prepare the vast cluster of genes encoding scaffold proteins from lymphocytes; consequently, antibodies have been widely used in medical chemistry [4], imaging [5], and proteomics [6,7].

The presence of the vast gene cluster enables us to obtain valuable binding proteins using selection methodology, and recent structural visualization of candidate proteins by X-ray or NMR structural analyses and the construction of artificial libraries allow constructive selection and functionalization not only of antibody fragments, but also of small, nonantibody proteins (Fig. 1). Accurate structural descriptions of protein–protein interaction provide support for strategies to replace binding site sequences between proteins and library construction in specific areas to increase the density of libraries.

This minireview series describes the methodology for elucidating protein–protein interactions and selecting specific binders to novel target proteins, and the first and second minireviews focus on the detection of protein–protein interactions [8,9]. In this third minireview, we focus on the molecular evolutional methodology for generating and screening binding proteins on the basis of tertiary structures visualized by X-ray and NMR analyses. We describe local library approaches as go-between techniques for grafted foreign peptide sequences and small scaffold proteins, and as methods for designing high-quality libraries of small scaffold proteins.

## Functionalization of small scaffold proteins by peptide grafting

The design of chimeric proteins, in which specific segments are replaced with functional sequences derived from other proteins, can give new binding abilities to scaffold proteins. A new chimeric protein can be generated by replacing the amino acid sequence in an exposed surface area with a fragment that binds a target molecule from another protein.

To generate a small binding protein by grafting, we need to visualize the tertiary structures of donor and recipient proteins in detail. In particular, visualization facilitates the identification of fragments with binding ability. The RGD motif (Arg-Gly-Asp) is a well-known fragment with binding ability. It is found in cell adhesion molecules such as fibronectin, and its interaction with a cell surface receptor called integrin has been analyzed from a structural viewpoint [10–12]. Its short sequence is attractive for generating small binding proteins by grafting. Grafting of the motif with its neighboring sequences from fibronectin into an exposed loop in lysozyme functionalized lysozyme without inactivating its enzyme function [13]. The grafting gave lysozyme low binding affinity for cell surface receptors, and X-ray and NMR structural analyses demonstrated high flexibility and exposure of the grafted motif [13].

Drakopoulou *et al.* [14] noted the resemblance of loop structures with binding ability between scorpion charybdotoxin (with affinity for potassium ion channel protein) and snake toxin α (with affinity for acetylcholine receptor), and replaced a loop sequence of charybdotoxin with one of toxin α to express a new binding function. Comparison of the X-ray crystal structures between charybdotoxin and toxin α showed the structural resemblance of the β-hairpin loop with binding function between toxins. The grafting of the toxin α loop structure into charybdotoxin caused little structural change, and gave charybdotoxin affinity for the

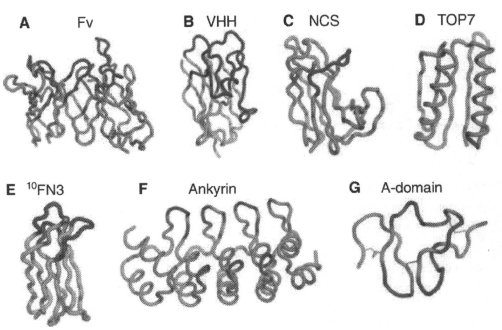


**Fig. 1.** Structures of small scaffold proteins as specific binders. Red loops are the appropriate locations that can have binding functions through peptide-grafting or local library approaches.

acetylcholine receptor instead of the potassium ion channel protein, albeit with lower binding affinity, as seen above with the grafting of RGD into lysozyme.

Recently, stable, small scaffold proteins with surface loop structures that can bind to another protein have been reported. Neocarzinostatin (NCS), found in *Streptomyces neocarzinostaticus*, is a candidate scaffold protein with a hydrophilic and IgG-like structure (Fig. 1C) [15,16]. Visualization of antigen–antibody complexes by X-ray crystallography shows that hypervariable complementarity-determining region (CDR) loops on a fragment of the variable region (Fv) of antibodies recognize specific antigen surfaces (red loops in Fig. 1A) [17–19]. Nicaise *et al.* [20] searched for the most suitable location in NCS for grafting the CDR loop of the single variable heavy chain of a heavy-chain camel antibody (VHH) (Fig. 1B) by comparing topologies between VHH and NCS (Fig. 2A); grafting of the CDR 3 loop of antilysozyme VHH functionalized NCS without denaturation, although the thermal stability was decreased and the affinity for lysozyme was weaker than in the original VHH.

