelated patient’s blood sample.

<table>
<thead>
<tr>
<th>Chromosome:Position (Mb; hg38)</th>
<th>Cytoband</th>
<th>Copy Number Loss/Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0-27,636,501</td>
<td>p36.33 – p35.3</td>
<td>loss</td>
</tr>
<tr>
<td>2:237,762,632-238,486,412</td>
<td>q37.3</td>
<td>loss</td>
</tr>
<tr>
<td>4:0-169,230,689</td>
<td>p16.3 - q33</td>
<td>loss</td>
</tr>
<tr>
<td>6:112,662,364-170,805,979</td>
<td>q21 – q27</td>
<td>loss</td>
</tr>
<tr>
<td>8:558,052-15,238,333</td>
<td>p23.3 – p22</td>
<td>gain</td>
</tr>
<tr>
<td>9:13,808,337-138,394,717</td>
<td>p23 – q34.3</td>
<td>loss</td>
</tr>
<tr>
<td>10:0-133,797,422</td>
<td>p15.3 – q26.3</td>
<td>loss</td>
</tr>
<tr>
<td>11:0-1,369,711</td>
<td>p15.5</td>
<td>loss</td>
</tr>
<tr>
<td>11:3,959,836-5,227,107</td>
<td>p15.4</td>
<td>loss</td>
</tr>
<tr>
<td>11:78,639,222-80,827,017</td>
<td>q14.1</td>
<td>loss</td>
</tr>
<tr>
<td>12:0-41,345,528</td>
<td>p13.33 – q12</td>
<td>gain</td>
</tr>
<tr>
<td>12:57,594,810-132,634,466</td>
<td>q13.3 – q24.33</td>
<td>gain</td>
</tr>
<tr>
<td>13:18,798,543-114,364,328</td>
<td>q11 – q34</td>
<td>loss</td>
</tr>
<tr>
<td>14:17,200,000-107,043,718</td>
<td>q11.1 – q32.2</td>
<td>gain</td>
</tr>
<tr>
<td>15:17,000,502-101,991,189</td>
<td>p11.2 – q26.3</td>
<td>gain</td>
</tr>
<tr>
<td>16:0-90,338,345</td>
<td>p13.3- q24.3</td>
<td>loss</td>
</tr>
<tr>
<td>17:0-21,227,316</td>
<td>p13.3 – p11.2</td>
<td>loss</td>
</tr>
<tr>
<td>17:41,732,953-82,478,901</td>
<td>q21.2 – q25.3</td>
<td>gain</td>
</tr>
<tr>
<td>18:49,738,286-51,622,721</td>
<td>q21.1 – q21.2</td>
<td>loss</td>
</tr>
<tr>
<td>19:0-12,643,882</td>
<td>p13.3- p13.13</td>
<td>loss</td>
</tr>
<tr>
<td>19:36,259,172-58,617,616</td>
<td>q13.12 – q13.43</td>
<td>gain</td>
</tr>
<tr>
<td>21: 12,000,000-46,709,983</td>
<td>q11.1 – q22.3</td>
<td>loss</td>
</tr>
</tbody>
</table>
Figure 5.4 Copy number profile of RT4 R1 normalised to RT4 parental. The copy number plot was generated in Nexus Copy Number 8.0 and shows log$_2$ ratio of the read counts of RT4 R1 normalised to parental RT4 for chromosomes 1-22 (GRch38 reference). Each point represents the log$_2$ ratio for a section of DNA of window size 1000 bp. Copy number gains (above the zero line) and losses (below the zero line) are highlighted with arrows and are also detailed in Table 5.5.
Table 5.5 Copy number differences between RT4 parental and RT4 R1. Copy number alterations were identified with low-pass whole genome sequencing and a patient blood sample was used as the reference sample which RT4 parental and RT4 R1 were examined against.

<table>
<thead>
<tr>
<th>Chromosome:Position (Mb; hg38)</th>
<th>Cytoband</th>
<th>Copy Number Loss/Gain</th>
<th>Potential Candidate Genes</th>
<th>Number of Genes in Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:0-48,800,000</td>
<td>p15.33 – q11.1</td>
<td>gain</td>
<td>LIFR, OSMR</td>
<td>136</td>
</tr>
<tr>
<td>5: 50,518,687-181,538,259</td>
<td>q11.1 – q35.3</td>
<td>loss</td>
<td>IL6ST, IL31RA, MAP3K1, SMAD5, FGF1, RASGEFC, MAP9</td>
<td>721</td>
</tr>
</tbody>
</table>
5.2.3 Whole exome sequencing of RT112 R1

In order to identify genetic changes which could be inducing resistance, whole exome sequencing was conducted in RT112 parental, R1 and R3. To be called, a mutation needed to satisfy the following two criteria: 1) a variant allele frequency of greater than 20% and 2) an absence of the mutation in RT112 parental. All variants were visualised in Integrative Genomics Viewer (IGV) and any considered to be artefacts were removed from variant lists. No insertions or deletions in RT112 R1 or RT112 R3 met these criteria. There were forty-three single nucleotide variants (SNVs) which met the criteria in RT112 R1. These are listed in Table C.1, Appendix C. Twenty-one mutations (49%) were non-synonymous. The mutations in RT112 R1 which were thought to be most likely to be inducing resistance, as they were predicted to induce a nonsense mutation, predicted to be deleterious by Polymorphism Phenotyping v2 (PolyPhen) and Sorting Intolerant from Tolerant (SIFT) or as previous research had implicated the mutated gene in cancer, are detailed in Table 5.6.

A missense mutation in MIB2 resulting in a Q898R amino acid change was observed in RT112 R1 (Table 5.6). MIB2 encodes Mindbomb E3 Ubiquitin Protein Ligase 2 which ubiquitinates Delta, a Notch ligand, inducing its removal from the cell membrane via endocytosis (Koo et al., 2005). Transfection of wildtype MIB2 into melanoma cell lines reduced MET protein expression and significantly reduced invasion in vitro and in vivo. (Takeuchi et al., 2006). Immunoblot analysis conducted in Chapter 4 showed that there was an increase in phosho-MET in R3 cultured without PD and in R1, R2 and R3 cultured with PD compared to RT112 parental acutely treated with PD (Fig. 4.11).

A missense mutation in ATF2 resulting in an E349K amino acid change was observed in RT112 R1 and was predicted to be deleterious by SIFT and possibly damaging by PolyPhen (Table 5.6). ATF2 encodes activating transcription factor 2 which elicits its activity by forming homodimers or by heterodimerizing with other activating protein 1 (AP1) family members. ATF2 regulates genes which control the cell cycle, inflammation and cell death (Watson et al., 2017).

A missense mutation in HACL1 resulting in a S15P amino acid change was observed in RT112 R1 and was predicted to be deleterious by SIFT (Table 5.6). HACL1 encodes 2-hydroxyacyl-CoA lyase which is involved in the α-oxidation pathway which breaks down branched fatty acid and 2-hydroxylated fatty acids into aliphatic aldehydes in the peroxisome. The resulting aldehyde is then able to be degraded with the β-oxidation pathway (Foulon et al., 2005).
A nonsense mutation (Q144*) in SLC35G2 was observed in RT112 R1 (Table 5.6). SLC35G2 encodes solute Carrier Family 35 Member G2. SLC35 family members are involved in the transport of nucleotide sugars or adenosine 3'-phospho 5'-phosphosulfate into the Golgi apparatus. Little is known about the SLCG35 subfamily (Song, 2013).

A nonsense mutation (K143*) in TRIM51 was observed in RT112 R1 (Table 5.6). TRIM51 encodes Tripartite Motif-Containing 51. A number of the TRIM family of RING type E3 ubiquitin ligases are involved in the regulation of the p53 pathway and the regulation of nucleotide receptors such as the thyroid receptor and the oestrogen receptors (Hatakeyama, 2011).

A nonsense mutation (E76*) was observed in SESN3 in RT112 R1 (Table 5.6). SESN3 encodes sestrin 3. Sestrins 1-3 are reported to act as negative regulators of mTORC1 via their interaction with the GATOR2 complex which occurs most strongly during amino acid deprivation. Sestrins 1-3 appear to be functionally redundant (Chantranupong et al., 2014).

A missense mutation in GRIP1 resulting in a G188E amino acid change was observed in RT112 R1 and was predicted to be deleterious by SIFT and probably damaging by PolyPhen (Table 5.6). GRIP1 encodes glutamate receptor interacting protein 1 which mediates the localisation of AMPA-type glutamate receptors at the synapse (Tan et al., 2015).

A missense mutation in TMPRSS15 resulting in a R583T amino acid change was observed in RT112 R1 and was predicted to be deleterious by SIFT and probably damaging by PolyPhen (Table 5.6). TMPRSS15 encodes Transmembrane Protease Serine 15, also known as Enteropeptidase, which cleaves the pancreatic precursor protrypsin to trypsin (Holzinger et al., 2002).

All the RT112 R1 SNVs detailed in Table 5.6 had a variant allele frequency of approximately 30%. This may imply the presence of a subclone in RT112 R1.

### 5.2.4 Whole exome sequencing of RT112 R3

There were twenty-five SNVs which met the criteria in RT112 R3. These are listed in Table C.2, Appendix C. Thirteen mutations (52%) were non-synonymous. The mutations which were thought to be most likely to be inducing resistance in RT112 R3, as they were predicted to induce a nonsense mutation, predicted to be deleterious by PolyPhen and SIFT or as previous research had implicated the mutated gene in cancer, are detailed in Table 5.7.
Table 5.6 Selected mutations identified by whole exome sequencing in RT112 R1. The mutations met the following criteria: the mutation was absent from RT112 parental and had a variant allele frequency of greater than 20%.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ensembl transcript ID</th>
<th>Chromosome: Position (Mb; hg38)</th>
<th>Variant Effect Predictor (VEP) consequence</th>
<th>Coding DNA sequence change (position from start codon)</th>
<th>Amino acid change</th>
<th>COSMIC ID</th>
<th>Sorting Intolerant From Tolerant (SIFT) Prediction</th>
<th>Polymorphism Phenotyping v2 (PolyPhen) Prediction</th>
<th>Variant allele frequency R1 (%)</th>
<th>Variant allele frequency R3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIB2</td>
<td>ENST00000355826</td>
<td>1:1629393</td>
<td>missense variant</td>
<td>c.A2693G</td>
<td>p.Q898R</td>
<td>tolerated</td>
<td>benign</td>
<td></td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>HACL1</td>
<td>ENST00000321169</td>
<td>3:15601421</td>
<td>missense variant</td>
<td>c.T43C</td>
<td>p.S15P</td>
<td>deleterious</td>
<td>benign</td>
<td></td>
<td>33</td>
<td>4</td>
</tr>
<tr>
<td>SLC35G2</td>
<td>ENST00000393079</td>
<td>3:136854890</td>
<td>stop gained</td>
<td>c.C430T</td>
<td>p.Q144*</td>
<td>-</td>
<td>-</td>
<td></td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td>TRIM51</td>
<td>ENST00000449290</td>
<td>11:55886138</td>
<td>stop gained</td>
<td>c.A427T</td>
<td>p.K143*</td>
<td>-</td>
<td>-</td>
<td></td>
<td>27</td>
<td>4</td>
</tr>
<tr>
<td>SESN3</td>
<td>ENST00000278499</td>
<td>11:95185375</td>
<td>stop gained</td>
<td>c.G226T</td>
<td>p.E76*</td>
<td>-</td>
<td>-</td>
<td></td>
<td>32</td>
<td>7</td>
</tr>
<tr>
<td>TMPRSS15</td>
<td>ENST00000284885</td>
<td>21:18329201</td>
<td>missense variant</td>
<td>c.G1748C</td>
<td>p.R563T</td>
<td>deleterious</td>
<td>probably damaging</td>
<td></td>
<td>29</td>
<td>4</td>
</tr>
</tbody>
</table>
A missense mutation in CD55 resulting in a Y245F amino acid change was observed in RT112 R3 and was predicted to be deleterious by SIFT and probably damaging by PolyPhen (Table 5.7). CD55, also known as decay accelerating factor, is an inhibitor of the complement system. CD55 inhibits complement dependent cytotoxicity of cancer cells (Reis et al., 2018).

A nonsense mutation (E2243*) was observed in XIRP2 in RT112 R3 (Table 5.7). XIRP2 encodes Xin Actin Binding Repeat Containing 2 which is expressed in skeletal and cardiac muscle. XIRP2 stabilises the actin cytoskeleton via binding and crosslinking actin filaments (Pacholsky et al., 2004).

A missense mutation in UGT3A1 resulting in a V244E amino acid change was observed in RT112 R3 and was predicted to be deleterious (low confidence) by SIFT and possibly damaging by PolyPhen (Table 5.7). UGT3A1 transfers N-acetylglucosamine to the bile acid ursodeoxycholic acid from UDP N-acetylglucosamine (Mackenzie et al., 2008).

A missense mutation in PCSK5 resulting in a W681S amino acid change was observed in RT112 R3. PCSK5 is a Calcium-dependent serine protease which cleaves and activates Pro-renin, Integrin α subunit and MMP14 (Klein-Szanto and Bassi, 2017). The COSMIC database v83 catalogues this mutation as having occurred in head and neck cancer cell lines and lung, kidney and colon cancers (Forbes et al., 2017). This missense mutation was predicted to have an unknown effect by PolyPhen and SIFT did not predict the effect of this mutation. According to the Ensembl database v91, the only transcript that this variant has coding effects in (ENST00000376767) is poorly supported by experimental evidence (Zerbino et al., 2018).

A missense mutation in ZNF114 resulting in a E362K amino acid change was observed in RT112 R3. This mutation was predicted to be possibly damaging by Polyphen. ZNF114 encodes zinc finger protein 114 which is involved in epigenetic repression of pro-differentiation genes to maintain a pluripotent state (Oleksiewicz et al., 2017).

It was decided that the genetic alteration most likely to be inducing resistance in RT112 R3 was the HRAS G12S mutation. The HRAS G12S mutation has been previously reported and it is known to act as a gain of function mutation (Hobbs et al., 2016). RAS mutations are usually mutually exclusive with FGFR3 mutations in bladder cancer suggesting the mutations have an overlapping function.
(Jebar et al., 2005). The HRAS G12S mutation was present in 131 out of 179 (73%) reads in RT112 R3 and was not present in any reads in RT112 parental or R1 (both samples had 151 reads at HRAS cDNA position 34) (Table 5.7, Fig. 5.5). This mutation was selected for further investigation. The other variants in RT112 R1 and R3 were not investigated further due to time limitations.

5.2.5 Analysis of HRAS G12S mutation

Sanger sequencing was conducted to confirm the presence of the HRAS G12S mutation in RT112 R3 and absence in RT112 parental, R1 and R2 (Fig. 5.6). Sanger sequencing identified both a guanine and adenine at position 34 (relative to the first base of the ATG start codon) in exon 1 of the HRAS gene in RT112 R3. This confirmed that R3 contained both wildtype and G12S mutant HRAS.

As whole exome sequencing and Sanger sequencing found that the RT112 R3 population contained both wildtype and G12S mutant HRAS, it was unclear if individual RT112 R3 cells contained both HRAS alleles or if this was a mixed population. The HRAS genomic coordinates on chromosome 11 are 532,636-534,322 Mb (hg38). Copy number analysis revealed that RT112 parental, R1 and R3 all exhibited loss of chromosome 11 from 0-8,295,312 Mb (Fig. 5.1, Table 5.1). As RT112 is diploid, this suggests that RT112 parental, R1 and R3 have only one copy of HRAS. SNaPshot analysis of 36 single cell RT112 R3 clones, conducted by Dr Julie Burns, identified 21 single cell clones with the HRAS G12S mutation, 14 with wildtype HRAS and 1 heterozygous/mixed clone (data not shown). It is most likely that the heterozygous/mixed clone was not of single cell origin. Therefore, it can be concluded that RT112 R3 is a mixed population of cells with some cells homozygous for wildtype HRAS and others homozygous for G12S mutant HRAS.
**Table 5.7 Selected mutations identified by whole exome sequencing in RT112 R3.** The mutations met the following criteria: the mutation was absent from RT112 parental and had a variant allele frequency of greater than 20%.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ensemble transcript ID</th>
<th>Chromosome: Position (Mb;hg38)</th>
<th>Variant Effect Predictor (VEP) consequence</th>
<th>Coding DNA sequence change (position from start codon)</th>
<th>Amino acid change</th>
<th>COSMIC ID</th>
<th>Sorting Intolerant From Tolerant (SIFT) Prediction</th>
<th>Polymorphism Phenotyping v2 (PolyPhen) Prediction</th>
<th>Variant allele frequency R3 (%)</th>
<th>Variant allele frequency R1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD55</td>
<td>ENST00000314754</td>
<td>1:207331177</td>
<td>missense variant</td>
<td>c.A734T</td>
<td>p.Y245F</td>
<td>deleterious</td>
<td>probably damaging</td>
<td></td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>XIRP2</td>
<td>ENST00000409195</td>
<td>2:167248119</td>
<td>stop gained</td>
<td>c.G6727T</td>
<td>p.E2243*</td>
<td>deleterious</td>
<td>possibly damaging</td>
<td></td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>UGT3A1</td>
<td>ENST00000333811</td>
<td>5:35962902</td>
<td>missense variant</td>
<td>c.T731A</td>
<td>p.V244E</td>
<td>deleterious low confidence</td>
<td>possibly damaging</td>
<td></td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>PCSK5</td>
<td>ENST00000376767</td>
<td>9:76175271</td>
<td>missense variant</td>
<td>c.G2042C</td>
<td>p.W681S</td>
<td>COSM4139532</td>
<td>unknown</td>
<td></td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>HRAS</td>
<td>ENST00000311189</td>
<td>11:534289</td>
<td>missense variant</td>
<td>c.G34A</td>
<td>p.G12S</td>
<td>COSM1644659, COSM1746299, COSM3931342, COSM480, COSM481, COSM482</td>
<td>deleterious tolerated</td>
<td>73</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.5 Screenshot of the HRAS G12S mutation in RT112 R3 visualised in the Integrative Genomics Viewer (IGV). IGV (version 2.3.59) is a computer application which enables the visualisation of next-generation sequencing data (Thorvaldsdottir et al., 2013). The HRAS gene is encoded on the reverse strand therefore the C>T mutation located on chr11 at 534289Mb (hg38) is a G>A mutation in the HRAS coding sequence. The wildtype sequences for RT112 parental and R1 are also shown.
A SNaPshot assay was conducted with RT112 R3 at passages 63, 73 and 76 maintained in PD. Although SNaPshot analysis is only semi-quantitative, the proportion of mutant HRAS could be determined from these results (Fig. 5.7). The proportion of mutant HRAS in the R3 population increased with increased passage number and therefore suggests that the HRAS G12S provided a selective advantage.

In order to examine whether mutant HRAS could confer resistance to PD, RT112 parental was retrovirally transduced with a construct encoding HRAS G12V and selection of transduced cells was conducted. A HRAS G12V construct was used because we did not have a HRAS G12S available and time was limited. It was observed that these cells had reduced sensitivity to PD (Fig. 5.8). This provides evidence that an activating HRAS mutation can induce resistance to FGFR inhibition.
5.2.6 Examination of EGFR mutation status in RT4 parental and R1

Immunoblot analysis conducted in Chapter 4 found that RT4 R1 had increased total and phosphorylated EGFR compared to RT4 parental (Fig. 4.6). RT4 parental and R1 were examined for the presence of activating EGFR mutations by The Leeds Genetics Laboratory (St James’s Hospital, Leeds) using NGS. This assay covered the following genomic regions (hg38 assembly):

1) 7:55173971-55174022 – This region accounts for the majority of exon 18.
2) 7:55174756-55174820 – This region accounts for the majority of exon 19.
3) 7:55181308-55181337 – This region is in exon 20.
4) 7:55181360-55181410 – This region is in exon 20.
5) 7:55191804-55191846 – This region is in exon 21.

EGFR exons 18-24 encode the tyrosine kinase domain of this RTK (da Cunha Santos et al., 2011). The genomic regions examined by the Leeds Genomics Laboratory include the most common sites of EGFR (ENST00000275493) mutation in lung cancer: 7:55174013-7:55174015 which encodes G719 in exon 18 is a common site of missense mutations, 7:55174769-55174795 which encodes I744P753 in exon 19 is a region in which deletions such as the A746_750del commonly occur, 7:55181312-55181329 encodes S768-V774 in exon 20 which is a region in which insertions commonly occur, 7:55181378 is the site of the T790M exon 20 activating mutation implicated in resistance to EGFR TKIs and 7:55191822-55191831 encodes L858-L861 in exon 21 which is a site in which missense mutations occur, notably the L858R mutation (Tsiambas et al., 2016). Exon 19 deletions and L858 mutations account for greater than 90% of mutations in lung adenocarcinoma (Sholl, 2016). No EGFR mutations were detected in RT4 R1 in the regions listed above (data not shown). A mutation in EGFR in RT4 R1 could have occurred in the regions not examined by this assay or the increased activation of EGFR in RT4 may not be due to an activating EGFR mutation.
Figure 5.7 Electropherograms from the SNaPshot analysis of HRAS cDNA position 34 in RT112 parental and RT112 R3 at passage 63, 73 and 76. Orange peaks represent Genescan-120LIZ size standards, green peaks represent adenine, blue peaks represent guanine. The proportion of wildtype and mutant HRAS in R3 was determined from SNaPshot analysis using the area under the wildtype and mutant curves for each passage.
5.3 Discussion

Our RT112 resistant derivatives were produced via long-term culture, for a total of 21 passages, in PD. As RT112 parental is not a clone, any genetic differences identified could have been present in a small fraction of cells prior to PD treatment or acquired during culture in PD. RT4 R1 was produced by long-term culture, for 16 passages, of RT4 parental in PD. This RT4 parental cell line was previously cloned and therefore genetic differences identified between RT4 R1 and the parental line are likely to have been acquired during the long-term culture in PD. In both RT112 and RT4, genetic differences between the parental line and the resistant derivatives could have been selected for by the culture in PD or may be passenger mutations.

Copy number alterations in RT112 and RT4 were generally consistent with previous examinations of the copy number changes in these cell lines (Hurst et al., 2004; Williams et al., 2005). Deletion of 2q37.2-q37.3 was observed in RT112 R1 but not RT112 parental or RT112 R3. Distal 2q deletions are associated with a higher histological stage and grade in urothelial carcinoma (Hurst et al., 2012; Lindgren et al., 2012; Nishiyama et al., 2011). It is unclear whether this deletion contributed to the resistant phenotype. The following genes in the 2q37.2-q37.3

![Viability in 1\(\mu\)M PD](image)

Figure 5.8 Cell viability of RT112 parental and parental HRAS G12V in 1\(\mu\)M PD173074. Viability of cells was assayed following 120 h treatment with PD173074 and normalised to vehicle control. Error bars show standard error of the mean. This assay was repeated twice and a representative example is shown.
region were lost in RT112 R1: SH3BP4, AGAP1, GBX2, ASB18, IQCA1, ACKR3, COPS8, COL6A3, MIR6811, MLPH, PRLH, RAB17, LRRFIP1, RBM44, RAMP1, UBE2F, UBE2F-SCLY, SCLY and ESPNL. SH3BP4 modulates the amino acid mediated activation of mTORC1 by binding and inhibiting the activity of the Rag GTPase complex. When amino acids are present, the Rag GTPase complex, in its active guanine triphosphate (GTP) bound form, recruits mTORC1 to the lysosome enabling its activation. SH3BP4 directly binds the Rag GTPase complex stabilising it in its guanine diphosphate (GDP) bound, inactive form preventing the interaction between the Rag GTPase complex and mTORC1. SH3BP4 deletions have been reported in a number of cancers including NSCLC, renal and breast cancers and SH3BP4 has been mooted as a tumour suppressor (Kim and Kim, 2013). Its loss of function could allow mTORC1 activation in the presence of FGFR3 inhibition. Whether the loss of SH3BP4 contributed to PD resistance in RT112 R1 could be tested by inducing SH3BP4 expression in RT112 R1 or knockdown of SH3BP4 expression in RT112 parental and examining if these alterations increased or reduced sensitivity to PD respectively. Whole exome sequencing identified a nonsense mutation in SESN3, which encodes the negative regulator of mTORC1 sestrin 3. This mutation could cooperate with the deletion of SH3BP4 to enable greater activation of mTORC1. However, as sestrin 1-3 are functionally redundant (Chantranupong et al., 2014), it was thought that this mutation was unlikely to be inducing resistance to PD in RT112 R1.

Both RT112 R1 and RT112 R3 had gain of 8p11.23-q11 which contains FGFR1. However, increased copy number of FGFR1 is unlikely to be the cause of resistance to PD as FGFR1 protein expression was low in RT112 R1 and R2 (Fig. 4.2). Phosphorylation of this receptor was not examined. Copy number analysis showed that there was a loss of 4p16.3-q22.1 in RT112 R1. A possible loss of 4p16.3 - p11 was also observed in RT112 R3, however, the level of this loss did not reach the threshold of -0.25 required for this region to be classified as a loss. FGFR3 is located in cytoband 4p16.3. Immunoblot analysis conducted in Chapter 3 showed that expression of FGFR3 was reduced in RT112 R1, R2 and R3 cultured in PD compared to RT112 parental cultured out of PD (Fig. 3.11). Culture of the RT112 resistant derivatives without PD for 4 passages induced an increase in expression of both wildtype FGFR3 and the FGFR3-TACC3 fusion. However, expression of both wildtype FGFR3 and the FGFR3-TACC3 fusion remained lower than in RT112 parental cultured without PD (Fig. 3.11). As there is differential expression of wildtype FGFR3 and the FGFR3-TACC3 fusion in the RT112 resistant derivatives depending on exposure to PD, this suggests that the altered
expression is due to transient regulation of gene expression rather than loss of 4p16.3.

