6 Castanotto, D., Rossi, J. J. and Sarver, N. (1994) *Adv. Pharmacol.* 25, 289–317
7 Muzyczka, N. (1992) *Curr. Top. Microbiol. Immunol.* 158, 97–123
8 Chatterjee, S., Johnson, P. R. and Wong, K. K., Jr (1992) *Science* 258, 1485–1488
9 Rossi, J. J., Elkins, D., Zaia, J. A. and Sullivan, S. (1992) *AIDS Res. Hum. Retroviruses* 8, 183–189
10 Heiser, W. C. (1994) *Anal. Biochem.* 217, 185–196
11 Zhao, J. J. and Pick, L. (1993) *Nature* 365, 448–451
12 Gossen, M. and Bujard, H. (1992) *Proc. Natl Acad. Sci. USA* 89, 5547–5551
13 Gossen, M., Bonin, A. L. and Bujard, H. (1994) *Trends Biotechnol.* 12, 58–62
14 Furth, P. A. *et al.* (1994) *Proc. Natl Acad. Sci. USA* 91, 9302–9306
15 Efrat, S., Fusco-DeMane, D., Lemberg, H., Emran, O. A. and Wang, X. (1995) *Proc. Natl Acad. Sci. USA* 92, 3576–3580
16 Efrat, S. *et al.* (1994) *Proc. Natl Acad. Sci. USA* 91, 2051–2055
17 Larsson, S. *et al.* (1994) *Nucleic Acids Res.* 22, 2242–2248
18 Zhou, C., Bahner, I. C., Larson, G. P., Zaia, J. A., Rossi, J. J. and Kohn, D. B. (1994) *Gene* 149, 33–39
19 Yu, M. *et al.* (1993) *Proc. Natl Acad. Sci. USA* 90, 6340–6344
20 Yamada, O., Yu, M., Yee, J-K., Kraus, G., Looney, D. and Wong-Staal, F. (1994) *Gene Therapy* 1, 38–45
21 Noonberg, S. B., Scott, G. K., Garovoy, M. R., Benz, C. C. and Hunt, C. A. (1994) *Nucleic Acids Res.* 22, 2830–2836
22 Cagnon, L., Cucchiarini, M., Lefebvre, J-C. and Doglio, A. *J. AIDS* (in press)
23 Sullenger, B. A. and Cech, T. R. (1993) *Science* 262, 1566–1569
24 Rosbash, M. and Singer, R. H. (1993) *Cell* 75, 399–401
25 Ding, D. and Lipshitz, H. D. (1993) *BioEssays* 15, 651–658
26 Bentley, L. and Singer, L. (1986) *Cell* 45, 407–415
27 Kislauskas, E. H., Li, Z., Singer, R. H. and Taneja, K. L. (1993) *J. Cell Biol.* 123, 165–172
28 Chen, C-J., Banerjea, A. C., Harmison, G. G., Haglund, K. and Schubert, M. (1992) *Nucleic Acids Res.* 20, 4581–4589
29 Ruffner, D. E., Stormo, G. D. and Uhlenbeck, O. C. (1990) *Biochemistry* 29, 10695–10702
30 Perriman, R., Delves, A. and Gerlach, W. L. (1992) *Gene* 113, 157–163
31 Dreyfuss, G., Matunis, M. J., Seraffin, P-R. and Burd, C. G. (1993) *Annu. Rev. Biochem.* 62, 289–321
32 Bertrand, E. L. and Rossi, J. J. (1994) *EMBO J.* 13, 2904–2912
33 Herschlag, D., Khosla, M., Tsuchihashi, Z. and Karpel, R. L. (1994) *EMBO J.* 13, 2913–2924
34 Tsuchihashi, Z., Khosla, M. and Herschlag, D. (1993) *Science* 262, 99–102

Intracellular antibodies: development and therapeutic potential

Jennifer H. Richardson and Wayne A. Marasco

Single-chain antibodies, synthesized by the cell and targeted to a particular cellular compartment, can be used to interfere in a highly specific manner with cell growth and metabolism. Recent applications of this technology include the phenotypic knockout of growth-factor receptors, the functional inactivation of $p21^{ras}$ and the inhibition of HIV-1 replication. Intracellular antibodies are likely to have a widespread impact in biological research as a simple and effective alternative to other forms of gene inactivation; they demonstrate clear potential as reagents for cancer therapy and for the control of infectious diseases.