The computer-designed TOP protein is an $\alpha/\beta$-protein composed of 93 amino acids without disulfide linkages (Fig. 1D) [21]. This artificial protein is so thermophilic that it is not denatured at 98 °C, and it can be expressed at a high level in *Escherichia coli*. Boschek *et al.* [22] grafted the CDR 1-containing loop of the heavy chain (CDR H1) of antibody against CD4 into a loop structure of TOP that was identified by molecular dynamics simulation as a suitable location without denaturation (Fig. 2B). CDR-grafted TOP had affinity for CD4 receptor, and was not denatured even at 95 °C.

## Combining grafting and local library approaches for high-affinity scaffold proteins

The grafting results demonstrate the utility of the structural information supplied by X-ray and NMR analyses for functionalizing small scaffold proteins. However, this structural information is not enough to support the complete transfer of functions.

Fv of antibodies is a well-studied small scaffold protein. Fv has a flexible and stable framework with hypervariable sequences and lengths in the six-loop CDR (Fig 1A) that bind to the antigen. The first study of grafting into the CDR replaced the CDR loops in a human antibody with those from a mouse antibody to avoid immunogenicity of the antibody framework from a different species [23–25]. The success of the series of studies shows that the stable framework structure of Fv enables the transfer of function by means of CDR replacement.

Barbas *et al.* first designed new functional antibody fragments by grafting the RGD motif in CDR loops [26,27]. Recognizing that functionalization by grafting RGD needs designs for adjusting the orientation of the RGD motif, they grafted XXXRGDXXX peptide sequences, in which the X positions were randomized, into the CDR 3 loop in the heavy chain (CDR H3) of Fv to select sufficiently functionalized Fab fragments by using phage display methods (Fig. 3A); clone Fab 9 had a low equilibrium dissociation constant ($K_d$) of 0.25 nM, comparable to that of vitronectin. This result implies that the library approach is important for the design of edge sequences neighboring to the grafted peptide fragment to fully functionalize scaffold proteins.

Fab 9 was also attractive as a supplier of the peptide sequence with affinity for a specific molecule. Smith *et al.* [28] reported the grafting of a CDR fragment into a loop structure of a small scaffold protein. When the CDR H3 loop of Fab 9 was grafted into a long, surface-exposed loop structure in a human tissue-type plasminogen activator with affinity for fibrin, the new plasminogen activator had comparable affinity for integrin to that of Fab 9, with no loss of fibrin-binding function.

Although peptide fragments have often been grafted into CDR H3, because its length and amino acid

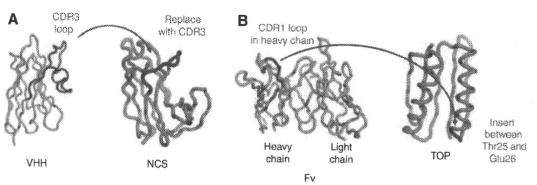


**Fig. 2.** Functionalization of small scaffold proteins by replacing a loop of the scaffold protein with a CDR loop of antibody fragments. (A) Replacement of the candidate location in NCS for grafting with the CDR 3 loop of VHH. (B) Insertion of the CDR 1-containing loop of the heavy chain in Fv into the candidate location in TOP.

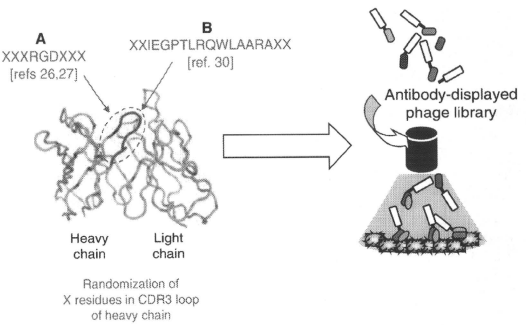


**Fig. 3.** Combination of grafting and local library approaches in CDR 3 loops of the heavy chain to select high-affinity Fv by using the phage display method.