RT112 R1 and RT4 R1 exhibited a gain of copy number of 5p15.33-q11.1. As this change in copy number was observed in two separate resistant lines it was thought that this was the most likely copy number alteration to be causing PD resistance. This region contains the genes OSMR and LIFR which encode the oncostatin M receptor and leukaemia inhibitory family receptor respectively. These receptors are part of the OSM signalling pathway which induces activation of the STAT and MAP kinase pathways (Hermanns, 2015). RT112 R1 also exhibited a gain of copy number of 5q11.1-q11.2 which contains the gene IL6ST which encodes glycoprotein 130, another receptor in the OSM signalling pathway (Hermanns, 2015). It is possible that the signalling via the OSM pathway induces resistance via the activation of these pathways in the RT112 and RT4 resistant lines. The OSM pathway is examined in greater detail in Chapter 6 section 6.2.5 following transcriptome analysis of RT112 and RT4 parental and resistant lines.

SNVs in ATF2, SLC35G2, TRIM51, SESN3, HACL1, GRIP1 and TMPRSS15 were identified in RT112 R1. These SNVs were also present in RT112 R3 at a low variant allele frequency. This suggests that these mutations were present in a subclone of parental RT112 or that the mutation arose prior to the point at which the RT112 cells were cultured as separate lines during the derivation of the RT112 resistant derivatives (Chapter 3, Fig. 3.1). Some mutations identified in RT112 R1 and R3 have not yet been catalogued in the COSMIC database. However, this does not mean these mutations are not important as they may be novel mutations which contribute to FGFR TKI resistance. Out of all the mutations identified in RT112 R1 and R3, only the PCSK5 W681S and the HRAS G12S mutations in R3 have been curated in the COSMIC database. The PCSK5 W681S mutation (COSM4139532) has been observed in head and neck cancer (Martin et al., 2014), colorectal cancer (van de Wetering et al., 2015), gastric cancer (COSMIC study ID COSU371), acute myeloid leukemia (COSU544), lung cancer (COSU583), prostate adenocarcinoma (COSU537) and renal cell cancer (COSU588). Mutations in the RAS family of GTPases occur in many cancers including pancreatic, colorectal and lung adenocarcinomas, multiple myeloma and bladder cancer (Scott et al., 2016; Robertson et al., 2017).

It was thought that of the SNVs identified in RT112 R1 by whole exome sequencing, E349K ATF2 and Q898R MIB2 were the most likely to be inducing resistance. ATF2 encodes activating transcription factor 2 which elicits its activity by
forming homodimers or by heterodimerizing with other activating protein 1 (AP1) family members. ATF2 regulates genes which control the cell cycle, inflammation and cell death (Watson et al., 2017). The mutation observed in RT112 R1 induces an amino acid substitution, p.E349K, in one of the nuclear localisation signals (NLS) in this protein. The NLS contains two bipartite NLS motifs, both of which are capable of inducing nuclear localisation. One NLS motif is encoded by amino acids 342-358 and the second is encoded by amino acids 356-372 (Liu et al., 2006). A suggested consensus sequence of a bipartite NLS is as follows: BBX10-12BBBBB, where B represents the basic amino acids lysine and arginine and X represents any amino acid. Only 3 residues out of the 5 basic amino acids in the second cluster are required to be lysine or arginine (Robbins et al., 1991). Kosugi et al. reported that the introduction of basic amino acids near the centre of the linker region impaired the function of an artificial NLS (Kosugi et al., 2008). Therefore, it is likely that the ATF2 E349K mutation would impair the function of the NLS motif. However, as ATF2 possesses a second functional NLS this mutation is not likely to impair ATF2 function (Liu et al., 2006). Therefore, it is most likely that this mutation is not inducing PD resistance in RT112 R1.

It was thought that the Q898R mutation observed in MIB2 in RT112 R1, could be inducing resistance to PD (Table 5.6). Takechui et al. observed hypermethylation of the MIB2 promoter in 6 out of 31 invasive melanoma samples whilst hypermethylation was not observed in 25 benign nevi or five non-invasive superficial spreading melanomas. It was also reported that transfection of wildtype MIB2 into melanoma cell lines reduced MET protein expression and significantly reduced invasion in vitro and in vivo. (Takeuchi et al., 2006). Total and phospho-MET protein expression was examined in the RT112 resistant derivatives in Chapter 4 (Fig. 4.11). An increase in total MET expression was not observed in RT112 R1 compared to RT112 parental. An increase in phospho-MET was observed in R3 cultured without PD and in R1, R2 and R3 cultured with PD compared to RT112 parental acutely treated with PD. However, as MET phosphorylation was reduced in RT112 R1 and R2 upon culture without PD for 4 passages, the increase in phospho-MET appears to be transient, and suggests that it is not due to a genetic alteration such as MIB2 mutation.

The resistance mechanism in RT112 R1 may be epigenetic, and could be due to the copy number gain of 5p15.33-q11.1 or loss of 2q37.2-q37.3 or could arise due to one of the SNVs identified in this line. Further investigation of the genetic alterations observed in RT112 R1 was not conducted due to time limitations. As whole exome sequencing and copy number analysis were not
conducted with RT112 R2, whether there is a genetic cause of PD resistance in this resistant line is unknown. However, due to the similar phenotypes of RT112 R1 and R2 it is thought that these lines may have similar mechanisms of resistance to PD. The plasticity of the RT112 R1 and R2 phenotype suggests these lines have an epigenetic mechanism of resistance. Possible resistance mechanisms in RT112 R1 and R2 include the activation of ERBB3 and MET as an increase in phosphorylation of these RTKs was observed in Chapter 4 (Fig. 4.8 and Fig. 4.11).

The RT112 R3 HRAS G12S mutation detected by whole exome sequencing was confirmed by Sanger sequencing. Retroviral transduction of RT112 parental with HRAS G12V indicated that a HRAS mutation can induce resistance to PD. The mutual exclusivity of mutant RAS and FGFR3 in urothelial carcinoma suggests that the mutations perform a similar function (Jebar et al., 2005). Immunoblot analysis conducted in Chapter 4 found that phospho-ERK and phospho-AKT were expressed in RT112 parental cells cultured without PD and the RT112 resistant derivatives in and out of PD. Phospho-ERK and phospho-AKT expression was reduced in RT112 parental cultured in PD for 24 h (Fig. 4.12). The reduced phosphorylation in RT112 parental acutely treated with PD showed that activation of the MAP kinase and PI3 kinase pathways in parental RT112 is dependent on FGFR3 activation. As the RAS family are also able to activate the MAP kinase and PI3 kinase pathways, the HRAS G12S mutation in RT112 R3 is likely to be inducing the phosphorylation of ERK and AKT observed in RT112 R3 cultured in PD (Castellano and Downward, 2011). FGFR3 and RAS mutations occur more frequently in bladder cancers of a lower stage (Hurst et al., 2017; Kimura et al., 2001). In Chapter 3, it was shown that RT112 R3 cultured in PD had a reduced rate of proliferation and cell density at confluence compared to RT112 parental cultured without PD. This suggests that the HRAS mutation, whilst providing resistance to PD, does not confer exactly the same intracellular signals to induce cell proliferation as FGFR3.

SNaPShot analysis of RT112 R3 single cell clones indicated that R3 consists of a mixed population of cells, some with wildtype HRAS and others with G12S mutant HRAS. The increase in prevalence of G12S HRAS in this population over time suggests that G12S HRAS confers a selective advantage to the cells with the mutation. The survival and proliferation of the RT112 R3 cells with wildtype HRAS may be mediated by the activation of ERBB3 and MET observed by immunoblot in Chapter 4.
RAS proteins activate the MAP kinase, PI3 kinase, RAL and PLCε pathways inducing cell survival and proliferation. RAS proteins activate downstream signalling only when bound to GTP (Stephen et al., 2014). The RAS family of proteins are GTPases and upon GTP hydrolysis to GDP RAS enters its inactive form. Guanine exchange factors elicit the return of RAS to its GTP bound active form and GTPase activating proteins aid the hydrolysis of GTP (Scott et al., 2016). The RAS family of GTPases contain three domains: residues 1-86 of RAS proteins encode the effector lobe, residues 87-166 encode the allosteric lobe and residues 167-188/189 encode the hypervariable region. The effector lobe contains switch 1, encoded by residues 30-40, and switch 2, encoded by residues 60 to 76. Switch 1 and 2 undergo a conformational change depending on the binding of GTP or GDP. When bound to GTP, switch 1 and 2 are able to bind and activate a range of effector molecules due to their flexible structure. These effector molecules include RAF, PI3K, RALGEF and PLCε. Whilst the sequence of the effector lobe is conserved between RAS family members, the sequence of the allosteric and hypervariable regions varies. The allosteric region is involved in RAS dimerization, which is thought be important in activation of the MAP kinase pathway. Post translational modification of the hypervariable region targets RAS to the cell membrane (Spencer-Smith and O’Bryan, 2017). The majority of mutations in HRAS, KRAS and NRAS occur at residues G12, G13 and Q61. These residues are in the RAS G domain which binds to GTP. This domain is also responsible for the hydrolysis of GTP to GDP. Mutations at residues G12, G13 and Q61 disrupt this GTPase activity which results in constitutively active RAS (Hobbs et al., 2016). Mutations in the RAS family of GTPases occur in many cancers including pancreatic, colorectal and lung adenocarcinomas and multiple myeloma (Scott et al., 2016). Robertson et al. examined a cohort of 412 MIBCs and found that HRAS, KRAS and NRAS were mutated in 5, 4 and 2% of tumours respectively (Robertson et al., 2017). KRAS and NRAS mutations are known to occur in colorectal cancer patients as a mechanism of intrinsic and acquired resistance to EGFR-targeted therapy (Sforza et al., 2016; Van Emburgh et al., 2016). Bockorny et al. reported that the long-term culture of the FGFR1-amplified, NSCLC cell line NCI-H2077 produced a resistant derivative with an NRAS Q61R mutation. Treatment of the resistant cells with BGJ398 and the MEK inhibitor trametinib reduced cell proliferation to a greater extent than treatment with either BGJ398 as a single agent. It was observed that the combination of BGJ398 and trametinib was well tolerated, significantly slowed tumour progression and increased progression-free survival in a mouse NCI-H2077 xenograft model (Bockorny et al., 2018).
Development of a potent small molecule inhibitor which inhibits RAS for the treatment of RAS mutant cancers has so far been unsuccessful. Attempted methods of inhibition have included inhibiting the lipid modification of RAS to prevent the translocation of RAS to the membrane and preventing the exchange of RAS-bound GDP for GTP via inhibition of guanine nucleotide exchange factor interaction with RAS (Scott et al., 2016). Development of inhibitors which target molecules downstream of RAS has been more successful. For example, the MEK inhibitor trametinib has been approved by the FDA and EMA for the treatment of BRAF mutant melanoma and NSCLC (Cheng and Tian, 2017). Inhibitors of MEK, RAF, PI3 kinase, AKT and mTOR, have entered clinical trials in patients with RAS mutant tumours (Scott et al., 2016).

The likely mechanism of resistance in RT4 R1 is the activation of EGFR identified in Chapter 4. Therefore, examination of RT4 focused on identifying an EGFR mutation or amplification in this line. No EGFR mutation or amplification was found and so EGFR-upregulated expression and activation probably occurs by a non-genetic mechanism. Herrera-Abreu et al. reported that RT112 increased activation of EGFR as a mechanism of short-term survival to treatment with PD. They reported that PD treatment induced EGFR accumulation at the plasma membrane and the increased activation of EGFR in RT112 was as a result of reduced MAP kinase pathway repression (Herrera-Abreu et al., 2013).

In conclusion, of the genetic alterations identified in RT112 and RT4 resistant derivatives in this Chapter, the most likely to be inducing PD resistance are the gain of copy number of OSM family receptors in RT112 R1 and RT4 R1, the HRAS G12S mutation identified in RT112 R3 and the E349K ATF2 and Q898R MIB2 mutations identified in RT112 R1.

<table>
<thead>
<tr>
<th>RT112 R1</th>
<th>RT112 R3</th>
<th>RT4 R1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gain of 5p15.33–q11.1</td>
<td>HRAS G12S</td>
<td>Gain of 5p15.33–q11.1</td>
</tr>
<tr>
<td>ATF2 E349K</td>
<td>No EGFR amplification</td>
<td></td>
</tr>
<tr>
<td>MIB2 Q898R</td>
<td>No EGFR mutation</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.9 Key genetic alterations identified in RT112 and RT4 resistant derivatives. The rational as to why the ATF2, MIB2 and HRAS mutations and the gain of 5p15.33–q11.1 were thought to be the most likely genetic alterations to be inducing resistance is outlined in the Chapter 5 discussion.
Chapter 6
Expression analysis of Parental and Resistant lines

6.1 Introduction

In Chapter 5 a HRAS G12S mutation was identified as the probable determinant of resistance in RT112 R3. However, RT112 R1, RT112 R2 and RT4 R1 have most likely developed resistance to PD through non-genetic mechanisms. It is probable that RT4 R1 developed resistance due to the overexpression and phosphorylation of EGFR observed in Chapter 4 (Fig. 4.6). Genetic analysis in Chapter 5 uncovered no amplification or mutation of EGFR, indicating that this resistance is not caused by a genetic alteration to EGFR in RT4 R1. Culture of RT4 R1 without PD for 4 passages re-sensitized this line to PD (Chapter 3, Fig. 3.10). Therefore, RT4 R1 did not maintain an epigenetic state which allowed it to respond to PD treatment. Culture of RT4 R1 for a greater period of time in PD may have led to the resistant line acquiring an epigenetic state which enabled it to maintain its PD-resistant state.

A genetic mechanism of resistance in RT112 R1 was not identified in Chapter 5 through exome sequencing or copy number analysis. It was thought that as RT112 R1 and R2 are phenotypically similar, these lines may employ a similar method of resistance to PD. The resistance to PD in RT112 R1 and R2 may be induced by the increased phosphorylation of ERBB3 and MET observed in Chapter 4 (Fig. 4.8 and 4.11). RT112 R1 and R2 retained resistance despite reverting to a phenotype similar to parental RT112 following culture without PD for 4 passages (Fig. 3.9 and 3.5). Therefore, the gene expression profile of RT112 R1 no PD and R2 no PD could provide insight into the mechanism of PD resistance in RT112 R1 and R2. Expression analysis with DNA microarray was conducted to examine the following: 1) other possible determinants of resistance, 2) the potential downstream effect(s) of the HRAS G12S mutation on gene expression in RT112 R3 and 3) whether the resistant lines exhibit gene expression features associated with having undergone an EMT.

A well-reported mechanism of resistance to inhibition of a RTK is the activation of an alternative RTK. This was discussed in section 1.2.2 of the Introduction. Increased expression of a RTK in a resistant line cultured in PD may
indicate that the receptor is a possible mediator of resistance. Therefore, expression of RTKs will be examined in this Chapter. The EMT phenotype is of interest as a mesenchymal phenotype is associated with greater cell migration and invasion. Therefore, if FGFR inhibitors induce an EMT in patients this could lead to tumour metastasis (Singh et al., 2017).

Expression analysis was conducted with DNA microarrays. Gene expression changes could be induced due to culture in PD, could be ‘passenger’ gene expression changes which are present in cells with a selective advantage or could be caused by unintended experimental differences such as cell culture conditions. Once important gene expression changes had been identified, these changes were validated with qRT-PCR and immunoblotting.

6.2 Results

Expression analysis, with Affymetrix® GeneChip® Human Transcriptome Array 2.0, was conducted with the following experimental conditions in triplicate: RT112 Parental + PD 24 h, RT112 Parental no PD, RT112 R1 + PD, RT112 R1 no PD, RT112 R2 + PD, RT112 R2 no PD, RT112 R3 + PD, RT112 R3 no PD, RT4 Parental no PD, RT4 R1 + PD and RT4 R1 no PD. Resistant line ‘no PD’ samples were cultured out of drug for 4-6 passages. This was long enough for RT112 R1, RT112 R2 and RT4 R1 to regain a faster proliferation rate and morphology similar to their parental line, enabling the identification of non-transient gene expression changes induced by PD. Appendix D summarises the quality control and normalisation of microarray data. Principal component analysis (PCA) was conducted and lists of genes which had undergone a significant gene expression change (p<0.05, one-way analysis of variance (ANOVA), 2-fold change) between experimental conditions were generated. These gene lists (included as supplementary data) were analysed with MetaCore™ pathway analysis.

6.2.1 Principal component analysis (PCA)

PCA was conducted on the full RT112 microarray dataset to examine which RT112 samples had similar gene expression (Fig. 6.1). The repeats of each experimental condition clustered close together suggesting that none of the samples were anomalous. The three parental + PD replicates clustered separately from the other
samples. The R1 + PD and R2 + PD replicates formed a second cluster. A third cluster was formed containing the following samples: parental no PD, R1 no PD, R2 no PD, R3 + PD and R3 no PD. PCA1 separated parental + PD, R1 + PD and R2 + PD from the other samples. PCA2 separated parental + PD from R1 + PD and R2 + PD. This indicated that gene expression in RT112 parental + PD was distinct from expression in other experimental conditions. It also indicated that R1 + PD and R2 + PD had similar gene expression profiles to each other, distinct from the other experimental conditions. The remaining experimental conditions have similar gene expression. This analysis fits with RT112 R1 and R2 + PD exhibiting a mesenchymal morphology in contrast to the epithelial morphology of RT112 parental no PD, R1 no PD, R2 no PD R3 + PD and R3 no PD (Chapter 3, Fig. 3.4 and Fig. 3.5). It is logical that RT112 parental + PD formed a separate cluster as this line exhibited greater sensitivity to PD than the RT112 resistant derivatives in Chapter 3 (Fig. 3.8).

![Figure 6.1 Principal component analysis of RT112 expression data. This analysis was conducted in Affymetrix Expression Console™.](image)

PCA was conducted on the full RT4 microarray dataset (Fig. 6.2). The repeats of each experimental condition clustered close together suggesting that none of the samples were anomalous. Parental no PD, R1 + PD and R1 no PD replicates each separated into a different cluster. PCA1 separated parental no PD and R1 no PD from R1 + PD. PCA2 separated R1 no PD replicates from other samples. This indicates that the expression profile of each RT4 experimental condition was
distinct from the others. In PCA analysis the first component accounts for the greatest amount of variability in the data. Therefore, as RT4 parental and RT4 R1 no PD were separated from each other by PCA2 rather than PCA1, the expression in these two conditions was more similar to each other than the gene expression in RT4 R1 + PD. This fits with the observation that RT4 R1 cultured out of PD and RT4 parental had a similar morphology (Chapter 3, Fig. 3.6).

Figure 6.2 Principal component analysis of RT4 expression data. This analysis was conducted in Affymetrix Expression Console™.

6.2.2 Identification of significantly differentially expressed genes

Table 6.1 details the numbers of significantly differentially expressed probes for comparisons of RT112 experimental conditions. The threshold for significant differential expression was a greater than two-fold expression change and a p<0.05 in a one-way ANOVA. Additionally, a false-discovery rate p-value was calculated. The lists of the genes which were differentially expressed between experimental conditions are included as supplementary data. The greatest number of significantly differentially expressed probes was found for the comparisons of parental no PD vs parental + PD, R1 + PD, and R2 + PD and parental + PD vs R3 + PD (Table 6.1). This suggests that gene expression is most different between these experimental conditions. The comparisons of parental no PD vs R3 + PD, R1 no PD, R2 no PD and R3 no PD had the least number of significantly differentially expressed probes, each with less than 1500. This suggests that the gene expression of parental no PD
is most similar to R3 + PD and the resistant derivatives cultured out of PD. The results are concordant with the PCA analysis which found similar gene expression between RT112 parental no PD, R1 no PD, R2 no PD, R3 + PD and R3 no PD and found expression in Parental + PD, R1 + PD and R2 + PD to be distinct from expression in other experimental conditions.

Table 6.1 Number of probes which detected significantly different gene expression (ANOVA p<0.05, 2-fold) in comparisons of RT112 experimental conditions.
Identification of differentially expressed probes was conducted with the Affymetrix Transcriptome Analysis Console.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Number of probes increased in 1st condition</th>
<th>Number of probes increased in 2nd condition</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental + PD vs Parental No PD</td>
<td>1728</td>
<td>1928</td>
<td>3716</td>
</tr>
<tr>
<td>Parental No PD vs R1 + PD</td>
<td>1910</td>
<td>1861</td>
<td>3771</td>
</tr>
<tr>
<td>Parental No PD vs R2 + PD</td>
<td>1706</td>
<td>1962</td>
<td>3668</td>
</tr>
<tr>
<td>Parental No PD vs R3 + PD</td>
<td>639</td>
<td>621</td>
<td>1260</td>
</tr>
<tr>
<td>Parental + PD vs R1 + PD</td>
<td>1255</td>
<td>1444</td>
<td>2699</td>
</tr>
<tr>
<td>Parental + PD vs R2 + PD</td>
<td>1428</td>
<td>1229</td>
<td>2657</td>
</tr>
<tr>
<td>Parental + PD vs R3 + PD</td>
<td>1983</td>
<td>1811</td>
<td>3795</td>
</tr>
<tr>
<td>R1 No PD vs Parental No PD</td>
<td>289</td>
<td>624</td>
<td>913</td>
</tr>
<tr>
<td>R2 No PD vs Parental No PD</td>
<td>726</td>
<td>772</td>
<td>1498</td>
</tr>
<tr>
<td>R3 No PD vs Parental No PD</td>
<td>467</td>
<td>349</td>
<td>815</td>
</tr>
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</table>

Table 6.2 details the numbers of significantly differentially expressed probes for comparisons of RT4 experimental conditions. As with the analysis of RT112, the threshold for significant differential expression was a greater than two-fold expression change and a p-value <0.05 in one-way ANOVA. The number of significantly differentially expressed probes was greatest in the comparison of R1 + PD vs Parental no PD followed by R1 + PD vs R1 no PD and finally R1 no PD vs Parental no PD. This is concordant with the PCA analysis which found RT4 R1 + PD to be separated from parental no PD and R1 no PD by PCA1. Lists of differentially expressed gene lists are included as supplementary data.
Table 6.2 Number of probes which detected significantly different gene expression (ANOVA p<0.05, 2-fold) in comparisons of RT4 experimental conditions. Identification of differentially expressed probes was conducted with the Affymetrix Transcriptome Analysis Console.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Number of probes increased in 1st condition</th>
<th>Number of probes increased in 2nd condition</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 + PD vs Parental No PD</td>
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<td>1251</td>
<td>2100</td>
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<tr>
<td>R1 + PD vs R1 No PD</td>
<td>774</td>
<td>1022</td>
<td>1796</td>
</tr>
<tr>
<td>R1 No PD vs Parental No PD</td>
<td>366</td>
<td>494</td>
<td>860</td>
</tr>
</tbody>
</table>

6.2.3 Hierarchical cluster analysis of significantly differentially expressed genes

Hierarchical cluster analysis enables the visualisation of expression data from multiple probes and microarray samples and indicates which samples have a similar gene expression profile. Hierarchical cluster analysis was performed for probes significantly differentially expressed between RT112 parental no PD samples and the other RT112 experimental conditions (one-way ANOVA p<0.01, 2-fold) (Fig. 6.3). The samples separated into two clusters, one containing parental + PD, R1 + PD and R2 + PD and a second containing all other experimental conditions. The cluster containing parental + PD, R1 + PD and R2 + PD consisted of 2 smaller clusters: one containing parental + PD samples and another containing R1 + PD and R2 + PD samples, indicating that R1 + PD and R2 + PD have more similar gene expression to each other than to parental + PD. This fits well with the results of the RT112 PCA (Fig. 6.1).

Hierarchical clustering was performed for probes differentially expressed in RT4 experimental conditions (Fig. 6.4). Parental no PD and R1 no PD samples clustered together whilst R1 + PD samples formed a separate cluster. This fits well with the results of the RT4 PCA (Fig. 6.2).

6.2.4 Identification of differentially expressed pathways with MetaCore™

The lists of genes which were significantly differentially expressed between RT112 experimental conditions (ANOVA p<0.05, 2-fold) were analysed with MetaCore™. The comparison of RT112 parental + PD versus parental no PD was conducted to examine the effect of acute treatment on RT112 parental. Eight of the ten most significantly differentially expressed pathway maps for the comparison of RT112
parental + PD versus parental no PD relate to the cell cycle (Fig. 6.5). This reflects the slow growth of parental RT112 when acutely treated with PD, as observed during the derivation of the RT112 resistant lines (described in Chapter 3). The other pathways in the top ten significantly differentially expressed pathways are ‘ATM/ATR regulation of G1/S checkpoint’ and ‘Sirtuin 6 regulation and functions’. ATM and ATR kinases are activated by DNA damage inducing the phosphorylation of targets involved in DNA repair, cell cycle arrest and apoptosis (Maréchal and Zou, 2013). Genes differentially expressed in the ‘ATM/ATR regulation of G1/S checkpoint’ MetaCore™ pathway included ATR and genes involved in the regulation of cell proliferation, such as members of the cyclin family and MYC. Expression of these genes was lower in parental + PD. Sirtuin 6 activates PARP1 via mono-ADP ribosylation, stimulating repair of DNA double strand-breaks (Mao et al., 2011). Many of the genes differentially expressed in the ‘Sirtuin 6 regulation and functions’ MetaCore™ pathway are involved in the regulation of lipid metabolism such as FASN and SCD which encode fatty acid synthase and stearoyl-CoA desaturase-1, respectively.

