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Phage Display Libraries as a Source of Tumour-Targeting Agents

Francesca Viti, Patrick Pedrioli, Barbara Mitta, and Dario Neri*

Abstract: One way to improve the therapeutic index of anti-cancer agents would be to target them to the tumour site, thereby sparing normal tissues. Tumour targeting approaches heavily rely on the availability of specific binding molecules, recognising markers which are selectively expressed in the tumour environment. The display of antibody repertoires on the surface of filamentous phage, together with efficient selection-amplification schemes, allows the isolation of good-quality human antibodies against virtually any molecular target. This article illustrates how antibody phage technology has been used in our laboratory for the isolation of tumour targeting agents. Furthermore, we present recent developments in the display of repertoires of small globular proteins on filamentous phage, aimed at the isolation of specific binding molecules with molecular weight < 10000 Dalton.

Keywords: Oncofoetal fibronection · Phage display · Protein engineering · Recombinant antibodies · Tumour targeting

1. Introduction

Cancer chemotherapy relies on the expectation that anti-cancer drugs will preferentially kill rapidly proliferating tumour cells, rather than normal cells. Since a large portion of the tumour cells has to be killed in order to obtain and maintain a complete remission, large doses of drugs are typically used, with significant toxicity towards proliferating non-malignant cells. The development of more selective anti-cancer drugs, with better discrimination between tumour and non-tumour cells, is possibly the most important goal of modern anti-cancer research.

Tumour targeting, *i.e.* the selective delivery of molecules to the tumour site, typically by intravenous injection, is a promising approach to tumour imaging and therapy [1][2]. This approach relies on the availability of good-quality markers, which are abundantly expressed at the tumour site but absent in normal tissues, and of targeting agents (*e.g.* antibodies), with high affinity for the target and suitable pharmacokinetic properties. Traditionally, murine monoclonal antibodies isolated by hybridoma technology have been used in tumour targeting applications. However, these antibodies are immunogenic in humans, limiting the possibility of repeated administration. The field has advanced, first with the development of methods for the humanisation of murine antibodies [3], then with the advent of methods for the production of fully human monoclonal antibodies, in particular antibody phage display technology [4], transgenic mouse antibody technology [5] and iterative filter colony screening [6].

Phage display was invented in 1985 by Smith [7], who described the use of non-lytic filamentous bacteriophage fd for the display of specific binding peptides fused to the minor coat protein pIII. In 1990 and following years, the group of Greg Winter at the Cambridge Centre for Protein Engineering demonstrated that antibody fragments can be functionally displayed on filamentous phage. Furthermore, it was shown that naïve libraries of millions of human antibody fragments displayed on phage can rapidly yield specific binders against virtually any target, without immunisation and by-passing hybridoma technology [8]. The principle of biopanning experiments with antibody phage libraries is schematically illustrated in Fig. 1. Specifically-binding phage antibodies are physically isolated by virtue of their affinity to an antigen immobilised on a solid support (*e.g.* antigen-coated immunotubes or antigen-coated magnetic beads), while irrelevant phage are washed away. The selected binding specificities can be eluted from the affinity support and amplified by bacterial infection. After a few rounds of biopanning, phage antibodies are typically used to infect bacteria, which are plated on selective agar plates. The corresponding bacterial colonies contain potentially different monoclonal antibodies, which can be expressed as soluble proteins in bacteria or in other organisms.

In our laboratory, we have constructed and validated a synthetic antibody phage library (the 'ETH-2 library'), containing > 6 x 10^8 different human antibody fragments displayed on the surface of filamentous phage [9]. The antibody format chosen was the single-chain Fv format ('scFv'), in which a variable heavy and a variable light antibody domain are joined together *via* a flexible polypeptide linker [10]. The ETH-2 library is a reliable source of good-quality monoclonal antibodies, and has been distributed to several dozens of academic

*Correspondence: Prof. Dr. D. Neri
Institute of Pharmaceutical Sciences
Department of Applied Biosciences
Swiss Federal Institute of Technology
Winterthurerstrasse 190
CH-8057 Zurich
Tel.: +41 1 635 60 63
Fax: +41 1 635 68 86
E-Mail: neri@pharma.anbi.ethz.ch