The specific and high-affinity-binding properties of immunoglobulin molecules have long been used in biomedical science as *in vitro* tools for the identification, purification, or functional manipulation of target antigens. A recent series of papers has demonstrated the potential for using antibodies to interfere with biological processes inside the cell in a highly specific manner. It is well established that preformed antibodies can transiently inactivate a target protein when they are introduced into the cell by microinjection[1]. The concept of stably transfecting cells with the antibody gene, coupled with advances in antibody engineering, have allowed the power of intracellular antibodies to be fully realized. This review summarizes recent experiments in which single-chain antibodies that have been synthesized by the cell and targeted to the relevant cellular compartment, have been used to downregulate growth-factor receptors, to inactivate

J. H. Richardson and W. A. Marasco are at the Division of Human Retrovirology, Dana Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA.

 0167 – 7799/95/$9.50

oncoproteins and to interfere with the replication of cytopathic viruses. The potential therapeutic applications, and the merits and disadvantages of intracellular antibodies are discussed. Other biotechnological applications that are not discussed here include the use of single-chain antibodies in the construction of pathogen-resistant plant strains[2].

Antibody design

Despite evidence that the heavy and light chains of immunoglobulin can functionally associate in the cytosol[3,4], initial attempts to manipulate cells by the intracellular expression of separate heavy- and light-chain genes met with limited success[3–6]. A key factor contributing to the success of the recent studies has been the use of single-chain antibodies (usually single-chain variable region fragments – scFvs – see Fig. 1) in which the heavy- and light-chain variable domains are synthesized as a single polypeptide. The two domains are separated by a flexible linker peptide, and the constant portion of the immunoglobulin molecule, which has no role in antigen binding, is dispensed with entirely. The result is a small (~28 kDa) molecule with high-affinity ligand-binding capability and minimal assembly requirements. The antibody can be directed to the relevant cellular compartment using classical intracellular-trafficking signals. Functional studies[2,7,8–10] have illustrated that single-chain antibodies are able to fold and assemble correctly in the cytoplasm, even though (1) the chaperones that would normally assist in this process are localized in the endoplasmic reticulum (ER) and, therefore, unavailable, and (2) the reducing environment does not favor the formation of disulfide bonds. However, it has been generally found that cytosolic antibodies (whether they are intact chains, Fab or scFv fragments) exhibit shorter half-lifes than their secretory counterparts.

Applications of intracellular antibodies

In principle, the high affinity and selective-binding properties of intracellular antibodies, or intrabodies, can be used to modulate cellular physiology and metabolism by a wide variety of mechanisms. For example, binding by an intrabody may be used to:

- block or stabilize macromolecular (e.g. protein–protein or protein–DNA) interactions;
- modulate enzyme function by occluding an active site, sequestering substrate, or fixing the enzyme in an active (on) or inactive (off) conformation;
- divert proteins from their usual cellular compartment, for example, by sequestering transcription factors in the cytoplasm, or by retention in the ER of proteins that are destined for the cell surface.

Some specific examples that have therapeutic potential are described in the following sections.

Downregulation of growth-factor receptors

Single-chain antibodies that are targeted to the lumen of the ER provide a simple and effective mechanism for inhibiting the transport of plasma membrane or secreted proteins to the cell surface (Fig. 2). The tubular architecture of the ER and the directional flow of proteins through the secretory system combine to make the capture mechanism extremely effective; even highly abundant cell-surface receptors have been reduced to undetectable levels using this method. Intrabodies that are intended for localization in the ER are generally equipped with a leader peptide and a C-terminus ER retention signal (the KDEL amino acid motif – Lys-Asp-Glu-Leu). In practice, the KDEL tag is not always necessary – a number of single-chain antibodies designed for secretion appear to be 'export incompetent', and remain in the ER for reasons that are, at present, unclear[11–13].