MetaCore™ analysis was used to compare the gene expression of RT112 parental acutely treated with PD with the expression of resistant derivatives cultured in PD. These differences in gene expression could be critical to the PD-resistant phenotype of the derivatives. Pathway maps related to cytoskeletal remodelling, EMT and TGF signalling pathways were differentially expressed in RT112 R1 and R2 + PD compared to parental + PD (Fig. 6.6 and Fig. 6.7). This reflects the fact that RT112 R1 and R2 have a mesenchymal morphology in contrast to the epithelial morphology of the parental line (Chapter 3, Fig. 3.4).

Eight of the ten most significantly differentially expressed pathway maps for the comparison of RT112 R3 + PD versus parental + PD relate to the cell cycle (Fig. 6.8). This reflects that RT112 R3 is the most PD-resistant RT112 derivative whilst RT112 parental is sensitive to PD (Chapter 3, Fig. 3.8). The other pathways in the top ten significantly differentially expressed pathways are ‘ATM/ATR regulation of G1/S checkpoint’ and ‘BRCA1 as a transcription factor’. Many of the genes differentially expressed in the ‘ATM/ATR regulation of G1/S checkpoint’ MetaCore™ pathway are involved in the regulation of cell proliferation, such as members of the cyclin family and MYC. Expression of these genes was higher in R3 + PD. Genes differentially expressed in the pathway ‘BRCA1 as a transcription factor’ included ATM, ATR, BRCA1 and MYC. Expression of BRCA1 and ATR was higher in R3 + PD, expression of ATM was higher in parental + PD. BRCA1 is a
tumour suppressor which is involved in the homologous recombination of DNA double strand breaks (Scully et al., 1997).

Figure 6.3 Hierarchical clustering of significantly differentially expressed genes between RT112 parental No PD and other RT112 experimental conditions (one-way ANOVA p<0.01, 2-fold change). The log₂ gene expression was standardised between 0 and 1. The expression profiles of samples and genes were clustered using Euclidean distance and complete linkage. Standardisation and hierarchical cluster analysis were conducted in Partek® Genomics Suite® 6.6. Scale bar indicates the normalised log₂ gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).
Figure 6.4 Hierarchical cluster analysis of significantly differentially expressed genes between RT4 experimental conditions (one-way ANOVA p<0.05, 2-fold change). The Log₂ gene expression was standardised between 0 and 1. The expression profiles of samples and genes were clustered using Euclidean distance and complete linkage. Standardisation and hierarchical cluster analysis were conducted in Partek® Genomics Suite® 6.6. Scale bar indicates the normalised Log₂ gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).
Figure 6.5 The 10 most significantly differentially expressed MetaCore™ pathway maps for RT112 parental + PD vs parental no PD.

Figure 6.6 The 10 most significantly differentially expressed MetaCore™ pathway maps for RT112 R1 + PD vs parental + PD.
Figure 6.7 The 10 most significantly differentially expressed MetaCore™ pathway maps for RT112 R2 + PD vs parental + PD.

Figure 6.8 The 10 most significantly differentially expressed MetaCore™ pathway maps for RT112 R3 + PD vs parental + PD.
The resistant derivatives retained their resistance following culture without PD for 4 passages whilst exhibiting a morphology similar to the parental line. Therefore, the genes differentially expressed between RT112 parental no PD and R1, R2 and R3 no PD could be causative of this poised resistant state. MetaCore™ analysis was used to compare the gene expression of RT112 parental not cultured in PD with that of the resistant derivatives not cultured in PD. Pathway maps relating to OSM signalling are among the top ten most significantly differentially expressed pathways for R1 and R2 no PD vs parental no PD but not for R3 no PD vs parental no PD (Fig. 6.9, 6.10 and 6.11). The pathway map ‘regulation of RAC1 activity’ is differentially expressed in R3 no PD vs parental no PD. Rho-like GTPases such as RAC1 regulate EMT, cell migration and cell proliferation (Ungefroren et al., 2017). The most significantly differentially expressed MetaCore™ pathway maps for the comparisons RT112 R1 no PD and parental + PD, R2 no PD and parental + PD and R3 no PD and parental + PD are detailed in Figures D.6, D.7 and D.8, respectively, in Appendix D.

Figure 6.9 The 10 most significantly differentially expressed MetaCore™ pathway maps between RT112 R1 no PD vs parental no PD.
Figure 6.10 The 10 most significantly differentially expressed MetaCore™ pathway maps for RT112 R2 no PD vs parental no PD.

Figure 6.11 The 10 most significantly differentially expressed MetaCore™ pathway maps for RT112 R3 no PD vs parental no PD.
As observed for the MetaCore™ comparisons of RT112 resistant lines with RT112 parental no PD, pathways relating to EMT and cell adhesion were significantly differentially expressed for RT4 R1 + PD vs RT4 parental no PD (Fig. 6.12). The most significantly enriched pathway map for this comparison is ‘ENaC regulation in normal and CF airways’. Genes differentially expressed in this pathway include SCNN1A and SCNN1B, which encode subunits of the epithelial sodium channel ENaC. Expression of these genes was higher in parental no PD. The ENaC sodium channel permits the diffusion of sodium ions across the apical membrane of epithelial cells. ENaC is an important mediator of homeostasis of electrolytes and water. Genes in the ‘immune response IFN-alpha/beta signaling via JAK/STAT’ pathway map which were differentially expressed included interferon inducible proteins and guanylate binding proteins regulated by STAT signalling. Interferons are antiviral cytokines produced in response to cellular stress which reduce proliferation and induce apoptosis in cancer cells (Snell et al., 2017). Differential expression of other pathways in the list of the top 10 most differentially expressed pathway maps, such as ‘effect of H. pylori infection on gastric epithelial cell proliferation’ and ‘B cell antigen pathway’ appears to reflect differential regulation of cell proliferation between RT4 R1 + PD and parental no PD.

Figure 6.12 The 10 most significantly differentially expressed MetaCore™ pathway maps for RT4 R1 + PD vs parental no PD.
RT4 R1 no PD did not retain resistance to PD following culture without PD for 4 passages. Therefore, the gene expression in this experimental condition did not reflect a resistant state although persistent gene expression changes caused by FGFR inhibition may be present in this line. Increased expression of IL-1 alpha and beta in R1 no PD features in a number of pathways for the comparison of RT4 R1 no PD vs parental no PD (Fig. 6.13). The IL-1 family of cytokines are pleiotropic, can induce migration, apoptosis and proliferation and are pro-inflammatory (Striz, 2017). For the comparison of RT4 R1 no PD vs parental no PD (Fig. 6.13) and RT4 R1 + PD vs RT4 parental no PD (Fig. 6.14) there was differential expression of the ‘immune response IFN-alpha/beta signaling via JAK/STAT’ pathway map.

For the comparison of RT4 R1 + PD vs R1 no PD the significantly-enriched pathways relate to the cell cycle, which is logical due to the faster growth rate of R1 no PD, interferon signalling and the hippo pathway (Fig. D.9).

6.2.5 Oncostatin M signalling and downstream pathways

MetaCore™ pathway analysis showed the pathway map ‘Oncostatin M signalling via MAPK in human cells’ to be in the top 10 differentially expressed pathways for the RT112 comparisons: Parental no PD vs R1 no PD (Fig. 6.9), Parental no PD vs R2 no PD (Fig. 6.10), Parental no PD vs RT112 R1 + PD (Fig. D.6) and Parental no PD vs RT112 R2 + PD (Fig. D.7). Additionally, copy number analysis showed that RT112 R1 has gain of 5p15.33 - q11.1 which contains LIFR and OSMR and gain of 5q11.1 - q11.2 which contains IL6ST compared to RT112 parental (Chapter 5, Fig. 5.2, Table 5.2). Therefore, it was considered that signalling via the Oncostatin M (OSM) pathway could be inducing resistance to PD in RT112 R1 and RT112 R2. Signalling downstream of OSM is summarised in Fig. 6.15. OSM binds the receptor glycoprotein 130 (gp130), and then either the Oncostatin M receptor (OSMR) or Leukaemia inhibitory factor receptor (LIFR) are recruited to the complex. The OSM pathway primarily signals via the Janus kinase (JAK) family of non-receptor tyrosine kinases which phosphorylate and activate the signal transducer and activator of transcription (STAT) family of transcription factors. This is known as the JAK/STAT pathway. OSM signalling also activates the MAPK pathway, JNK, the AKT/PI3K pathway and PKCδ. Activation of these pathways could contribute to the resistant phenotype of these cells.
Figure 6.13 The 10 most significantly differentially expressed MetaCore™ pathway maps for RT4 R1 no PD vs parental no PD.

Figure 6.14 The 10 most significantly differentially expressed MetaCore™ pathway maps for RT4 R1 + PD and R1 no PD.
Figure 6.15 Signalling pathways activated by OSM. Orange denotes the MAP kinase pathway, magenta denotes the JAK/STAT pathway, green denotes the PI3 kinase pathway. Blue denotes the gp130 and OSMR/LIFRβ heterodimer and yellow denotes OSM. ‘P’s represent the intracellular phosphorylation of gp130 and OSMR/LIFRβ. Image adapted from Hermanns et al, 2015.

*IL6ST, OSMR and LIFR*, which encode receptors in the OSM signalling pathway, were amongst the genes in the MetaCore™ pathway ‘Oncostatin M signalling via MAPK in human cells’ which were differentially expressed in RT112 R1 + PD and RT112 R2 + PD compared to RT112 parental no PD. The *IL6ST* gene encodes the receptor glycoprotein 130 (gp130). Microarray analysis found that *IL6ST* expression was highest in R1 + PD and R2 + PD where it was approximately 20-fold and 9-fold higher respectively than in RT112 parental no PD (Fig. 6.16). qRT-PCR analysis of *IL6ST* confirmed that expression was significantly increased in R1 + PD and R1 no PD (Fig. 6.16). Microarray analysis showed that *OSMR* expression was significantly increased in R1 + PD, R1 no PD, R2 + PD and R2 no PD and significantly decreased in parental + PD compared to parental no PD (Fig. 6.17). qRT-PCR analysis confirmed the increase in *OSMR* expression in R1 + PD and R1 no PD (Fig. 6.17). Microarray analysis showed that *LIFR* expression was approximately 3, 5 and 2-fold higher in R1 + PD, R2 + PD and R3 + PD respectively than in parental no PD (Fig. 6.18). qRT-PCR confirmed that *LIFR* was significantly increased in R1 + PD and also showed that expression was significantly increased in R1 no PD (Fig. 6.18). The observed increase in expression of *IL6ST, OSMR* and *LIFR* could
be mediating an increase in activation of the OSM signalling pathway in RT112 resistant lines. The RT112 R1 5p15.33 - q11.1 and 5q11.1 - q11.2 gains may be mediating the increase in expression of *IL6ST, OSMR* and *LIFR* observed in RT112 R1 no PD compared to parental no PD. However, the 5p gains cannot account for the increased expression of *IL6ST, OSMR* and *LIFR* in RT112 R1 + PD compared to R1 no PD.

**Figure 6.16 Expression of *IL6ST* in RT112 determined by microarray and qRT-PCR analysis.** A) microarray analysis. B) qRT-PCR analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. For microarray: asterisks indicate the experimental conditions in which *IL6ST* is differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For qRT-PCR: asterisks indicate the experimental conditions in which *IL6ST* is differentially expressed compared to parental no PD (Mann-Whitney U, 2-tailed test, p<0.05).

**Figure 6.17 Expression of *OSMR* in RT112 determined by microarray and qRT-PCR analysis.** A) microarray analysis. B) qRT-PCR analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which *OSMR* was differentially expressed compared to parental no PD. Microarray statistical test: ANOVA p<0.05, 2-fold expression change. qRT-PCR statistical test: Mann-Whitney U 2-tailed test, p<0.05.
Figure 6.18 Expression of LIFR in RT112 determined by microarray and qRT-PCR analysis. A) microarray analysis. B) qRT-PCR analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which LIFR was differentially expressed compared to parental no PD. Microarray statistical test: ANOVA p<0.05, 2-fold expression change. qRT-PCR statistical test: Mann-Whitney U 2-tailed test, p<0.05.

Microarray analysis showed that the OSM signalling pathway ligands OSM and IL31 were not significantly differentially expressed between RT112 parental no PD and the other RT112 experimental conditions (Appendix D, Fig. D.10). Therefore, if activation of the OSM signalling pathway is increased in RT112 it is unlikely to be due to increased presence of ligand.

In RT4, MetaCore™ pathway analysis did not suggest that the OSM signalling pathway was differentially expressed in RT4 parental and RT4 R1 (Fig. 6.13, 6.14 and D.9). However, as this pathway had been identified as a possible mediator of resistance to PD in RT112 R1 and R2, it was examined in RT4. Additionally, copy number analysis showed that RT4 R1 had copy number gain of the region 5p15.33 - q11.1 which contains LIFR and OSMR and loss of the region 5q11.1 - q35.3 which contains IL6ST. IL6ST, OSMR and LIFR were not significantly differentially expressed in RT4 R1 + PD compared to RT4 parental no PD (Appendix D, Fig. D.11). OSM and IL31 were not significantly differentially expressed between RT4 experimental conditions (Appendix D, Fig. D.12). Therefore, differential expression of these receptors or ligands is unlikely to be contributing to resistance in RT4 R1 and the copy number alterations on chromosome 5 observed in RT4 R1 are not significantly altering expression of IL6ST, OSMR and LIFR.

As previously stated, OSM signals primarily via the JAK/STAT pathway (Fig. 6.15). For this reason, it was decided to examine the microarray data for expression of the STATs in RT112 experimental conditions (Fig. 6.19). Expression of STAT1,
STAT3 and, to a lesser extent, STAT5B was increased in RT112 R1 + PD and R2 + PD compared to RT112 parental no PD. STAT5A expression was similar between the RT112 experimental conditions. The increase in STAT3 expression in R1 + PD was validated by qRT-PCR (Fig. 6.19).

Phosphorylation of STAT family members induces the translocation of these molecules to the nucleus where they carry out their function as transcription factors (Hermanns, 2015). For this reason, phosphorylation of STAT1 and STAT3 was examined in RT112 parental and resistant lines (Fig. 6.20 and 6.21). A431 treated with EGF was selected as a positive control for STAT1 and STAT3 as previous research has shown that A431 treated with EGF expresses total and phosphorylated STAT1 and STAT3 (Grudinkin et al., 2007). Phosphorylated STAT1 and STAT3 could not be detected by immunoblot in any RT112 samples. Therefore, STAT1 and STAT3 are not mediating resistance in RT112 resistant lines. As the STAT family of transcription factors are commonly activated when OSM and OSMR signalling is active, it is unlikely that the increased expression of OSMR, LIFR and IL6ST is inducing resistance in RT112 R1 and RT112 R2 via STAT activation. However, it is possible that OSMR, LIFR and IL6ST are mediating resistance via activation of the MAP kinase pathway without activating the STAT family of transcription factors. Phosphorylation of STAT1 or STAT3 was not examined in RT4 as the microarray data indicated that there was no significant increase in OSM, IL31, IL6ST, OSMR, LIFR, STAT1, STAT3, STAT5A or STAT5B expression in RT4 R1 compared to parental.

As previously stated, OSM signalling can activate the MAPK pathway (Fig. 6.15) which regulates the activity of the activator protein 1 (AP-1) transcription factor. Microarray analysis indicated that expression of JUN, which encodes AP-1 family member JUN, was significantly increased in RT112 R1 + PD, R2 + PD and R2 no PD compared to RT112 parental (Fig. 6.22). This increase was confirmed for R1 + PD and R1 no PD by qRT-PCR. Jun can homodimerize or heterodimerize with other AP-1 family members to form the AP-1 transcription factor. Jun is a positive regulator of cell proliferation (Shaulian, 2010). A key difference between RT112 parental acutely treated with PD and the RT112 resistant lines is the maintenance of ERK phosphorylation in the resistant lines (Chapter 4, Fig. 4.12). OSM pathway signalling could be activating AP-1 via the MAPK pathway in RT112 R1 and R2. JUN expression was also significantly increased in RT4 R1 + PD and non-significantly increased in R1 no PD compared to parental no PD (Fig. 6.22). This may induce cell proliferation in RT4 R1 + PD. JUN protein expression was not examined due to time limitations.
Expression of STATs in RT112 determined by microarray and qRT-PCR analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the probe and experimental condition for which significantly different expression was detected compared to parental no PD. Microarray statistical test: ANOVA p<0.05, 2-fold expression change. qRT-PCR statistical test: Mann-Whitney U 2-tailed test, p<0.05. For genes with specificity to more than one probe, all probes are shown with a legend indicating the probe’s Affymetrix IDs.
Figure 6.20 Immunoblot analysis of phospho-STAT1 and total STAT1 protein expression in RT112. Expression was examined in parental, parental treated with PD173074 for 24 h, R1, R2 and R3 cultured with PD173074 and R1, R2 and R3 cultured without PD173074 for 4 passages. A431 + EGF was included as a positive control for phospho-STAT1 and total STAT1 and β-actin was used as a loading control.

Figure 6.21 Immunoblot analysis of phospho-STAT3 and total STAT3 protein expression in RT112. Expression was examined in parental, parental treated with PD173074 for 24 h, R1, R2 and R3 cultured with PD173074 and R1, R2 and R3 cultured without PD173074 for 4 passages. A431 + EGF was included as a positive control for phospho-STAT3 and total STAT3 and β-actin was used as a loading control.
Figure 6.22 Expression of JUN in RT112 and RT4 determined by microarray and qRT-PCR analysis. A) microarray analysis of JUN in RT112. B) qRT-PCR analysis of JUN in RT112. C) microarray analysis of JUN in RT4. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. For microarray and qRT-PCR, asterisks indicate the experimental conditions in which JUN was differentially expressed compared to parental no PD. Microarray statistical test: ANOVA p<0.05, 2-fold expression change. qRT-PCR statistical test: Mann-Whitney U 2-tailed test, p<0.05.

6.2.6 Expression of regulators of lipid homeostasis

The regulation of fatty acid synthesis by FGFR3 has been studied in RT112. Du et al. reported that FGFR3 knockdown in RT112 reduced expression of genes involved in fatty acid and sterol biosynthesis and metabolism (Du et al., 2012). The transcription factors SREBP1 and SREBP2, encoded by the genes SREBF1 and SREBF2, preferentially regulate synthesis of unsaturated fatty acids and cholesterol, respectively (Hagen et al., 2010). In RT112, microarray analysis showed that expression of SREBF1 was significantly differentially downregulated in parental + PD, R1 + PD, R2 + PD and R2 no PD compared to parental no PD. Additionally, SREBF1 was non-significantly reduced in R1 no PD, R3 + PD and R3 no PD. SREBF2 was not significantly differentially expressed in the other RT112
experimental conditions compared to parental no PD (Fig. 6.23). qRT-PCR validation found significantly reduced expression of SREBF1 in parental + PD, R1 + PD, R1 no PD, R2 + PD and R2 no PD compared to parental no PD (Fig. 6.24). In contrast to the microarray analysis, qRT-PCR analysis indicated that SREBF1 expression in R3 no PD was significantly increased (Fig. 6.24). Therefore, the synthesis of fatty acids could be reduced in RT112 parental + PD and in RT112 resistant lines. Cholesterol synthesis may also be reduced despite the reduction in SREBF2 expression not being statistically significant.

Full-length SREBP1 and SREBP2 are endoplasmic reticulum-bound precursor proteins which are cleaved to produce their mature form (Hagen et al., 2010). Immunoblot analysis showed that expression of mature SREBP1 was reduced in RT112 parental + PD, R1 + PD, R2 + PD, R3 + PD and R3 no PD (Fig. 6.25). Stearoyl-CoA desaturase-1 (SCD1) is the rate-limiting enzyme in the production of mono-unsaturated fatty acids from saturated fatty acids and increases lipogenesis (Igal, 2016). Immunoblot analysis showed that SCD1 protein expression was highest in RT112 parental no PD and R2 no PD, lower in RT112 R1 no PD, R3 + PD and R3 no PD and lowest in RT112 parental + PD, R1 + PD and R2 + PD (Fig. 6.25). These results suggest there is a reduction in mono-unsaturated fatty acid synthesis and lipogenesis in RT112 parental and the RT112 resistant derivatives when cultured in PD. Figure 6.26 illustrates the effects that SREBP1 induces on the synthesis of fatty acids.
Figure 6.24 qRT-PCR validation of *SREBF1* in RT112. A) parental + PD. B) R1. C) R2. D) R3. Error bars indicate standard error of the mean. Signal intensity is given relative to RT112 no PD. Asterisks indicate the experimental conditions in which *SREBF1* was differentially expressed compared to parental no PD. qRT-PCR statistical test: Mann-Whitney U 2-tailed test, p<0.05.
Figure 6.25 Immunoblot analysis of full length SREBP1, mature SREBP1 and SCD1 protein expression in RT112. Expression was examined in parental, parental treated with PD173074 for 24 h, R1, R2 and R3 cultured with PD173074 and R1, R2 and R3 cultured without PD173074 for 4 passages. β-actin was used as a loading control. Image Lab software was used to determine the intensity of the β-actin, mature SREBP1 and SCD1 protein bands and normalise mature SREBP1 and SCD1 expression relative to β-actin. Mature SREBP1 and SCD1 expression were quantified relative to RT112 parental no PD.
Du et al. reported a set of 33 genes involved in fatty acid and sterol biosynthesis and metabolism that were downregulated upon knockdown of FGFR3 in RT112 (Du et al., 2012). Unsupervised hierarchical cluster analysis was performed in RT112 microarray samples for this set of genes (Fig. 6.27). Expression of this cohort of genes was predominantly reduced in RT112 + PD compared to RT112 no PD. A cluster with generally low expression of these genes contained the samples RT112 R1 + PD, R2 + PD and parental + PD. A second cluster was formed containing the samples RT112 parental no PD, R3 + PD, R1 no PD, R2 no PD and R3 no PD with predominantly high expression of this cohort of genes. Overall, the differential expression of this cohort of genes indicated that fatty acid and sterol biosynthesis and metabolism was likely to be reduced in RT112 parental + PD, RT112 R1 + PD and RT112 R2 + PD compared to the other RT112 experimental conditions. Therefore, the mechanism of resistance to PD in RT112 R1 and R2 did not re-establish the expression of fatty acid and sterol biosynthesis genes whereas the mechanism of resistance in RT112 R3, the HRAS G12S mutation, did. However, the mutant HRAS in RT112 R3 did not activate fatty acid synthesis to the same extent as the signalling via FGFR3-TACC3 and wildtype FGFR3 in parental no PD. Du et al. observed that treatment with a MEK inhibitor reduced FGF1-induced expression of mature SREBP1 (Du et al., 2012). Therefore, it is logical to suggest that the activation of the MAP kinase pathway via the acquisition of a HRAS G12S mutation in RT112 R3 induced the maintenance of fatty acid and sterol biosynthesis genes expression in the presence of PD.
Figure 6.27 Unsupervised hierarchical cluster analysis of Du et al. cohort of fatty acid and sterol biosynthesis and metabolism genes in RT112 microarray samples. The log2 gene expression was normalised by dividing each expression value by the probe’s mean log2 gene expression. Following log2 gene expression normalisation, the expression profiles of samples and genes were clustered in Partek® Genomics Suite® 6.6 using Euclidean distance and complete linkage. Scale bar indicates the normalised log2 gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).
Immunoblot analysis of FGFR3 conducted in RT112 in Chapter 3 (Chapter 3, Fig. 3.11) found reduction in FGFR3 expression in the RT112 resistant lines. Examination of phospho-FGFR3 expression via immunoprecipitation was unsuccessful. A reduction in FGFR3 expression in RT112 R1 + PD, R1 no PD, R2 + PD, R2 no PD, R3 + PD and R3 no PD compared to parental no PD was observed with microarray analysis and qRT-PCR analysis (Fig. 6.28). In contrast to the immunoblot and microarray analysis, qRT-PCR showed a significant increase in FGFR3 expression in parental + PD (Fig. 6.28). The low expression of FGFR3 in R3 + PD indicates that R3 maintains expression of the Du et al. cohort of genes when cultured in PD by a mechanism other than signalling via FGFR3.

As was observed for RT112 experimental conditions, genes involved in lipid homeostasis were differentially expressed between RT4 experimental conditions (Supplementary data, 2-fold change lists). Microarray analysis showed that expression of SREBF1 and SREBF2 was non-significantly reduced in RT4 R1 compared to parental no PD. qRT-PCR analysis found that SREBF1 mRNA was significantly downregulated in RT4 R1 + PD and R1 no PD (Fig. 6.29).

