Co-targeting of FAK and MDM2 triggers additive anti-proliferative effects in mesothelioma via a coordinated reactivation of p53

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Background: Improved mesothelioma patient survival will require development of novel and more effective pharmacological interventions. TP53 genomic mutations are uncommon in mesothelioma, and recent data indicate that p53 remains functional, and therefore is a potential therapeutic target in these cancers. In addition, the tumour suppressor NF2 is inactivated by genomic mechanisms in more than 80% of mesothelioma, causing upregulation of FAK activity. Because FAK is a negative regulator of p53, NF2 regulation of FAK–p53–MDM2 signalling loops were evaluated.

Methods: Interactions of FAK–p53 or NF2–FAK were evaluated by phosphotyrosine-p53 immunoaffinity purification and tandem mass spectrometry, and p53, FAK, and NF2 immunoprecipitations. Activation and/or expression of FAK, p53, and NF2 were also evaluated in mesotheliomas. Effects of combination MDM2 and FAK inhibitors/shRNAs were assessed by measuring mesothelioma cell viability/growth, expression of cell cycle checkpoints, and cell cycle alterations.

Results: We observed constitutive activation of FAK, a known negative regulator of p53, in each of 10 mesothelioma cell lines and each of nine mesothelioma surgical specimens, and FAK was associated with p53 in five of five mesothelioma cell lines. In four mesotheliomas with wild-type p53, FAK silencing by RNAi induced expression and phosphorylation of p53. However, FAK regulation of mesothelioma proliferation was not restricted to p53-dependent pathways, as demonstrated by immunobLOTS after FAK knockdown in JMN1B mesothelioma cells, which have mutant/inactivated p53, compared with four mesothelioma cell lines with nonmutant p53. Additive effects were obtained through a coordinated reactivation of p53, by FAK knockdown/inhibition and MDM2 inhibition, as demonstrated by immunobLOTS, cell viability, and cell-cycle analyses, showing increased p53 expression, apoptosis, anti-proliferative effects, and cell-cycle arrest, as compared with either intervention alone. Our results also indicate that NF2 regulates the interaction of FAK–p53 and MDM2–p53.

Conclusions: These findings highlight novel therapeutic opportunities in mesothelioma.

Mesothelioma is a locally aggressive and highly lethal neoplasm, linked epidemiologically to asbestos exposure (Craighead and Mossman, 1982; Jongsm a et al, 2008), and SV40 virus infection (Carbone et al, 1994), in which the neoplastic proliferation originates from pleural, peritoneal, or – rarely – pericardial mesothelial cells. Pathologically, mesothelioma histological...
subtypes are epithelioid, spindle-cell, and mixed (epithelioid and spindled; Janne et al, 2002), of which the spindled type has the worst prognosis. Conventional chemotherapies and radiation therapy have limited efficacy against mesothelioma, and substantial improvements in survival will require development of novel and more effective pharmacological interventions. A better understanding of mesothelioma biology — including the function of highly recurrent mutations such as NF2 (Bianchi et al, 1995) and BAP1 (Bott et al, 2011; Testa et al, 2011), and key growth factor signalling pathways — will likely be useful in identifying biologically rational targets for novel therapies.

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that mediates signalling through several downstream pathways, leading to cell migration, growth factor signalling, cell cycle progression, and cell survival (Lim et al, 2008; Ding et al, 2010; Huang et al, 2010; Long et al, 2010). Furthermore, FAK has a prominent role in integrin and receptor tyrosine kinase (RTK) signalling (Parsons, 2003). Although FAK itself has not been demonstrated to be an oncogene, FAK overexpression has been reported in tumours of various tissue origins, especially in invasive and metastatic tumours (Gabarra-Niecko et al, 2003). The effects of FAK on tumour growth in mice suggest that FAK may influence the formation, growth, and metastasis of tumours (Gabarra-Niecko et al, 2003; Slack-Davis et al, 2009). There is increasing evidence to indicate that FAK has an important role in regulating cell cycle progression through cyclin D1 transcription, p27 expression, and MAPK activation (Zhao et al, 2001; Ding et al, 2005). The tumour suppressor p53 was first linked with FAK by Ilic et al (Ilic et al, 1998) and a p53 binding site has been identified in the FAK promoter (Golubovskaya et al, 2004). Recently, studies of direct interaction of the N-terminal domain of FAK with the N-terminal transactivation domain of p53, particularly at the 7-amino-acid site in the proline-rich region of the p53 N-terminal domain, indicated that FAK can suppress p53-mediated apoptosis and inhibit the transcriptional activity of p53 (Golubovskaya et al, 2005; Golubovskaya et al, 2008). FIP200, a FAK inhibitor, has been shown to induce cell cycle arrest by increasing the expression and phosphorylation of p53 in human breast cancer cells (Melkoumian et al, 2005). FAK inactivates p53 in a kinase-independent manner through the FAK FERM domain, which acts as a scaffold to enhance MDM2-dependent p53 ubiquitination. Importantly, p53 regulation is dependent on FAK nuclear translocation (Lim et al, 2008). These observations suggest that FAK regulates cell cycle progression in a p53-dependent manner. However, it is still unclear whether the FAK–p53 cell survival pathway may also have a role in promoting tumour progression.