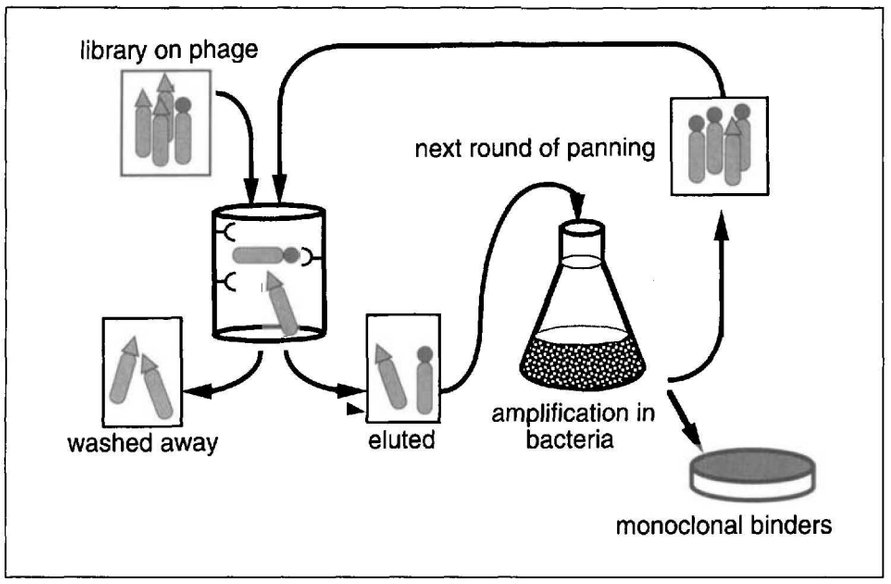

Fig. 1. Schematic representation of a biopanning experiment using a library of globular proteins (*e.g.* antibody fragments) displayed on the surface of filamentous phage. After one or more rounds of panning, phage can be used to infect bacteria, which can be plated onto selective agar plates. Individual colonies correspond to potentially different monoclonal antibodies.

laboratories world-wide. Nonetheless, the facile production of human monoclonal antibodies using the ETH-2 library leaves open the decision about which tumour marker to use in antibody-based targeting applications. Markers of angiogenesis appear to be particularly interesting targets for molecular intervention.

Angiogenesis, *i.e.* the proliferation of new blood vessels from pre-existing ones, is a characteristic feature of aggressive solid tumours and of relevant disorders, such as age-related macular degeneration, diabetic retinopathy and rheumatoid arthritis [11]. Molecules capable of inhibiting angiogenesis, or of selectively targeting and destroying new blood vessels, would be promising agents for the treatment of angiogenesis-related diseases. A good-quality marker for both tumoural and non-tumoural neovasculature is the extra-domain B (ED-B) of fibronectin (a sequence of 91 amino acids identical in mouse, rat, rabbit, dog and man) that can be inserted into the fibronectin molecule by a mechanism of alternative splicing [12]. Fibronectin containing ED-B (B-FN) accumulates around neovascular structures in aggressive tumours and other tissues undergoing angiogenesis, such as the endometrium in the proliferative phase and some ocular structures in pathological conditions, but is otherwise undetectable in normal adult tissues [13–15].

To date, the production of monoclonal antibodies directly recognising the ED-B domain in B-FN has not been possible using hybridoma technology, because of tolerance. This problem, however, has been overcome using antibody phage technology with large synthetic antibody repertoires [16]. Several antibody fragments specific for the ED-B domain of fibronectin have recently been generated. These antibody fragments stain vascular structures in tumour sections and selectively target tumour neovasculature, as shown in tumour-bearing mice using infrared fluorescence and radioactive techniques [17][18]. Increased binding affinity leads to improved targeting of tumoural angiogenesis, as demonstrated by biodistributions studies performed using the L19 antibody fragment with affinity for the ED-B domain in the picomolar range and L19 mutants with reduced affinity [19]. Fig. 2 shows the results of an immunophotodetection experiment, in which scFv(L19) and scFv(HyHEL-10) (a negative control antibody fragment), labelled with the infrared fluorophore Cy7, have been injected into tumour-bearing mice and imaged with an infrared fluorescence imager [20]. Twenty-four hours after intravenous injection, a selective antibody localisation on the tumour can be observed