Hierarchical cluster analysis was performed in RT4 microarray samples for the Du et al. cohort of fatty acid and lipid synthesis genes (Fig. 6.30). One RT4 parental no PD sample did not cluster with any of the other RT4 samples. This sample passed the microarray quality control (Appendix D, Fig. D.1, D.2 and D.3), however, in the PCA analysis this sample was separate from the other two parental no PD samples (Fig. 6.2). The remaining two RT4 parental no PD replicates and R1 no PD samples formed a cluster characterised by a predominantly high expression of this gene cohort relative to the other RT4 experimental conditions. R1 + PD samples formed a cluster of samples with predominantly low expression of this gene cohort. Expression of some genes was maintained or upregulated in RT4 R1 + PD relative to parental no PD (FGFR3, PDSS1, HMGCR, ELOVL5, SDR16C5, ACER3 and PCSK9). Therefore, RT4 R1 + PD may maintain some fatty acid and sterol biosynthesis and metabolism. However, as expression of this cohort of genes was predominantly reduced in RT4 R1 + PD, including SCD which encodes SCD1, this suggests that fatty acid and sterol biosynthesis and metabolism was reduced in RT4 R1 + PD.
Figure 6.28 Expression of FGFR3 in RT112 determined by microarray and qRT-PCR analysis. A) microarray analysis in all RT112 experimental conditions. B) qRT-PCR analysis in parental. C) qRT-PCR analysis in R1. D) qRT-PCR analysis in R2. E) qRT-PCR analysis in R3. Error bars indicate standard error of the mean. Signal intensity is given relative to RT112 no PD. For microarray and qRT-PCR, asterisks indicate the probe and experimental condition for which significantly different FGFR3 expression was detected compared to parental no PD. Microarray statistical test: ANOVA p<0.05, 2-fold expression change. qRT-PCR statistical test: Mann-Whitney U 2-tailed test, p<0.05. For genes with specificity to more than one probe, all probes are shown with a legend indicating the probe's Affymetrix IDs.
Immunoblot analysis in Chapter 3 found FGFR3 to be expressed at the same level in RT4 parental and R1 + PD (Fig. 3.13). Microarray analysis showed there was a non-significant, approximately 1.5-fold increase in FGFR3 expression in RT4 R1 + PD and a non-significant decrease in FGFR3 expression in R1 no PD (Fig. 6.31). qRT-PCR analysis showed that FGFR3 expression was significantly increased approximately 2-fold in R1 + PD and not significantly different in R1 no PD compared to parental no PD (Fig. 6.31). However, it is likely that RT4 R1 does not signal via FGFR3 when cultured in PD as activation of EGFR has been identified as a likely mechanism of resistance to PD in RT4 R1 (Chapter 4, Fig. 4.6).
Figure 6.30 Unsupervised hierarchical cluster analysis of Du et al. cohort of fatty acid and sterol biosynthesis and metabolism genes in RT4 microarray samples. The log₂ gene expression was normalised by dividing each expression value by the probes mean log₂ gene expression. Following log₂ gene expression normalisation, the expression profiles of samples and genes were clustered in Partek® Genomics Suite® 6.6 using Euclidean distance and complete linkage. Scale bar indicates the normalised log₂ gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).
Figure 6.31 Expression of FGFR3 in RT4 determined by microarray and qRT-PCR analysis. A) microarray analysis B) qRT-PCR analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which FGFR3 is differentially expressed compared to parental no PD. Microarray statistical test: ANOVA p<0.05, 2-fold expression change. qRT-PCR statistical test: Mann-Whitney U, 2-tailed test, p<0.05. For genes with specificity to more than one probe, all probes are shown with a legend indicating the probe's Affymetrix IDs.

6.2.7 Expression of epithelial-mesenchymal transition markers

MetaCore™ pathway analysis found EMT and cytoskeletal remodelling pathways to be differentially expressed between RT112 parental + PD and R1 + PD (Fig. 6.6) and R2 + PD (Fig. 6.7) and between parental no PD and R1 no PD (Fig. 6.9), R2 no PD (Fig. 6.10), R1 + PD (Appendix D, Fig. D.6), R2 + PD (Appendix D, Fig. D.7) and R3 + PD (Appendix D, Fig. D.8). It had previously been observed that RT112 R1 and R2 cultured in PD have a mesenchymal morphology (Chapter 3, Fig. 3.4).

Immunoblot analysis found that expression of the epithelial marker E-cadherin was constant between RT112 parental no PD, R1 + PD and R2 + PD and that N-cadherin expression was increased in RT112 R1 and R2 + PD, and to a lesser extent RT112 R3 + PD compared to RT112 parental no PD (Chapter 3, Fig. 3.12). Microarray analysis of expression of CDH1, which encodes E-cadherin, was concordant with this and showed that CDH1 was not significantly differentially expressed in any experimental condition compared to RT112 parental no PD (Fig. 6.32). Microarray analysis showed that expression of the CDH2 gene, which encodes N-cadherin, was increased in RT112 R1, R2 and R3 + PD, and by a smaller magnitude in RT112 R1 and R2 no PD compared to parental no PD. This increase was only significant for RT112 R2 + PD (Fig. 6.32). A small increase in protein expression of another mesenchymal marker, vimentin, was observed in RT112 R1 and R2 compared to the parental line (Chapter 3, Fig. 3.12). Concordant with this, a small but non-significant increase in expression of the gene that
encodes vimentin, *VIM*, was seen in RT112 parental, R1, R2 and R3 + PD compared to RT112 parental no PD (Fig. 6.32).

Microarray analysis showed expression of *FN1*, which encodes the mesenchymal marker fibronectin, was significantly increased in RT112 R1 + PD, R1 no PD and R2 + PD approximately 27-fold, 3-fold and 19-fold, respectively, compared to RT112 parental no PD. A smaller, non-significant increase in *FN1* expression was seen in R1 and R2 no PD and R3 + PD (Fig. 6.32). *SNAI2* encodes snail family transcriptional repressor 2 which promotes a mesenchymal phenotype (Alves *et al.*, 2009). Microarray analysis found *SNAI2* expression to be significantly increased in RT112 R1 + PD, R2 + PD, R2 no PD and R3 + PD compared to RT112 parental no PD (Fig. 6.32). *ELF5* encodes E74-like ETS transcription factor 5, which is reported to repress transcription of *SNAI2*, inhibiting EMT (Chakrabarti *et al.*, 2012). Expression of *ELF5* was significantly reduced in RT112 R1 + PD, R2 + PD, R2 no PD, R3 + PD and R3 no PD compared to RT112 parental no PD (Fig. 6.32). The expression changes observed in *FN1*, *SNAI2* and *ELF5* were not validated by qRT-PCR or immunoblot due to time limitations. The microarray analysis, in conjunction with the morphological and immunoblot analysis in Chapter 3 indicate that RT112 R1, R2 and possibly R3 have undergone a partial EMT during their derivation which is reversed upon culture out of PD for 4 passages.

MetaCore™ pathway analysis also showed that genes in the pathway map ‘regulation of EMT’ were differentially expressed between RT4 parental no PD and RT4 R1 + PD (Fig. 6.12). Immunoblot analysis conducted in Chapter 3 found that expression of N-cadherin and vimentin was low in RT4 parental and R1. E-cadherin expression was found to be increased in RT4 R1 compared to the parental line (Chapter 3, Fig. 3.13). Consistent with this, *CDH1*, *CDH2* and *VIM* were not significantly differentially expressed between parental no PD, R1 + PD and R1 no PD (Fig. 6.33). However, *FN1* expression was significantly increased by approximately 6 in R1 + PD and non-significantly increased by approximately 11-fold in R1 no PD (Fig. 6.33). A non-significant increase in *SNAI2* expression of approximately 2 and 1.5-fold was observed in RT4 R1 + PD and RT4 R1 no PD, respectively (Fig. 6.33). Expression of *ELF5* was non-significantly reduced in RT4 R1 no PD to approximately half the expression observed in RT4 parental no PD (Fig. 6.33). The microarray analysis, in conjunction with the morphological and immunoblot analysis in Chapter 3 suggests that RT4 R1 did not undergo an EMT during its derivation.
Figure 6.32 Expression of CDH1, CDH2, VIM, FN1, SNAI2 and ELF5 in RT112 determined by microarray analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which CDH1, CDH2, VIM, FN1, SNAI2 and ELF5 were differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change).
Figure 6.33 Expression of CDH1, CDH2, VIM, FN1, SNAI2 and ELF5 in RT4 determined by microarray analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which genes were differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change).

6.2.8 Expression of luminal cell markers in RT112

Bladder cancers can be classified into basal and luminal subtypes (Choi et al. 2014, 2014a; Dadhania et al., 2016; Warrick et al., 2016), as discussed in Chapter 1, section 1.1.2. It was observed that basal and luminal markers were differentially
expressed between RT112 parental no PD and RT112 R1 + PD, R2 + PD and R3 + PD (Supplementary data; 2-fold gene lists). Basal tumours are more aggressive than luminal tumours, although they are typically sensitive to chemotherapy (Dadhania et al., 2016). Choi et al. reported that p53-like subtype tumours were typically resistant to chemotherapy (Choi et al., 2014a). Both RT112 and RT4 have been previously classified as luminal (Warrick et al., 2016). If the resistant cells have undergone a switch from a luminal to a basal or p53-like subtype during their derivation, the resistant line could be more aggressive or could have reduced sensitivity to treatment. If FGFR inhibitors induce a similar switch subtype in patients, this could result in worse patient survival.

Unsupervised hierarchical cluster analysis was performed with RT112 microarray expression data for the luminal subtype markers reported by Choi et al. (Fig. 6.34). Compared to parental no PD, expression of ERBB2, ERBB3, FGFR3, GPX2 and CYP2J2 was increased in RT112 parental + PD. RT112 R1 + PD and R2 + PD had increased expression of ERBB2, ERBB3 and KRT7 and reduced expression of FGFR3, FOXA1, GPX2 and CYP2J2. Expression of the luminal markers ERBB2, ERBB3 and KRT7 was generally low in RT112 R1 no PD, R2 no PD, R3 no PD, and R3 + PD (Fig. 6.34). This analysis suggests that acute treatment with PD induced a more luminal phenotype in parental RT112. The expression of luminal markers in R1 and R2 culture in PD is more complex with expression of some markers increased whilst other markers exhibit decreased expression. The low expression of luminal markers in R1 and R2 when cultured out of PD suggests that these lines become less luminal upon the removal of PD.

In order to validate the changes in expression of luminal markers observed in the RT112 microarray data, qRT-PCR was performed using assays specific for a selected panel of these markers. Expression of the luminal marker KRT20 was non-significantly reduced in all RT112 microarray samples compared to parental no PD. qRT-PCR analysis showed that KRT20 expression was significantly reduced in R1 + PD and R1 no PD (Fig. 6.35).

Expression of the transcription factor PPARG, which promotes a luminal phenotype in MIBC, was non-significantly reduced in all RT112 microarray samples compared to parental no PD (Choi et al., 2014a). This reduction was found to be significant in R1 + PD and R1 no PD by qRT-PCR (Fig. 6.36). The transcription factors GATA3 and FOXA1 work with PPARG to promote a luminal phenotype in bladder cancer cell lines (Warrick et al., 2016). Microarray analysis showed GATA3 expression was significantly increased in R1 + PD compared to parental no PD.
The increase in R1 + PD GATA3 expression was confirmed by qRT-PCR and GATA3 expression in R1 no PD was also found to be significantly reduced (Fig. 6.36). Microarray analysis showed that expression of FOXA1 was reduced in RT112 R1 + PD, R1 no PD, R2 + PD, R2 no PD, R3 + PD and R3 no PD compared to RT112 parental no PD. This reduction was only significant in RT112 R1+ PD and R2 + PD. Reduced expression of this luminal marker was confirmed in RT112 R1 + PD and R1 no PD by qRT-PCR (Fig. 6.36).

Figure 6.34 Unsupervised hierarchical cluster analysis of Choi et al. cohort of luminal markers in RT112 microarray samples. The Log2 gene expression was normalised by dividing each expression value by the probe’s mean Log2 gene expression. Following Log2 gene expression normalisation, the expression profiles of samples and genes were clustered in Partek® Genomics Suite® 6.6 using Euclidean distance and complete linkage. Scale bar indicates the normalised Log2 gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).
Figure 6.35 Expression of KRT20 in RT112 determined by microarray and qRT-PCR analysis. A) microarray analysis. B) qRT-PCR analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. For microarray: asterisks indicate the experimental conditions in which KRT20 is differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For qRT-PCR: asterisks indicate the experimental conditions in which KRT20 is differentially expressed compared to parental no PD (Mann-Whitney U, 2-tailed test, p<0.05).

FGFR3 is a luminal marker (Choi et al., 2014). As discussed in section 6.2.6, microarray and qRT-PCR analysis showed that compared to RT112 parental no PD, expression of FGFR3 was increased in parental + PD and reduced in the resistant derivatives cultured with and without PD (Fig. 6.28). Microarray expression of ERBB3, another luminal marker, was significantly increased in RT112 parental + PD and non-significantly increased in RT112 R1 and R2 + PD compared to RT112 parental no PD. qRT-PCR showed ERBB3 to be significantly increased in RT112 parental + PD, R1 + PD, R1 no PD, R1 + PD and R2 + PD compared to RT112 parental no PD (Fig. 6.37). ERBB3 is a possible mediator of resistance in RT112 R1 and R2 as an increase in phospho-ERBB3 expression was seen in these resistant lines (Chapter 4, Fig. 4.8).

Uroplakins, encoded by UPK1A, UPK2, UPK3A and UPK3B, are luminal markers expressed at the apical surface of the urothelium (Hu et al., 2000). Microarray analysis showed that UPK1A expression was significantly increased in RT112 parental + PD, R1 + PD and R2 + PD and significantly reduced in R1 no PD, R2 no PD and R3 no PD (Fig. 6.38). UPK2 expression was significantly increased in RT112 parental + PD and R1 + PD and significantly decreased in R2 no PD. UPK3A expression was not differentially expressed and UPK3B expression was significantly decreased in R2 + PD and R2 no PD (Fig. 6.38). Microarray analysis found that expression of the transcription factor and luminal marker ELF3 (Bock et
al., 2014) was significantly increased in RT112 parental + PD and non-significantly reduced in R1 no PD and R2 no PD compared to parental no PD (Fig. 6.38).

**Figure 6.36** Expression of *PPARG, GATA3* and *FOXA1* in RT112 determined by microarray and qRT-PCR analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in the genes were differentially expressed compared to parental no PD. Microarray analysis: ANOVA p<0.05, 2-fold expression change. qRT-PCR analysis: Mann-Whitney U, 2-tailed test, p<0.05. For genes with specificity to more than one probe, all probes are shown with a legend indicating the probe’s Affymetrix IDs.
Figure 6.37 Expression of ERBB3 in RT112 determined by microarray and qRT-PCR analysis. A) microarray analysis in all RT112 experimental conditions. B) qRT-PCR analysis in parental. C) qRT-PCR analysis in R1. D) qRT-PCR analysis in R2. E) qRT-PCR analysis in R3. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the probe and experimental condition for which ERBB3 was significantly differentially expressed compared to parental no PD. Microarray statistical test: ANOVA p<0.05, 2-fold expression change. qRT-PCR statistical test: Mann-Whitney U 2-tailed test, p<0.05. For genes with specificity to more than one probe, all probes are shown with a legend indicating the probe’s Affymetrix IDs.
Figure 6.38 Expression of *UPK1A*, *UPK2*, *UPK3A*, *UPK3B*, and *ELF3* in RT112 determined by microarray analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to RT112 no PD. Asterisks indicate the experimental conditions in which genes were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe’s Affymetrix IDs.
The expression of luminal markers in R1 + PD and R2 + PD does not follow a set pattern with some markers exhibiting an increase in expression compared to parental no PD, whilst others exhibit a decrease or no change in expression. The microarray analysis suggests that luminal markers are not differentially expressed between R3 + PD and parental no PD. The expression of a number of luminal markers is significantly reduced in R1 no PD, R2 no PD and R3 no PD, supporting the hypothesis that expression of luminal markers is reduced upon culture of the resistant lines without PD.

The Choi et al. p53-like subtype of MIBCs had an activated wildtype TP53 gene expression signature (Choi et al., 2014). Unsupervised hierarchical cluster analysis was performed with RT112 microarray expression data for the p53-like subtype markers reported by Choi et al. These markers were not differentially expressed between RT112 experimental conditions (Appendix D, Fig. D.13). Therefore, it is unlikely that the RT112 resistant lines have adopted a p53-like phenotype.

6.2.9 Expression of basal cell markers in RT112

Unsupervised hierarchical cluster analysis performed with the basal subtype markers reported by Choi et al. showed that basal markers were differentially expressed in RT112 microarray samples (Fig. 6.39).

In order to validate the changes in basal marker expression observed in the microarray data, qRT-PCR was performed using assays specific for a selected panel of these markers. Microarray analysis found expression of KRT6C to be significantly reduced, by approximately 70%, in RT112 parental + PD and significantly increased approximately 13-fold in R1 no PD compared to parental no PD (Fig. 6.40). qRT-PCR analysis showed significantly higher KRT6C expression in R1 + PD and R1 no PD (Fig. 6.40). Microarray analysis showed expression of KRT5 was increased in RT112 R1 no PD compared to RT112 parental no PD (Fig. 6.40). The expression data generated from one microarray probe indicated that there was an approximate 6-fold significant increase whilst the second microarray probe indicated that there was an approximate 1.4-fold non-significant increase (Fig. 6.40). KRT5 expression was significantly reduced for at least one probe in RT112 parental + PD, R1 + PD, R3 + PD and R3 no PD compared to RT112 parental no PD. qRT-PCR analysis showed that KRT5 was significantly reduced in RT112 R1 + PD and significantly increased in R1 no PD (Fig. 6.40). Microarray analysis showed that expression of the basal marker KRT14, which is associated
with squamous differentiation in the bladder (Sjodahl et al., 2013), was non-significantly increased in RT112 R1 + PD, R1 no PD, R2 + PD and R2 no PD and non-significantly decreased in R3 + PD and R3 no PD compared to RT112 parental no PD (Fig. 6.40). qRT-PCR showed that there was a significant increase in KRT14 expression in RT112 R1 + PD and R1 no PD (Fig. 6.40).

Figure 6.39 Unsupervised hierarchical cluster analysis of Choi et al. cohort of basal markers in RT112 microarray samples. The Log2 gene expression was normalised by dividing each expression value by the probe’s mean Log2 gene expression. Following Log2 gene expression normalisation, the expression profiles of samples and genes were clustered in Partek® Genomics Suite® 6.6 using Euclidean distance and complete linkage. Scale bar indicates the normalised Log2 gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).
Figure 6.4 Expression of KRT6C and KRT5 in RT112 determined by microarray and qRT-PCR analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which genes were significantly differentially expressed compared to parental no PD. Microarray analysis statistical test: ANOVA p<0.05, 2-fold expression change. qRT-PCR statistical test: Mann-Whitney U, 2-tailed test, p<0.05. For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe's Affymetrix IDs.
Immunoblot analysis showed that expression of cytokeratin 5/6 was much lower in RT112 samples than in the positive control SCaBER. The sample with the highest expression of cytokeratin 5/6 was RT112 parental no PD (Fig. 6.41). Therefore, treatment with PD, or subsequent removal of PD, did not induce an increase in cytokeratin 5/6 protein expression. The discrepancy between the KRT5 and KRT6C mRNA and cytokeratin 5/6 protein levels could be due to post-transcriptional regulation of the mRNA.

**Figure 6.41 Immunoblot analysis of cytokeratin 5 and 6 protein expression in RT112.** Expression was examined in parental, parental treated with PD173074 for 24 h, R1, R2 and R3 cultured with PD173074 and R1, R2 and R3 cultured without PD173074 for 4 passages. SCaBER was included as a positive control for cytokeratin 5 and 6 and β-actin was used as a loading control.

Microarray analysis showed that expression of the basal marker CD44 was significantly decreased in RT112 parental + PD and R2 + PD compared to parental no PD (Fig. 6.42). However, no CD44 protein expression was detected in any RT112 samples with immunoblot (Fig. 6.43). This indicates that despite the observed differential expression of basal marker mRNA in the RT112 experimental conditions, this may be insufficient to induce detectable changes in protein expression. As the immunoblot analysis conducted to examine cytokeratin 5/6 and CD44 did not show that protein expression of these basal markers was increased in the RT112 resistant line, it is unlikely that the cells underwent a luminal to basal subtype switch.
Figure 6.42 Expression of CD44 in RT112 determined by microarray analysis. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which CD44 was significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). The data generated from each probe with specificity to CD44 is shown separately with a legend indicating the probe’s Affymetrix IDs.

Figure 6.43 Immunoblot analysis of CD44 protein expression in RT112. Expression was examined in parental, parental treated with PD173074 for 24 h, R1, R2 and R3 cultured with PD173074 and R1, R2 and R3 cultured without PD173074 for 4 passages. TCCSUP was included as a positive control for CD44 and β-actin was used as a loading control.

6.2.10 Expression of luminal cell markers in RT4

It was noted that some basal and luminal markers were differentially expressed in RT4 microarray data (2-fold change lists). Additionally, an increase in activation of EGFR was observed in RT4 R1 (Chapter 4, Fig. 4.6) which is characteristic of the basal subtype in MIBC (Kiselyov et al., 2016). Unsupervised hierarchical cluster analysis was performed with RT4 microarray expression data for the luminal subtype markers reported by Choi et al. (Fig. 6.44). Compared to RT4 parental no PD, expression of luminal markers was predominantly reduced in RT4 R1 no PD.
Figure 6.44 Unsupervised hierarchical cluster analysis of Choi et al. cohort of luminal markers in RT4 microarray samples. The Log₂ gene expression was normalised by dividing each expression value by the probe’s mean Log₂ gene expression. Following Log₂ gene expression normalisation, the expression profiles of samples and genes were clustered in Partek® Genomics Suite® 6.6 using Euclidean distance and complete linkage. Scale bar indicates the normalised Log₂ gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).

Expression of the luminal markers FOXA1 and GPX2 was significantly reduced in RT4 R1 + PD and RT4 R1 no PD to approximately half the level expressed in RT4 parental no PD (Fig. 6.45). Expression of KRT20 was non-significantly reduced in RT4 R1 + PD and R1 no PD to approximately half the level expressed in parental no PD (Fig. 6.45). GATA3 and PPARG were not significantly differentially expressed across RT4 experimental conditions (Fig. 6.45). Compared to RT4 parental no PD expression of the luminal marker CYP2J2 was significantly
increased approximately 4-fold and non-significantly increased approximately 2-fold in R1 + PD and R1 no PD respectively (Fig. 6.45). qRT-PCR analysis found expression of FGFR3 to be significantly increased in RT4 R1 + PD compared to RT4 parental no PD (Fig. 6.31). However, immunoblot analysis of FGFR3 in Chapter 3 found FGFR3 to be expressed at similar levels in RT4 parental and R1 + PD (Chapter 3, Fig. 3.13). Microarray analysis found a non-significant decrease in expression of ERBB3 in RT4 R1 no PD compared to RT4 parental no PD (Fig. 6.46). qRT-PCR analysis showed that ERBB3 expression was significantly reduced in RT4 R1 no PD to approximately half the expression level in RT4 parental no PD (Fig. 6.46). As expression of FOXA1, GPX2 and KRT20 was reduced in RT4 R1 + PD it is possible that RT4 R1 gained a less luminal phenotype during its derivation.

Microarray analysis showed that expression of UPK1A and UPK2 was significantly increased and expression of UPK3A, UPK3B and ELF3 was non-significantly increased in RT4 R1 + PD compared to PD (Fig. 6.47). Overall, microarray analysis showed that, as for RT112 R1 + PD and R2 + PD, expression of some luminal markers was increased in RT4 R1 + PD compared to the parental line, whilst other luminal markers exhibited a decrease or no change in expression.

p53-like subtype markers were not significantly differentially expressed across RT4 experimental conditions (Appendix D, D.14 and Fig. D.15) therefore it is unlikely that RT4 R1 has gained a more p53-like phenotype.

6.2.11 Expression of basal cell markers in RT4

Unsupervised hierarchical cluster analysis was performed on RT4 microarray samples with Choi et al. basal markers (Fig. 6.48). This suggested that expression of CD44, KRT14 and CDH3 could be differentially expressed between RT4 experimental conditions. Further examination of the microarray data showed that KRT14 and CDH3 were not significantly differentially expressed (Fig. 6.49). Expression of CD44 was significantly lower in R1 + PD than in parental and R1 no PD (Fig. 6.49). As the microarray analysis showed that expression of basal markers was not significantly increased in RT4 R1 + PD compared to RT4 parental, it is unlikely that RT4 R1 underwent a luminal to basal subtype switch.
Figure 6.45 Expression of FOXA1, GPX2, KRT20, GATA3, PPARG and CYP2J2 in RT4 determined by microarray analysis. A) FOXA1 B) GPX2 C) KRT20 D) GATA3 E) PPARG and F) CYP2J2. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which FOXA1, GPX2, KRT20, GATA3, PPARG and CYP2J2 were differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe’s Affymetrix IDs.
6.2.12 Expression of receptor tyrosine kinases

Examination of the expression and phosphorylation of RTKs previously implicated in resistance to FGFR inhibition in RT112 was conducted in Chapter 4. This showed that resistance to PD is not likely to have arisen in RT112 R1 and R2 via an increase in the activation of EGFR and ERBB2 (Chapter 4, Fig. 4.5 and Fig. 4.7). However, an increase in phosphorylation of ERBB3 was observed in RT112 R1 + PD and R2 + PD which may contribute to their resistance to PD. Immunoblot analysis found that total MET expression was reduced in RT112 R1 + PD and R2 + PD compared to parental no PD. However, MET phosphorylation was increased in RT112 R1 + PD, R2 + PD and R3 + PD supporting the notion that this RTK is mediating resistance to PD (Chapter 4, Fig. 4.11).