Mouse double minute 2 (MDM2) is an oncoprotein, the primary E3 ubiquitin ligase responsible for ubiquitination and degradation of p53 (Brooks and Gu, 2006). The structure of MDM2 bound to p53 and the molecular mechanisms of the interaction between MDM2 and p53 have been well characterised (Kussie et al, 1996; Schon et al, 2002; Chene, 2003; White et al, 2006). This interaction of the MDM2-p53 interaction by small molecules is an attractive therapeutic opportunity in oncology. Nutlin-3, a specific small-molecule inhibitor of MDM2, suppresses the interaction of MDM2 with wild-type p53, activating its transactivation activity (Kojima et al, 2006; Tovar et al, 2006; Van et al, 2009; Moran and Maki, 2010). Nutlin-3 shows strong antitumour effects in mice, with few side effects on normal tissues (Brummelkamp et al, 2006), indicating that tumours with wild-type p53 may be susceptible to inhibition of the MDM2–p53 interaction. Recently, we found that wild-type p53 depletion by histone deacetylase inhibitors was MDM2 amplification-dependent (Ou et al, 2015), and inhibition of PI3K/AKT was also associated with MDM2–p53 cell cycle regulation (Zhou et al, 2014).

TP53 mutations are seen infreqently in mesothelioma (Metcalf et al, 1992; Papp et al, 2001). Recent data also indicate that p53 is functional in the absence of p14ARF and, if reactivated, can induce apoptosis in mesothelioma (Hopkins-Donaldson et al, 2006). Suppressor of cytokine signalling 3 (SOCS3) overexpression showed anti-proliferative effects in mesotheliomas via multiple signalling pathways including JAK/STAT3 and FAK/p53 (Iwahori et al, 2011). Increasing p53 expression enhances cytotoxicity of the first-line chemotherapeutics cisplatin or pemetrexed in p53-wild-type mesothelioma (Li et al, 2012).

The NF2 (merlin) is a tumour-suppressor protein, inactivated by genomic mechanisms in more than 80% of mesothelioma; these are crucial events in mesothelioma pathogenesis, and result in hyperactivation of FAK (Bianchi et al, 1995; Poulikakos et al, 2006). Recent studies showed that NF2 deficiency predicts increased sensitivity of mesothelioma cells to a FAK inhibitor, VS-4718 (Shapiro et al, 2014). In addition, previous data have demonstrated that NF2 neutralises the inhibitory effect of MDM2 on p53 (Kim et al, 2004).

In the present study, we investigated interaction of FAK and p53 through a functional proteomic approach including phosphotyrosine and p53 immunoprecipitation double immunoaffinity purification, and tandem mass spectrometry. The expression and phosphorylation of FAK and p53 were also evaluated in mesothelioma cell lines and primary tumours. We evaluated the effects of coordinated reactivation of p53, and our findings highlight novel therapeutic opportunities in mesothelioma.

**Materials and Methods**

**Antibodies and reagents.** Polyclonal antibody to FAK, and monoclonal antibodies to phosphotyrosine (PY99), p53, PARP, and NF2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal mouse antibodies to MDM2 and p21, and polyclonal antibody to phospho-FAK (Y397) were from Zymed Laboratories (Invitrogen Life Technologies, Carlsbad, CA, USA). Polyclonal antibodies to phospho-p53 (Ser15) and phospho-NF2 (Ser518) were from Cell Signaling Technology (Beverly, MA, USA). Phospho-FAK (Y861) was obtained from Biosource (Invitrogen Life Technologies). We obtained monoclonal mouse antibodies to cyclin A from Novocastra (Newcastle upon Tyne, UK), and β-actin and GAPDH from Sigma-Aldrich (St Louis, MO, USA).