sequence are highly variable, a few studies of grafting into other CDR loops have also been reported. Simon *et al.* [29] grafted the receptor-binding site sequence of somatostatin, which binds to somatostatin receptor 5, into the CDR 1 and CDR 2 loops in the light chain (CDR L1 and CDR L2) to study the potential of Fv as a scaffold protein for grafting. They investigated deviations in the amino acid sequences of the CDRs of 1330 human light chains to identify the candidate residues important in the light chain conformation. Peptide grafting into locations with no significance for light chain folding functionalized the antibody fragments, but expression of the fragment was decreased and the binding affinity was weakened. This might imply the importance of library approaches in specific local areas to overcome the problems not resolved by visualized structural information alone.

The stability of Fvs as scaffold proteins also enables the design of new functional antibody fragments from peptide sequences selected from peptide libraries. A peptide with high affinity for thrombopoietin (TPO), which was selected from a peptide library by the use of phage display method, was grafted into the CDR H3 loop in human Fabs [30]. Grafting of the TPO-binding peptide with two randomized residues at the edge terminus enabled selection of a high-affinity Fab (Fig. 3B), demonstrating the utility of the grafting of functional peptides with randomized edge sequences for optimizing the orientation of the grafted peptide on a scaffold protein. In addition, when the combination of grafting and local library approaches was applied to other CDR loops in Fabs with TPO-binding peptide grafted onto CDR H3, a clone of the double-grafted Fabs had not only higher affinity, but also bivalent function [30]: the grafted Fab had agonist activity caused by the dimerization of the TPO-binding peptide.

The combination of grafting and local library methods is suitable for generating binding proteins. In particular, bispecific small proteins, such as Fabs with dual affinity for human epidermal growth factor receptor 2 and vascular endothelial growth factor (VEGF) [31], might be achievable by grafting two different functional peptide sequences. Recently, several peptides with affinity for inorganic material surfaces have been selected from a peptide library, and the replacement of material-binding peptide with the CDR 1 loop of VHH and the local library approach in the CDR 3 loop generated the VHH fragments with high affinity for specific inorganic material surfaces [32]. The combination of grafting and local library methods might also be suitable for generating specific binders against unexplored targets.

## Local artificial library in a small scaffold protein

Detailed tertiary structural information obtained by X-ray and NMR techniques not only enables grafting approaches for the functionalization of small scaffold proteins, but also opens the way to direct functionalization of scaffold proteins by the use of artificial libraries. Functionalizing a small scaffold protein by a library approach requires large-scale, high-quality libraries with correctly folded variants of scaffold proteins. If the rate of correctly folded variants in a library were low, the number of functional variants in the library would be extremely low. Native libraries of antibodies, such as immune and naive libraries, are considered to hold correctly folded variants; but for the construction of artificial libraries,

randomized locations in scaffold proteins and diversity of amino acids in libraries should be carefully considered.

In the use of artificial libraries for generating binding proteins, Fvs of antibodies are most commonly used as scaffold proteins. In the case of single-chain Fvs and Fabs, artificial libraries of CDR loops have been constructed from synthetic DNA fragments with randomized sequences and lengths. The first attempt with artificial libraries did not provide high-affinity antibody fragments [33], but increasing the library scale to $\sim 10^{11}$ enabled the selection of fragments with high affinity for various protein antigens and haptens [34]. The construction of very large libraries is effective, because it increases the number of correctly folded variants [35]. To decrease the number of misfolded, unfolded and aggregated variants in the libraries, efficient libraries mimicking the frequency of amino acids in native CDR loops have been constructed on one or more frameworks [36,37].

Recently, amino acid-restricted libraries, in which CDR loops were randomized using only the amino acids frequently found in native CDR, have been constructed to increase the density of libraries (Fig. 4A). Fabs with high affinity for human VEGF were selected from a restricted library constructed from only Tyr, Ser, Asp, and Ala, and X-ray structural analysis demonstrated the importance of Tyr residues [38]. The construction of more restricted libraries from only Tyr and Ser residues (YS binary code libraries) also enabled the selection of high-affinity antibodies [39]: one Fab had high affinity for human VEGF ($K_d$ = 60 nM). X-ray structural analysis of the complex of another Fab and human death receptor 5 confirmed the importance of Tyr residues in the antigen–antibody interface.

