Human Combinatorial Fab Library yielding specific and functional antibodies against the human fibroblast growth factor receptor 3

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Running title: Biologically active Fabs from HuCAL®-Fab1 against FGFR3

Abbreviations: CDR, complementarity-determining region; FCS, fetal calf serum; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HAMA, human anti-mouse antibodies; HuCAL®, Human Combinatorial Antibody Library; Ig, immunoglobulin; IL, interleukin; mAb, monoclonal antibody; POD, peroxidase; RTK, receptor tyrosine kinase; scFv, single-chain Fv;

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Summary

The Human Combinatorial Antibody Library Fab 1 (HuCAL®-Fab 1), was generated by transferring the heavy and light chain variable regions from the previously constructed scFv library (1), diversified in both complementarity determining regions 3, into a novel Fab display vector, yielding $2.1 \times 10^{10}$ different antibody fragments. The modularity has been retained in the Fab display and screening plasmids ensuring rapid conversion into various antibody formats as well as antibody optimization using pre-built maturation cassettes.

HuCAL®-Fab 1 was challenged against the human fibroblast growth factor receptor 3, a potential therapeutic antibody target, against which, to the best of our knowledge, no functional antibodies could be generated so far. A unique screening mode was designed utilizing recombinant functional proteins and cell lines differentially expressing fibroblast growth factor receptor isoforms diversified in expression and receptor dependence.

Specific Fab fragments with sub-nanomolar affinities were isolated by selection without any maturation steps as determined by fluorescence flow cytometry. Some of the selected Fab fragments completely inhibit target mediated cell proliferation, rendering them the first monoclonal antibodies against fibroblast growth factor receptors having significant function blocking activity. This study validates HuCAL®-Fab 1 as valuable source for the generation of target specific antibodies for therapeutic applications.
Introduction

The development of the hybridoma technology opened the application of monoclonal antibodies for research and human therapy (2). A major drawback of these first generation mAbs, especially for clinical application, was their murine origin, which often caused immune response in human and lead to the generation of human anti-mouse antibodies (HAMA reaction), limiting efficacy in long term and repeated administration (3). Phage display and antibody library technologies have evolved as a powerful alternative for the generation of human antibody fragments for research, clinical and therapeutic applications (for review, see 4), since it was shown that peptides (5) and antibody fragments (6) can be displayed on the surface of filamentous bacteriophage, and functional antibody fragments can be expressed in the periplasm of E. coli cells (7, 8).

To date, a variety of different antibody libraries has been generated, which range from immune to naïve and even synthetic antibody libraries. Immune libraries derived from IgG genes of immunized donors (9) are useful if immunized patients are available, but have the disadvantage that antibodies can only be made against the antigens used for immunization. In contrast, antibodies against virtually any antigen, including self, non-immunogenic or toxic antigens, can be isolated from naïve or synthetic libraries. Naïve libraries from non-immunized donors have been generated by PCR-cloning Ig repertoires from various B-cell sources (10-13). Semi-synthetic libraries were built by in vitro assembly of PCR-amplified antibody genes derived from human germline genes and randomized only in the CDR3 regions (14-16) or derived from a single V-gene with complete randomization of all CDRs (17, 18). The affinities of the antibodies derived from these libraries are assumed to be dependent on the size of the library (19). Therefore many attempts have been undertaken to make the library size as big as possible and site-specific recombination
systems have been created to overcome the library size limitations given by the conventional cloning strategies (20, 21). Beside library generation, also the panning process itself limits the library size that can be handled conveniently. Therefore it is important to generate libraries with a high quality of the displayed antibodies and thus emphasizing on the functional library size and not only on the apparent library size. With the recently described HuCAL® (Human Combinatorial Antibody Library) concept, a synthetic library in the scFv format was created focusing on a high number of correct antibody fragments (1, 22).