Mouse anti-phosphotyrosine (PY)-sepharose 4B, Protein A- and Protein G-sepharose beads were from Zymed Laboratories. NOVEX Coomassie colloidal blue stain kit, NuPAGE TM 4–12% Bis-Tris Gel, Lipoectamin and Plus reagent were obtained from Invitrogen Life Technologies. Phenyl phosphate and polybrene were from Sigma. Nutlin-3 and PF562271 were obtained from LC Labs (Woburn, MA, USA) and Symanesis (Auckland, NZ, USA), respectively. Lentiviral NF2 shRNAs were from Sigma. NF2 shRNAs: 5'-CCGGCCGGGCTTTGTTTCCTTCTTTACTCGAGTAAGAAAGGAAACAAAGCCCGTTTTTG-3'. NF2 shRNA2: 5'-CCGGCCGGGCTTTGTTTCCTTCTTTACTCGAGTAAGAAAGGAAACAAAGCCCGTTTTTG-3'.

**Mesothelioma cell lines and frozen tumour specimens.** Eight mesothelioma cell lines were established from surgical materials from previously untreated patients, as reported previously (Ou et al, 2011). The MESO59, MESO257, MESO542, and MESO924 cell lines were established from epithelial-type mesotheliomas, MESO296, MESO589, and MESO647 from mixed-histology mesotheliomas, and MESO428 from a spindle-cell mesothelioma. An additional mesothelioma cell line, JMN1B, was established from an epithelial-type mesothelioma (Demetri et al, 1989). Derivation of each cell line from the corresponding surgical specimen was corroborated by STR profiling (Supplementary Table 1). All mesothelioma frozen tumour specimens were discarded tissues, obtained from Brigham and Women’s Hospital. The Brigham and Women’s Hospital.
Phosphotyrosine-p53 immunoaffinity purification and tandem mass spectrometry. Tyrosine kinases (TKs) were immunoprecipitated from mesothelioma cell lysates using a p53 monoclonal antibody, and subsequently immunoblotted and stained for phosphotyrosine (PY99) to identify the molecular mass of putative activated TKs, which interacted with p53. Candidate-activated TKs were then characterised from the MESO257 cell line by mass spectrometry. Activated proteins were purified on a phosphotyrosine (PY) immunoaffinity column in which 0.5 ml of sepharose-conjugated anti-PY was loaded in a 10 ml polypropylene column and washed with a 5X volume of 1× PBS containing 0.02% sodium azide. The column was pre-equilibrated with a 5X volume of PBS buffer overnight at 4°C. The flow-through fraction was collected and reloaded on the column 10 times. The column was then washed with 1 ml lysis buffer five times, and the tyrosine-phosphorylated proteins were eluted with 1 ml 100 mM phenyl phosphate in lysis buffer 10 times. The eluates were dialysed against 1 litre of 1× PBS overnight at 4°C. The sample was then concentrated to ~100 µl of final volume using centrifugation filters (Millipore Corporation, Bedford, MA, USA). Ninety-six percent of the eluates were immunoprecipitated by antibody to p53. Finally, 4% of the eluates and 15% of the PY-p53 fractions were immunoblotted and stained for phosphotyrosine, while the remaining 85% of the PY-p53 fractions were gel electrophoresed and stained using a NOVEX Coomassie colloidal blue stain kit protocol. A strongly phosphorylated protein band at 130 kDa was excised from the Coomassie gel, and protein sequences were determined by mass spectrometry. The blots were then stripped and restained with specific antibodies to validate candidate tyrosine kinases.

Western blotting analysis. Whole-cell lysates were prepared using lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 100 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium molybdate, 5 mM EDTA, and 2 mM sodium orthovanadate) containing protease inhibitors (10 µg ml⁻¹ aprotinin, 10 µg ml⁻¹ leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Frozen tumour samples were diced into small pieces in cold lysis buffer on dry ice and homogenised using a Tissue Tearor (Model 398, Biospec Products, Inc., Fort Collins, CO, USA) and restained with specific antibodies to validate candidate tyrosine kinases.
Statistical analysis. Student’s t-tests were performed on data from cells treated with control DMSO or inhibitors, as well as cells treated with empty vector or FAK shRNA. Statistically significant differences between untreated control and treatment were defined as *P < 0.05, **P < 0.01, and ***P < 0.001.