Unsupervised hierarchical cluster analysis was performed using expression array data for a selected panel of RTKs for RT112 microarray samples (Fig. 6.50). Expression of KIT was increased in parental + PD compared to parental no PD but not increased in the RT112 resistant derivatives therefore resistance is unlikely to be mediated in these lines by an increase in KIT expression. Expression of ERBB2, ERBB3 and INSR was increased in RT112 parental + PD and R1 + PD and to a lesser extent R2 + PD compared to the other RT112 experimental conditions. qRT-PCR validation confirmed that ERBB3 expression was increased in parental + PD, R1 + PD and R2 + PD (Fig. 6.37). Hierarchical clustering of microarray data also
identified an increase in *IGF1R*, *EGFR* and *MET* expression in R1 + PD and R2 + PD compared to the other RT112 samples (Fig. 6.50).

**Figure 6.47 Expression of **UPK1A, UPK2, UPK3A, UPK3B and ELF3** in RT112 determined by microarray analysis.** A) *UPK1A*. B) *UPK2*. C) *UPK3A*. D) *UPK3D*. E) *ELF3*. Error bars indicate standard error of the mean. Where error bars are not present this is due to the error being too small to plot. Signal intensity is given relative to RT112 no PD. Asterisks indicate the experimental conditions in which genes were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe’s Affymetrix IDs.
Figure 6.48 Unsupervised hierarchical cluster analysis of Choi et al. cohort of basal markers in RT4 microarray samples. The Log₂ gene expression was normalised by dividing each expression value by the probe’s mean Log₂ gene expression. Following Log₂ gene expression normalisation, the expression profiles of samples and genes were clustered in Partek® Genomics Suite® 6.6 using Euclidean distance and complete linkage. Scale bar indicates the normalised Log₂ gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).
Figure 6.49 Expression of KRT5, KRT6C, KRT14, CDH3 and CD44 in RT4 determined by microarray analysis. A) KRT5. B) KRT6C. C) KRT14. D) CDH3. E) CD44. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which KRT5, KRT6C, KRT14, CDH3 and CD44 were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe’s Affymetrix IDs.
Further examination of the microarray data showed that for IGF1R expression was significantly increased in R1 + PD. One of the two IGF1R specific probes reported a significant increase in IGF1R expression in R2 + PD (Fig. 6.51). Immunoblot analysis was not conducted to examine IGF1R expression due to time limitations. The microarray analysis showed that MET expression was significantly increased in RT112 R1 + PD and iR3 + PD, and non-significantly increased in R2 + PD (Fig. 6.51). Resistance to PD in RT112 R1 and R2 could be mediated via increased signalling of IGF1R or MET and whether this is the case requires further investigation.

Increased expression of a RTK ligand could induce increased activation of an RTK and downstream signalling pathways, and therefore cause resistance to PD. As IGF1R, MET and ERBB3 had been identified as possible mediators of resistance to PD in RT112 R1 and R2, the expression of the ligands with specificity to these receptors was examined. Microarray analysis showed that expression of IGF1, which encodes the IGF1R ligand insulin-like growth factor 1, HGF, which encodes the MET ligand hepatocyte growth factor, EREG and NRG2, which encode the ERBB3 ligands epiregulin and neuregulin 2 respectively, were not significantly differentially expressed in the RT112 resistant derivatives compared to the parental line (Appendix D, Fig. D.16). Expression of IGF2 which encodes the IGF1R ligand insulin-like growth factor 2 was significantly reduced in R1 + PD and R2 + PD (Fig. 6.52). One microarray probe set (TC08000250.hg.1) showed expression of NRG1 was significantly increased in RT112 R1 + PD and R2 + PD. However, this increase in expression was of a small magnitude and the other probe sets with specificity to NRG1 did not identify an increase in expression of this gene (Fig. 6.52). Therefore, it is most probable that R1 and R2 did not gain their PD-resistant phenotype due to an increase in expression of any of the ligands examined.

Microarray analysis showed that expression of FGFR2 and FGFR3, was increased in RT4 R1 + PD (Fig. 6.53), however these RTKs are unlikely to be inducing resistance as these receptors are inhibited by PD. Expression of EPHA3, which encodes ephrin receptor A3, was significantly increased approximately 2-fold in RT4 + PD compared to the parental line (Fig. 6.53 and Fig. 6.54).The Ephrin family of RTKS are implicated in angiogenesis (Pitulescu and Adams, 2014). Expression of EGFR family members was increased in R1 + PD (Fig. 6.53), including a significant increase in EGFR expression of approximately 3-fold in RT4 R1 + PD and R1 no PD (Fig. 6.54). The immunoblot analysis of phosphorylation of EGFR, ERBB2, ERBB3 in RT4 parental no PD and RT4 R1 + PD suggested that
EGFR could be inducing resistance to PD in RT4 R1 (Chapter 4, Fig. 4.6 and Fig. 4.9). Microarray analysis showed that expression of ERBB4 was non-significantly increased in RT4 R1 + PD (Fig. 6.54), however, it is possible that increased expression of this receptor could contribute to resistance to PD in RT4 R1. EGFR activation remains the most likely mechanism of resistance in RT4 R1 + PD.

Figure 6.50 Unsupervised hierarchical cluster analysis of receptor tyrosine kinases in RT112 microarray samples. The Log2 gene expression was normalised by dividing each expression value by the probe's mean Log2 gene expression. Following Log2 gene expression normalisation, the expression profiles of samples and genes were clustered in Partek® Genomics Suite® 6.6 using Euclidean distance and complete linkage. Scale bar indicates the normalised Log2 gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).
Figure 6.51 Expression of IGF1R and MET in RT112 determined by microarray analysis. A) IGF1R. B) MET. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which IGF1R and MET were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe’s Affymetrix IDs.

Figure 6.52 Expression of IGF2 and NRG1 in RT112 determined by microarray analysis. A) IGF2. B) NRG1. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which IGF2 and NRG1 were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe’s Affymetrix IDs.

As EGFR activation was the most likely mechanism of resistance in RT4 R1 + PD, microarray analysis was used to examine expression of EGFR family ligands in RT4 experimental conditions. Expression of EREG, HBEGF, EGF and EPGN, which encode EGFR ligands, was not significantly increased in RT4 R1 + PD (Appendix D, Fig. D.17). Expression of NRG1 and NRG2 which encode the EGFR family ligands neuregulin 1 and neuregulin 2, was not significantly increased in RT4
R1 + PD compared to parental no PD (Appendix D, Fig. D.18). However, expression of *AREG* and *BTC*, which encode the EGFR ligands amphiregulin and betacellulin respectively, was significantly increased in RT4 R1 + PD compared to parental no PD (Fig. 6.55). Thus the increased activation of EGFR in RT4 R1 may be mediated by the increased expression of these ligands.

**Figure 6.53** Unsupervised hierarchical cluster analysis of receptor tyrosine kinases in RT4 microarray samples. The Log₂ gene expression was normalised by dividing each expression value by the probe’s mean Log₂ gene expression. Following Log₂ gene expression normalisation, the expression profiles of samples and genes were clustered in Partek® Genomics Suite® 6.6 using Euclidean distance and complete linkage. Scale bar indicates the normalised Log₂ gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low green.
Figure 6.54 Expression of EPHA3, EGFR and ERBB4 in RT4 determined by microarray analysis. A) EPHA3. B) EGFR. C) ERBB4. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions and probes for which genes were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe’s Affymetrix IDs.

6.2.13 Expression of KDM5A and KDM6A

Sharma et al. generated derivatives of the NSCLC line PC9 tolerant to EGFR TKIs by culturing parental PC9 in gefitinib for 9 days. These cells mediated their resistance by activation of IGF1R. An increase in expression of histone demethylase KDM5A was observed in the resistant cells and the drug tolerant state of these cells was dependent upon KDM5A expression (Sharma et al., 2010). It was observed that, upon culture without an EGFR TKI, resistant cells maintained their resistant phenotype for approximately 30 passages before regaining gefitinib sensitivity (Sharma et al., 2010). It was considered that increased expression of KDM5A may be mediating resistance to PD in RT112, however, microarray analysis showed that KDM5A mRNA was not differentially expressed across RT112 conditions suggesting that this is unlikely (Fig. 6.56).
RT112 has a heterozygous P1139fs mutation in KDM6A. It was noted that expression of the histone demethylase KDM6A was significantly increased in the RT112 resistant derivatives cultured in PD compared to parental no PD (Fig. 6.56). KDM6A expression was also non-significantly increased in parental + PD, R1 no PD, R2 no PD and R3 no PD compared to parental no PD. It is possible that this increase in expression mediates the resistant phenotype.

Microarray analysis showed that expression of KDM5A was not significantly differentially expressed across RT4 experimental conditions (Fig. 6.56). Expression of the tumour suppressor KDM6A was significantly increased in RT4 R1 no PD and non-significantly increased in RT4 R1 no PD. Therefore, an increase in KDM6A expression may contribute to the resistant phenotype in RT4 R1.

Figure 6.55 Expression of AREG and BTC in RT4 determined by microarray analysis. A) AREG. B) BTC. Error bars indicate standard error of the mean. Signal intensity is given relative to RT4 no PD. Asterisks indicate the experimental conditions in which genes were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe’s Affymetrix IDs.
Figure 6.56 Expression of KDM5A and KDM6A in RT112 and RT4 determined by microarray analysis. A) KDM5A expression in RT112. B) KDM6A expression in RT112. C) KDM5A expression in RT4. D) KDM6A expression in RT4. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions and Affymetrix probes for which genes were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change).

6.3 Discussion

An online cancer microarray database such as Oncomine (Rhodes et al., 2007) could have been used to identify genes differentially expressed in urothelial carcinoma compared to normal urothelium. It would then have been possible to use the list of genes differentially expressed in urothelial carcinoma to filter our lists of significantly differentially expressed genes. This would have removed genes whose differential expression has not previously been implicated in urothelial carcinoma, therefore, removing potentially unimportant genes from our lists of significantly differentially expressed genes. However, it is possible that this would have removed gene expression changes implicated in resistance to FGFR inhibition that have yet to be reported in urothelial carcinoma.
Microarray analysis suggested that the OSM pathway could be mediating resistance to PD in RT112 resistant lines as a significant increase in the expression of OSMR, LIFR and IL6ST was observed in RT112 R1 + PD, R1 no PD and R2 + PD compared to parental no PD. A significant increase in expression of LIFR and IL6ST was also observed in RT112 R3 + PD. The increase in expression in RT112 R1 no PD may be due an increase in the copy number of these genes as copy number analysis showed that RT112 R1 has a gain of 5p15.33 - q11.1 which contains LIFR and OSMR and a gain of 5q11.1 - q11.2 which contains IL6ST, compared to RT112 parental (Chapter 5, Fig. 5.2, Table 5.2). However, the 5p gains cannot account for the increased expression of IL6ST, OSMR and LIFR in RT112 R1 + PD compared to R1 no PD. These copy number alterations were not observed in RT112 R3 (Chapter 5, Fig. 5.3, Table 5.3). Copy number analysis was not conducted in RT112 R2. qRT-PCR validated the increased expression of these receptors in RT112 resistant lines.

Microarray analysis showed that the ligands which activate the OSMR pathway, OSM and IL31, were not differentially expressed between RT112 experimental conditions. The protein expression levels of OSMRβ, LIFRβ and gp130 was not examined. STAT phosphorylation is commonly used as a readout of activation of the OSM pathway (Moidunny et al., 2016; Zhang et al., 2017). However, immunoblot analysis found that phosphorylation of STAT1 and STAT3 remained low in RT112 resistant derivatives. Therefore, the OSM signalling pathway is not activating STAT1 and STAT3 in the RT112 resistant derivatives. However, immunoblot analysis conducted in Chapter 4 showed that ERK was phosphorylated in RT112 resistant derivatives but not RT112 parental acutely treated with PD (Fig. 4.12). It is possible that the OSM signalling pathway is inducing resistance in RT112 resistant lines via activation of the MAP kinase pathway. No significant increase in OSM pathway ligands or receptors was observed in the microarray analysis of RT4 R1.

Microarray analysis showed that genes which regulate the synthesis of cholesterol and fatty acids were differentially expressed in RT112 and RT4 experimental conditions. It was thought that the resistance mechanism in the PD resistant lines might maintain the expression of fatty acid synthesis genes. Alternatively, the resistant lines could be proliferating despite reduced fatty acid synthesis. It was previously reported that expression of a cohort of genes regulating fatty acid and sterol biosynthesis was reduced in RT112 upon FGFR3 knockdown for 48 h (Du et al., 2012). Our microarray analysis found that expression of the majority of genes in this cohort was reduced in RT112 acutely treated in PD for 24
h compared to RT112 parental no PD. SCD1 is the rate-limiting enzyme in the production of mono-unsaturated fatty acids from saturated fatty acids and increases lipogenesis (Igal, 2016). As expression of this protein was reduced in RT112 resistant derivatives this suggests that the resistance mechanism employed in these lines does not restore fatty acid synthesis to the level observed in RT112 parental.

The HRAS mutation identified in RT112 R3 did not restore mSREBP1 or SCD1 expression to the level observed in the parental line. This indicates that the signalling via the mutant HRAS in RT112 R3 did not activate fatty acid synthesis to the same extent as the signalling via FGFR3-TACC3 and wildtype FGFR3 in RT112 parental no PD. However, expression of SCD1 was higher in RT112 R3 + PD than in R1 + PD and R2 + PD. Additionally, RT112 R3 + PD maintained expression of many of the Du et al. cohort of genes regulating fatty acid and sterol biosynthesis whilst expression was not maintained in R1 + PD and R2 + PD. This suggests that the HRAS mutation may have enabled RT112 R3 + PD to maintain fatty acid and sterol biosynthesis to a greater extent than it was maintained in RT112 R1 + PD and R2 + PD. The resistance mechanism in R3 may enable fatty acid and sterol biosynthesis and, therefore, could be the reason that R3 exhibits greater resistance to PD than RT112 R1 or R2 (Chapter 3, Fig. 3.8). Alternatively, the increased expression of genes regulating fatty acid and sterol biosynthesis in RT112 R3 may be due to the greater PD resistance exhibited by this line.

Expression of SCD1 and SREBP1 was not examined by immunoblot in RT4 experimental conditions but expression of the Du et al. gene cohort was predominantly reduced in RT4 + PD compared to RT4 parental. This suggests that there was reduced fatty acid and sterol biosynthesis in RT4 + PD. The effect of the reduced expression of genes regulating fatty acid and sterol biosynthesis on lipid metabolism in resistant lines could be investigated by measuring the incorporation of $^{14}$C labelled acetate into fatty acids as conducted by Du et al. (Du et al., 2012). Alternatively, cellular lipid composition of parental and resistant lines could be examined with mass spectrometry as conducted by Griffiths et al. (Griffiths et al., 2013).

Wang et al. reported that RT112 cells cultured long-term in the FGFR TKIs BJG398 and ponatinib, to produce TKI-resistant derivatives, exhibited a mesenchymal morphology. It was found that removal of the FGFR TKIs resulted in the derivatives regaining an epithelial morphology within 2-4 weeks (Wang et al., 2014). In Chapter 3 it was observed that RT112 R1 and R2 cultured in PD had a
mesenchymal morphology and had increased expression of the mesenchymal marker N-cadherin (Chapter 3, Fig. 3.4 and Fig. 3.11). These cells had low N-cadherin expression and regained an epithelial morphology following culture without PD for 4 passages (Chapter 3, Fig. 3.5 and Fig. 3.11). The mesenchymal phenotype is associated with greater cell migration and invasion. Therefore, if FGFR TKIs induce an EMT in patients this could lead to tumour metastasis (Singh et al., 2017). EMT has been observed in NSCLC patients with acquired resistance to EGFR TKIs (Sequist et al., 2011). The microarray analysis showed that expression of some epithelial and mesenchymal markers, such as the epithelial marker CDH1, remained similar in all RT112 experimental conditions whilst other markers were differentially expressed, such as the mesenchymal marker FN1. This supports the previously-held hypothesis that RT112 R1 and R2 underwent a partial EMT during their production. MetaCore™ pathway analysis also showed that R3 had undergone some EMT-like expression changes compared to parental. The evidence for an EMT in RT4 R1 is limited compared to the evidence in RT112 R1 and R2. However, MetaCore™ pathway analysis did find enrichment of the pathway ‘regulation of epithelial to mesenchymal transition (EMT)’ for the comparison RT4 R1 + PD and RT4 parental no PD (Fig. 6.12). EMT-like gene expression changes could pose a problem for patients treated with FGFR inhibitors if this facilitates tumour metastasis.

Basal bladder cancers tend to be more aggressive than luminal bladder cancers (Choi et al., 2014a; Kiselyov et al., 2016). Microarray and qRT-PCR analysis indicated that expression of some basal markers was increased and expression of some luminal markers was reduced in RT112 resistant derivatives. Expression of GATA3 and UPK1A was increased in R1 + PD and R2 + PD whilst expression of FOXA1 was reduced in R1 + PD and R2 + PD. Immunoblot analysis would have confirmed whether the differences in mRNA expression resulted in differential protein expression. This was no conducted due to time limitations. Microarray and qRT-PCR analysis suggested that KRT5, KRT6C and CD44 were differentially expressed between RT112 experimental conditions. However, as the expression of CD44 and cytokeratin 5/6 protein remained low it appears that mRNA expression changes did not result in corresponding changes in protein expression. Therefore, it is unlikely that RT112 resistant derivatives underwent a luminal to basal subtype switch.

As microarray analysis showed that expression of basal markers was not significantly increased in RT4 R1 + PD or R1 no PD, and expression of some luminal markers was increased in RT4 R1 + PD, it is unlikely that RT4 R1
underwent a luminal to basal switch during the derivation of this resistant line or upon culture without PD. Luminal/basal expression changes were not examined with qRT-PCR or immunoblot in RT4 due to time limitations. Expression of p53-like markers was not significantly different between RT4 or RT112 experimental conditions, therefore the RT4 and RT112 resistant derivatives did not undergo a luminal to p53-like subtype switch.

Sharma et al. produced PC9 cells resistant to EGFR-TKIs, which mediated their resistance via activation of IGF1R, via culture in gefitinib for 9 days. It was observed that the resistant cells had increased expression of KDM5A and that KDM5A knockdown did not reduce the proliferation of parental PC9 cells but did reduce the production of resistant cells upon treatment with gefitinib (Sharma et al., 2010). Gale et al. developed the KDM5A inhibitor YUKA1 and observed that treatment with this inhibitor reduced the formation of gefitinib-resistant colonies following the culture of PC9 in gefitinib for 35 days. Culture with YUKA1 as a single agent did not reduce the growth of PC9 (Gale et al., 2016). Sharma et al. observed that, upon culture without an EGFR TKI, resistant cells maintained their resistant phenotype for approximately 30 passages before regaining gefitinib sensitivity (Sharma et al., 2010). Microarray analysis showed that KDM5A was not differentially expressed between RT112 and RT4 parental cells and their resistant derivatives. Therefore, it is unlikely that PD resistance is mediated by an increase in KDM5A expression.

KDM6A mutations are present in approximately 25% of MIBCs and 40% of NMIBCs (Pietzak et al., 2017; Robertson et al., 2017). KDM6A demethylates lysine 27 of histone H3. One consequence of the histone demethylation activity of KDM6A in fibroblasts is an increase in expression of retinoblastoma binding proteins required for the function of RB1 as a tumour suppressor (Wang et al., 2010). Its role in epithelial cells, including the urothelium, is unknown. Microarray analysis showed that expression of KDM6A was increased in the RT112 and RT4 resistant derivatives when cultured in PD. Whether the resistant derivatives are dependent upon KDM6A expression to maintain their PD resistance could be tested by examining whether knockdown of KDM6A re-sensitizes these cells to PD.

Resistance to RTK inhibition often arises via the activation of an alternative RTK as discussed in Chapter 1, section 1.2.2. Ma et al. reported that treatment with the IGF1R ligand IGF1 induced gefitinib resistance in glioblastoma cell lines. The combination of gefitinib and the small molecule inhibitor linsitinib, which has specificity for IGF1R and insulin receptor (InsR), reduced the cell viability of the
glioblastoma cell lines GBM39 and GBM76 more effectively than treatment with either inhibitor as a single agent (Ma et al., 2016). Harris et al. found that breast cancer patients whose tumours expressed IGF1R were less likely to respond to the combination of trastuzumab and the chemotherapeutic agent vinorelbine (Harris et al., 2007). Wang et al. reported that IGF1R knockdown induced greater sensitivity to gemcitabine in the bladder cancer cell line 5637. Additionally, a meta-analysis showed that bladder cancer patients with high expression of IGF1R mRNA have a lower overall survival time and lower time to disease recurrence (Wang et al., 2017a). IGF1R activates the MAP kinase and PI3 kinase pathways (Hartog et al., 2007). Immunoblot analysis in Chapter 4 showed that signalling via these pathways was reduced in RT112 parental acutely treated with PD whereas these pathways were activated in RT112 resistant lines cultured in PD (Fig. 4.12). Therefore, IGF1R could be mediating resistance via activation of the MAP kinase and PI3 kinase pathways in RT112 R1 and R2. Whether IGF1R protein expression is increased in the RT112 resistant lines and whether signalling via IGF1R is active in parental RT112 and the resistant lines could be confirmed with immunoblot analysis. A cell viability assay with the IGF1R inhibitor linsitinib will be described in Chapter 7 to examine the sensitivity of parental RT112 and the RT112 resistant lines to IGF1R inhibition.

Microarray analysis identified an increase in MET expression in the RT112 resistant derivatives. This is discrepant with the immunoblot analysis in Chapter 4, in which total MET protein expression was reduced in R1+ PD and R2 + PD (Fig. 4.11). As the immunoblot analysis identified an increase in the phosphorylation of MET, this RTK may be mediating resistance to PD. MET signalling has been reported as a mechanism of short term survival in response to FGFR inhibition (Harbinski et al., 2012).

qRT-PCR analysis identified an increase in ERBB3 expression in RT112 parental acutely treated with PD, R1 + PD and R2 + PD. An increase in phospho-ERBB3 expression was observed in Chapter 4 (Fig. 4.8) but examination of total ERBB3 protein expression was unsuccessful. Signalling via ERBB3, together with its dimerization partner ERBB2, has been previously reported as a resistance mechanism to FGFR inhibition in RT112 (Wang et al., 2014). ERBB3 remains a possible mediator of resistance in the RT112 resistant derivatives. MET has been reported to heterodimerize with ERBB3 (Pérez-Ramírez et al., 2015; Tanizaki et al., 2011), therefore it is possible that these two RTKs act together to induce PD resistance.
**EPHA3** mRNA expression was significantly increased in RT4 + PD compared to RT4 R1 no PD. Conflicting evidence has found the EPH family of RTKs to be both oncogenic and tumour-suppressive. Agonistic and antagonistic targeted agents specific for EPH family members, such as ifabotuzumab which is a EphA3 agonist, have entered early stage clinical trials (Lodola et al., 2017). Expression of **ERBB4** was non-significantly increased in RT4 R1 + PD. Canfield et al. showed that knockdown of **ERBB4** induced apoptosis in ERBB2-positive breast cancer cell lines with acquired resistance to the EGFR, ERBB2 and ERBB3 TKI lapatinib and the ERBB2-specific monoclonal antibody trastuzumab (Canfield et al., 2015). Whether RT4 R1 exhibits sensitivity to inhibition or knockdown of EphA3 and ERBB4 is worthy of further examination.

The increased expression of **EGFR** in RT4 R1 + PD observed in the microarray analysis is concordant with the previously observed increase in EGFR protein expression and phosphorylation. This increased activation of EGFR remains the most likely mediator of resistance in RT4 R1. Herrera-Abreu et al. showed that EGFR mediated short-term survival in RT112 treated with PD by re-activating the MAP kinase pathway (Herrera-Abreu et al., 2013). Harbinski et al. screened a secreted protein cDNA library and identified that TGF-α, which activates EGFR signalling, was able to induce resistance to the FGFR TKI BGJ398 in RT112 (Harbinski et al., 2012; Singh and Coffey, 2014). EGFR is reported to induce intrinsic resistance to BRAF inhibition via re-activation of the MAP kinase pathway in cell line models of colorectal cancer (Corcoran et al., 2012; Prahallad et al., 2012). Approximately 6% of MIBC tumours exhibit gain of copy number of 7p11.2 which contains **EGFR** (Robertson et al., 2017). Approximately 60% of bladder cancer tumours express membranous EGFR (Ibrahim et al., 2009). These tumours could be intrinsically resistant to FGFR inhibition.

In summary, the targets most worth examining with drug testing in RT112 are IGF1R, MET, ERBB3 and KDM6A, and in RT4 are EGFR, ERBB4, EPHA3 and KDM6A.
Chapter 7
Screening targeted agents to overcome PD173074 resistance

7.1 Introduction

The experimental work in this study so far was conducted with the aim of identifying the mechanisms of resistance in the PD resistant derivatives. Altered expression of several RTKs was identified by immunoblot and transcriptome analysis. Experiments described in this Chapter were performed in order to test whether these RTKs could be acting as mediators of resistance to PD.

Immunoblot analysis in Chapter 4 examined the phosphorylation of RTKs previously implicated in resistance to FGFR inhibition. An increase in phospho-ERBB3 was identified in RT112 R1 and R2. However, phosphorylation of ERBB2 and EGFR remained low in these lines. An increase in phospho-MET was also observed in the RT112 resistant lines. Total and phospho-EGFR expression was increased in RT4 R1 but expression of phospho-ERBB2 and phospho-ERBB3 remained low.

In Chapter 6, qRT-PCR showed ERBB3 expression to be increased in RT112 parental acutely treated with PD, R1 and R2. Microarray analysis identified a significant increase in IGF1R mRNA in RT112 R1 and R2. Microarray analysis also showed EGFR mRNA expression to be increased in RT4 R1, concordant with the increase in total EGFR observed by immunoblot in Chapter 4.