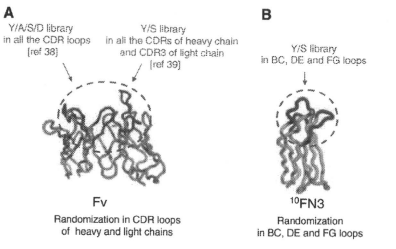


**Fig. 4.** Local artificial library design in (A) CDR loops of Fv and (B) BC, DE and FG loops of $^{10}$FN3 to select high-affinity small scaffold proteins.

Artificial library approaches are also effective with nonantibody proteins when the tertiary structures of scaffold proteins are analyzed in detail. The 10th fibronectin type III domain ($^{10}$FN3) of human fibronectin (Fig. 1E), which is a component of the extracellular matrix, is a monomer with a similar β-sandwich structure to the IgG fold, and has three loops [12]. Koide *et al.* [40] reported the construction of nonantibody-binding proteins, called monobodies, by randomizing the sequences of the loops in $^{10}$FN3 (Fig. 4B). Monobodies with a wide range of affinities (picomolar to micromolar $K_d$ values) have been reported. Xu *et al.* [41] selected a monobody with a $K_d$ of 20 pM for tumor necrosis factor-α by mRNA display from an extremely large library ($10^{12}$ unique clones). Lipovšek *et al.* [42] selected anti-lysozyme monobodies with a low $K_d$ value of 350 pM by yeast surface display from a small library ($10^7$–$10^9$ unique clones). A YS binary code library has also allowed selection of monobodies with affinity for maltose-binding protein and small ubiquitin-like modifier [43], indicating the effectiveness of the amino acid-restricted library approach even with nonantibody scaffold proteins. X-ray structural analysis of monobodies selected from the YS binary library again indicated the importance of Tyr residues for binding to target molecules [43]. Tyr residues might play an important role in molecular recognition independently of scaffold proteins. The generation of recombinant binding proteins by library approaches will supply new insights into protein–protein interactions, and the information might suggest novel designs for high-quality artificial libraries.

## Construction of high-affinity-binding proteins by multispecific design

Tertiary structural information on antibody fragments and nonantibody small scaffold proteins from X-ray and NMR analyses enables the design of and screening for small binding proteins. The preparation of the small binding proteins with binding function further allows us to increase the binding strength by multibinding approaches, constructing multispecific proteins from two small proteins with different epitopes in a target molecule [44,45].

Neri *et al.* [44] created a bispecific antibody fragment with two single-chain Fvs (scFvs), each of which binds to a nonoverlapping epitope in lysozyme, called chelating recombinant antibody (CRAb). The polypeptide linker via which the two scFvs were tandemly connected was designed by computer graphic modeling, using tertiary structures of the antigen–antibody complexes, with the result that the CRAb with D1.3 and

mutant HyHEL-10 scFvs had 100-fold the affinity of either of the scFvs alone. Local library approaches have also been attempted for the design of appropriate polypeptide linkers with a repeat unit of $(XGGGS)_n$, in which the residues at X were randomized and the linker length ($n$) was intermittently varied from 11 to 54 (Fig. 5A) [46]. Selection from the tandem-scFv-displayed phage libraries led to the enrichment of CRAbs with linker lengths comparable to those obtained with computer graphic modeling. The linker library approach has potential for the design of CRAbs when the exact relative positions of two epitopes are indefinite, and for application to nonantibody scaffold proteins.

Several recent studies have reported the simultaneous operation of generating small binding polypeptide units and incrementing the units to achieve multibinding on a target molecule. Designed ankyrin repeat protein (DARPin) is a protein constructed from the ankyrin repeat unit (Fig. 1F) [47]. The unit has 33 amino acids, without internal disulfide linkages, and it forms a β-turn followed by two antiparallel helices and a loop reaching the β-turn of the next repeat. The number of replications is changed so that small binding proteins with appropriate multibinding effects can be generated from units recognizing different epitopes. The randomization of six amino acids in the loop and helix structures without Cys, Gly or Pro enabled the selection of DARPin variants with high affinity for maltose-binding protein [48], Her2 [49,50], and mitogen-activated protein kinase (Fig. 5B) [51].

