

CONTEMPORARY STRATEGIES FOR CANCER THERAPY: THE p53 GENE AS A PARADIGM

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Abstract

Since its discovery in 1979, p53 has become one of the most intensively studied genes of all time (over 15,000 articles published so far). However, some issues concerning p53 biology remained unresolved. What is in no doubt is the important role that p53 plays in regulating the response of cells to adverse stresses such as DNA damage. Indeed, this control mechanism is lost in over 50% of human cancers due to mutation of the p53 gene. A large number of therapies have been devised with p53 in mind, ranging from genetic therapies to more conventional drugs. Crucially, it is still controversial as to whether p53 mutants (*i.e.* the dysfunctional proteins produced upon mutation of the p53 gene) have additional tumour-promoting (oncogenic) properties that are independent of wild-type p53 inhibition. Our work has provided further evidence that mutant p53 proteins do portray such 'gain of function' activities. We have identified a novel protein that interacts in both a physical and functional manner with certain mutant forms of the p53 protein. This protein was assigned the term MBP1 - for **M**utant **p**53 **B**inding **P**rotein **1**. MBP1 is the fourth member of the emerging fibulin family. It displays both mutant p53-dependent and -independent oncogenic properties. As such, MBP1 may be useful as a target for cancer therapy, along with being informative in terms of determining patient prognosis.

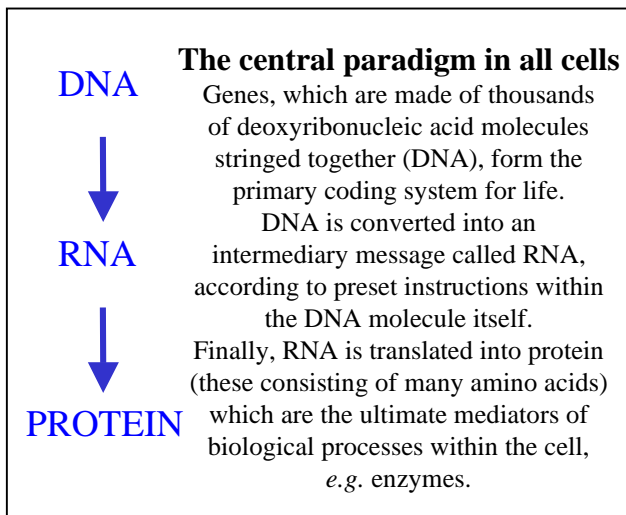
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1 INTRODUCTION

1.1 Cancer therapy today

Treatment of human cancer is presently undergoing a process of radical change, both in terms of drug design and implementation within the clinic [1,2]. The original cancer chemotherapeutics largely arose from chance observations and through the use of relatively non-specific drug screens which were carried out with only marginal rationality. Modification of these agents, mostly low molecular weight compounds, in efforts to improve limiting pharmacological characteristics, such as solubility and stability, has led to an extensive range of analogues, some of which have exhibited heightened anti-tumour efficacy in pre-clinical models and patients.

However, although patients with selected tumour types (*e.g.* leukaemia and testicular cancer) benefit considerably from certain existing therapeutic regimens, the vast majority of human cancers still remain quite intractable in terms of patient response [3,4]. This is primarily due to either the intrinsic or acquired resistance of cancers to presently used agents. To meet this pressing demand for more effective forms of chemotherapy, a host of novel anti-tumour biotherapies have been devised by pioneering investigators world-wide [5,6]. Detailed exploration of the molecular events involved in cancer formation has provided us with an extensive contingent of biological processes (targets) upon which one may act. Today, we are in a position to exploit our refined knowledge of tumour biology in the creation of more innovative therapeutics, designed to better combat a disease that has approximately 200 different subtypes.



1.2 The p53 gene and cancer

Huge strides have been made in understanding the complex biology surrounding the p53 gene [7]. Normal, otherwise known as wild-type (wt), p53 functions as a suppressor of tumour development (tumourigenesis). It does this by positively modulating crucial cellular events such as apoptosis (a form of programmable cell death) and cell cycle arrest (see Figure 1). Wt p53 may also regulate DNA replication and DNA repair. In response to stress signals, such as DNA damage and hypoxia (a low oxygen environment), wt p53 becomes activated and mediates the afore-mentioned cellular effects by either turning on or off a set of p53-responsive genes (*i.e.* the p53 protein is a transcription factor and can bind DNA in a sequence-specific manner). Wt p53 also interacts in a physical manner with a variety of cellular proteins, which may in turn act as effectors of the p53 stress response pathway.