HuCAL® is a fully human antibody library wherein each VH and VL subfamily frequently used in human is represented by a consensus framework, resulting in 7 VH and 7 VL master genes giving 49 different combinations. The master genes were optimised for expression and folding; furthermore, a high functional quality of the library is guaranteed by diversifying both CDR3 regions with trinucleotide mixtures (23) reflecting the natural amino acid composition of CDR3. The modular design of the library with unique restriction sites flanking the CDR and framework regions, as well as compatible vector modules facilitate (i) conversion into different antibody formats, (ii) addition of effector functions and (iii) further antibody optimisation by exchanging the CDR regions of selected binders by pre-built CDR libraries (24). Here, we describe the generation of a second version of the HuCAL® library (HuCAL®-Fab 1), wherein we combined all the characteristics of the HuCAL® concept with the Fab format. Whereas scFv fragments have a high tendency to form multimers (25-27) Fab fragments tend to stay completely monomeric, allowing selection for affinity in contrast to selection for avidity. The monomeric behavior even in crude periplasmic extracts allows a rapid off-rate screening for affinity ranking of the antibody fragments without time-consuming purifications. Furthermore, the Fab format ensures conversion into complete immunoglobulins without loss of function, thus, being optimally suited for therapeutic applications.
HuCAL®-Fab 1 was challenged against FGFR3, so far resistant to generation of function blocking antibodies. FGFR3 belongs to a family of highly homologous cell surface expressed receptor tyrosine kinases (RTKs), currently including five members (FGFR1-5) (reviewed in 28; 29 for FGFR5). The FGFRs are glycoproteins comprising 2 or 3 immunoglobulin (Ig)-like extracellular domains, a hydrophobic transmembrane domain and a cytoplasmic region that contains the tyrosine kinase domain (30-32). Binding of a complex of fibroblast growth factor (FGF) ligand and heparin to FGFR3 leads to its dimerization, resulting in autophosphorylation of the intracellular kinase domain and a downstream activation of intracellular signalling cascades (33). So far, more than 20 ligands (34) have been identified varying in their specificity for individual FGFRs, e.g., FGF2, showing a very promiscuous binding pattern, whereas FGF9 shows a high preference for FGFR2 and FGFR3 (35). FGFs and their receptors regulate a multitude of cellular processes, including cell growth, differentiation, migration, and survival (reviewed in 36, 37).

FGFR3 is mainly expressed during embryogenesis and development of bones, brain, lungs, and spinal cord (38, 39). Different pathological disorders such as dwarfism or tumor genesis were linked to increased FGFR3 activity (reviewed in 40, 41). The transforming potential of FGFR3 harbouring an activating mutation was especially shown for tumor progression of ectopically expressed FGFR3 in NIH3T3 or mouse bone marrow cells (42, 43). Correlation of FGFR3 expression and tumor genesis was demonstrated for multiple myeloma patients with the t(4;14) translocation (42, 44), and in bladder carcinoma and cervical cancer (45-47). A prominent mutation is the exchange of glycine to arginine at position 380 (FGFR3G380R). The increased receptor activity caused by this amino acid change manifests itself in retarded bone growth, causing the most common form of achondroplasia and dwarfism (48, 49).
Accordingly, high affinity human antibodies that block FGFR3 activity could be of great therapeutic benefit in treating FGFR3 mediated skeletal disorders and tumor genesis.

Here, we report the generation of the high quality, large size (2.1 x 10^{10}) HuCAL\textsuperscript{®}-Fab 1 library based on the already described HuCAL\textsuperscript{®}-scFv library. HuCAL\textsuperscript{®}-Fab 1 display and screening vectors were optimized for protein expression to overcome the problem that Fab fragments are often less optimally produced than scFv proteins (11). We show the generation of high affinity HuCAL\textsuperscript{®}-Fab fragments with specificity for FGFR3, using a differential whole cell panning approach. Several selected antibodies completely inhibit FGFR3-mediated cell proliferation, some of which possess sub-nanomolar affinities without any maturation step. To the best of our knowledge, these are the first monoclonal antibodies to FGFRs that have function-blocking activity, thus, being promising candidates for therapeutic application.
Materials and Methods

1. Enzymes, antibodies and growth factors

DNA restriction and modification enzymes, as well as polymerases, were purchased from New England Biolabs (Beverly, MA, USA) and Roche Diagnostics (Mannheim, Germany). Goat anti-human IgG (Fcγ specific) (109-005-098), R-phycoerythrin conjugated F(ab’)2 fragment of goat anti-human IgG (109-116-088), FITC conjugated goat anti-rabbit IgG (111-095-144), and POD-conjugated goat anti-human IgG (F(ab’)2 fragment specific) (109-035-097) were supplied by Jacksons (West Grove, PN, USA). POD-conjugated goat anti-human IgG (Fcγ fragment specific) (A-0170), POD-conjugated goat anti-rabbit IgG (A-0545), POD-conjugated sheep anti-mouse IgG (whole molecule) (A-6782) and mouse anti-FLAG M2 antibody (F-3165) were purchased at Sigma (St. Louis, MO, USA). Recombinant IL3 was supplied by PeproTech (London, UK).

Recombinant FGF2 was expressed in E. coli. Cells were sonicated in PBS supplemented with a cocktail of proteinase inhibitors (Complete, purchased from BM) and cleared lysate was batch incubated with heparin-sepharose (Pharmacia). The resin was washed with PBS and bound protein was eluted with PBS, 2M NaCl. The partially purified material was diluted 10 times with PBS and subjected to a second purification step by FPLC using a pre-packed heparin-sepharose column (Pharmacia). NaCl gradient was employed to elute the FGF2, typically released from the heparin at 1 M NaCl.