The A-domain is a small scaffold protein that can be used as a repeat unit (Fig. 1G) [52–54]. A-domains consisting of ~ 35 amino acids occur in strings of multiple domains in several cell surface receptors, and are connected via several amino acid linkers. Each A-domain in the multimer binds to different epitopes in a target, generating avidity [55]. Twelve amino acids that form disulfide linkages and coordinate calcium ions are conserved in ~ 200 human A-domains, but other residues are highly variable [56]. By repeating randomization of the variable residues, selection of A-domain variants with affinity for a target, and connection between the selected variants (Fig. 5C), Silverman *et al.* [57] selected avidity multimers called avimers with two or three A-domains with high affinity (nanomolar $K_d$) for interleukin-6, CD40L, and CD28.

## Conclusions and outlook

Accurate structural descriptions of protein–protein complexes provide support for the replacement of binding site sequences and thus binding function between structurally similar proteins. Functionalization by grafting is not perfect, because structural information derived only from X-ray and NMR analyses is not enough to avoid the decrease in affinity, but some local library approaches can compensate. The identification of the binding site on a protein from visualized tertiary structures can lead to the construction of an efficient library with a low probability of denatured variants, and its combination with the design for library diversity opens the way to increasing the size of the amino acid sequence that can be randomized without decreasing the density of the library. Detailed tertiary structural analyses of protein–protein complexes further accurately describe epitope locations, enabling the design of and screening for bispecific high-affinity proteins recognizing different epitopes in a target molecule.

The recent explosive increase in new genomic and protein structural information has revealed various

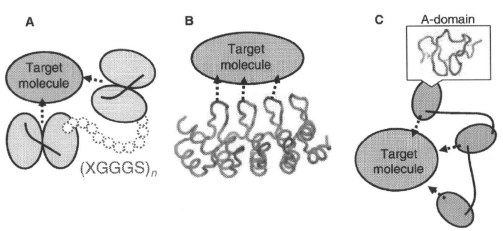


**Fig. 5.** Selection of multispecific binders with multiple binding sites for different epitopes. The red loops are randomized to select high-affinity binders with the binding sites for multiepitopes (black arrows). (A) Tandem scFv: two scFvs were tandemly connected via a repeat unit of $(XGGGS)_n$ in which the X residues were randomized and the linker length ($n$) was intermittently varied. (B) DARPin: six amino acids in the loop and helix structures are randomized. (C) A-domains: variable residues in each A-domain are repeatedly randomized.

small scaffold proteins of a size suitable for *in vitro* selection methods such as phage display [58,59]. The generation of recombinant binding proteins from small scaffold proteins will also help to explain the mechanism of protein–protein interactions. Consequently, analysis might suggest novel designs for high-quality artificial libraries.

Binding proteins can be used in research, diagnosis, and therapy. In particular, their therapeutic use could supply novel protein medicines that could be efficiently produced in bacterial hosts; many successful therapeutic antibodies with large and multidomain IgG formats are difficult and expensive to manufacture. However, the immunogenicity of small scaffold proteins and their very short serum half-life, owing to their small molecular size, must be overcome. Library approaches might serve the dual purposes of increasing both affinity and size.

## References


1 Winter G, Griffiths AD, Hawkins RE & Hoogenboom HR (1994) Making antibodies by phage display technology. *Annu Rev Immunol* **12**, 433–455.

2 Watanabe H, Tsumoto K, Taguchi S, Yamashita K, Doi Y, Nishimiya Y, Kondo H, Umetsu M & Kumagai I (2007) A human antibody fragment with high affinity for biodegradable polymer film. *Bioconjug Chem* **18**, 645–651.

3 Watanabe H, Nakanishi T, Umetsu M & Kumagai I (2008) Human anti-gold antibodies: biofunctionalization of gold nanoparticles and surfaces with anti-gold antibodies. *J Biol Chem* **283**, 36031–36038.