RESULTS

FAK non-receptor tyrosine kinase is strongly activated and interacts with p53 in mesothelioma. Phosphotyrosine eluates, phosphotyrosine-p53 fractions, and p53 immunoprecipitations were prepared from the MESO257 cell line, and phosphotyrosine stains demonstrated ~130 kDa proteins in immunoblot preparations (Figure 1). Mass spectrometry analysis of the ~130 kDa band cut from a Coomassie-stained gel identified the FAK non-receptor tyrosine kinase. This finding was confirmed by subsequent stripping of the immunoblots and restaining for FAK (Figure 1). The p53 immunoblotting was not shown because of overlay of p53 and immunoglobulin heavy chain.

Using MESO924 as an internal standard, the phosphorylation and expression of FAK and p53 were evaluated by immunoblotting in mesothelioma cell lines and primary tumours. FAK and p53 were expressed in most mesotheliomas, with especially strong expression of p53 in JMN1B and one frozen tumour, both of which have been found to contain TP53 missense mutations (JMN1B: G245S; MESO96–975: A159V) (Figure 2). Focal adhesion kinase (phospho-FAK Y397) was constitutively activated in all mesotheliomas. The NF2 was weakly expressed in most frozen tumours, in which the residual NF2 expression likely originated from nonneoplastic cells in the specimens, whereas expression was nearly undetectable in mesothelioma cell lines except for MESO257 (Figure 2).

We investigated the FAK–p53 interaction in mesothelioma cell lines by FAK and p53 immunoprecipitations, followed by FAK immunostaining (Figure 3). The p53 immunoprecipitations revealed a dominant FAK 130 kDa band in five mesothelioma cell lines, which was confirmed by FAK immunostaining in FAK immunoprecipitations (Figure 3).

FAK shRNA knockdown or kinase inhibition results in upregulation of p53. The FAK shRNA-mediated inhibition resulted in ~60–70% inhibition of FAK protein expression in MESO257 at 48 h and 96 h post infection (Figure 4A). Treatment with FAK inhibitor PF562271 decreased expression of phospho-FAK, phospho-MDM2, and MDM2 in MESO257 and MESO296 in a dose-dependent manner (Figure 4B and Supplementary Figure 1). Interestingly, FAK shRNA knockdown or activity inhibition induced two- to three-fold upregulation of phospho-p53 and p53, indicating that FAK regulates p53 (Figure 4). The p53 expression quantifications were normalised to control transfections using empty vector pLKO or DMSO.

Combination inhibition of the FAK–p53 and MDM2–p53 interactions suppressed cell viability in mesothelioma. Additive effects on p53 expression were obtained through coordinated dysregulation of p53, as demonstrated by immunoblots, after FAK shRNA knockdown and MDM2 inhibition. Inhibition of FAK–p53 and MDM2–p53 interactions were evaluated in five mesothelioma cell lines including MESO924, MESO257, MESO296, MESO428, and JMN1B, at 48 h after treatment with nutlin-3 and/or at 72 h post infection with FAK shRNA (Figure 5A). JMN1B is a control cell line, which has mutant/inactivated p53. The expression of MDM2, p53, and phospho-p53 increased in a dose-dependent manner after treatment with nutlin-3 in cells with wild type p53 (MESO924, MESO257, MESO296, and MESO428). Similarly, p53 expression and phosphorylation increased after FAK shRNA knockdown in these cells. Phospho-p53 and total p53 expression quantifications were normalised to DMSO-treated cells. Combination of FAK knockdown and MDM2–p53 inhibition by nutlin-3 induced greater p53, phospho-p53, and CDK inhibitor p21 expression, and inhibited proliferation marker cyclin A expression.
more effectively compared to either intervention alone (Figure 5A). By contrast, expression and phosphorylation of p53 did not increase in JMN1B after treatment with nutlin-3 and FAK shRNA either alone or in combination. However, FAK shRNA knockdown inhibited cyclin A proliferation marker expression in JMN1B cells (Figure 5A).

Additive effects on cell viability and cell cycle inhibition were obtained through coordinated dysregulation of p53 after FAK and MDM2 inhibition. FAK knockdown in wild-type p53 mesothelioma cell lines MESO924, MESO257, MESO296, and MESO428 resulted in ~15–35% inhibition of cell viability at 3 days after FAK silencing, compared with an empty vector control (Figure 5B). In p53 wild-type cells, the most striking reduction in cell viability (20–50%) was seen after treatment with nutlin-3 (Figure 5B). However, viability was little affected in p53 mutant JMN1B cells after treatment with nutlin-3 (Figure 5B). To determine whether FAK kinase activity regulates mesothelioma viability, the FAK kinase was inactivated using a small molecule inhibitor, PF562271. Treatment with PF562271 showed profound anti-proliferative effects at day 6 in all the five mesothelioma cell lines (Figure 5C). Combination of FAK shRNA (day 3) or FAK inhibition (PF562271, day 6) and MDM2 inhibition resulted in 20–77% or 80–90% reduction in viability for these lines, respectively (Figure 5B and C). Interestingly, FAK shRNA knockdown or FAK kinase inhibition resulted in ~90% or 50% viability reduction in JMN1B cells, respectively (Figure 5B and C).