The EGFR family, MET and IGF1R can be targeted with small-molecule inhibitors which have specificity to a small number of RTKs. In this Chapter, cell viability assays conducted with small-molecule inhibitors will be presented. These were carried out to test the dependency of resistant lines on RTKs for which an increase in expression or phosphorylation has been identified. This was carried out with the aim of determining which RTKs may be mediating resistance and to suggest which drug combinations could be used in the clinic to overcome resistance to FGFR inhibition. Cell viability assays were conducted with these small-molecule inhibitors, both as single agents and in combination with PD, in order to determine whether the continued inhibition of FGFR3 would elicit a greater reduction in cell proliferation and survival.
Exome sequencing, in Chapter 5, identified a HRAS G12S mutation in approximately 75% of RT112 R3 cells. Retroviral transduction of RT112 parental with mutant HRAS and subsequent cell viability assays showed that mutation of this gene alone induces resistance to PD. Inhibition of RTKs is thus unlikely to reduce cell viability in RT112 R3 cells with mutant HRAS as the constitutively active HRAS would be able to maintain cell proliferation and survival. The sub-population of RT112 R3 which does not possess the HRAS mutation may, however, be sensitive to RTK inhibition.

7.2 Results

7.2.1 Cell viability assays with RT112 and the MET TKI capmatinib

An increase in phosphorylation of MET was observed in RT112 R1, R2 and R3 by immunoblot analysis (Fig. 4.11). MET activation has been reported to overcome sensitivity to FGFR inhibition in RT112 (Harbinski et al., 2012). Capmatinib is a selective, ATP-competitive inhibitor of MET. Liu et al. conducted a cell-free assay which showed that capmatinib inhibited MET with an IC50 of 0.13nM, whereas 2μM of capmatinib inhibited the other kinases in the panel tested by no more than 30%. Capmatinib inhibited cell viability in the MET-amplified gastric cancer cell line SNU-5 and the mouse fibroblast cell line S114, which expresses a high level of MET and HGF, with IC50s of 1.2 and 12.4 nM respectively. Capmatinib was inactive in the gastric cancer cell line SNU-1 and in the kidney cell line HEK293 which express no and a low level of MET respectively (Liu et al., 2011). Capmatinib is currently being assessed in clinical trials for the treatment of EGFR TKI-resistant NSCLC (Wu et al., 2017). As sensitivity to capmatinib had been previously reported at concentrations between 2 and 13nM in MET-dependent cell lines (Liu et al., 2011), it was thought that 1μM capmatinib was a sufficiently high concentration to determine if the RT112 cells were sensitive to MET inhibition. Therefore, sensitivity to 1μM capmatinib was examined by cell viability assay (Fig. 7.1). 1μM capmatinib did not reduce cell viability in RT112 parental, R1, R2 or R3. The sensitivity of RT112 parental and resistant lines to a range of capmatinib concentrations in combination with 1μM PD was also tested (Fig. 7.2). Treatment with capmatinib + PD did not reduce cell viability any more than was observed with PD alone.
Therefore, RT112 parental and resistant lines are not dependent upon signalling via MET.

Expression of MET has previously been reported in the urothelial carcinoma cell line 5637 and activation of MET signalling by hepatocyte growth factor (HGF) has been reported to increase invasiveness in 5637 (Shintani et al., 2017; Wang et al., 2007). Immunoblot analysis showed that 5637 expressed phospho-MET and MET (Chapter 4, Fig 4.11). Therefore, 5637 was selected as a positive control to determine if capmatinib was successfully inhibiting MET. However, 5637 was insensitive to treatment with capmatinib (Fig. 7.3). This could indicate that capmatinib was not inhibiting MET at the concentrations used in this assay. One alternative explanation is that, whilst 5637 expresses phospho-MET and the activation of MET signalling increases the invasiveness of 5637, MET inhibition does not reduce 5637 cell proliferation or survival. It is also possible that MET could be promoting cell proliferation and survival in 5637 via crosstalk with another receptor, and when MET is inhibited activation of the other receptor compensates for the reduced MET activity. There was insufficient time to conduct immunoblot analysis to examine whether capmatinib inhibited MET phosphorylation in the RT112 resistant derivatives or 5637. This experiment would have clarified whether RT112 parental and the resistant derivatives were resistant to MET inhibition or capmatinib was not inhibiting MET activation. Possible explanations for insensitivity to capmatinib are discussed in greater detail later in this Chapter.

7.2.2 Cell viability assays with RT112 and the ERBB family TKI sapitinib

qRT-PCR analysis established that ERBB3 was upregulated in RT112 parental acutely treated with PD, RT112 R1, R2 and R3 when compared to parental no PD (Fig. 6.46). Phosphorylation of ERBB3 was identified by immunoblot in R1 and R2 (Chapter 4, Fig. 4.8) whereas increased phosphorylation of EGFR and ERBB2 was not observed in R1 and R2 (Chapter 4, Fig. 4.5 and Fig. 4.7). Wang et al. reported activation of ERBB2 and ERBB3 as a mechanism of resistance in RT112 cultured in FGFR inhibitors (Wang et al., 2014). Herrera-Abreu et al. reported EGFR activation as a mechanism of short term survival in response to treatment with PD in RT112 (Herrera-Abreu et al., 2013).
Figure 7.1 Cell viability of parental RT112 and resistant derivatives in capmatinib (MET TKI). Viability of cells was assayed using CellTiter-Blue following 120 h treatment with capmatinib and normalised to vehicle control. Error bars show the standard error of the mean. This assay was repeated twice and a representative example is shown.

Figure 7.2 Cell viability of parental RT112 and resistant derivatives in capmatinib (MET TKI) + 1µM PD173074. Viability of cells was assayed using CellTiter-Blue following 120 h treatment with a range of capmatinib concentrations + 1µM PD173074 and normalised to vehicle control. Error bars show standard error of the mean; error bars are absent where the error was too small to plot. This assay was repeated twice and a representative example is shown.
To determine if ERBB3 activation, or activation of an alternative EGFR family member, was contributing to the resistant phenotype of the RT112 resistant derivatives, cell viability was tested in RT112 parental and resistant derivatives when treated with sapitinib. Sapitinib is a reversible ATP-competitive TKI with specificity for EGFR, ERBB2 and ERBB3. A cell-free assay found sapitinib inhibited EGFR and ERBB2 with IC50s of 0.012 and 0.014μM respectively. No activity was observed at concentrations up to 10μM for other RTKs which were not members of the EGFR family. Sapitinib inhibited EGFR phosphorylation with an IC50 of 4nM in the buccal carcinoma cell line KB (Hickinson et al., 2010). Sapitinib inhibited ERBB2 and ERBB3 phosphorylation in the breast adenocarcinoma cell line MCF7 with IC50s of 3 and 4nM respectively (Hickinson et al., 2010). Sapitinib has been assessed in phase I and II clinical trials with breast cancer patients with limited success (Johnston et al., 2016; Kurata et al., 2014).

Single agent treatment with sapitinib did not appreciably reduce cell viability in RT112 parental or the resistant derivatives (Fig. 7.4). The combination of sapitinib and PD reduced the cell viability of the RT112 resistant derivatives to 50-60% of the vehicle control and viability in parental RT112 was reduced to approximately 20% (Fig. 7.5). It was observed in Chapter 3 that single agent treatment with PD reduced the viability of RT112 parental to 33%, R1 to 53%, R2 to 67% and R3 to 90% of the vehicle control (Fig. 3.8). Hence the combination of
Sapitinib and PD was not effective in overcoming the resistance to PD in the RT112 resistant lines.

To confirm that the batch of sapitinib used to treat the RT112 parental and resistant lines was successfully inhibiting its target RTKs, it was decided to assay a cell line as a positive control for sensitivity to EGFR family inhibition. The urothelial carcinoma cell line DSH1, which expresses high levels of ERBB2 and is sensitive to ERBB2 inhibition, was selected as a positive control (de Martino et al., 2014) (Fig. 7.6). The cell viability of DSH1 cultured in 1μM sapitinib was 34% of the vehicle control. As DSH1 was sensitive to sapitinib we can conclude that the sapitinib was inhibiting EGFR family members in the viability assays conducted with RT112 resistant derivatives. Therefore, these resistant derivatives displayed resistance to inhibition of the EGFR family.

Figure 7.4 Cell viability of parental RT112 and resistant derivatives in sapitinib (EGFR, ERBB2 and ERBB3 TKI). Viability of cells was assayed using CellTiter-Blue following 120 h treatment with sapitinib and normalised to vehicle control. Error bars show the standard error of the mean. This assay was repeated twice and a representative example is shown.
Figure 7.5 Cell viability of parental RT112 and resistant derivatives in sapitinib (EGFR, ERBB2 and ERBB3 TKI) + 1µM PD173074. Viability of cells was assayed using CellTiter-Blue following 120 h treatment with a range of sapitinib concentrations + 1µM PD. Cell viability was normalised to vehicle control. Error bars show the standard error of the mean; error bars are absent where the error was too small to plot. Sigmoidal dose response curves were plotted in GraphPad Prism®. This assay was repeated twice and a representative example is shown.

Figure 7.6 Cell viability of DSH1 in sapitinib (EGFR, ERBB2 and ERBB3 TKI). Viability of cells was assayed using CellTiter-Blue following 120 h treatment with 1µM sapitinib and normalised to vehicle control. Error bars show standard error of the mean. This assay was conducted only once.
7.2.3 Cell viability assays with RT112 and the IGF1R TKI linsitinib

Transcriptome analysis showed that *IGF1R* expression was upregulated in RT112 R1 and R2 (Chapter 6, Fig. 6.51). Linsitinib is a small-molecule ATP-competitive kinase inhibitor developed to inhibit IGF1R. A cell-free assay found that linsitinib inhibited IGF1R, the insulin receptor (IR) and insulin receptor-related protein (INSRR) with IC50s of 0.035, 0.075 and 0.075μM respectively but that concentrations greater than 10μM were required to inhibit other RTKs (Mulvihill *et al.*, 2009). Linsitinib has been assessed in clinical trials: for example, a phase III clinical trial was conducted with patients with locally advanced or metastatic adrenocortical carcinoma but was not found to increase patient survival (Fassnacht *et al.*, 2015).

To examine whether signalling via upregulated IGF1R might be inducing resistance to PD, sensitivity to linsitinib was assayed in RT112 parental and RT112 resistant derivatives (Fig. 7.7). The IC50s of linsitinib in RT112 parental, R1, R2 and R3 were 0.8, 0.4, 0.9 and 3.3μM respectively. This indicated that all lines were sensitive to linsitinib. The sensitivity of RT112 parental to linsitinib was unexpected as this cell line is able to signal via FGFR3 to promote cell growth and survival. It was also surprising that RT112 R3 was sensitive to linsitinib as it was hypothesised that the *HRAS* mutation in this line would enable constitutive activation of cell proliferation and cell survival. However, the IC50 values indicated that RT112 R3 was less sensitive to linsitinib than RT112 parental, R1 or R2.

RT112 parental and RT112 R1 were then assayed for their sensitivity to linsitinib in combination with 1μM PD (Fig. 7.8). RT112 parental and R1 treated with a range of linsitinib concentrations in combination with 1μM PD, had IC50s of 0.11μM and 0.34μM respectively. Therefore, in RT112 parental inhibition of IGF1R with linsitinib was more efficacious in combination with FGFR inhibition than treatment with either PD or linsitinib alone. In contrast, in RT112 R1, inhibition of IGF1R with linsitinib was not more efficacious in combination with FGFR inhibition than as a single agent.
Figure 7.7 Cell viability of parental RT112 and RT112 resistant lines in linsitinib (IGF1R TKI). Viability of cells was assayed using CellTiter-Blue following 120 h treatment with a range of linsitinib concentrations and normalised to vehicle control. Error bars show standard error of the mean; error bars are absent where the error was too small to plot. Sigmoidal dose response curves were plotted in GraphPad Prism®. This assay was repeated twice and a representative example is shown.

Figure 7.8 Cell viability of parental RT112 and RT112 R1 in linsitinib (IGF1R TKI) + 1µM PD173074. Viability of cells was assayed using CellTiter-Blue following 120 h treatment with a range of linsitinib concentrations + 1µM PD and normalised to vehicle control. Error bars show standard error of the mean; error bars are absent where the error was too small to plot. Sigmoidal dose response curves were plotted in GraphPad Prism®. This assay was repeated twice and a representative example is shown.
7.2.4 Cell viability assays with RT4 and the EGFR TKI erlotinib

Transcriptome analysis identified that expression of EGFR RNA was significantly increased in RT4 R1 cultured with and without PD compared to RT4 parental (Chapter 6, Fig. 6.52). Immunoblot analysis confirmed that both phospho-EGFR and total EGFR expression were increased in RT4 R1 compared to parental RT4 (Chapter 4, Fig. 4.5). Erlotinib is an ATP-competitive inhibitor with selectivity for EGFR (Moyer et al., 1997). Moyer et al. examined the selectivity of erlotinib on purified RTKs and found that erlotinib inhibited EGFR with an IC50 of 2nM and exhibited over 1000-fold greater selectivity for EGFR than for the other kinases examined: SRC and ABL. Immunoblot analysis and densitometry showed that erlotinib inhibited the phosphorylation of EGFR following treatment with EGF in the head and neck squamous cell carcinoma cell line HN5 with an IC50 of 20nM (Moyer et al., 1997). Erlotinib inhibited the proliferation of the colorectal carcinoma cell line DiFi with an IC50 of 100nM in an 8 day proliferation assay (Moyer et al., 1997). Erlotinib is approved for treatment of NSCLC and pancreatic cancer (Mosquera et al., 2016; Singh and Jadhav, 2017). To determine if activation of EGFR was mediating resistance to PD in RT4 R1, RT4 parental and RT4 R1 were assayed for their sensitivity to erlotinib (Fig. 7.9). RT4 parental had an IC50 of 6.1μM and RT4 R1 had an IC50 of 10μM. Erlotinib reduced viability in RT4 parental and RT4 R1 to approximately 15% and 20% of the vehicle control respectively. RT4 parental and RT4 R1 were then assayed for their sensitivity to erlotinib in combination with 1μM PD (Fig. 7.10). In combination with 1μM PD RT4 parental had an IC50 of 0.07μM and RT4 R1 had an IC50 of 0.37μM and viability was reduced in RT4 parental and RT4 R1 to approximately 15% and 6% of the vehicle control respectively. Therefore, simultaneous inhibition of the FGFRs and EGFR reduced cell viability in RT4 parental and RT4 R1 with greater efficacy than single agent treatment with erlotinib.
Figure 7.9 Cell viability of parental RT4 and R1 in erlotinib (EGFR TKI). Viability of cells was assayed using CellTiter-Blue following 120 h treatment with a range of erlotinib concentrations and normalised to vehicle control. Error bars show the standard error of the mean; error bars are absent where the error was too small to plot. Sigmoidal dose response curves were plotted in GraphPad Prism®. This assay was repeated twice and a representative example is shown.

Figure 7.10 Cell viability of parental RT4 and R1 in erlotinib (EGFR TKI) + 1μM PD173074. Viability of cells was assayed using CellTiter-Blue following 120 h treatment with a range of erlotinib concentrations + 1μM PD173074 and normalised to vehicle control. Error bars show standard error of the mean; error bars are absent where the error was too small to plot. Sigmoidal dose response curves were plotted in GraphPad Prism®. This assay was repeated twice and a representative example is shown.
7.3 Discussion

It has been reported that the addition of HGF, which binds and upregulates MET, was able to rescue cell growth in RT112 treated with the FGFR TKI BGJ398 (Harbinski et al., 2012). Additionally, immunoblot analysis in Chapter 4 showed that phospho-MET was expressed in RT112 R1, R2 and R3 cultured in PD (Fig. 4.11). It was therefore thought that MET could be mediating resistance to PD in the RT112 resistant derivatives. However, the resistant lines were not sensitive to treatment with the MET inhibitor capmatinib. It was hoped that treatment of 5637, which expresses phospho-MET, with capmatinib would demonstrate that the capmatinib used in the cell viability assays was successfully inhibiting MET and was not defective. Cell viability was not reduced in 5637 upon treatment with capmatinib. There was insufficient time to conduct immunoblot analysis to determine if capmatinib was inhibiting phosphorylation of MET in 5637 or the RT112 resistant derivatives. As we did not demonstrate that capmatinib was capable of reducing cell viability in a MET-dependent cell line or reducing MET phosphorylation it is possible that our batch of capmatinib was defective.

There are a number of other explanations which could explain the insensitivity of 5637 to capmatinib. It is possible that 5637 is not sensitive to MET inhibition under the conditions used in the viability assay. Previous research such as that conducted by Shintani et al. has examined the effect of stimulating MET signalling in 5637 via treatment with HGF, whereas the 5637 in this study 5637 was not cultured with exogenous HGF (Shintani et al., 2017). It may be that exogenous HGF is required to induce cell proliferation mediated by MET in 5637. Therefore, as the 5637 cell viability assay was conducted in the absence of HGF this may be the reason that capmatinib did not reduce cell viability. It has been reported that HGF induced invasion in 5637 but cell proliferation was not examined (Wang et al., 2007). Therefore, it is possible that signalling via MET in 5637 may induce invasion but not affect cell proliferation. An alternative explanation for the insensitivity of 5637 to capmatinib is that 5637 may express an ATP-binding cassette transporter such as P-glycoprotein which can induce drug resistance by transporting TKIs out of cancer cells (He and Wei, 2012). It was considered that 5637 may possess a gatekeeper mutation in MET which renders the line resistant to capmatinib. However, the exome of 5637 was previously sequenced by Nickerson et al. and a mutation in MET was not detected (Nickerson et al., 2017). Finally, it is possible that MET could be promoting cell proliferation and survival in 5637 via crosstalk with another receptor, and when MET is inhibited activation of the other receptor
compensates for the reduced MET activity. Therefore, whether capmatinib was defective or RT112 parental and the resistant derivatives were resistant to MET inhibition not was not confirmed. Immunoblot analysis to examine whether capmatinib inhibited MET phosphorylation in the RT112 resistant derivatives or 5637 would have clarified this.

Cell viability was examined in RT112 parental and R1 for the combined treatment of the IGF1R inhibitor linsitinib and PD and for each drug as a single agent. RT112 parental was sensitive to linsitinib single agent treatment. This suggests that IGF1R signalling is active in RT112 parental cells rather than being activated as a mechanism of acquired resistance in the resistant derivatives. The RT112 resistant derivatives also displayed sensitivity to the IGF1R TKI linsitinib. In RT112 parental a greater sensitivity was displayed to the combination of linsitinib and PD than to either drug as a single agent. This suggests that if either FGFR3 or IGF1R is inhibited in parental RT112, signalling via the other receptor maintains some cell viability.

IGF1R has been implicated in resistance to inhibition of EGFR in NSCLC, ERBB2 in breast cancer and BRAF in melanoma (Harris et al., 2007; Huang and Fu, 2015; Peled et al., 2013; Villanueva et al., 2010). Targeted agents specific for IGF1R are under clinical development. IGF1R expression has been associated with higher stage and grade in colorectal cancer and IGF1R targeted agents have been assessed in clinical trials for the treatment of this cancer (Hakam et al., 1999; Shali et al., 2016). A phase II/III clinical trial tested addition of the monoclonal antibody dalotuzumab specific for IGF1R to the standard treatment of irinotecan and cetuximab in metastatic colorectal cancer patients but found no increase in survival (Sclafani et al., 2015). A phase III clinical trials in patients with non-adenocarcinoma NSCLC with the monoclonal antibody figitumumab, which inhibits IGF1R, indicated that the addition of this monoclonal antibody to treatment with chemotherapy or erlotinib did not increase patient survival (Langer et al., 2014; Scagliotti et al., 2015). The results in this Chapter suggest that single agent treatment with an inhibitor of IGF1R could overcome resistance to FGFR-targeted agents in some bladder cancers.

Single agent sapitinib had very little effect on the viability of RT112 resistant derivatives. The combination of sapitinib and PD did have some efficacy in RT112 parental and resistant derivatives but not enough to determine an IC50 in these lines. This suggests that ERBB family signalling is activated and does contribute to
cell proliferation or survival in RT112 parental and the RT112 resistant derivatives when these lines are cultured in PD.

The reduced cell viability in RT112 R3 was unexpected as exome sequencing and single cell cloning in Chapter 5 revealed that a HRAS G12S mutation was present in 73% of RT112 R3 cells. As HRAS is downstream of RTKs, it was thought that this mutation would constitutively promote cell proliferation and survival in RT112 R3 despite FGFR3 and EGFR family inhibition. It is unclear whether EGFR family signalling contributes to the cell proliferation or survival of all or only a subset of the RT112 parental and RT112 resistant derivative cells. It may be that the combinatorial treatment of sapitinib and PD primarily reduces the cell viability of RT112 R3 due to efficacy in the subpopulation of RT112 R3 cells which do not possess the HRAS G12S mutation.

It was considered that only a small reduction in cell viability observed in RT112 parental and resistant derivatives could be due to the batch of sapitinib being less active. For this reason the efficacy of the sapitinib was assessed in DSH1, which has been previously reported to be sensitive to the TKI lapatinib which exhibits specificity for EGFR and ERBB2 (de Martino et al., 2014). DSH1 was found to be sensitive to single agent treatment with sapitinib. Therefore, it can be concluded that inhibition of the EGFR family of RTKs did not overcome PD resistance in the RT112 resistant lines.

Wang et al. generated RT112 derivatives resistant to FGFR inhibition via long term culture with the FGFR TKIs BGJ398 and ponatinib. These resistant derivatives had become resistant via activation of ERBB2 and ERBB3 (Wang et al., 2014). Single agent treatment with sapitinib reduced the viability of the BGJ398 resistant derivatives to approximately 70% of the untreated control. They reported that treatment with sapitinib in combination with the FGFR TKI BGJ398 reduced cell viability to 20-30% of the untreated control in these resistant derivatives (Wang et al., 2014). It can be concluded that the RT112 PD resistant derivatives in this study have become resistant to FGFR inhibition by a different mechanism than the mechanism observed by Wang et al. in their RT112 resistant derivatives.

IGF1R has been reported to heterodimerise with EGFR family members inducing resistance to EGFR family targeted agents. Morgillo et al. produced an EGFR TKI resistant derivative of the NSCLC cell line H460 via long term culture in erlotinib. Immunoblot analysis showed that this resistant line had increased expression of phospho-IGF1R compared to parental line. Coimmunoprecipitation showed that there was an increased binding of EGFR to IGF1R in the H460
erlotinib-resistant derivative, and in parental H460 acutely treated with erlotinib compared to the parental line cultured without erlotinib. Treatment with erlotinib and the IGF1R inhibitor AG1024 reduced colony formation in both parental H460 and the H460 erlotinib-resistant derivative to a greater extent than treatment with either drug as a single agent (Morgillo et al., 2006). Nahta et al. produced trastuzumab resistant cells from the breast cancer cell line SKBR3 via long-term culture of the parental line in trastuzumab. These resistant cells had increased expression of phospho-IGF1R and coimmunoprecipitation of ERBB2 and IGF1R was observed in the resistant cells but not the parental line. Stimulation of the resistant cells with the IGF1R ligand IGF1 induced ERBB2 phosphorylation in the resistant cells but not parental SKBR3 (Nahta et al., 2005). IGF1R has also been reported to interact with ERBB3 and ERBB4 in gefitinib-resistant cells derived from breast cancer cell line MCF-7 (Jones et al., 2006). It is possible that IGF1R is heterodimerizing with EGFR family members in the RT112 parental and RT112 resistant derivatives and that this induces some sensitivity to the combination of sapitinib and PD.

Sensitivity to erlotinib was tested in RT4 parental and R1 as an increase in total and phosphorylated EGFR in RT4 R1 was observed via immunoblot analysis in Chapter 4. Additionally, microarray analysis showed an increase in EGFR mRNA in RT4 R1 compared to the parental line. Both these lines exhibited sensitivity to erlotinib which confirms that both RT4 parental and R1 are dependent on EGFR signalling. The inhibition of EGFR by erlotinib in parental RT4 and RT4 R1 could be confirmed by immunoblot analysis. The efficacy of erlotinib was potentiated by the addition of PD. This suggests that targeting the FGFR family and EGFR in combination could be an effective treatment for urothelial carcinoma. Signalling via EGFR has previously been implicated in resistance to FGFR-targeted agents in bladder cancer cell lines. An siRNA screen which identified EGFR as limiting the sensitivity to PD in three bladder cancer cell lines with mutant FGFR3, RT4, RT112 and MGH-U3 (Herrera-Abreu et al., 2013). RT112 and 639V, a bladder cancer cell line with an FGFR3 point mutation, showed increased phosphorylation of EGFR upon acute treatment with PD. A RT112 xenograft mouse model showed that treatment with PD and gefitinib reduced tumour volume to a greater extent than either of the drugs given separately (Herrera-Abreu et al., 2013).

EGFR-targeted agents are already in use as treatments for treat lung and pancreatic and colorectal cancer (Lubner et al., 2017; Mosquera et al., 2016; Singh and Jadhav, 2017). EGFR-targeted agents may be efficacious in a subset of urothelial carcinoma patients who have relapsed on FGFR-targeted therapy. Approximately 60% of bladder cancer tumours express membranous EGFR and
expression does not correlate with the stage or grade of tumours (Ibrahim et al., 2009). Approximately 6% of MIBC tumours exhibit gain of copy number of 7p11.2 which contains EGFR (Robertson et al., 2017). TKIs targeting the EGFR family have entered clinical trials in urothelial carcinomas with EGFR or ERBB2 overexpression and have generally been found not to significantly benefit patients (Choudhury et al., 2016; Miller et al., 2016; Petrylak et al., 2010; Powles et al., 2017). It may be that combinatorial treatment with an EGFR and an FGFR family inhibitor would be beneficial in those patients who have FGFR3 alterations.