4 Adams GP & Weiner LM (2005) Monoclonal antibody therapy of cancer. *Nat Biotechnol* **23**, 1147–1157.

5 Sharkey RM, Cardillo TM, Rossi EA, Chang CH, Karacay H, McBride WJ, Hansen HJ, Horak ID & Goldenberg DM (2005) Signal amplification in molecular imaging by pretargeting a multivalent, bispecific antibody. *Nat Med* **11**, 1250–1255.

6 Carter P & Merchant AM (1997) Engineering antibodies for imaging and therapy. *Curr Opin Biotechnol* **8**, 449–454.

7 Holt LJ, Enever C, de Wildt RM & Tomlinson IM (2000) The use of recombinant antibodies in proteomics. *Curr Opin Biotechnol* **11**, 445–449.

8 Ishii J, Fukuda N, Tanaka T, Ogino C & Kondo A (2010) Protein–protein interactions and selection: yeast-based approaches that exploit guanine nucleotide-binding protein signaling. *FEBS J* **277**, 1982–1995.

9 Tomizaki K, Usui K & Mihara H (2010) Protein–protein interactions and selection: array-based techniques for screening disease-associated biomarkers in predictive/early diagnosis. *FEBS J* **277**, 1996–2005.

10 Pierschbacher MD & Ruoslahti E (1984) Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**, 30–33.

11 Leahy DJ, Hendrickson WA, Aukhil I & Erickson HP (1992) Structure of a fibronectin type III domain from tenascin phased by MAD analysis of the selenomethionyl protein. *Science* **258**, 987–991.

12 Main AL, Harvey TS, Baron M, Boyd J & Campbell ID (1992) The three-dimensional structure of the tenth type III module of fibronectin: an insight into RGD-mediated interactions. *Cell* **71**, 671–678.

13 Yamada T, Matsushima M, Inaka K, Ohkubo T, Uyeda A, Maeda T, Titani K, Sekiguchi K & Kikuchi M (1993) Structural and functional analyses of the Arg-Gly-Asp sequence introduced into human lysozyme. *J Biol Chem* **268**, 10588–10592.

14 Drakopoulou E, Zinn-Justin S, Guenneugues M, Gilqin B, Menez A & Vita C (1996) Changing the structural context of a functional beta-hairpin. Synthesis and characterization of a chimera containing the curaremimetic loop of a snake toxin in the scorpion alpha/beta scaffold. *J Biol Chem* **271**, 11979–11987.

15 Adjadj E, Quiniou E, Mispelter J, Favaudon V & Lhoste JM (1992) Three-dimensional solution structure of apo-neocarzinostatin from Streptomyces carzinostaticus determined by NMR spectroscopy. *Eur J Biochem* **203**, 505–511.

16 Gao X (1992) Three-dimensional solution structure of apo-neocarzinostatin. *J Mol Biol* **225**, 125–135.

17 Braden BC & Poljak RJ (1995) Structural features of the reactions between antibodies and protein antigens. *FASEB J* **9**, 9–16.

18 Davies DR & Cohen GH (1996) Interactions of protein antigens with antibodies. *Proc Natl Acad Sci USA* **93**, 7–12.

19 Kondo H, Shiroishi M, Matsushima M, Tsumoto K & Kumagai I (1999) Crystal structure of anti-Hen egg white lysozyme antibody (HyHEL-10) Fv–antigen complex. Local structural changes in the protein antigen and water-mediated interactions of Fv–antigen and light chain–heavy chain interfaces. *J Biol Chem* **274**, 27623–27631.

20 Nicaise M, Valerio-Lepiniec M, Minard P & Desmadril M (2004) Affinity transfer by CDR grafting on a non-immunoglobulin scaffold. *Protein Sci* **13**, 1882–1891.

21 Kuhlman B, Dantas G, Ireton GC, Varani G, Stoddard BL & Baker D (2003) Design of a novel globular protein fold with atomic-level accuracy. *Science* **302**, 1364–1368.

22 Boschek CB, Apiyo DO, Soares TA, Engelmann HE, Pefaur NB, Straatsma TP & Baird CL (2009) Engineering an ultra-stable affinity reagent based on Top7. *Protein Eng Des Sel* **22**, 325–332.