To date, the technique has been used to functionally inactivate three cell-surface receptors that are implicated in human cancer. An ER-targeted intrabody was used to downregulate the α subunit of the receptor for human interleukin 2, IL-2Rα (Ref. 12). IL-2Rα plays a key role in T-cell-mediated immune responses, and is constitutively overexpressed in some T- and B-cell leukemias, most notably in adult T-cell leukemia. T-cell lines that stably expressed an ER-targeted single-chain antibody (scFvTac) against IL-2Rα exhibited a complete loss of cell-surface IL-2Rα expression and were no longer responsive to IL-2. The receptor chain could be detected inside the cells as an immature form that was sensitive to endoglycosidase H; this finding is consistent with its retention in a pre-Golgi compartment[12]. Intrabodies, such as scFvTac, offer significant potential for immunomodulation and for the control of IL-2R-dependent tumor-cell growth *in vivo*, especially when used in combination with targeted gene-delivery systems that should allow the genetic manipulation of specific cell types[14–16].

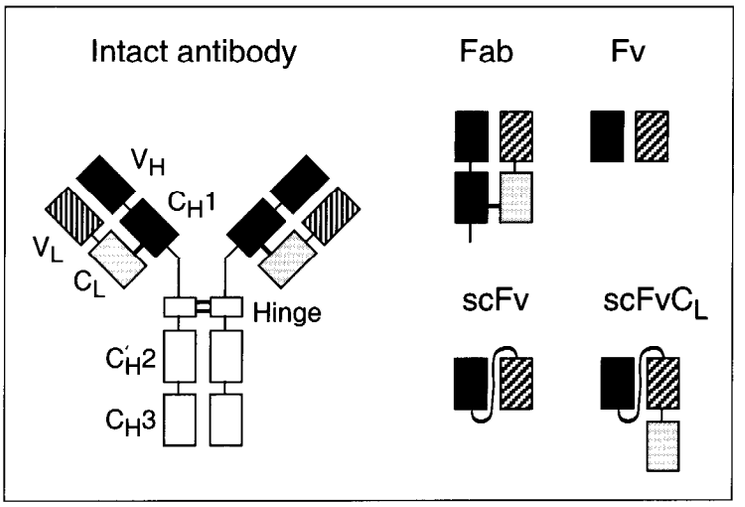

Figure 1

Structures of an intact antibody and antibody fragments. Abbreviations: V_H, heavy-chain variable domain; C_H, heavy-chain constant domain; V_L, light-chain variable domain; C_L, light-chain constant domains; Fv, variable-region fragment; scFv, single-chain variable region fragment; scFvC_L, single-chain variable region fragment with C_L domain.