The FAK knockdown in all the five mesothelioma cell lines resulted in G1 arrest. The G1 peaks were 54% (MESO924), 52% (MESO257), 61% (MESO296), 48% (MESO428), and 36% (JMN1B) in the empty vector-treated cells compared with 64% (MESO924), 62% (MESO257), 70% (MESO296), 65% (MESO428), and 40% (JMN1B) in FAK shRNA knockdown cells (Figure 5D and Table 1). The cell cycle analyses also demonstrated a G1 block with a decrease in the S-phase population after MDM2 inhibition by nutlin-3 in wild-type p53 mesothelioma cell lines (Figure 5D and Table 1). The combination of FAK shRNA and MDM2 inhibition resulted in a G1/G2 block in cell cycle for these lines (Figure 5D and Table 1). The FAK knockdown also induced apoptosis in JMN1B: nuclear fragmentation was demonstrated in 4.6% cells treated with empty vector control, but in 13% cells treated with FAK shRNA (Figure 5D and Table 1).

NF2 regulates the FAK–p53 and MDM2–p53 interaction. The observations of mild upregulation of p53 by FAK shRNA knockdown in MESO257 (Figure 5A) with strong NF2 expression

![Figure 3. The FAK–p53 complex in mesothelioma cell lines. FAK–p53 interactions were evaluated by p53 immunoprecipitation followed by FAK immunoblotting.](image)

![Figure 4. FAK silencing or inhibition induces p53 expression. (A) Left panel: Lentiviral FAK shRNA knockdown resulted in upregulation of p53 in MESO257 at 48 h and 96 h post infection. Right panel: quantification of p53 protein levels. The experiments were performed in triplicate, and statistically significant differences between vector control and FAK shRNA are defined as *P < 0.05, **P < 0.01. (B) Expression of FAK, phospho-FAK, p53, phospho-p53, MDM2, and phospho-MDM2 was evaluated by immunoblotting in MESO257 and MESO296 after incubating cells for 6 h with FAK inhibitor PF562271 (1 and 5 μM) in serum-free medium. Actin staining is a loading control.](image)
Figure 5. Additive effects of coordinated dysregulation of p53, as demonstrated by immunoblotting (A), cell viability (B and C) and cell cycle analyses (D), showing that FAK and MDM2 inhibition induced greater p53 expression, anti-proliferative effects, and cell cycle arrest, as compared to either intervention alone. (A) FAK, MDM2, phospho-p53, p53, p27, and cyclin A were evaluated by immunoblotting at 72 h post-infection with FAK shRNA and/or 48 h after treatment with nutlin-3. Actin staining is a loading control. Phospho-p53 and p53 expression quantitations are standardised to the DMSO or empty vector control. (B and C) Cell viability was evaluated by a Cell-Itter Glo ATP-based luminescence assay in wild type p53 mesothelioma cell lines (MESO924, MESO257, MESO296, and MESO428), and in mutant p53 mesothelioma cell line (JMN1B), at 72 h post-infection with FAK shRNA and/or 48 h after treatment with nutlin-3 (B) or 6 days after treatment with nutlin-3 and PF562271 (C). Data were normalised to empty lentivirus infections or DMSO, and represent the mean values (± s.d.) from quadruplicate cultures. Statistically significant differences between untreated control and treatments or between empty vector control and FAK shRNA are presented as *P < 0.05, **P < 0.01, ***P < 0.001. (D) Cell cycle analyses were performed 72 h after transduction with lentiviral FAK shRNA constructs and/or 48 h after treatment with nutlin-3. MESO924, MESO257, MESO296, MESO428, and JMN1B cells show substantial G1 and G2-block after FAK silencing and inhibition of the MDM2–p53 interaction, as compared with empty vector or DMSO control. JMN1B shows substantial nuclear fragmentation after FAK silencing.