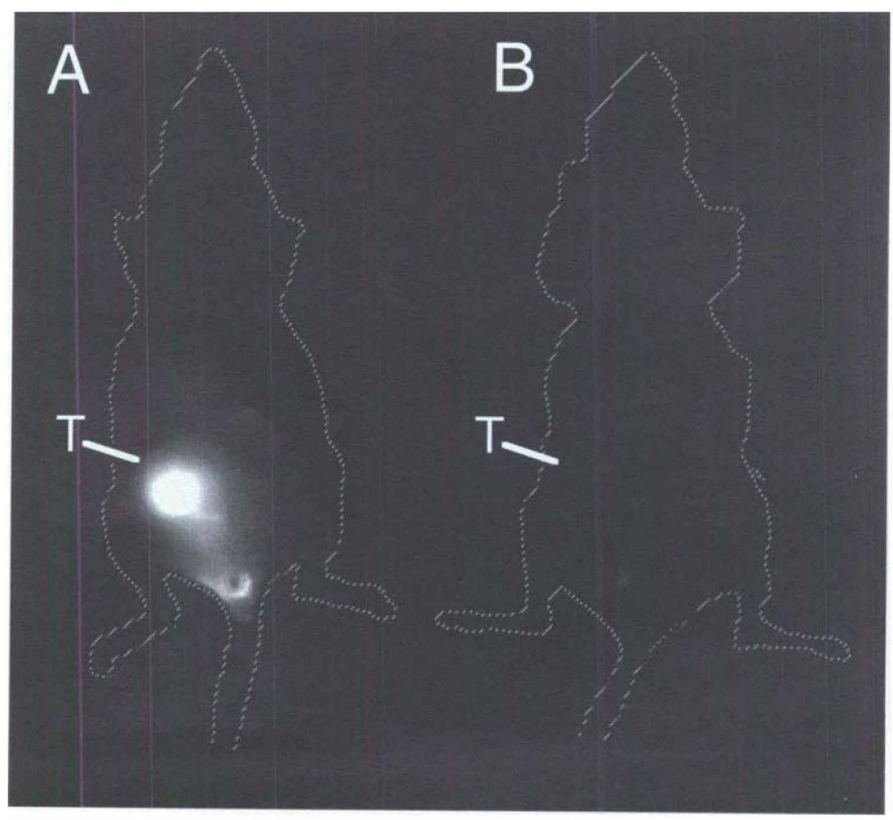

Fig. 2. Immunophotodetection of recombinant antibody fragments, labelled with the infrared fluorophore Cy7, 24 h after intravenous injection in mice bearing a subcutaneously-implanted murine F9 teratocarcinoma. (A), scFv(L19), specific for the ED-B domain of fibronectin; (B) scFv(HyHEL-10), specific for an antigen irrelevant in this experimental context (hen egg lysozyme). Only the scFv(L19) selectively localises on the tumour.

with scFv(L19), but not with scFv (Hy HEL-10).

The human antibody fragment scFv (L19) has recently been fused to the procoagulant protein tissue-factor (residues 1-219), with the aim to promote the selective intra-luminal blood coagulation in tumour blood vessels. This fusion protein mediated the complete and selective infarction of three different types of solid tumours in mice. At the highest doses administered, complete tumour eradication was observed in 30% of the mice treated, without apparent side effects [21].

2. Results

2.1. *Human Antibodies against Tumour-associated Antigens*

In addition to the ED-B domain of fibronectin, a number of other tumour-associated components of the modified extracellular matrix could be considered for tumour targeting applications. Radiolabeled antibodies against tenascin-C, for example, have been used in clinical trials for the immunoscintigraphic detection and therapy of brain tumours [22–24]. Like fibronectins, tenascins show a wide distribution in normal adult tissues. Tenascin-C exists in several polymorphic isoforms due to alternative splicing of nine fibronectin-like type III repeats [25]. Large tenascin-C isoforms are present in almost all normal adult tissues but are upregulated in foetal, regenerating and neoplastic tissues to a much greater extent. One of the repeats present in the large tenascin-C isoforms is domain D.