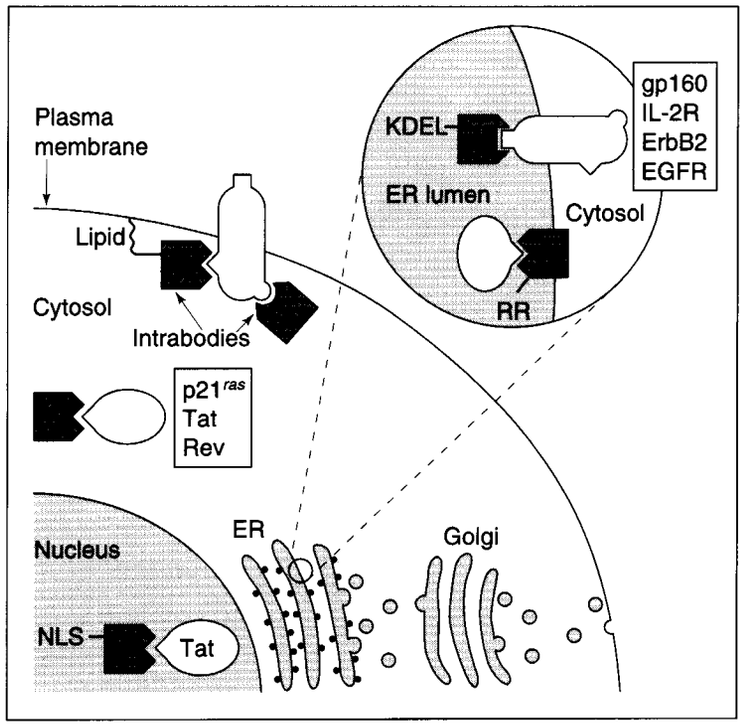

Figure 2

Potential sites of intrabody action. Cytosolic intrabodies could be used to bind and inactivate any cytosolic structure, including the cytoplasmic domains of transmembrane proteins. Interactions taking place at the cell surface may be enhanced by tethering the intrabody to the inner surface of the plasma membrane by means of a lipidmoiety. Intrabodies can be directed into the nucleus by addition of a nuclear localization signal (NLS), or into the endoplasmic reticulum (ER) using a signal peptide. Intrabodies retained in the lumen of the ER by means of a KDEL retention signal (Lys-Asp-Glu-Leu) can inhibit the transport of membrane and secreted proteins. Membrane-anchored intrabodies that are held in the ER owing to the presence of an N-terminus double-arginine motif[28] could also be used to retain or inactivate ER luminal proteins.

Two groups have reported the use of ER-targeted single-chain antibodies to inhibit the expression of the ErbB2 transmembrane protein[17,18]. ErbB2 is a member of the epidermal growth factor receptor (EGFR)-related family of receptor tyrosine kinases. It is amplified or overexpressed in a variety of human tumors, including breast and ovarian carcinoma, where it correlates with an unfavorable prognosis. Two KDEL-tagged intrabodies have been shown to markedly decrease the cell-surface expression of ErbB2 in NIH3T3 fibroblasts that express an oncogenically activated form of ErbB2 (Ref. 17). Functional inactivation of the receptor was suggested by a reduction in the phosphotyrosine content of the cells and, more importantly, by the reversion of the cells to a non-transformed phenotype. In a complementary study, an ER-directed single-chain antibody was used to down-regulate ErbB2 in the human ovarian-carcinoma cell line SKOV3 (Ref. 18). Transient expression of the intrabody gene in SKOV3 cells led to the loss of ErbB2 expression at the cell surface, and induced a temporary arrest of cellular proliferation. Furthermore, it was difficult to establish SKOV3 clones that stably expressed the anti-ErbB2 intrabody, suggesting that constitutive downregulation of ErbB2 is incompatible with the long-term survival of these cells. In a separate study, a single-chain antibody directed against EGFR was shown to reduce EGF-dependent colony formation in NIH3T3 cells that had been stably transfected with the human EGFR gene[19]. The implied tumoricidal properties of intrabodies that functionally inactivate an oncogenic receptor is of obvious clinical interest, and could be augmented if bystander effects were invoked *in vivo*. The problem of genetically modifying enough cells to have an impact on the tumor load remains one of the greater challenges with this approach to tumor therapy.

Apart from their therapeutic importance, these types of intrabodies could have a considerable impact on basic research into growth-factor signaling. In one study of this kind, intrabody-mediated downregulation of ErbB2 has been used to demonstrate that ErbB2 co-expression is important for signal transduction by other members of the EGFR-related family, possibly as a result of heterodimerization and trans-phosphorylation[20].

Inactivation of cytosolic oncoproteins

Single-chain antibodies that have been adapted for expression in the cytosol by the removal of the leader sequence have been used to perturb the function of $p21^{ras}$, a guanine nucleotide-binding protein that is strategically involved in the control of cell growth and differentiation. Members of the *ras* gene family have been implicated in many types of human cancer. When the mRNA encoding an anti-$p21^{ras}$ intrabody was microinjected into *Xenopus* oocytes, it was shown to inhibit insulin-induced meiotic maturation of the cell, a process known to be $p21^{ras}$-dependent[6,7]. Meiotic maturation was not inhibited in oocytes that co-expressed cytosolic forms of the separate heavy and light chains, presumably reflecting the more stringent assembly requirements of a native four-chain antibody[6]. Intrabodies that interfere with the function of cytosolic kinases, GTPases or other molecules involved in signal transduction could prove extremely valuable in unravelling the complex and interconnecting pathways that serve to deliver extracellular signals to the nucleus.