The results of this Chapter have some limitations. There was large variability between individual cell viability assays, therefore, it would have been beneficial to have assayed the resistant lines for their sensitivity to PD as a single agent, as a control, at the same time as examining the sensitivity of the resistant lines to the combinatorial treatment of each small-molecule inhibitor + PD. This would have enabled a direct comparison of the efficacy of single agent PD and with the efficacy of each drug combination. Additionally, for each cell viability experiment it would have been more reliable to have presented the average of the cell viability assay repeats, rather than presenting one representative example. This would have enabled a statistical test to have been conducted to determine whether observed differences in viability were significant.
Chapter 8
Final Discussion

This project was conducted with the aim of examining the differences between the RT112 and RT4 resistant derivatives and their parental lines. It was hoped that examining these differences would identify the mechanisms by which the resistant derivatives have acquired reduced sensitivity to FGFR inhibition. The identification of these mechanisms would allow drugs to be tested for their ability to re-sensitize the resistant cells to FGFR inhibition.

Initial characterisation of the resistant lines identified that RT112 R1, RT112 R2 and RT4 R1 had a different morphology to their parental lines but that these changes in morphology were reversed upon culture without PD. RT112 resistant derivatives maintained resistance to PD following culture without PD and phenotypic reversion, whereas RT4 R1 did not. Reduced expression of FGFR3 was observed in the RT112 resistant derivatives indicating that FGFR3 overexpression or mutation or drug efflux is not the cause of resistance in these cells. Increased expression of N-cadherin was observed in RT112 R1 and R2 cultured in PD, suggesting these cells may have undergone an EMT. Following the initial characterisation of the parental and resistant cells, expression and phosphorylation of a number of RTKs was examined with immunoblot analysis. This identified increased expression of phospho-MET in the RT112 resistant derivatives, phospho-ERBB3 in RT112 R1 and R2 and phospho-EGFR in RT4 R1. Exome sequencing identified a HRAS G12S mutation in RT112 R3. Retroviral transfection of HRAS G12V into parental RT112 demonstrated that gain of constitutively active HRAS induces resistance to PD. Copy number analysis showed that EGFR was not amplified in RT4 R1 and a NGS assay which screens for common EGFR mutations did not identify an EGFR mutation in this line. Transcriptome analysis identified EMT-associated gene expression changes in the RT112 resistant derivatives compared to parental RT112 and identified a reduction in expression of genes which regulate fatty acid synthesis in RT112 and RT4 resistant derivatives. Immunoblot analysis confirmed reduced expression of mature SREBP1, a transcription factor which regulates the synthesis of unsaturated fatty acids, and SCD1, the rate limiting enzyme in the production of monounsaturated fatty acids, in RT112 resistant derivatives. Additionally, transcriptome analysis identified an
increase in expression of IGF1R in RT112 R1 and R2 and increase in expression of KDM6A in RT112 and RT4 resistant derivatives. Finally, a range of TKIs were tested for their efficacy at reducing the viability of resistant cells. The IGF1R TKI linsitinib and the EGFR TKI erlotinib proved effective at reducing viability in RT112 and RT4 resistant lines respectively.

A key finding of this project was the identification of the HRAS G12S mutation in RT112 R3 and the confirmation that the introduction of mutant HRAS into RT112 parental induces resistance to PD. This suggests that mutation of HRAS may occur in urothelial carcinoma patients as a mechanism of acquired resistance to FGFR-targeted agents. RAS and FGFR3 mutations are mutually exclusive in urothelial carcinoma (Jebar et al., 2005). This suggests that these mutations have a similar function and therefore there is no selective pressure for a urothelial carcinoma to gain activating mutations in both these genes. It appears that the inhibition of FGFR3 induces a selective pressure to gain a mutation of similar functionality. RAS activates the MAP kinase and PI3 kinase pathways: two pathways also activated by FGFR3 (Klint et al., 1999; Rodriguez-Viciana et al., 1994; van Weering et al., 1998; Zhang et al., 1993). As activation of both these pathways was reduced in RT112 parental acutely treated with PD but not in RT112 resistant derivatives, the reactivation of these pathways may be key to inducing resistance to PD.

Despite decades of research, development of direct inhibitors of RAS has proved difficult due to RAS lacking deep hydrophobic binding pockets to which small molecule inhibitors could bind and the low affinity of RAS for GTP (Spencer-Smith and O'Bryan, 2017). Attempts to inhibit RAS with small molecule inhibitors have included inducing GTP hydrolysis with a GTP analogue which is more effectively hydrolysed by the mutant RAS than GTP (Ahmadian et al., 1999). Another possible way to directly inhibit RAS may be by inhibiting the interaction of RAS with guanine exchange factors. For example, Patgiri et al. developed a mimetic of the guanine exchange factor SOS which reduced the interaction of GDP-bound mutant RAS with SOS, thereby preventing the mutant RAS from binding GTP and reducing the activation of downstream signalling (Patgiri et al., 2011). Monoclonal antibodies have been developed which directly inhibit RAS, although full size antibodies cannot cross the plasma membrane to target intracellular molecules (Furth et al., 1982). A monoclonal antibody, RT11, has been developed which was able to enter the cytosol via clathrin-mediated endocytosis and bind to the protein-protein interface of the active GTP-bound form of RAS. This inhibited downstream signalling. Upon further engineering of RT11 so that it could bind
tumour-associated integrins, this antibody was able to inhibit the growth of RAS mutant xenografts in mice (Choi et al., 2014; Shin et al., 2017). An alternative approach taken to reduce the activity of mutant RAS is by reducing the presence of RAS at the cell membrane. This can be achieved with farnesyltransferase and PDEδ inhibitors. Farnesyltransferase inhibitors disrupt the post-translational modifications of RAS which results in reduced RAS localisation at the cell membrane (Choy et al., 1999; Reiss et al., 1990). PDEδ inhibitors inhibit the binding of PDEδ to KRAS, reducing the trafficking of KRAS to the cell membrane (Zimmermann et al., 2013).

The development of inhibitors specific for molecules downstream of RAS has been more successful than direct RAS inhibition. MEK is a kinase in the MAP kinase pathway. The MEK inhibitor trametinib is approved by the EMA and FDA for the treatment of V600 mutant melanoma and NSCLC and cobimetinib is approved for the treatment of BRAF V600 mutant melanoma (Cheng and Tian, 2017). Inhibition of MEK has so far been unsuccessful as a mechanism of treating KRAS mutant colorectal cancer (Bahrami et al., 2018). A number of ongoing phase I and II trials are examining the efficacy of MEK inhibitors in combination with chemotherapy or other targeted agents, including PI3 kinase inhibitors, in KRAS mutant NSCLC (Tomasini et al., 2016). Bockorny et al. reported that the long-term culture of the FGFR1-amplified NSCLC cell line, NCI-H2077, produced a resistant derivative with an NRAS Q61R mutation. Treatment of the resistant cells with the pan-FGFR TKI BGJ398 and the MEK inhibitor trametinib reduced cell proliferation to a greater extent than treatment with BGJ398 as a single agent. It was observed that the combination of BGJ398 and trametinib was well tolerated, significantly slowed tumour progression and increased progression-free survival in a mouse NCI-H2077 xenograft model (Bockorny et al., 2018). Treatment with a MEK inhibitor such as trametinib may overcome the resistance to PD observed in RT112 R3 and could overcome resistance to FGFR inhibitors in urothelial carcinomas if they had gained mutations in the MAP kinase pathway. Examination of whether MEK inhibition would overcome PD resistance in RT112 R3 was not conducted due to time limitations.

Inhibition of EGFR with erlotinib overcame resistance to PD in RT4 R1. However, erlotinib was more effective in parental RT4 than RT4 R1 suggesting that EGFR inhibition may be more effective as a first line rather than second line treatment. Treatment of RT4 R1 with the combination of erlotinib and PD reduced cell viability to a greater extent than either TKI alone. Isobologram analysis would have determined if treatment with erlotinib and PD was additive in RT4 parental and
R1 but was not conducted due to time limitations. Herrera-Abreu et al. reported that
activation of EGFR was a mechanism of short term survival in response to PD in
RT112 that could be overcome by combined treatment with PD and the small
molecule inhibitor of EGFR, gefitinib. Immunoblot analysis revealed that treatment
with the MEK inhibitor CI-1040 induced an increase in EGFR phosphorylation and
that PD treatment induced EGFR accumulation at the cell membrane and giant
early endosomes. The mechanism by which expression of EGFR and phospho-
EGFR was increased in RT4 R1 is unknown. Small molecule inhibitors and
monoclonal antibodies which inhibit EGFR are approved for the treatment of lung,
pancreatic and colorectal cancer (Lubner et al., 2017; Mosquera et al., 2016; Singh
and Jadhav, 2017).

In addition to signalling as a homodimer, EGFR heterodimerises with other
EGFR family members (Roskoski, 2014), therefore it is possible that other EGFR
family members are implicated in the resistance to PD in RT4 R1. Wang et al.
found RT112 activated ERBB2 and ERBB3 signalling as a mechanism of
resistance to the FGFR TKIs BGJ398 and ponatinib upon long-term culture in these
inhibitors (Wang et al., 2014). Canfield et al. showed that knockdown of ERBB4
induced apoptosis in ERBB2-positive breast cancer cell lines with acquired
resistance to the EGFR, ERBB2 and ERBB3 TKI lapatinib and the ERBB2-specific
monoclonal antibody trastuzumab (Canfield et al., 2015). Immunoblot analysis
showed that expression of phospho-ERBB2 remained low in RT4 R1, whereas,
phospho-ERBB3 expression was increased in RT4 R1. Immunoblot analysis of total
ERBB4 and phospho-ERBB4 expression was not conducted with RT4 parental or
R1 due to time limitations. Microarray analysis showed that expression of ERBB4
was non-significantly increased in RT4 R1 + PD compared to parental no PD. It is
possible that activation of ERBB3 or ERBB4 contributes to the RT4 R1 resistant
phenotype via dimerisation with EGFR. Whether this is the case could be tested by
examining whether knockdown of ERBB3 or ERBB4 expression or treatment with
an ERBB3- or ERBB4-specific inhibitor re-sensitised RT4 R1 to PD.

Microarray analysis showed that expression of AREG and BTC, which
encode the EGFR ligands amphiregulin and betacellulin respectively, was
significantly increased in RT4 R1 + PD compared to parental no PD. Therefore, the
increased activation of EGFR in RT4 R1 may be mediated by the increased
expression of these ligands. Whether protein expression of amphiregulin and
betacellulin was increased in RT4 R1 could have been examined with immunoblot
analysis. Whether these ligands mediate PD resistance could be determined by
examining whether knockdown of AREG or BTC expression re-sensitised RT4 R1.
to PD. Additionally, whether treatment with amphiregulin or betacellulin induced resistance to PD in parental RT4 could be examined. These experiments were not conducted due to time limitations.

Urothelial carcinomas with expression or mutation of EGFR, ERBB2 or ERBB3 could be intrinsically resistant to FGFR-targeted agents as they may be able to signal via these EGFR family members. The sensitivity of urothelial carcinomas to EGFR-targeted agents has been assessed in clinical trials but unfortunately these have mainly been disappointing. A phase II clinical trial conducted by Pruthi et al. examined erlotinib as a neoadjuvant prior to radical cystectomy. Erlotinib was well tolerated and the results indicated that erlotinib may be efficacious as a single agent (Pruthi et al., 2010). A phase II clinical trial with gefitinib in metastatic urothelial carcinoma patients who had failed previous chemotherapy concluded that further study with this TKI in this setting was not justified (Petrylak et al., 2010). Another phase II clinical trial showed that the addition of gefitinib to treatment with chemotherapy did not significantly increase time to progression, the primary endpoint, in patients with advanced or metastatic urothelial carcinoma (Miller et al., 2016). Choudhury et al. conducted a phase II study in platinum-refractory metastatic urothelial carcinoma patients with the EGFR and ERBB2 TKI afatinib. They observed that treatment with afatinib showed significant activity in patients with ERBB2 amplification and ERBB3 mutations but EGFR amplification was not indicative of a response to afatinib (Choudhury et al., 2016). Powles et al. conducted a phase III clinical trial which found that treatment with the EGFR and ERBB2 TKI lapatinib following on from chemotherapy did not significantly improve outcomes in urothelial carcinoma patients with EGFR or ERBB2 overexpression (Powles et al., 2017). The findings in this project and the research conducted by Herrera-Abreu et al. indicate that EGFR-targeted agents may benefit urothelial carcinoma patients with resistance to FGFR-targeted agents. To my knowledge, clinical trials have not been conducted in urothelial carcinoma patients whose tumours are FGFR3 mutant or overexpress FGFR3 with the combination of EGFR- and FGFR-targeted agents. Similarly, the efficacy of EGFR-targeted agents has not been assessed in urothelial carcinoma patients who have acquired resistance to FGFR-targeted agents. Whether the combination of FGFR and EGFR-targeted agents would be tolerated in patients with urothelial carcinoma is unknown.

Microarray analysis showed that EPHA3 mRNA expression was significantly increased in RT4 R1 + PD compared to RT4 parental no PD. Conflicting evidence has found the EPH family of RTKs to be both oncogenic and tumour-suppressive.
Agonistic and antagonistic targeted agents specific for EPH family members, such as ifabotuzumab which is a EphA3 agonist, have entered early stage clinical trials (Lodola et al., 2017). Immunoblot analysis could have been used to confirm if expression of EphA3 and phospho-EphA3 was increased in RT4 R1. Whether the increase in expression of EPHA3 contributes to the resistant phenotype in RT4 R1 could be tested by examining whether knockdown of EPHA3 expression re-sensitised RT4 R1 to PD. This was not conducted due to time limitations.

Inhibition of IGF1R with linsitinib was efficacious in RT112 parental, R1, R2 and R3. As linsitinib reduced cell viability in parental RT112, signalling via IGF1R did not arise in the RT112 resistant lines as a mechanism of resistance to PD, rather the dependency on IGF1R signalling was pre-existing. It was surprising that RT112 R3 exhibited sensitivity to IGF1R, as this line had a HRAS mutation, resulting in a constitutively active protein, and it was thought that this mutation would diminish the dependency on RTK activation in RT112. Isobologram analysis could have been conducted to determine if treatment with linsitinib and PD was additive in RT112 parental and resistant derivatives, but was not due to time limitations. Immunoblot analysis to determine the expression of IGF1R and phospho-IGF1R in RT112 parental and the resistant lines was not conducted during this project due to time limitations. This would have determined whether the resistant lines cultured in PD or parental RT112 acutely treated with PD had increased activation of IGF1R, suggesting that IGF1R activation compensated for the loss of FGFR signalling. Alternatively, IGF1R may be activated at a similar level in RT112 parental and the RT112 resistant derivatives. Higher expression of IGF1R RNA correlates with a lower overall survival time in MIBC patients (Wang et al., 2017). Sun et al. reported that treatment of the urothelial carcinoma cell line, T24, with IGF1 significantly reduced mitomycin-induced apoptosis. Furthermore, depletion of IGF1R by treatment with an antisense oligodeoxynucleotide, in combination with mitomycin, induced apoptosis and cell proliferation to a greater extent than single agent treatment with either therapy (Sun et al., 2001).

Unfortunately, the results of clinical trials with targeted agents with specificity for IGF1R have been disappointing (Janssen and Varewijck, 2014). A phase III clinical trial was conducted with linsitinib in patients with advanced or metastatic adrenocortical carcinoma, but linsitinib did not increase overall survival (Fassnacht et al., 2015). EGFR is reported to transactivate IGF1R and signalling via IGF1R has been implicated in resistance to EGFR-targeted therapy in NSCLC (Burgaud and Baserga, 1996; Li et al., 2017; Morgillo et al., 2007). For this reason, phase II trials in NSCLC patients have compared treatment with erlotinib to
treatment with linsitinib in combination with erlotinib. Unfortunately, these trials have found that linsitinib did not improve patient outcomes (Ciuleanu et al., 2017; Leighl et al., 2017). Phase II/III clinical trials in colorectal cancer patients have examined the addition of the monoclonal antibody dalotuzumab, which has specificity for IGF1R, to treatment with the EGFR-specific monoclonal antibody cetuximab and chemotherapy. These trials have indicated that this targeted agent is ineffective at treating colorectal cancers. However, expression levels of IGF1R, IGF1 or IGF2 may indicate which patients would benefit from IGF1R-targeted therapy (Sclafani et al., 2015; Sclafani et al., 2017). As IGF1R has been implicated in resistance to EGFR-targeted agents it is logical that this RTK could also induce resistance to inhibition of other RTKs. Whilst IGF1R-targeted agents are still in the clinical trial stage, and the results of clinical trials have so far been disappointing, the use of biomarkers to stratify who receives IGF1R-targeted agents may yield more promising results.

The activation of RTKs in RT112 resistant lines was examined with a phospho-RTK array (PathScan® RTK signaling antibody array Kit #7982). However, as the results of this array were disappointing with a low signal detected from RTK specific spots, the results of this analysis were not presented in this thesis. An alternative phospho-RTK array from a different supplier was identified. However, analysis was not conducted with this array due time limitations. This analysis may have identified other RTKs which were activated in RT112 R1 and R2.

The initial examination of RT112 R1 and R2 revealed that these cells had lost the parental RT112 epithelial morphology, gained a mesenchymal morphology and increased protein expression of N-cadherin. A complete EMT was not observed in these lines as E-cadherin expression remained constant between RT112 parental, R1 and R2. N-cadherin expression was not increased in parental RT112 acutely treated with PD and microarray analysis showed that EMT markers were not significantly differentially expressed between parental RT112 cultured without PD and parental RT112 acutely treated with PD. MetaCore™ analysis of microarray data found that pathways relating to EMT maps were upregulated in R1, R2 and R3 compared to RT112 parental and in RT4 R1 compared to RT4 parental. This is similar to what was reported by Wang et al. whose FGFR TKI-resistant RT112 derivatives, produced by long term culture in either ponatinib or BGJ398, reverted to a mesenchymal morphology and had increased expression of CDH2 and FN1 (Wang et al., 2014). EMT-like changes have been reported in in vitro models of adaptive resistance to other targeted therapies such as EGFR, ERBB2 and ALK TKIs (Brown et al., 2016; Gower et al., 2016; Lee et al., 2017).
mesenchymal phenotype is associated with greater cell migration and invasion. It would be undesirable for treatment with a FGFR TKI to induce these characteristics in a patient as this could result in a greater risk of metastasis (Singh et al., 2017). This project has not determined whether the EMT-like changes observed in the resistant lines were fundamental to, or a by-product of, the resistant phenotype. Li et al. reported that activation of signalling via IGF1R in gefitinib resistant derivatives of the EGFR-mutant NSCLC cell lines PC9 and HCC827 induced an EMT. Treatment of the PC9 gefitinib resistant derivatives with the IGF1R inhibitor picropodophyllin increased sensitivity to gefitinib and reduced expression of mesenchymal markers (Li et al., 2017). Signalling via FGFR3 is associated with an epithelial morphology in urothelial carcinoma cell lines (Cheng et al., 2013). It is possible that, upon culture with PD, the resistant cells lose the signals from FGFR3 that induce an epithelial phenotype and gain a more mesenchymal phenotype. Signalling via EGFR has also been associated with EMT (Chang et al., 2012; Serrano et al., 2014). However, EGFR did not induce an EMT in RT4 R1 as this resistant derivative did not exhibit a mesenchymal morphology, exhibited low expression of N-cadherin and vimentin and maintained expression of E-cadherin. Cell migration and invasion was not examined in the parental lines and their resistant derivatives so the extent to which long term culture in PD induces these characteristics has not been established.

Bladder cancers can be classified as 'luminal' if their gene expression is more similar to the superficial or intermediate layers of the urothelium or 'basal' if their gene expression is more similar to the basal layer of the urothelium. Basal tumours exhibit upregulation of p63 target genes and are more likely to have mutations in TP53 (Choi et al., 2014b; Robertson et al., 2017). Luminal tumours exhibit upregulation of PPARγ target genes and are more likely to have mutations in FGFR3, ELF3, CDKN1A, and TSC1. Basal MIBC are more aggressive and patients with basal tumours have shorter survival times than patients with luminal tumours (Dadhania et al., 2016). Therefore, if urothelial tumours switch towards a more basal phenotype upon being treated with FGFR-targeted agents this could result in a worse prognosis for patients. Both RT112 and RT4 have been previously classified as luminal (Warrick et al., 2016). Microarray and qRT-PCR analysis suggested that the expression of some basal markers was increased and expression of some luminal markers was decreased in RT112 resistant derivatives compared to the parental line. However, immunoblot analysis showed that expression of the basal markers cytokeratin 5/6 and CD44 was low in RT112
parental and the RT112 resistant derivatives. Therefore, it is unlikely that the RT112 resistant derivatives have gained a basal phenotype.

Previous research has shown that fatty acid synthesis is reduced in RT112 upon FGFR3 knockdown due to FGFR3 regulation of the transcription factor SREBP1 (Du et al., 2012). The weak base chloroquine induces lysosomal cell death by diffusing into lysosomes and raising the lysosomal pH. This induces lysosomal swelling, cathepsin leakage and cathepsin-mediated activation of caspases. King et al. reported that inhibition of FGFRs, PI3Kα, AKT or mTOR potentiated the lysosomal cell death induced by chloroquine in RT112. This was due to a reduction in the biosynthesis of cholesterol which can promote the integrity of lysosomal membranes (King et al., 2016). Here, microarray analysis indicated that there was differential expression of genes regulating lipid metabolism between RT112 experimental conditions and between RT4 experimental conditions. Immunoblot analysis indicated that expression of mature SREBP1, the transcription factor which regulates expression of fatty acid synthesis genes, was reduced in RT112 parental acutely treated with and RT112 R1 cultured in PD. Mature SREBP1 expression was reduced to a lesser extent in R2 and R3 cultured in PD. SCD1 is the rate-limiting enzyme in the production of mono-unsaturated fatty acids from saturated fatty acids (Igal, 2016). Immunoblot of SCD1 showed that expression of this protein was reduced in RT112 parental acutely treated with PD and R1 and R2 cultured in PD. Expression was also reduced but to a lesser extent in R3 + PD. These results confirmed that fatty acid synthesis was reduced in RT112 resistant derivatives. Protein expression of SCD1 and SREBP1 was not examined in RT4 parental and R1. However, microarray analysis indicated that genes involved in fatty acid synthesis were downregulated in RT4 R1 compared to RT4 parental. This indicates that the reduced sensitivity to PD exhibited by RT112 and RT4 resistant derivatives is not mediated by the restoration of fatty acid synthesis during FGFR3 inhibition. Even the HRAS mutation in RT112 R3 did not induce the return of fatty acid synthesis markers to the expression level observed in RT112 parental. This highlights that, although the two mutations are mutually exclusive, they do not have an identical function in the context of driving urothelial carcinoma. Further examination of fatty acid synthesis could have been conducted by examining the incorporation of 14C-labelled acetate into fatty acids as conducted by Du et al. Alternatively, cellular lipid composition of parental and resistant lines could be examined with mass spectrometry as conducted by Griffiths et al. (Griffiths et al., 2013). This could have confirmed whether the observed changes in gene expression resulted in an overall reduction in fatty acid synthesis.
As RT4 R1, RT112 R1 and RT112 R2 exhibited morphological and gene expression changes which were reversible upon culture without PD, it was thought that these lines were in a resistant state mediated via epigenetic or gene expression changes rather than via a genetic mechanism. RT112 R1 and R2 maintained their resistance following culture without PD for 4 passages, despite reverting to a morphology similar to parental RT112. This was in contrast to the RT112 ponatinib and BGJ398 resistant derivatives produced by Wang et al. which regained sensitivity to FGFR inhibition following culture without drug for two to four weeks (Wang et al., 2014). It is not known whether culture of RT112 R1 and R2 for a longer period of time out of PD would have re-sensitized the lines to PD. Although the epigenetic state of RT112 R1 and R2 was not examined, these lines may have gained epigenetic alterations which enable the cells to maintain their resistance to FGFR inhibition.

As it was thought that the resistant state in RT4 R1, RT112 R1 and RT112 R2 was mediated via epigenetic or gene expression changes rather than via a genetic mechanism, the inhibition of epigenetic modifiers could be a useful strategy to overcome resistance FGFR TKIs. Examination of whether knockdown of epigenetic modifiers or treatment with inhibitors of epigenetic modifies re-sensitizes the resistant lines to PD could identify epigenetic alterations which facilitate PD resistance and therefore highlight an alternative therapeutic approach to overcoming PD resistance.

Epigenetic modifiers have been targeted to overcome resistance to targeted therapy. Gastrointestinal stromal tumours (GISTs) often have activating mutations in RTKs KIT and PDGFRA (Heinrich et al., 2003; Hirota et al., 1998). Mühlengberg et al. reported that treatment with the non-selective histone deacetylase inhibitor vorinostat and imatinib were additive in KIT-positive GIST cell lines. Vorinostat reduced KIT mRNA expression and increased acetylation of HSP90, a KIT chaperone, inducing KIT degradation (Mühlenberg et al., 2009). Bauer et al. conducted a phase I clinical trial which assessed the efficacy of imatinib in combination with the non-selective histone deacetylase inhibitor panobinostat in overcoming resistance in patients with gastrointestinal stromal tumours refractory to the combination of imatinib and the multitargeted TKI sunitinib. One out of the 11 patients showed a partial response, 7 had stable disease and 3 patients had progressive disease (Bauer et al., 2014).