23 Jones PT, Dear PH, Foote J, Neuberger MS & Winter G (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* **321**, 522–525.
24 Co MS, Deschamps M, Whitley RJ & Queen C (1991) Humanized antibodies for antiviral therapy. *Proc Natl Acad Sci USA* **88**, 2869–2873.
25 Makabe K, Nakanishi T, Tsumoto K, Tanaka Y, Kondo H, Umetsu M, Sone Y, Asano R & Kumagai I (2008) Thermodynamic consequences of mutations in vernier zone residues of a humanized anti-human epidermal growth factor receptor murine antibody, 528. *J Biol Chem* **283**, 1156–1166.
26 Barbas CF 3rd, Languino LR & Smith JW (1993) High-affinity self-reactive human antibodies by design and selection: targeting the integrin ligand binding site. *Proc Natl Acad Sci USA* **90**, 10003–10007.
27 Smith JW, Hu D, Satterthwait A, Pinz-Sweeney S & Barbas CF 3rd (1994) Building synthetic antibodies as adhesive ligands for integrins. *J Biol Chem* **269**, 32788–32795.
28 Smith JW, Tachias K & Madison EL (1995) Protein loop grafting to construct a variant of tissue-type plasminogen activator that binds platelet integrin alpha IIb beta 3. *J Biol Chem* **270**, 30486–30490.
29 Simon PJ, Brogle KC, Wang B, Kyle DJ & Soltis DA (2005) Display of somatostatin-related peptides in the complementarity determining regions of an antibody light chain. *Arch Biochem Biophys* **440**, 148–157.
30 Frederickson S, Renshaw MW, Lin B, Smith LM, Calveley P, Springhorn JP, Johnson K, Wang Y, Su X, Shen Y *et al.* (2006) A rationally designed agonist antibody fragment that functionally mimics thrombopoietin. *Proc Natl Acad Sci USA* **103**, 14307–14312.
31 Bostrom J, Yu SF, Kan D, Appleton BA, Lee CV, Billeci K, Man W, Peale F, Ross S, Wiesmann C *et al.* (2009) Variants of the antibody herceptin that interact with HER2 and VEGF at the antigen binding site. *Science* **323**, 1610–1614.
32 Hattori T, Umetsu M, Nakanishi T, Togashi T, Yokoo N, Abe H, Ohara S, Adschiri T & Kumagai I (2010) High-affinity anti-inorganic-material antibody generation by integrating graft and evolution technologies: the potential of antibodies as biointerface molecules. *J Biol Chem* **285**, 7784–7793.
33 Nissim A, Hoogenboom HR, Tomlinson IM, Flynn G, Midgley C, Lane D & Winter G (1994) Antibody fragments from a 'single pot' phage display library as immunochemical reagents. *EMBO J* **13**, 692–698.
34 Griffiths AD, Williams SC, Hartley O, Tomlinson IM, Waterhouse P, Crosby WL, Kontermann RE, Jones PT, Low NM, Allison TJ *et al.* (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J* **13**, 3245–3260.
35 Christ D, Famm K & Winter G (2006) Tapping diversity lost in transformations – in vitro amplification of ligation reactions. *Nucleic Acids Res* **34**, e108, doi:10.1093/nar/gkl605.
36 Lee CV, Liang WC, Dennis MS, Eigenbrot C, Sidhu SS & Fuh G (2004) High-affinity human antibodies from phage-displayed synthetic Fab libraries with a single framework scaffold. *J Mol Biol* **340**, 1073–1093.
37 Knappik A, Ge L, Honegger A, Pack P, Fischer M, Wellnhofer G, Hoess A, Wolle J, Plückthun A & Virnekas B (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J Mol Biol* **296**, 57–86.
38 Fellouse FA, Wiesmann C & Sidhu SS (2004) Synthetic antibodies from a four-amino-acid code: a dominant role for tyrosine in antigen recognition. *Proc Natl Acad Sci USA* **101**, 12467–12472.
39 Fellouse FA, Li B, Compaan DM, Peden AA, Hymowitz SG & Sidhu SS (2005) Molecular recognition by a binary code. *J Mol Biol* **348**, 1153–1162.
40 Koide A, Bailey CW, Huang X & Koide S (1998) The fibronectin type III domain as a scaffold for novel binding proteins. *J Mol Biol* **284**, 1141–1151.
41 Xu L, Aha P, Gu K, Kuimelis RG, Kurz M, Lam T, Lim AC, Liu H, Lohse PA, Sun L *et al.* (2002) Directed evolution of high-affinity antibody mimics using mRNA display. *Chem Biol* **9**, 933–942.
42 Lipovšek D, Lippow SM, Hackel BJ, Gregson MW, Cheng P, Kapila A & Wittrup KD (2007) Evolution of an interloop disulfide bond in high-affinity antibody mimics based on fibronectin type III domain and selected by yeast surface display: molecular convergence with single-domain camelid and shark antibodies. *J Mol Biol* **368**, 1024–1041.
43 Koide A, Gilbreth RN, Esaki K, Tereshko V & Koide S (2007) High-affinity single-domain binding proteins with a binary-code interface. *Proc Natl Acad Sci USA* **104**, 6632–6637.
44 Neri D, Momo M, Prospero T & Winter G (1995) High-affinity antigen binding by chelating recombinant antibodies (CRAbs). *J Mol Biol* **246**, 367–373.
45 Zhou HX (2003) Quantitative account of the enhanced affinity of two linked scFvs specific for different epitopes on the same antigen. *J Mol Biol* **329**, 1–8.
46 Wright MJ & Deonarain MP (2007) Phage display of chelating recombinant antibody libraries. *Mol Immunol* **44**, 2860–2869.
47 Binz HK, Stumpp MT, Forrer P, Amstutz P & Plückthun A (2003) Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J Mol Biol* **332**, 489–503.