Inhibition of virus replication

Intracellular antibodies have important therapeutic potential in the defence against microbial pathogens, particularly the human immunodeficiency virus (HIV-1). The exploration of genetic approaches to combat HIV-1 infection has gained impetus following the clinical failure of reverse transcriptase and protease inhibitors[21]. Other approaches that were initially promising, such as the use of soluble CD4 to block virus entry into cells, have similarly not translated into an effective therapy. A single-chain antibody directed against the HIV-1 exterior-envelope glycoprotein gp120 has been shown to interfere with virus assembly in HIV-1-infected cells[11]. The envelope protein mediates the attachment of the virus to its cellular

receptor (the CD4 molecule), and is required both for cell-free and cell-to-cell transmission of the virus. Expression of the scFv105 intrabody in the ER of infected cells led to intracellular retention of the envelope precursor gp160, indicating that cell-surface translocation and proteolytic cleavage of the precursor were inhibited as a result of its interaction with scFv105 in the ER. The biological consequences of this were a marked reduction in the envelope-mediated cytopathic effect (syncytium formation) and a drop in infectivity (by three orders of magnitude) of the virus particles released[11,22]. While intrabody research is still at an early stage, and *in vivo* efficacy is so far untested, two features of the scF105 intrabody make it an attractive candidate for therapeutic use. First, the antibody is directed against a highly conserved epitope of gp160/120, and is, therefore, able to neutralize a broad spectrum of HIV-1 strains. Second, scFv105 is based on a naturally occurring human antibody, and unlikely to be recognized as foreign by the immune system. An extension of this work has been the development of a Fab version of scF105, which is partially secreted and is able to provide intracellular and extracellular protection against the virus[23].

Equally promising results have been obtained by using single-chain antibodies directed against the HIV-1 regulatory proteins Tat and Rev. The Rev protein shuttles between the nucleus and cytoplasm of infected cells and is required for the nuclear export of a subset of HIV-1 mRNAs that encode the structural proteins. A cytosolic single-chain antibody directed against the C-terminus-effector domain of Rev has been demonstrated to possess significant anti-HIV activity[8,9]. The intrabody strongly antagonized Rev function when stably expressed in Hela T4 cells, and led to a sustained inhibition of virus replication. Cells expressing the intrabody were resistant to challenge by multiple strains of HIV-1, and they exhibited an RNA profile typical of a Rev-minus virus. Rev protein was detected exclusively in the cytoplasm of intrabody-expressing cells, suggesting that sequestration of Rev protein in the cytoplasm may be the primary mechanism of inhibition.

The effects of two single-chain antibodies directed against the Tat protein have been examined[10]. In the nucleus, Tat acts as a potent transactivator of viral-gene expression by activating transcription from the HIV-1 long terminal repeat (LTR). A cytosolic intrabody (scFvTat1) directed against the N-terminal-activation domain of Tat efficiently blocked the transport of Tat into the nucleus and inhibited Tat-mediated transactivation of the HIV-1 LTR. The antiviral activity of scFvTat1 was significantly enhanced by the addition of the human κ-type light-chain constant domain at the C-terminus (Fig. 1). This appeared to improve the stability of the molecule, but it may also promote dimerization. T-lymphocyte lines that were stably transfected with the modified gene encoding scFvTat1C_κ demonstrated long-term resistance to challenge by HIV-1. An alternative single-chain antibody directed against the C-terminus of Tat was unable to suppress HIV-1 replication, indicating that the choice of antibody and/or epitope is critical.