Disruption of wt p53 function, through mutation of the gene itself or other means, occurs very frequently in many types of human cancer, *e.g.* lung, breast and colon cancer [8]. Moreover, tumours with a defective p53 pathway are often more aggressive and display evidence of early metastasis (*i.e.* the appearance of secondary tumours, with this spreading process generally being the lethal event in tumourigenesis). This provides for the strong correlation between loss of p53 function and reduced patient survival that is commonly seen [9]. Since the majority of clinically effective anti-cancer drugs target DNA, p53 is an important determinant of cellular responsiveness to such agents [10]. In summary, the status of p53 in a tumour cell has implications not only for tumour development but also for the treatment of emerging malignancies.

Indeed, the potential role of p53 in drug resistance interested me (WMG) considerably during my PhD studies, which were carried out at the CRC Beatson Laboratories in Glasgow, UK. While working on the various mechanisms of resistance that tumours present to chemotherapy [11,12], we further confirmed the association between loss of p53 function and acquired drug resistance in ovarian cancer [13]. Two and a half

years ago, I moved to Paris to begin working as a Marie Curie research Fellow with Rhone-Poulenc Rorer (RPR), the major French pharmaceutical company.

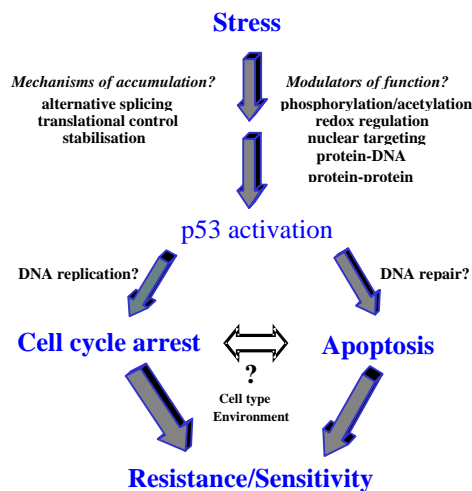


Figure 1: The p53-dependent stress response pathway.

Schematic representation of the signalling pathway involving p53 from the initial stress, *e.g.* DNA damage, to cellular responses, such as cell cycle arrest and apoptosis. The signalling events leading to p53 activation are still being deciphered and appear to involve a complex set of events. The exact response of cells to p53 activation is determined by both cell type and the environment in which the cell is exposed to. In keeping with the role of p53 in being an important determinant of cellular responsiveness to anti-cancer agents, alterations in the p53 pathway commonly occur during the development of drug resistance – with loss of the apoptotic function mediated by p53 generally leading to increased resistance to such agents. However, loss of the cell cycle arrest function mediated by p53 may provide added sensitivity in certain situations, this probably being due to defective/inappropriate DNA repair.

1.3 Cancer therapies based around p53

The main reason for my transfer to industry was to obtain a perspective of more applied research, again within the field of p53 biology, than I had been accustomed to. A host of p53-oriented cancer therapies are currently being evaluated by a large number of investigators world-wide [14]. Most of these therapies are still at the pre-clinical stage, with only a few having been applied to cancer patients. In the presence of mounting supportive evidence, gene replacement therapy using wt p53 is slowly becoming an accepted method in the fight against cancer, either as an alternative to or in combination with conventional chemotherapeutic drugs. The objective in this form of therapy is to replace the damaged or missing p53 protein present within cancer cells with an intact form, which hopefully should result in tumour suppression through apoptosis, cell cycle arrest or other means.

The Oncology Department at RPR is closely involved in developing wt p53 gene replacement therapies, and is one of the world leaders in this area. They are also ideally

placed to evaluate the potential efficacy of combining wt p53 gene therapy with other anti-cancer agents. Interestingly, the RPR Oncology Department have also developed a novel form of the p53 gene (called CTS1), which displays superior activities to the wt gene [15]. Several methods of wt p53 gene transfer are currently being evaluated, with delivery vehicles ranging from liposomes to viral particles. While viral delivery systems offer the advantage of a relatively high efficiency of gene transfer, adverse immune responses might engender some problems. Non-viral alternatives, while still much less efficient in terms of gene transfer, may hold the best promise for obviating unwanted side effects (*see Future Perspectives section*).