We cloned and purified the human domain D of tenascin-C expressed in *E.coli*. The resulting protein was then biotinylated and used to isolate specifically binding antibodies from the ETH-2 library. One of the clones isolated, 5H, bound to the recombinant domain D with a dissociation constant Kd = 2.8 x 10^{-7} M (k_{off} = 1.2 x 10^{-2} s^{-1}; k_{on} = 4.3 x 10^{4} $M^{-1}s^{-1}$), as determined by real-time interaction analysis using a BIAcore 3000 instrument. The sequence of the VH and VL domains of scFv(5H) is illustrated in Fig. 3. In order to improve the affinity of scFv(5H), a secondary affinity maturation phage display library was constructed (5 x 10^5 individual clones), in which residues 31, 32, 33, 50, 52, 54 of VH were combinatorially mutated (for the design of the affinity maturation library, see [16]). The resulting phage library was biopanned on the recombinant domain D, yielding several dozens of specific binders. One of them (clone D7), bound to the recombinant domain D with a dissociation constant Kd = 1.2 x 10^{-7} M (k_{off} = 1.1 x 10^{-3} s^{-1}; k_{on} = 8.8 x 10^{3} $M^{-1}s^{-1}$). Similar to what we had previously observed for anti-ED-B antibodies [19], we expect that the lower kinetic dissociation constant of D7 may result in longer residence times on the tumour in *in vivo* targeting experiments. The sequence of scFv(D7) is depicted in Fig. 3.

2.2. *Phage Display Libraries of Small Globular Proteins*

Many different libraries have been generated that display linear peptides or constrained peptides (containing two or more cysteine residues) on phage. These libraries have been a rich source of ligands for many targets [26]. Furthermore, they have allowed the identification of linear peptides that mimic small molecules, such as biotin or carbohydrate antigens.

The small size of peptides makes them attractive ligands, particularly for tumour targeting applications, in which tissue penetration is expected to improve with low molecular weight binders. However, several arguments exist for the preferential use of small globular proteins as scaffolds for combinatorial phage display libraries:

- higher affinities can be expected for structured proteins, because of the lower entropy loss upon binding;
- if the structure of the original protein is preserved in the mutants, the interpretation of interactions at the atomic level are facilitated;
- higher proteolytic stability can be expected for a folded protein, compared to a linear peptide, making it more suitable for recombinant production and for *in vivo* applications;

A number of combinatorial phage libraries of small protein mutants have been produced so far [27]. However, the dissociation constant of the ligands isolated was typically in the micromolar range.

We have constructed two phage display libraries (the '434 library' and the 'PED-B library') based on small stable globular proteins, devoid of cysteine residues, in which we have combinatorially mutated a number of amino acid positions. Fig. 4 shows the three-dimensional structure of the N-terminal domain of the phage 434 repressor (residues 1-63) and of the ED-B domain of fibronectin. The Cα and Cβ atoms of the amino acid residues which have been combinatorially mutated in the libraries are displayed as solid spheres. For comparison, the three-dimensional structure of the scaffold onto which the ETH-2 library was constructed is also indicated in Fig. 4. The N-terminal domain of the phage 434 repressor (residues 1-63) is an alpha-helical protein; the ED-B domain of fibronectin contains solely beta-sheets as secondary structure elements. Both structures have been determined at high resolution by NMR spectroscopy in solution [28][29].

VH (5H)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQ
APGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTL
YLQMNSLRAEDTAVYYCAK**GRSI**FDYWGQGTLVTVSS

VH (D7)
EVQLLESGGGLVQPGGSLRLSCAASGFTFS**GYT**MSWVRQ
APGKGLEWVS**TISGSR**GSTYYADSVKGRFTISRDNSKNTL
YLQMNSLRAEDTAVYYCAKGRSIFDYWGQGTLVTVSS

linker
GGGGSGGGGSGGGGS

VL (both 5H and D7)
SSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKP
GQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQA
EDEADYYCNSS**RPVNHK**VVFGGGTKLTVLG

Fig. 3. Amino acid sequence of the variable heavy and light chain domains of scFv(5H) and scFv(D7), specific for the D domain of tenascin-C. Residues in boldface are the ones which had been combinatorially mutated in the library, from which the antibody was selected.