Further studies are needed to determine how well intrabodies perform compared with other gene-based strategies that are aimed at inhibiting HIV-1 replication (reviewed in Ref. 24); these include antisense RNA, ribozymes, mutant viral proteins that interfere dominantly with the activity of the wild-type protein, and TAR or RRE decoys (RNA structures that act as decoy binding sites for the Tat and Rev proteins, respectively). Most of these strategies can provide short-term inhibition of HIV-1 replication *in vitro*, but may vary in other areas that are relevant to clinical development: such as the immunogenicity of the introduced gene; the capability for broad-range (cross-isolate) and long-term protection; and the ease with which the virus can escape by mutation or the selection of naturally occurring variants.

Perspectives

Intracellular antibodies represent new and versatile tools that have the potential to manipulate diverse biological processes ranging from the inactivation of cellular proteins to the neutralization of microbial pathogens. The results obtained to date suggest that intracellular antibodies represent a powerful alternative to other methods of gene inactivation, such as antisense RNA, dominant-negative mutants and targeted gene disruption. Unlike the dominant-negative approach, which can only be applied to certain types of protein, there are few theoretical constraints to the use of intracellular antibodies. Indeed, these may represent the only option in circumstances where the desired target has not been cloned, or is non-protein in nature (e.g. sugar, DNA, or a soluble metabolite). Owing to the diversity of the immunoglobulin V gene repertoire, antibody molecules have the capacity to recognize an enormous variety of structures, and the major hurdle in any given application may be that of identifying an antibody with the desired specificity and affinity. Hybridoma-cell lines represent an immediately available source of well-characterized and affinity-matured antibodies that can be readily converted to the single-chain form. Moreover, recent advances in antibody engineering allow the *de novo* construction of antibodies and the selection of a desired antibody specificity by the screening of phage-display libraries[25]. Improving the stability of intrabodies that are expressed outside of the secretory compartment (e.g. in the cytosol) is one area that needs attention. While several studies have demonstrated that relatively short-lived intrabodies can achieve the desired biological effect, the power of the technology would undoubtedly be enhanced by factors that reduce intrabody turnover. Intrabodies also have the potential, not only to disrupt protein function, but to act in a positive fashion, for example, by enhancing protein activity, by stabilizing protein–protein interactions, or by performing catalytic functions themselves.

As is the case for other gene-based therapies, certain issues need to be addressed before intrabody

technology can be applied in a clinical context. These include:

- developing gene-transfer systems that are capable of delivering the intrabody gene into a sufficient number of the correct cell type;
- expression of the intrabody gene will need to be sustained and high enough to achieve the desired clinical benefit;
- the intrabody protein should be nontoxic and nonimmunogenic.

The problems of efficient gene delivery and *in vivo* expression are not trivial but are shared by all gene-based-therapy approaches; these have been reviewed elsewhere[26]. At present, the immunogenic potential of intracellular murine antibodies is unknown. While these might be expected to elicit a cytotoxic T-cell response in humans, it is notable that cells transduced with the prokaryotic neomycin-resistance (*neo*) gene have remained in the circulation of cancer patients for over 18 months[27]. Ultimately, the use of human or humanized antibodies should circumvent the issue of immune recognition. The toxicity associated with intrabody expression appears to be minimal. To date, cells have been shown to tolerate high levels of intrabody expression with no apparent detriment to cell growth or phenotype. Therefore, intracellular antibodies represent a highly versatile and effective tool for the functional manipulation of cells, and could find widespread application, both in basic science and medicine.