The remaining p53-oriented therapies that are being tested involve restoration of p53 function by alternative means or specific targeting of the p53 dysfunction itself. This latter category inspired me (during my Marie Curie Fellowship) to tackle an area which may be of considerable use in the design of such therapies, *i.e.* the controversial subject of the mutant p53 ‘gain of function’ phenotype [16]. Mutation of the p53 gene may affect p53 protein function in one, or more, of the following ways: (a) loss of wt p53 function and consequent ablation of tumour suppression activity, (b) inhibition of wt p53 in a dominant-negative manner leading to acquired oncogenic potential, (c) gain of additional oncogenic properties not dependent on inhibition of wt p53 – the fundamental tenet of the ‘gain of function’ hypothesis.

2 RESULTS AND DISCUSSION

2.1 Searching for mutant p53-specific partners

My plan was to search for proteins that physically interacted with mutant, but not wt, p53 protein. If discovered, such factors might shed some light on this cryptic phenomenon (*i.e.* ‘gain of function’ properties) and, perhaps, open the way for more selective targeting of p53. Using a yeast two-hybrid screening approach [17], one can find proteins expressed from cDNA libraries (a catalogue of the genes expressed by a particular cell or tissue type) which interact in a physical manner with a protein of interest. As our bait in the so-called ‘interaction trap’, we selected a very commonly-found human tumour-derived mutant termed H175 (to indicate a single mutation at this amino acid position with arginine being substituted for histidine). Indeed, such missense mutations account for over 80% of mutations within the p53 gene. This striking predisposition for missense mutations to appear, instead of deletions or other types of mutations, has been put forward by some as circumstantial evidence for p53 being an oncogene, as well as a tumour suppressor gene.

During screening of various cDNA libraries, we identified several human proteins that interacted with mutant p53 [18]. These include ubiquitin conjugating enzyme 9 (involved in protein degradation) and PIAS1, an inhibitor of STAT1 signalling. However, these mutant

p53-interacting protein partners also bound efficiently to wt p53, perhaps reflective of additional functional pathways in the already complex profile of p53-related activities. Only one protein was found which physically interacted with mutant p53 protein solely (*i.e.* not with wt p53 protein also). This was a novel protein, termed MBP1 for **Mutant p53 Binding Protein 1**. At this stage, we only had the mouse form of MBP1, since the cDNA library was murine in origin. Later on, the human form of MBP1 (hMBP1) was isolated by both ourselves [19] and another group [20]. Interactions between mutant p53 protein and MBP1 were indicated to occur in mammalian cells [16, see section 2.3]. We have also shown that MBP1 gene expression is differentially regulated both temporally during development of the mouse embryo and in a tissue-specific manner within the adult mouse. Notably, a higher level of MBP1 gene expression is seen early in the developmental process within the mouse. Some tissues, such as the lung and testis, express increased levels of the MBP1 gene than others, *e.g.* the brain and liver.

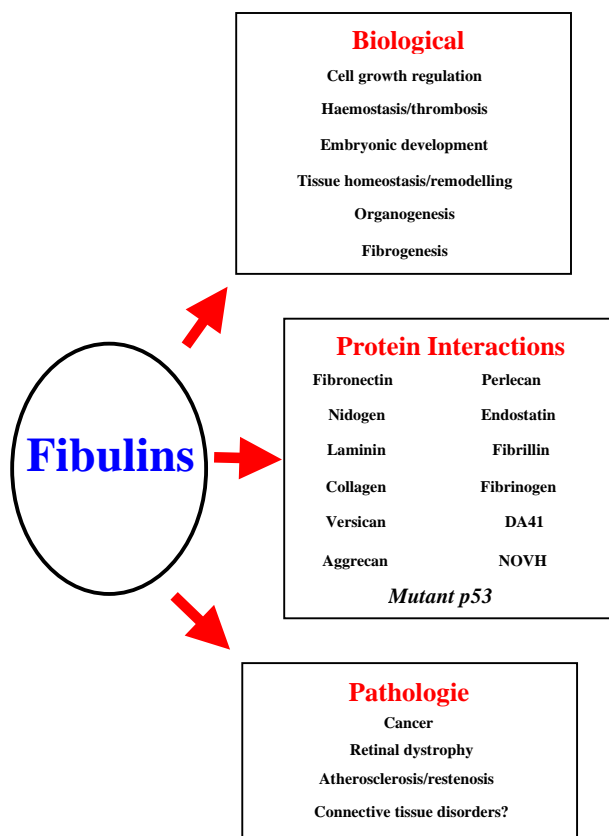


Figure 2: The fibulins.