Sharma et al. produced PC9 cells resistant to EGFR-TKIs, which mediated their resistance via activation of IGF1R, via culture in gefitinib for 9 days. It was
observed that the resistant cells had increased expression of KDM5A and that KDM5A knockdown did not reduce the proliferation of parental PC9 cells but did reduce the production of resistant cells upon treatment with gefitinib. Upon culture without an EGFR TKI, resistant cells maintained their resistant phenotype for approximately 30 passages before regaining gefitinib sensitivity (Sharma et al., 2010). Treatment with the KDM5A inhibitor YUKA1 reduced the formation of gefitinib-resistant colonies following the culture of PC9 in gefitinib for 35 days. Culture with YUKA1 as a single agent did not reduce the growth of PC9 (Gale et al., 2016). How long RT112 R1 and R2 would have to be cultured without PD to regain PD sensitivity is unknown. Hou et al. observed that expression of KDM5A was increased in the breast cancer lines SUM149 and SUM102 following 6 and 9 days culture in erlotinib. Stable knockdown of KDM5A in the breast cancer cell lines SUM149 and HCC1937 reduced the number of drug-tolerant SUM149 and HCC1937 cells following 30-day culture in erlotinib (Hou et al., 2012). Gale et al. cultured the breast cancer cell line BT474 with a low dose of trastuzumab for 35 days with and without YUKA1 and observed that YUKA1 reduced the formation of trastuzumab-resistant colonies. Culture with YUKA1 as a single agent did not reduce the growth of BT474 (Gale et al., 2016). Whether knockdown of KDM5A or treatment with a KDM5A inhibitor could re-sensitise RT112 R1 and R2 was not examined in this project due to time limitations. However, microarray analysis showed that KDM5A mRNA expression was not significantly differentially expressed between RT112 parental and the RT112 resistant lines. Expression of the histone demethylase KDM6A was significantly increased in the RT112 and RT4 resistant cells cultured in PD compared to their parental lines. Whether KDM6A contributes to the resistant phenotype could be tested by examining whether knockdown of KDM6A re-sensitizes these cells to PD.

Inhibition of the bromodomain and extraterimal (BET) family of chromatin readers has been previously examined in RT112 as a mechanism of increasing the efficacy of FGFR-targeted therapy. Binding of the BET bromodomain-containing protein BRD4 to MYC enhancers induces transcription of the oncogene MYC (Lovén et al., 2013). Mahe et al. observed that siRNA knockdown of the transcription factor MYC decreased FGFR3 expression in the bladder cancer cell lines MGH-U3, which has a Y375 FGFR3 activating mutation, and RT112. Treatment with PD reduced MYC expression in MGH-U3 and RT112 suggesting a MYC/FGFR3 positive feedback loop. In MGH-U3 and RT112, treatment with the PI3 kinase inhibitor LY294002 decreased phosphorylation of glycogen synthase kinase 3 β which induces the proteosomal degradation of MYC. Additionally, siRNA
knockdown of MAPK14, which encodes the MAP kinase protein p38α, reduced MYC mRNA expression. This demonstrated that both the MAP kinase and PI3 kinase pathway act downstream of FGFR3 to increase MYC expression. Treatment with the BET bromodomain inhibitor JQ1, in combination with PD, reduced cell viability in MGHU3 and RT112 in an additive fashion (Mahe et al., 2018). It is possible that treatment with a BET bromodomain inhibitor such as JQ1, in combination with an FGFR TKI might prevent the development of acquired resistance to FGFR3 inhibition.

A single cell clone of parental RT4 was used to generate RT4 R1, whereas a heterogenous population of RT112 cells, rather than a single cell clone, was used to generate the RT112 resistant derivatives. This was because a single cell clone of RT4 was available at the start of this project, whilst a single cell clone of RT112 was not. The use of a heterogenous population of RT112 cells made analysis of genetic and gene expression changes more challenging to interpret. For example, it is unclear whether the HRAS G12S mutation observed in RT112 R3 was present in a subpopulation of parental RT112 or was acquired by an RT112 cell during long-term culture in PD. Additionally a number of copy number differences were observed between parental RT112 and the RT112 R1 and between parental RT112 and the RT112 R3. These copy number differences may have been present in a subpopulation of cells prior to, or acquired during, the long-term culture of RT112 cells in PD. In contrast, as a single cell clone of parental RT4 was used to generate RT4 R1, the copy number differences observed between parental RT4 and RT4 R1 were likely to have been acquired during the long-term culture of RT4 cells in PD.

A limitation of the transcriptome analysis was that, as this involved examining expression of a large number of genes, a number of genes will have been falsely classified as differentially expressed between experimental conditions. Raising the threshold for classifying genes as differentially expressed may have reduced the number of false positives. However, this would have reduced the detection of true gene expression differences. The use of a greater number of repeats per experimental condition would have increased the reliability of the microarray data. In order to validate the microarray data, immunoblotting and qRT-PCR were used to confirm gene expression changes.

There are a number of experiments which would have been conducted had there been more time available to continue this project. A limitation of this study is that short tandem repeat profiling was last used to authenticate the cell line identity of the parental RT112 and RT4 in 2012 and that short tandem repeat profiling has not been used to authenticate the identity of the RT112 and RT4 resistant
derivatives. Had more time been available, short tandem repeat profiling would have been conducted to confirm the cell line identity of the RT112 and RT4 parental lines and resistant derivatives. Immunoblot analysis could have been conducted to determine the expression of IGF1R and phospho-IGF1R in RT112 parental and the resistant lines. Additionally, immunoblot analysis could have been used to show that treatment with capmatinib reduced MET phosphorylation in RT112 resistant lines, demonstrating the efficacy of this TKI. Microarray analysis identified an increase in expression of KDM6A mRNA expression in RT112 and RT4 resistant derivatives when cultured in PD. Additionally, microarray analysis identified an increase in ERBB4 and EPHA3 mRNA expression in RT4 R1 + PD. Immunoblot analysis could have determined whether these changes in mRNA expression induced a change in protein expression of KDM6A, ERBB4 and EphA3 and to examine whether ERBB4 and EphA3 were phosphorylated in RT4 R1. Whether KDM6A, ERBB4 or EPHA3 contributed to PD resistance could have been tested by examining whether knockdown of these genes, or treatment with a specific inhibitor, re-sensitised the resistant lines to PD. A cell viability assay could have been conducted with a MEK inhibitor to determine whether this treatment would overcome the resistance to PD observed in RT112 R3. Isobologram analysis could have been conducted to determine if treatment with erlotinib and PD was additive in RT4 parental and R1 and if treatment with linsitinib and PD was additive in RT112 parental and resistant derivatives.

In conclusion, this project has shown that mutations downstream of FGFR3, in this case a HRAS G12S mutation, can be acquired as a mechanism of resistance to FGFR inhibition. Currently RAS mutations cannot be directly targeted with therapeutic agents. As RAS mutations are frequent in cancer there is a requirement for effective treatments for RAS mutant tumours and research is ongoing to meet this need. As the RT112 resistant derivatives exhibited sensitivity to linsitinib, this suggests that it may be possible to target IGF1R to overcome resistance to FGFR inhibition. EGFR was identified as a mediator of acquired resistance to FGFR inhibition in RT4 R1. EGFR activation has been previously identified as a mechanism of short-term survival following treatment with PD in RT112 (Herrera-Abreu et al., 2013). A number of different RTKs have been reported in this study, and elsewhere, to induce resistance to FGFR-targeted agents in urothelial carcinoma cell lines. Therefore, the examination of the RTK expression profile of an FGFR-TKI resistant urothelial carcinoma, by a method such as immunohistochemistry, and screening for mutations in pathways activated downstream of FGFR3, may be key to treating refractory tumours.
Figure 8.1 Summary of key findings from immunoblot analysis, copy number analysis, whole exome-sequencing, transcriptome analysis and screen of targeted agents. A) Protein expression changes identified by immunoblot analysis. B) Genetic alterations identified by whole exome sequencing and copy number analysis thought to be most likely to be inducing resistance to PD. C) RNA expression changes identified by transcriptome analysis. D) Small-molecule inhibitors identified to efficaciously reduce cell viability as monotherapies.
Appendix A
Tissue culture media, buffers and solutions

RPMI-1640 Medium
500ml RPMI-1640 medium
50ml FCS
2mM GlutaMax

Trypsin/versene
dH2O
0.25% (w/v) Trypsin
0.02% (w/v) EDTA
HBSS minus Ca2+ and Mg2+

Ham’s F12 complete medium
500ml Ham’s F12 nutrient mixture
1% FCS
1% insulin-transferrin-selenium
1% minimal essential medium non-essential amino acids
25mM Hyrocortisone
2mM GlutaMax

Freezing medium
120ml Ham’s F12
15ml FCS
15ml DMSO

Blocking solution for Western blotting
10ml PBS
0.1% (v/v) Tween 20
4% non-fat milk

Antibody diluent for Western blotting
10ml PBS
0.1% (v/v) Tween 20
2% non-fat milk

SDS loading buffer (5x)
1.25ml 2M Tris-HCl pH 6.8
3ml 30% glycerol
2mg bromphenol blue
5.75ml molecular biology grade water (Sigma Aldrich)

DNA loading buffer
2.5g Ficoll
0.04g bromphenol blue
0.04g Xylene cyanole
10ml molecular biology grade water (Sigma Aldrich)
Stripping buffer for Western blotting
120.12g Urea
10ml 2M Tris-HCl pH 6.8
200ml dH$_2$O

RIPA lysis buffer
PBS
1% Triton x100
1mM EDTA
0.5% (w/v) Sodium deoxycholate
0.1% SDS
Appendix B
List of suppliers

Abcam
330 Cambridge Science Park, Cambridge, CB4 0FL, UK
http://www.abcam.com/

Adooq Bioscience
4000 Barranca Parkway, Suite 250, Irvine, California, 92604, USA
http://www.adooq.com/

Affymetrix
Supplied by Thermo Fisher Scientific
https://www.thermofisher.com/

Agilent
5301 Stevens Creek Boulevard, Santa Clara, California, 95051, USA
https://www.agilent.com

Applied Biosystems
Supplied by Life technologies
http://www.lifetechnologies.com

Apex Technology
4745 Sutton Park Court, Suite 402, Jacksonville, Florida, 32224, USA
http://www.apextechnology.com/

Beckman Coulter
Oakley Court, Kingsmead Business Park, London Road, High Wycombe, HP11 1JU, UK
https://www.beckmancoulter.com

Berthold Technologies
Calmbacher Strasse 22, 75323 Bad Wildbad, Germany
https://www.berthold.com

Bio-Rad
Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX, UK
http://www.bio-rad.com

Biosera
Rue de la Caille, 49340. Nuaille, France
http://www.biosera.com

Biovision
155 South Milpitas Boulevard, Milpitas, California, 95035, USA
https://www.biovision.com/

BMG labtech
8 Bell Business Park, Smeaton Close, Aylesbury, Buckinghamshire, HP19 8JR, UK
https://www.bmglabtech.com
Cambridge Bioscience
Munro House, Trafalgar Way, Bar Hill, Cambridge, CB23 8SQ, UK
https://www.bioscience.co.uk/

Cayman Chemical
Munro House Trafalgar Way Bar Hill, Cambridge, CB23 8SQ, UK
https://www.caymanchem.com

Cell Signalling Technology
3 Trask Lane, Danvers, Massachusetts, 01923, USA
https://www.cellsignal.com/

Corning Life Sciences B.V.
Fogostraat 12, 1060 LJ Amsterdam, The Netherlands, Amsterdam, 1060, The Netherlands
https://www.corning.com

Dako
Supplied by Agilent
https://www.agilent.com

Gibco
Supplied by Life technologies
http://www.lifetechnologies.com

Illumina
Chesterford Research Park, Little Chesterford, Near Saffron Walden, Essex, CB10 1XL UK
https://www.illumina.com/

Invitrogen
Supplied by Life technologies
http://www.lifetechnologies.com

Labtech international
Mytogen House, 11 Browning Road, Heathfield, East Sussex, TN21 8DB, UK
https://www.labtech.com/

Life Technologies
3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK
https://www.lifetechnologies.com

Medical Air Technologies (MAT)
Unit 2, Mercury Way, Trafford Park, Manchester, M41 7LY
http://www.medicalairtechnology.com

Merk Millipore
Suite 21, Building 6, Croxley Green, Business Park, Watford, Hertfordshire, WD18 8YH, UK
http://www.merckmillipore.com

New England Biolabs
New England Biolabs,75-77 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 0TY, UK
https://www.neb.com
Panreac AppliChem
Surechem products LTD. Lion Barn Industrial Estate, Needham Market, Suffolk, IP6 8NZ, UK
https://www.applichem.com

Promega
Delta House, Southampton Science Park, Southampton, SO16 7NS
http://www.promega.co.uk

Qiagen
Skelton House, Lloyd Street North, Manchester, M15 6SH, UK
https://www.qiagen.com

R&D Systems
614 McKinley Place NE, Minneapolis, Minnesota, 55413, USA
https://www.rndsystems.com

Santa Cruz Biotechnology
10410 Finnell Street, Dallas, Texas, 75220, USA
https://www.scbt.com/

Sanyo Limited
9 The Office Village, North Road, Loughborough, Leicestershire, LE11 1QJ, UK
http://sanyo-biomedical.co.uk/

Sarstedt
68 Boston Road, Beaumont Leys, Leicester, LE4 1AW, UK
https://www.sarstedt.com

Severn Biotech Limited
Unit 2, Park Lane, Kidderminster, Worcestershire, DY11 6TJ, UK
http://www.severnbiotech.com

Sigma Aldrich Limited
The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK
https://www.sigmaaldrich.com

Southern Biotech
Supplied by Cambridge Bioscience
https://www.bioscience.co.uk

Thermo Fisher Scientific
Bishop Meadow Road, Loughborough, LE11 5RG
https://www.thermofisher.com/
Appendix C

Copy number analysis and exome sequencing data

Figure C.1 Copy number profiles of chromosomes 8 and 9 for RT112 parental, R1 and R3 normalised to a patient blood sample. Copy number plots were generated in Nexus Copy Number 8.0. and show log2 ratio of the read counts of RT112 samples normalised to a patient blood sample for chromosomes 8 and 9 (GRch38 reference). Each point represents the log2 ratio for a section of DNA of window size 1000 bp. Copy number gains (above the zero line) and losses (below the zero line) shared by RT112 parental, R1 and R3 are highlighted with arrows and are also detailed in Table 5.1.
Table C.1 Mutations identified by whole exome sequencing in RT112 R1. The mutations met the following criteria: the mutation was absent from RT112 parental and had a variant allele frequency of greater than 20%

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ensembl transcript ID</th>
<th>Chromosome: Position (Mb; hg38)</th>
<th>Variant Effect Predictor (VEP) consequence</th>
<th>Coding DNA sequence change (position from start codon)</th>
<th>Amino acid change</th>
<th>COSMIC ID</th>
<th>Sorting Intolerant From Tolerant (SIFT) Prediction</th>
<th>Polymorphism Phenotyping v2 (PolyPhen) Prediction</th>
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<td>3:15601421</td>
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<td>3:136854890</td>
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<td>5:53646367</td>
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<td>synonymous variant</td>
<td>c.C1587G</td>
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<td>19:15615676</td>
<td>synonymous variant</td>
<td>c.G60T</td>
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<td>19:17821329</td>
<td>missense variant</td>
<td>c.A178G</td>
<td>p.T60A</td>
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<td>GDF1</td>
<td>ENST00000247005</td>
<td>19:18869363</td>
<td>missense variant</td>
<td>c.C353T</td>
<td>p.A118V</td>
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<td>CERS1</td>
<td>ENST00000623927</td>
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<td>missense variant</td>
<td>c.C353T</td>
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<td>tolerated benign</td>
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<td>Gene</td>
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<td>Chromosome: Position (Mb:hg38)</td>
<td>Variant Effect Predictor (VEP) consequence</td>
<td>Coding DNA sequence change (position from start codon)</td>
<td>Amino acid change</td>
<td>COSMIC ID</td>
<td>Sorting Intolerant From Tolerant (SIFT) Prediction</td>
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<td>c.T234C</td>
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<td>19:19279376</td>
<td>synonymous variant</td>
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<td>p.Y455=</td>
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<td>CILP2</td>
<td>ENST00000291495</td>
<td>19:19540331</td>
<td>synonymous variant</td>
<td>c.A291G</td>
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<td>TMPRSS15</td>
<td>ENST00000284885</td>
<td>21:18329201</td>
<td>missense variant</td>
<td>c.G1748C</td>
<td>p.R583T</td>
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<td>probably damaging</td>
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Table C.2 Mutations identified by whole exome sequencing in RT112 R3. The mutations met the following criteria: the mutation was absent from RT112 parental and had a variant allele frequency of greater than 20%.
<table>
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<th>Gene</th>
<th>Ensembl ID</th>
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<th>Variant Type</th>
<th>Variant Details</th>
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<td>9:93676737</td>
<td>synonymous variant</td>
<td>c.C2976A</td>
<td>p.T992=</td>
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<td>MUC5AC</td>
<td>ENST00000621226</td>
<td>11:1190532</td>
<td>synonymous variant</td>
<td>c.A12387G</td>
<td>p.T4129=</td>
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<td>GOLGA6L6</td>
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<td>15:20534648</td>
<td>missense variant</td>
<td>c.C1786T</td>
<td>p.R596W</td>
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<td>KRTAP4-16</td>
<td>ENST00000440582</td>
<td>17:41101693</td>
<td>missense variant</td>
<td>c.G517C</td>
<td>p.V173L</td>
<td>COSM5177690  unknown</td>
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</table>
Appendix D
Quality control, normalisation and analysis of transcriptomic data

This appendix contains the first stages of transcriptome analysis. Affymetrix GeneChip HTA 2.0 raw data files (.CEL files), were imported into Expression Console and the quality of the data was assessed using quality controls included on the microarray. Data was then normalised so that gene expression of different samples could be compared.

D.1 Quality control of expression data

Affymetrix Expression Console™ version 1.4 was used to assess the quality of the RNA microarray data. The area under the curve for the receiver operator curve for all microarray samples (Fig. D.1) was greater than or equal to 0.98 for microarray samples indicating a low false positive rate and a high true positive rate for the microarray positive and negative controls.

Figure D.1 The area under the curve for the receiver operator curve. The receiver operator curve plots the true positive rate against the false positive rate. The area under this curve gives an indication of how successfully positive and negative microarray controls were identified. The closer the AUC is to 1, the more accurate the identification of controls. This analysis was conducted in Affymetrix Expression Console™.
The Eukaryotic hybridisation controls were detected with signal values increasing in the order bioB, bioC, bioD and cre, matching the concentrations that these controls are spiked into the hybridisation cocktail (Fig. D.2). Affymetrix state that bioC, bioD and cre should be present in every array sample and bioB should present in a minimum of 70% of array samples. As the eukaryotic hybridisation controls are present as expected this indicates successful hybridisation.

Figure D.2 Eukaryotic hybridisation controls (spike controls). The transcripts bioB, bioC and bioD take their sequence from genes of the *E.coli* biotin synthesis pathway and the probe cre takes its sequence from the bacteriophage P1 recombinase gene (all non-eukaryotic genes so not expressed in human cells). These controls are spiked into the hybridisation cocktail at different, known concentrations to enable signal alignment during image analysis. This analysis was conducted in Affymetrix Expression Console™.

Figure D.3 Array labelling controls. The transcripts lys, phe, thr and dap take their sequence from *D.subtilis* genes, these genes are not expressed in eukaryotic cells. Different, known concentrations of these transcripts are amplified and labelled with the array samples to examine the labelling process. This analysis was conducted in Affymetrix Expression Console™.
D.2 Data normalisation

Normalisation of the microarray data was conducted in the Affymetrix Expression Console™ version 1.4. Due to experimental variation, such as that occurring during the fluorescent labelling of cDNA and hybridisation of cDNA to the microarray, there is some variability between the probe cell intensity data of the separate microarray samples, measured from CEL files, prior to normalisation (Fig. D.4).

![Log probe cell intensity box plot](image)

**Figure D.4 Log probe cell intensity.** The log probe cell intensity box plot shows the intensity of the signal from each array. As data normalisation is yet to be conducted, differences in signal intensity are expected. This analysis was conducted in Affymetrix Expression Console™.

To adjust for the variability in probe cell intensity data between microarray samples, Signal Space Transformation-Robust Multichip Average (SST-RMA) normalisation was conducted using the raw data from the CEL files generating CHP files. SST removes significant fold-change compression and RMA minimises probe variance (Irizarry et al., 2003). After normalisation, CHP file summarized probe set signal values have a reduced variability compared to the probe cell intensity values of microarray samples measured from CEL files (Fig. D.5). This indicates that the data normalisation was successful and array samples can now be compared.
Figure D.5 Log expression signal of CHP files generated from the CEL files through SST-RMA normalisation. This box plot shows the signal intensity of each array following the SST-RMA normalisation and therefore differences in signal intensities between arrays should not be present. This analysis was conducted in Affymetrix Expression Console™.

D.3 Supplementary expression analysis

Figure D.6 The 10 most significantly differentially expressed pathway maps between RT112 R1 no PD and parental + PD according to MetaCore™.
Figure D.7 The 10 most significantly differentially expressed pathway maps between RT112 R2 no PD and parental + PD according to MetaCore™.

Figure D.8 The 10 most significantly differentially expressed pathway maps between RT112 R3 no PD and parental + PD according to MetaCore™.
Figure D.9 The 10 most significantly differentially expressed pathway maps between RT4 R1 + PD vs R1 no PD according to MetaCore™.

Figure D.10 Expression of OSM and IL31 in RT112 determined by microarray analysis. A) OSM B) IL31. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. OSM and IL31 were not differentially expressed in any experimental conditions compared to parental no PD (ANOVA p<0.05, 2-fold expression change).
Figure D.11 Expression of \textit{IL6ST}, \textit{OSMR} and \textit{LIFR} in RT4 determined by microarray analysis. A) \textit{IL6ST}. B) \textit{OSMR}. C) \textit{LIFR}. Error bars indicate standard error of the mean. Signal intensity is given relative to RT112 no PD. Asterisks indicate the experimental conditions in which \textit{IL6ST}, \textit{OSMR} or \textit{LIFR} were differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change).

Figure D.12 Expression of \textit{OSM} and \textit{IL31} in RT4 determined by microarray analysis. Error bars indicate standard error of the mean. A) \textit{OSM}. B) \textit{IL31}. Signal intensity is given relative to parental no PD. \textit{OSM} and \textit{IL31} were not differentially expressed in any experimental conditions compared to parental no PD (ANOVA p<0.05, 2-fold expression change).
Figure D.13 Unsupervised hierarchical cluster analysis of Choi et al. cohort of p53-like markers in RT112 microarray samples. The Log₂ gene expression was normalised by dividing each expression value by the probe’s mean Log₂ gene expression. Following Log2 gene expression normalisation, the expression profiles of samples and genes were clustered in Partek® Genomics Suite® 6.6 using Euclidean distance and complete linkage. Scale bar indicates the normalised Log₂ gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).
Figure D.14 Unsupervised hierarchical cluster analysis of Choi et al. cohort of p53-like markers in RT4 microarray samples. The Log₂ gene expression was normalised by dividing each expression value by the probe’s mean Log₂ gene expression. Following Log₂ gene expression normalisation, the expression profiles of samples and genes were clustered in Partek® Genomics Suite® 6.6 using Euclidean distance and complete linkage. Scale bar indicates the normalised Log₂ gene expression with colour depicting the level of gene expression as high (red), intermediate (black) and low (green).
Figure D.15 Expression of *PGM5* and *ACTG2* in RT4 determined by microarray analysis. A) *PGM5*. B) *ACTG2*. Error bars indicate standard error of the mean. Signal intensity is given relative to parental no PD. Asterisks indicate the experimental conditions in which *PGM5* and *ACTG2* were differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change).

Figure D.16 Expression of *IGF1*, *HGF*, *EREG* and *NRG2* in RT112 determined by microarray analysis. A) *IGF1*. B) *HGF*. C) *EREG*. D) *NRG2*. Error bars indicate standard error of the mean. Signal intensity is given relative to RT112 no PD. Asterisks indicate the experimental conditions in which genes were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe’s Affymetrix IDs.
Figure D.17 Expression of *EREG, HBEGF, EGF, NRG2 and EPGN* in RT4 determined by microarray analysis. A) *EREG*. B) *HBEGF*. C) *EGF*. D) *NRG2*. E) *EPGN*. Error bars indicate standard error of the mean. Error bars are absent where the standard error of the mean was too small to plot. Signal intensity is given relative to RT4 no PD. Asterisks indicate the experimental conditions in which genes were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change).
Figure D.18 Expression of NRG1 and NRG2 in RT4 determined by microarray analysis. A) NRG1. B) NRG2. Error bars indicate standard error of the mean. Error bars are absent where the standard error of the mean was too small to plot. Signal intensity is given relative to RT4 no PD. Asterisks indicate the experimental conditions in which genes were significantly differentially expressed compared to parental no PD (ANOVA p<0.05, 2-fold expression change). For genes with specificity to more than one probe, the data generated from each probe is shown separately with a legend indicating the probe's affymetrix IDs.


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