**Table 1. Cell cycle analysis after treatment with 5 μM Nutlin-3, FAK shRNA, and combination in mesothelioma cell lines**

|          | MESO924 | MESO257 | MESO296 | MESO428 | JMN1B |
|----------|---------|---------|---------|---------|-------|
| G_{1/0}  | G_{2}   | S       | A       | G_{1/0} | G_{2} | S | A | G_{1/0} | G_{2} | S | A | G_{1/0} | G_{2} | S | A | G_{1/0} | G_{2} | S | A |
| DMSO     | 62      | 10      | 28      | 0.7     | 53    | 8  | 39 | 1.8 | 62       | 7   | 31 | 0.3 | 52    | 5  | 43 | 0.3 | 36       | 15 | 49 | 6.6 |
| Nutlin-3 | 68      | 8       | 24      | 0.8     | 55    | 24 | 21 | 2.2 | 79       | 6   | 15 | 0.6 | 50    | 2  | 48 | 1.1 | 36       | 16 | 48 | 5.1 |
| Vector   | 54      | 6       | 40      | 1.6     | 52    | 9  | 39 | 2.6 | 61       | 6   | 33 | 0    | 48    | 2  | 50 | 2.1 | 36       | 15 | 49 | 4.6 |
| FAK shRNA| 64      | 6       | 30      | 0.9     | 62    | 12 | 26 | 2.6 | 70       | 1   | 29 | 0.8 | 65    | 1  | 34 | 0.7 | 40       | 23 | 37 | 13 |
| DMSO & Vector | 54 | 6 | 40 | 2.1 | 51 | 9 | 40 | 2.0 | 63 | 5 | 32 | 1.5 | 52 | 3 | 45 | 3.1 | 37 | 10 | 53 | 4.5 |
| Nutlin-3 & FAK shRNA | 62 | 6 | 22 | 0.5 | 60 | 30 | 11 | 3.3 | 68 | 16 | 16 | 2.3 | 60 | 12 | 26 | 4.0 | 43 | 24 | 33 | 13 |

Abbreviations: DMSO = dimethyl sulfoxide; FAK = focal adhesion kinase; shRNA = short hairpin RNA. 'A': pre-G1. The data were obtained from Figure 5D and shown by percentage (%).

**Discussion**

Mesothelioma is a fatal malignancy most often associated with asbestos exposure. Currently available therapies, which involve aggressive surgery, radiation, and chemotherapy, have done little to improve the prognosis of patients with this cancer. Incomplete understanding of mesothelioma biology has hindered the development of more effective therapeutics. Evidence indicates that activation of tyrosine kinases is essential in the progression from non-neoplastic mesothelial progenitor cells to mesothelioma. p53, a tumour suppressor, has a crucial role in the cellular responses to a variety of stresses, such as hypoxia or DNA damage, through its accumulation in the nucleus (Levine, 1997; Oren, 1999). Inactivating TP53 mutations are found in approximately 50% of human tumours (Hollstein et al., 1991). However, TP53 mutations are seen less frequently in mesothelioma (Metcalfe et al., 1992; Papp et al., 2001). Previous studies have suggested that p53 activity is potentially lost in mesothelioma because of p14ARF inactivation and the effects of the SV40 large T antigen (Testa and Giordano, 2001; Yang et al., 2001). However, the role of SV40 in mesothelioma carcinogenesis and the frequency of SV40 presence in mesothelioma have been questioned (Lopez-Rios et al., 2004; Whiston and Kratzke, 2006). Furthermore, recent studies indicate that p53 is functional in the absence of p14ARF and can induce apoptosis in mesothelioma (Hopkins-Donaldson et al., 2006). Thus, the identification of TK–p53 interactions and their functional evaluation in mesothelioma provides an opportunity to identify novel therapeutic strategies.

The interaction of tyrosine kinases and the p53 family was first reported by investigating the association of c-Abl and p73 in apoptosis (Agami et al., 1999). In the present study, we identified a tyrosine kinase, FAK, that is highly expressed in mesothelioma, and investigated potential interactions of FAK with p53. Although interaction between FAK and p53 was shown previously by a biochemical approach (Golubovskaya et al., 2005), it is still unclear whether the FAK–p53 interaction may also have a crucial role in tumour biology. In previous studies, we developed and validated a functional proteomic approach including double phosphotyrosine and panRTK immuno-affinity purification, coupled to tandem mass spectrometry in various tumour tissues or cell lines (Ou et al., 2011). We therefore identified the interaction of FAK–p53 in the MESO257 cell line by the PY–p53 double purification and mass spectrometry approach (Figure 1), and validated this interaction in five mesothelioma cell lines by FAK and p53 immunoprecipitations (Figure 3). Immunoblotting evaluations of FAK and p53 expression and phosphorylation in mesothelioma cell lines and primary frozen tumours further demonstrated that FAK and p53 are ubiquitously expressed and constitutively activated in mesothelioma (Figure 2), indicating that FAK–p53 pathways have a role in mesothelioma growth. In addition, normal p53 expression in most mesothelioma samples confirmed that TP53 mutations are infrequent in these cancers (Figure 2).