The varied biological functions, physical interactions with other proteins and pathologies with which fibulins have been associated so far.

2.2 MBP1, a new member of the fibulin family

The amino acid sequence of MBP1 was compared against other currently known proteins. From this, we asserted that MBP1 is the fourth member of the emerging fibulin gene family. Fibulins have been implicated in a variety of biological processes [21], ranging from cell growth regulation to fibrogenesis (Figure 2). The first member of the fibulin gene family (fibulin-1) was discovered in 1989

[22], and later defined as a calcium-binding extracellular matrix and plasma glycoprotein (*i.e.* with carbohydrate chains attached to the main body of the protein). To date, there are five members of the fibulin family (fibulin-1 to -5), all of which are characterised by a homologous domain structure. We assert that MBP1 is fibulin-4. It is clear that fibulins are *generally* secreted from the cell, as these are mainly extracellular matrix proteins. This has important implications as to the physiological relevance of our observed physical interaction between mutant p53 protein and MBP1 (see section 2.4), which like other fibulins contains a topogenic sequence (a short sequence of amino acids found at the start of proteins which are destined for either secretion or integration into cellular membranes). However, there is increasing circumstantial evidence to suggest that fibulins may have functions in the cytoplasm (*i.e.* the fluid interior) of the cell as well.

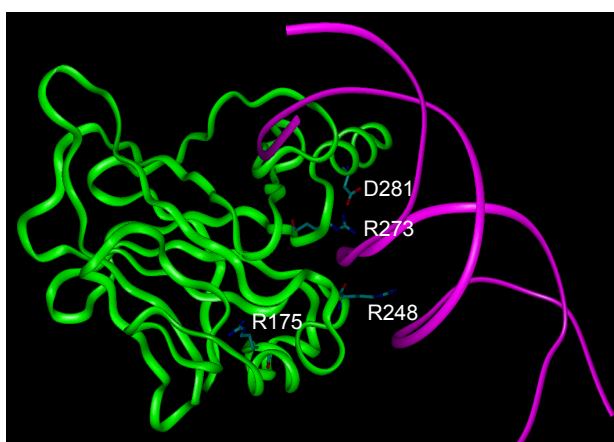


Figure 3: p53/DNA complex.

The sequence-specific DNA binding domain of wt p53 is shown in green, with a short segment of DNA being highlighted in purple. This is the part of the wt p53 protein that interacts with DNA in a specific manner.

2.3 Physical and functional interactions between MBP1 and mutant p53 protein

So far, we have only been able to demonstrate a physical interaction between mutant p53 protein and a deleted form of MBP1 (minus the topogenic sequence and which we called MBP1 Δ N). While it still has not been shown that the entire MBP1 protein interacts with mutant p53, our group has proven that a functional interaction between the entire MBP1 protein and mutant p53 does indeed exist. In more detail, MBP1 (both the deleted and full-length forms) displays oncogenic properties of its own, in that it can promote tumour cell growth and increase the rate of neoplastic transformation (*i.e.* the appearance of tumour-like characteristics). MBP1 does not require the presence of the mutant p53 gene for these effects to be realised. On the other hand, MBP1 and mutant p53 can also co-operate together in terms of oncogenic activity. In summary, MBP1 exhibits both mutant p53-dependent and -independent oncogenic properties.

We also examined the affinity of MBP1 Δ N protein towards 4 different p53 missense mutants (each of them being commonly-found or ‘hot-spot’ mutations). Figure 3 is a representation of a single p53 sequence-specific DNA binding domain (in green) interacting with a short sequence of DNA elements (in purple). This image is derived from the publicly accessible X-ray crystal structure resolved by Nikola Pavletich and co-workers in 1994 [23]. Four amino acid residues are highlighted (*e.g.* D281), these being the most common sites for mutation in p53.