The 434 library contains 9 x 10^8 individual clones. It has been biopanned against a variety of different antigens (including glutathione S-transferase, bovine serum albumin, human chorionic somatotropin, tenascin-C, ED-B domain of fibronectin, soybean trypsin inhibitor), overall with poor results. The PED-B library (3 x 10^8 individual clones) has so far been tested on four antigens: bovine serum albumin, recombinant CD-46, ED-B domain of fibronectin, domain D of tenascin-C. In parallel, selections against the same antigens have been performed using the ETH-2 library.

Fig. 5 shows the ELISA results obtained with bacterial supernatants of individual clones derived from the ETH-2 phage library (Fig. 5A) and the PED-B phage library (Fig. 5B), after different rounds of biopanning against the ED-B domain of fibronectin. Already after one round of biopanning, the ETH-2 library yielded specific binders, which recognise the target antigen immobilised on a streptavidin-coated microtitre plate, but which do not bind to the streptavidin-coated microtitre plate devoid of the antigen (black bars). By comparison, three rounds of panning were necessary to obtain specific ED-B binders from the PED-B library. Even though specific binders can be isolated from the PED-B library, their performance in ELISA and their specificity appear to be lower compared to the ones of scFv fragments isolated from the ETH-2 library.

3. Discussion

Large antibody phage libraries, such as the ETH-2 library, are rich sources of binding specificities. Human antibody fragments have been generated against a variety of tumour-associated antigens, which are now starting to be evaluated in preclinical and clinical studies. In our

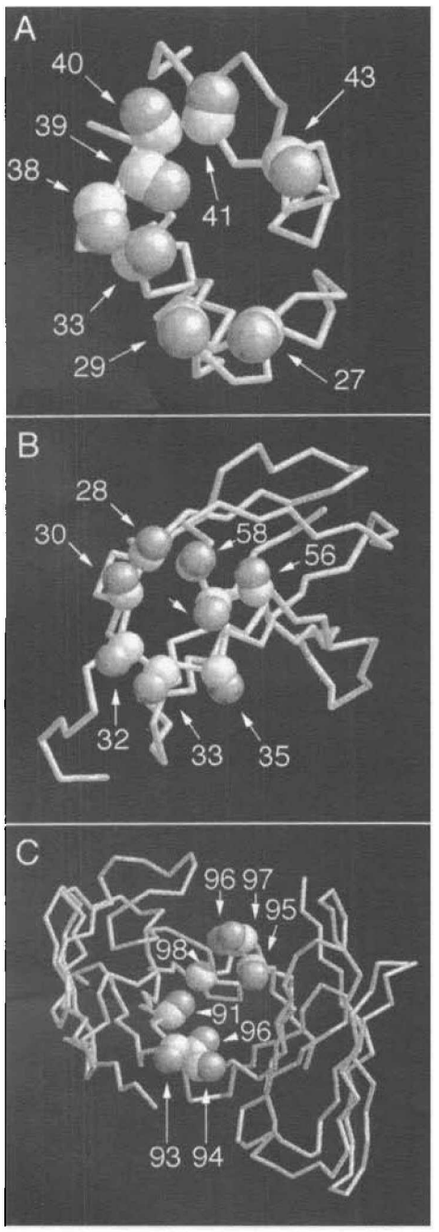

Fig. 4. Small globular proteins used as scaffold for the construction of phage display libraries. (A) N-terminal domain of the phage 434 repressor (residues 1-63); (B) ED-B domain of fibronectin; (C) scFv fragment. The Cα and Cβ atoms of the residues combinatorially mutated in the phage display library are indicated as light-grey and medium-grey spheres, respectively. Residue numbers are indicated.