48 Binz HK, Amstutz P, Kohl A, Stumpp MT, Briand C, Forrer P, Grutter MG & Plückthun A (2004) High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat Biotechnol* **22**, 575–582.

49 Zahnd C, Pecorari F, Straumann N, Wyler E & Plückthun A (2006) Selection and characterization of Her2 binding-designed ankyrin repeat proteins. *J Biol Chem* **281**, 35167–35175.

50 Zahnd C, Wyler E, Schwenk JM, Steiner D, Lawrence MC, McKern NM, Pecorari F, Ward CW, Joos TO & Plückthun A (2007) A designed ankyrin repeat protein evolved to picomolar affinity to Her2. *J Mol Biol* **369**, 1015–1028.

51 Amstutz P, Koch H, Binz HK, Deuber SA & Plückthun A (2006) Rapid selection of specific MAP kinase-binders from designed ankyrin repeat protein libraries. *Protein Eng Des Sel* **19**, 219–229.

52 Krieger M & Herz J (1994) Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu Rev Biochem* **63**, 601–637.

53 Gliemann J (1998) Receptors of the low density lipoprotein (LDL) receptor family in man. Multiple functions of the large family members via interaction with complex ligands. *Biol Chem* **379**, 951–964.

54 North CL & Blacklow SC (1999) Structural independence of ligand-binding modules five and six of the LDL receptor. *Biochemistry* **38**, 3926–3935.

55 Rettenberger PM, Oka K, Ellgaard L, Petersen HH, Christensen A, Martensen PM, Monard D, Etzerodt M, Chan L & Andreasen PA (1999) Ligand binding properties of the very low density lipoprotein receptor. Absence of the third complement-type repeat encoded by exon 4 is associated with reduced binding of Mr 40,000 receptor-associated protein. *J Biol Chem* **274**, 8973–8980.

56 Koduri V & Blacklow SC (2001) Folding determinants of LDL receptor type A modules. *Biochemistry (Mosc)* **40**, 12801–12807.

57 Silverman J, Liu Q, Bakker A, To W, Duguay A, Alba BM, Smith R, Rivas A, Li P, Le H *et al.* (2005) Multivalent avimer proteins evolved by exon shuffling of a family of human receptor domains. *Nat Biotechnol* **23**, 1556–1561.

58 Binz HK, Amstutz P & Plückthun A (2005) Engineering novel binding proteins from nonimmunoglobulin domains. *Nat Biotechnol* **23**, 1257–1268.

59 Hosse RJ, Rothe A & Power BE (2006) A new generation of protein display scaffolds for molecular recognition. *Protein Sci* **15**, 14–27.