Two of these residues, R248 and R273, interact directly with DNA. Variants of p53 with mutations at such sites are called ‘contact’ mutants. The two other residues, D281 and R175, do not directly interact with DNA but are involved in stabilising the structure of the DNA binding surface of p53. Mutations here give rise to what are known as ‘structural’ mutants. Mutation at either of these two types of critical residues results, to variable extents, in impairment of the sequence-specific DNA binding activity and, as a consequence, the transactivation function of p53. Transrepression by p53 is also affected upon mutation. Another intriguing feature is the observation that certain p53 mutants occupy a different conformational state in comparison to the wt p53 protein. In general, mutants of the ‘structural’ class are more different from the wt protein than those of the ‘contact’ type.

Interestingly, MBP1 Δ N showed a much higher affinity for ‘structural’, rather than ‘contact’, p53 mutants [18]. In more detail, MBP1 displayed the following order of binding affinities towards different p53 forms: H175 > G281 > H273 \geq W248 > wt p53. Similar results were obtained both in yeast two-hybrid experiments and with co-immunoprecipitation studies of overexpressed proteins in transfected mammalian cells. The data is in keeping with the concept that MBP1 is a mutant p53-specific protein partner. What remains to be seen is whether the physical interaction observed between mutant p53 and MBP1 Δ N, is responsible for their functional synergy.

2.4 How might a physical interaction between MBP1 and mutant p53 protein occur?

We have carried out *in vitro* transcription/translation of mouse MBP1. This experiment utilises cloned cDNA to artificially generate recombinant proteins. Using this particular method, we produced recombinant MBP1 protein of approximately 54KD in size, its predicted molecular weight. Interestingly, we also observed the production of a smaller species of protein. This smaller protein product may represent usage of an alternative downstream translation initiation site.

If the case, this would mean that the smaller protein would lack the N-terminal topogenic sequence and, thus, be localised intracellularly. This may be one of the ways in which mutant p53 may interact physically with endogenous MBP1 protein. Another way may be for mutant p53 to enter the lumen of the endoplasmic

reticulum, as proteins pass along the secretory pathway, thus interacting with MBP1 there. Indeed, mutant p53 has been previously reported to perform an equivalent feat [24]. This mutant p53-associated phenomenon still awaits further exploration.

In summation, this work has suggested several novel, perhaps even controversial, biological aspects. Firstly, genes encoding proteins that contain classical secretory signals may also code for alternative proteins which are missing these signals – these potentially having, as yet undiscovered, important physiological functions. Secondly, this work provides further evidence supporting a ‘gain of function’ role for certain p53 mutants. Finally, our work has pointed a way towards understanding, in more detail, the part played by fibulins in the pathogenesis of cancer, along with various biological processes and other pathologies.

3 FUTURE PERSPECTIVES

A primary objective of my present work is to further clarify the role of fibulins in cancer, in particular the involvement of MBP1 (fibulin-4). To achieve this, our group have already formed, and shall continue to form, close collaborative links with a variety of international research groups – both in the areas of fibulin and cancer biology. Firstly, fibulin expression/activity in tumours of various types will be correlated with patient outcome. This may shed some light on the current paradoxes surrounding the participation of fibulins in the tumorigenic process. I would also like to examine in more detail the physical and functional relationship between MBP1 and mutant p53. Such information may yet prove useful in the design of mutant p53-specific therapeutic agents. In addition, this added insight into the ‘gain of function’ phenotype of p53 mutants may have implications for gene replacement therapies involving the p53 gene.

One of my other research interests is to improve the delivery of both chemotherapeutic drugs and genes to diseased tissues. Our group has begun tackling this issue through the use of novel biomaterials that provide a molecular platform from which therapeutic agents can be released in a highly regulated and programmed fashion. These biomaterials are being designed by our partners in the *Irish Centre for Colloid Science and Biomaterials*, UCD, led by Professor Kenneth Dawson. Immediate objectives are to examine release of anti-proliferative/anti-migratory agents, *e.g.* taxol, from polymer films/matrices, with a view towards use in the localised treatment of certain pathologies such as restenosis (a disorder of the cardiovascular system) and cancer. We are also considering alternatives to virus-mediated gene delivery, notably through the use of lipid-based vehicles that have been generated ‘in-house’. We envisage wt p53 as being a prime candidate for such immune response-sympathetic gene delivery systems.

In summary, current research interests include cancer biology and therapy, drug resistance and

targeted/localised drug delivery. It should be noted that three peer-reviewed publications (including the present one) have already arisen from this particular EC-supported project, along with one patent submission (deposited Oct 1998). Another paper has been submitted to *Oncogene*, while two other manuscripts are currently in preparation.

4 ACKNOWLEDGEMENTS

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