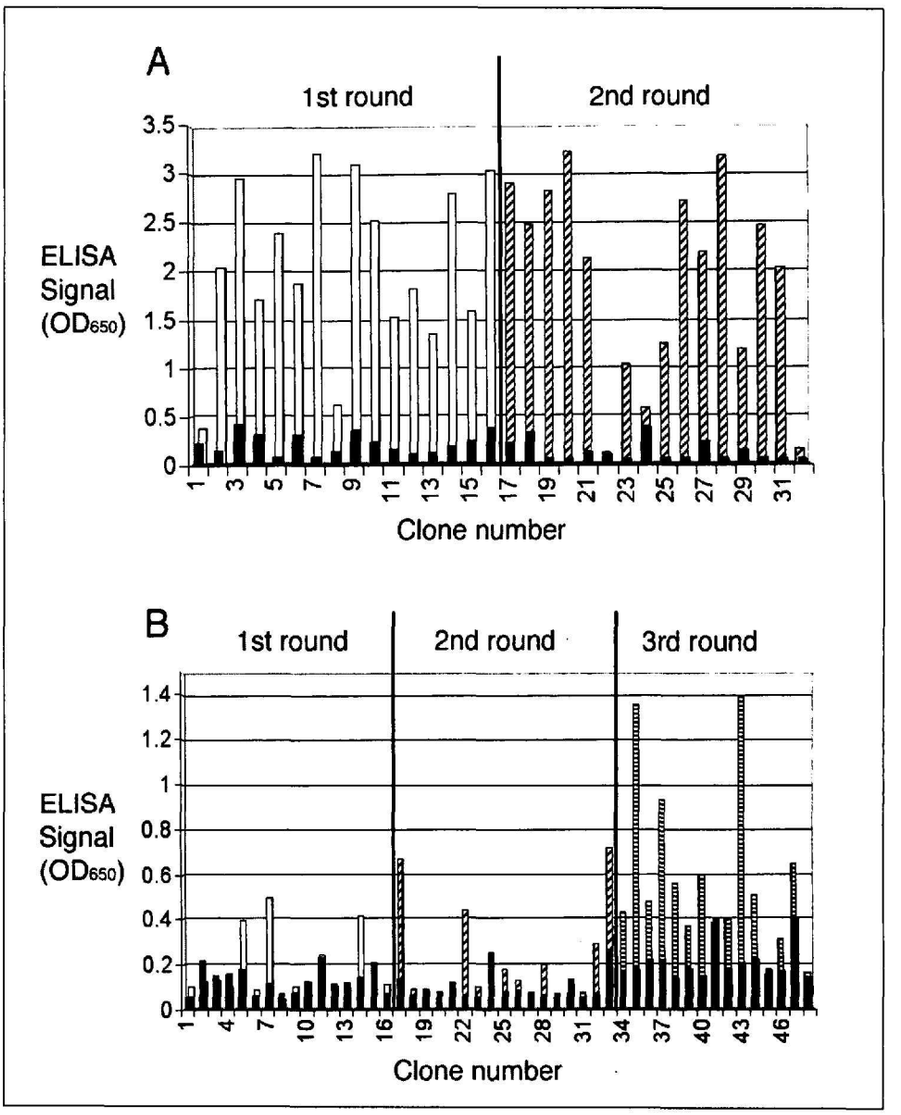

Fig. 5. ELISA results obtained with supernatants of individual clones derived from the ETH-2 phage library (A) and the PED-B phage library (B), after different rounds of biopanning against the ED-B domain of fibronectin, a marker of angiogenesis. In the ELISA assay, the biotinylated antigen had been coated onto streptavidin-coated microtitre plates. In the histogram, black bars indicate the ELISA signal obtained in negative control experiments, in which the biotinylated antigen had not been added to the streptavidin-coated plate.

laboratory, in collaboration with Prof. Dr. Luciano Zardi (National Cancer Institute, Genova, Italy), we have focused on the production of human antibodies specific for tumour-associated components of the modified extracellular matrix. High-affinity antibodies specific for the ED-B domain of fibronectin are among the most promising ones, because of their ability to selectively localise *in vivo* around new blood vessels, while sparing mature vessels and healthy tissues.

In principle, the availability of small globular proteins (< 10000 Daltons), endowed with a high binding affinity towards tumour markers, could open a number of useful tumour targeting opportunities, particularly for the scintigraphic imaging of cancer. In practice, advances in this field have been limited by the low affinity of the binders isolated from phage display libraries (typically in the micromolar range). Our results, presented in this article, are in line with observations made by other groups with phage display libraries of globular proteins of similar size [27], which typically yielded low affinity binders. It is still a matter of speculation why the antibody protein scaffold may offer a more versatile starting point for the isolation of variants, with high binding affinity towards a variety of different antigens.