References

1 Morgan, D. O. and Roth, R. A. (1988) *Immunol. Today* 9, 84–88
2 Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. and Galeffi, P. (1993) *Nature* 366, 469–472
3 Carlson, J. R. (1988) *Mol. Cell. Biol.* 8, 2638–2646
4 Biocca, S., Neuberger, M. S. and Cattaneo, A. (1990) *EMBO J.* 9, 101–108
5 Bowdish, K., Tang, Y., Hicks, J. B. and Hilvert, D. (1991) *J. Biol. Chem.* 266, 11901–11908
6 Biocca, S., Pierandrei-Amaldi, P., Campioni, N. and Cattaneo, A. (1994) *Bio/Technology* 12, 396–399
7 Biocca, S., Pierandrei-Amaldi, P. and Cattaneo, A. (1993) *Biochem. Biophys. Res. Commun.* 197, 422–427
8 Duan, L., Bagasra, O., Laughlin, M. A., Oakes, J. W. and Pomerantz, R. J. (1994) *Proc. Natl Acad. Sci. USA* 91, 5075–5079
9 Duan, L., Zhang, H., Oakes, J. W., Bagasra, O. and Pomerantz, R. J. (1994) *Hum. Gene Ther.* 5, 1315–1324
10 Mhashilkar, A. M., Bagley, J., Chen, S. Y., Szilvay, A. M., Helland, D. G. and Marasco, W. A. (1995) *EMBO J.* 14, 101–110
11 Marasco, W. A., Haseltine, W. A. and Chen, S. Y. (1993) *Proc. Natl Acad. Sci. USA* 90, 7889–7893
12 Richardson, J. H., Sodroski, J. G., Waldmann, T. A. and Marasco, W. A. (1995) *Proc. Natl Acad. Sci. USA* 92, 3137–3141
13 Jost, C. R., Kurucz, I., Jacobus, C. M., Titus, J. A., George, A. J. T. and Segal, D. M. (1994) *J. Biol. Chem.* 269, 26267–26273
14 Salmons, B. and Günzburg, W. H. (1993) *Hum. Gene Ther.* 4, 129–141
15 Russell, S. J., Hawkins, R. E. and Winter, G. (1993) *Nucleic Acids Res.* 21, 1081–1085
16 Michael, S. I. and Curiel, D. T. (1994) *Gene Ther.* 1, 223–232
17 Beerli, R. R., Wels, W. and Hynes, N. E. (1994) *J. Biol. Chem.* 289, 23931–23936
18 Deshane, J., Loechel, F., Conry, R. M., Siegal, G. P., King, C. R. and Curiel, D. T. (1994) *Gene Ther.* 1, 332–337
19 Beerli, R. R., Wels, W. and Hynes, N. E. (1994) *Biochem. Biophys. Res. Commun.* 204, 666–672
20 Graus-Porta, D., Beerli, R. R. and Hynes, N. E. (1995) *Mol. Cell. Biol.* 15, 1182–1191
21 Condra, J. H. *et al.* (1995) *Nature* 374, 569–571
22 Chen, S. Y., Bagley, J. and Marasco, W. A. (1994) *Hum. Gene Ther.* 5, 595–601
23 Chen, S. Y., Khouri, Y., Bagley, J. and Marasco, W. A. (1994) *Proc. Natl Acad. Sci. USA* 91, 5932–5936
24 Dropulic, B. and Jeang, K-T. (1994) *Hum. Gene Ther.* 5, 927–939
25 Winter, G., Griffiths, A. D., Hawkins, R. E. and Hoogenboom, H. R. (1994) *Annu. Rev. Immunol.* 12, 433–455
26 Mulligan, R. C. (1993) *Science* 260, 926–932
27 Brenner, M. K. *et al.* (1993) *Lancet* 342, 1134–1137
28 Schutze, M-P., Peterson, P. A. and Jackson, M. R. (1994) *EMBO J.* 13, 1696–1705

Erratum

In the recent article by Carlos Ibáñez ('Neurotrophic factors: from structure–function studies to designing effective therapeutics'; *Trends Biotechnol.* 13, pp. 217–227), an error was made in the last paragraph on page 222. This was inadvertently printed with an incorrect statement concerning neurotrophin binding to Trk receptors. The correct text follows:

'In NGF, studies of variable residues have shown that Arg103 is located on the binding surface (Fig. 3). This suggests that all neurotrophins make use of the same surface to bind Trk receptors. In addition, in most cases, point mutations of variable residues have not shown substantial losses of binding affinity to Trk receptors. Taken together, these data support a model in which conserved residues in beta-strands on the side of the dimer provide the contacts with the highest binding energy, while variable residues in turns and loop regions along this surface determine biological specificity. This specificity may be conferred either directly, by the variable residues contributing contacts of lower energy to cognate receptors or indirectly, by preventing interaction with inappropriate receptors.'

We apologize to the author, and to readers for any confusion this may have caused.