To evaluate whether FAK–p53 interaction has crucial functional roles in mesothelioma, FAK was silenced by lentiviral FAK shRNA in wild-type p53 mesothelioma cell lines (MESO924, MESO257, MESO296, and MESO428) and in the mutant p53 JMN1B line (Figures 4 and 5A). Interestingly, FAK shRNA knockdown induced one- to three-fold increases of p53 expression and phosphorylation in wild-type p53 mesothelioma cell lines, which is consistent with the level of p53 upregulation after FAK shRNA knockdown in human fibroblasts (Lim et al., 2008). The p53 expression did not increase after FAK inhibition at low concentrations of PF562271 (Supplementary Figure 1). Further studies showed that FAK inhibition at high concentration of PF562271 (5 μM) induced p53...
expression because of inhibition of MDM2 and phospho-MDM2 expression (Figure 4B). These results are consistent with a FAK kinase-independent effect for p53 expression. However, FAK shRNA knockdown had little effect on p53 levels as compared with vector-treated cells in mutant p53 JMN1B cells (Figure 5A). These findings indicate that FAK negatively regulates wild-type p53 expression.

The structure and mechanisms of the MDM2–p53 interaction have been extensively studied (Kussie et al., 1996; Chene, 2003; Brooks and Gu, 2006; White et al., 2006). MDM2 suppresses p53 activity by inducing nuclear export and degradation, and blocking its transcriptional activity (Chene, 2003). Therefore, inhibition of the wild-type p53–MDM2 interaction is an opportunity for cancer therapeutics (Kojima et al., 2006; Tovar et al., 2006; Van et al., 2009). In the current study, we show increased expression and phosphorylation of p53, and decreased viability in a dose-dependent manner after treatment with nutlin-3 in wild-type p53 cell lines (Figure 5A and B), indicating that inhibition of MDM2–p53

**Figure 6.** NF2 regulates the interaction of FAK–p53 and MDM2–p53. (A) The NF2–FAK complex was evaluated in MESO257 by NF2 and FAK immunoprecipitation followed by FAK and NF2 immunoblotting. (B) Nuclear localisation of NF2, FAK, p53, and MDM2 was evaluated in MESO257 by immunoblotting. Poly(ADP-ribose) polymerase (PARP) is a nuclear localisation control, and GAPDH is a cytoplasmic control. (C) In MESO257 with stable NF2 shRNA expression, p53, NF2, FAK, MDM2, and p21 expression were evaluated by immunoblotting after FAK shRNA knockdown for 72 h. β-Actin staining is a loading control. p53 expression quantifications are standardised to the empty vector control. (D) Expression of p53 and p21 was evaluated in MESO257 cell membrane, cytoplasm, and nucleus with stable NF2 shRNA expression by immunoblotting. (E) Cell viability was evaluated by a cell titre Glo ATP-based luminescence assay in MESO257 with stable expressed NF2 shRNA, at 72 h post-infection with FAK shRNA. Data were normalised to empty lentivirus infections, and represent the mean values (± s.d.) from quadruplicate cultures. Statistically significant differences between untreated control and treatments or between vector control and FAK shRNA or NF2 shRNA and NF2 + FAK shRNA are presented as *P < 0.05, **P < 0.01.
interaction has an important biological function in mesothelioma. It has been shown that FAK–p53 and MDM2–p53 form an autoregulatory feedback loop (Chene, 2003; Golubovskaya et al., 2005). The FAK shRNA knockdown induced expression of p53 and phospho-p53 (Figure 5A), which prevents association with MDM2 (Chene, 2003), but had little impact on MDM2 expression level (Figure 5A). By contrast, nutlin-3 treatment reduced MDM2 and p53 expression, but had little effect on FAK expression in wild-type p53 mesothelioma cell lines. Nuclear FAK acts as a scaffold to stabilise the MDM2–p53 complex, leading to p53 polyubiquitination and subsequent p53 degradation by nuclear or cytoplasmic proteasomes (Lim et al., 2008). Those observations suggest that a feedback loop regulates FAK–p53–MDM2 activity. In the future, we will further study this signalling feedback loop in the tumour microenvironment.