The tumour targeting performance of human antibodies appears to be dictated by a number of parameters. Antibody affinity, together with the accessibility, abundance and specificity of the target antigen, are probably the dominant features leading to improved tumour targeting. In this respect, the targeting of angiogenesis mediated by antibodies specific for the ED-B domain of fibronectin is a promising strategy to develop anti-cancer agents with improved therapeutic ratio, considering the restricted tissue distribution of the antigen and its specific localisation around tumour neo-vasculature.

4. Experimental Procedures

4.1. *Phage Display Libraries*

The construction and use of the ETH-2 human antibody library have been described elsewhere [9].

In the 434 repressor library, residues 27, 29, 33, 38, 39, 40, 41 and 43 of the DNA binding domain (residues 1 to 63) of the 434 repressor were combinatorially mutated in two PCR reactions performed with the oligo pairs 434baNco/434foXho (which introduce diversity at the level of the first three randomised residues) and 434baXho/434foNot (which introduce diversity at the level of the residues 38, 39, 40, 41) (Table 1). The two PCR products were sequentially cloned in the vector pCANTAB-6 (a pHEN1 derivative [4]), and a library of 9×10^8 potentially different clones was obtained by transformation of TG1 bacteria. Virtually 100% of the clones contained an insert of the correct size, as judged by PCR screening of bacterial colonies.

Table 1.

434banco:	catctagccatggcccagagtatttcttccagggtaaaaag
434foxho:	ctacggctcgagMNNctcgatcgaMNNctgMNNagtccccacctttgagcaag
434baxho:	ctacggctcgagaacggtNNKNNKNNKNNKccaNNKtttttaccagaacttgcgtcagc
434fonot:	gagtcattctgcggccgcggtgccattgagcagccagtcaac

In the PED-B library, residues 28, 30, 32, 33, 35, 56, 57 and 58 of the ED-B domain of fibronectin were combinatorially mutated in three PCR reactions, using the oligo pairs indicated in Table 2. As a template for this reaction, we used the ED-B domain of fibronectin, cloned in the NcoI/NotI sites of vector pDNEK [9]. The three resulting fragments were then PCR assembled; the gene product was then digested and cloned into the NcoI/NotI sites of the pDNEK vector. The resulting ligation mixture was electroporated into TG1 cells, yielding a library containing 3×10^8 independent clones. At least 95% of the clones contained an insert of the correct size, as judged by PCR screening of bacterial colonies.

Table 2.

LMB3long:	CAGGAAACAGCTATGACCATGATTAC
ED-B libfo2:	CGAACTACTGTGATGCGGTACCCMNNAAT(MNN)$_2$AGAMNNTAGMNNGGTCCACCTCAGGCCGAT
ED-B libba3b:	GGGTACCGCATCACAGTAGTTGCGGCA
ED-B libfo4:	CCCTGTGACTGTGTAGTATCCTAC(MNN)$_3$CACAAAATCTTCAAAAATAGG
ED-B libba5:	GTAGGATACTACACAGTCACAGGG
LMB5:	TGGCCTTGATATTCACAAACGAAT

4.2. *Biopanning Procedures*

Phage selections from the ETH-2, 434 and PED-B libraries were performed as described [9], using antigens biotinylated with NHS-SS-Biotin (Biotin disulfide N-hydroxy succinimide ester, Pierce), a cleavable biotin reagent. In short, approx. 10^{12} phages were pre-incubated for 30 min with an excess (typically, a 0.1 μM soln) of biotinylated antigen. After 30 min incubation at room temperature, binding phage were captured on streptavidin-coated paramagnetic beads (Dynabeads M-280 streptavidin, Dynal, Oslo, Norway), washed and eluted with 20 mM dithiothreitol. The eluted phage was used to infect exponentially growing TG1 bacteria. Soluble ELISA of bacterial supernatants obtained from single colonies was performed as described [9] with the biotinylated antigen immobilised on streptavidin-coated plates.

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