The NF2 inactivation by genomic mechanisms occurs in more than 80% of mesothelioma, as a critical step in mesothelioma pathogenesis, and upregulates FAK activity (Bianchi et al., 2001). After treatment with nutlin-3, the FAK inhibitor PF562271, or FAK shRNA knockdown, upregulation of p53 and phospho-p53, and reduction of cell viability were least pronounced in NF2-positive MESO257 as compared with NF2-inactivated cell lines (Figure 5A–C). NF2 knockdown resulted in the accumulation of p53-p21 in nucleus and cytoplasm (Figure 6D), and NF2-positive MESO257 cells showed increased sensitivity to FAK shRNA after NF2 knockdown (Figure 6E), indicating that the presence of NF2 regulates MDM2–p53 and FAK–p53 interactions. This is in keeping with previous data demonstrating that NF2 neutralises the inhibitory effect of MDM2 on p53 (Kim et al., 2004), that NF2 negatively regulates FAK activity (Poulikakos et al., 2006), and that NF2 deficiency predicts increased sensitivity of mesothelioma cells to a FAK inhibitor, VS-4718 (Shapiro et al., 2014). NF2-null mesothelioma cells have been found to have weak cell–cell adhesions (Shapiro et al., 2014), which results in FAK leaving focal contact sites, increasing the cytoplasmic pool of FAK and enhancing FAK nuclear accumulation (Lim et al., 2008). Nuclear FAK acts as a scaffold to stabilise MDM2–p53 complexes, leading to p53 polyubiquitination and subsequent p53 degradation by nuclear or cytoplasmic proteasomes (Lim et al., 2008). These observations establish mechanisms by which FAK knockdown or FAK kinase activity suppression affect the growth via minimising p53 degradation by MDM2 (Figure 4A and B, and Figure 5A), and have greater anti-proliferative effects in NF2-null mesothelioma cell lines (MESO296 and MESO428) than NF2-positive MESO257 cell lines (Figure 5B and C). Further, nuclear co-localisation of NF2, FAK, and p53 indicate that NF2 potentially regulates the interaction of FAK and p53 (Figure 6B). The Fak shRNA knockdown resulted in the upregulation of p53 and MDM2 in MESO257 (Figure 6C), indicating that NF2 regulates the interaction of MDM2 and p53, and that upregulation of p53 expression by MDM2 inhibition (nutlin-3) is NF2-dependent. Focal adhesion kinase inhibits p53 through FAK nuclear translocation, binding to p53, and enhanced MDM2-dependent p53 ubiquitination and degradation (Lim et al., 2008). Thus, additive effects were obtained through coordinated reactivation of p53 by FAK knockdown, which attenuated MDM2-dependent p53 degradation, and by NF2 knockdown, which attenuated the inhibitory effect of MDM2 on p53.

Our data indicate that FAK regulates cell survival via p53-dependent and -independent pathways, which was demonstrated by immunoblots after FAK knockdown in mutant p53 JMN1B cell line and in nonmutant p53 mesothelioma cell lines (MESO924, MESO257, MESO296, and MESO428; Figure 5). In addition, we found the dramatic anti-proliferative and apoptotic effects of FAK knockdown in mutant leiomyosarcoma cell lines (data not shown). The FAK (FAK shRNA or PF562271) and MDM2 (nutlin-3) inhibitors showed anti-proliferative effects in mesothelioma cell lines characterised by a decrease of cyclin A expression, CDK inhibitor p21 upregulation (Figure 5A), reduction of cellular viability (Figure 5B and C), and G1-phase arrest (Figure 5D). Interestingly, additive effects were obtained through a coordinated attack on p53, as demonstrated by immunoblots, cell viability, and cell cycle analyses, showing that FAK and MDM2 inhibition together induced greater p53 expression, cell apoptosis, anti-proliferative effects, and cell cycle arrest, as compared with either intervention alone. These findings highlight novel therapeutic opportunities in mesothelioma.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

W-BO and JAF designed the study. W-BO, ML, GE, JD, XM, YW, QS, and H-MZ performed the experiments and acquired the data. W-BO, ML, GE, JD, XM, YW, QS, H-MZ, and JAF analysed and interpreted the acquired data. W-BO, GE, and JAF participated in scientific discussion and drafting of the manuscript.

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