Recombinant FGF9 was a kind gift from Herbert Weich (GBF Braunschweig, Germany).

2. Construction and production of recombinant FGFR proteins

For cloning of an FGFR1-Fc fusion protein (FGFR1-371hFc), the primers 5’-CGGGGATCCGAGGTCATCAGTGCGGC-3’ and 5’-CGGGTACCGGGATGTGGAGCTGGAAGTG-3’
were used to amplify the FGFR1 sequence from amino acid 1 to 371 from human cDNA by PCR. Fragments were cloned via the *KpnI* and *BamHI* restriction sites into the pCXFc vector, which is a pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA, USA) vector with the sequence of human Fc between *BamHI* and *XbaI* sites. The FGFR3-Fc fusion protein (FGFR3<sup>1-369</sup>hFc) was cloned by amplifying FGFR3 from human cDNA by PCR with the primers 5’-CGGGATCCCCCGCCTCGTCAGCCTCC-3’ and 5’-CGGGGTACCGCGCGCTGCCTGAGG-3’ and cloning into pCXFc via the *KpnI* and *BamHI* restriction sites. cDNAs of both FGFR-Fc fusions were used to transfect 293T cells and conditioned medium was harvested after 2 and 4 days. A soluble his-tagged FGFR3 (FGFR3<sup>23-374</sup>TDis) was constructed by amplifying FGFR3 by PCR with the primers 5’-ACGTGCTAGCTGAGTCCTTGGGGACGGAGCAG-3’ and 5’-ACGTCTCGAGTTAATGGTGATGGTGATGGTGTGCATACACACAGCCCCGCTGC-3’ from human cDNA. The fragment was cut with *XhoI* and cloned into pBlueScript (Stratagene, La Jolla, CA, USA) digested with *XhoI* and *EcoRV* to obtain the plasmid (pBsFR3<sup>23-374</sup>TDis). Digesting this plasmid with *NheI* and *XhoI* resulted in a fragment encoding the extracellular domain of FGFR3, which was placed at the same sites in pCEP-Pu (50) generating pCEP-FR3<sup>23-374</sup>TDis. FGFR3<sup>23-374</sup>TDis was expressed transiently in 293E cells by transfection with pCEP-FR3<sup>23-374</sup>TDis and purified from supernatants with Ni-NTA beads (Qiagen, Hilden, Germany) followed by elution with a step gradient ranging from 20 mM to 0.5 M imidazol. SDS-PAGE and immunoblot analyses demonstrated peak amounts of purified FGFR3<sup>23-374</sup>TDis at 50 mM imidazol.

3. Cell lines and medium

293 cells were cultured in DMEM, 10% FCS, penicillin/streptomycin, glutamine (all Gibco, Rockville, MD, USA). Non-transformed rat chondrocytes (RCJ3.1C5.18) were transfected with full length FGFR1 (RCJ- FGFR1), FGFR3 (RCJ- FGFR3) and FGFR3<sup>G380R</sup> (RCJ- FGFR3<sup>G380R</sup>) as
described elsewhere (51). Expression of FGFRs was regulated by a tetracycline suppression system (Gibco, Rockville, MD, USA) expressing the receptor in the absence of tetracycline (-tet) and lacking the receptor if tetracycline was added to the culture medium (+tet).

The mouse myeloid progenitor cell line FDCP-1 was cultured in ISCOVES medium (Gibco, Rockville, MD, USA) supplemented with 10% FCS, penicillin, streptomycin, glutamine and 0.1 ng/ml IL3. FDCP-1 cells transfected with full length human FGFR1 (FGFR1 was a generous gift from Josef Schlessinger, New York University School of Medicine, USA) or human FGFR3 were grown in the same medium, but IL3 was substituted by 10 ng/ml FGF2 or FGF9, respectively.

4. Cloning of Fab-expression vectors containing cysteines for covalent linkage of heavy and light chains

Genes encoding the human C_{H1}- (sub type IgG1, Genbank accession number A49444), C_{\kappa}- and C_{\lambda}-domains (Genbank accession numbers P01834 and P01842, respectively) had been previously constructed by gene synthesis (1) without the region responsible for formation of the inter-molecular disulfide bond. Gene fragments containing the cysteine codon at the 3’-end of C_{H1}, C_{\kappa} or C_{\lambda}, respectively, were constructed by adding additional nucleotides to a \kappa- and \lambda-Fab in the expression vector pMORPH®X7_Fab_FS (22) containing a Flag- (52) and a Strep-tag (53) by PCR using Pwo-polymerase. 5’-GTGACGTTAGCTCAGCGTC-3’ and 5’-GATATCTGCAGAATTCGCAGCTTTTCGGTTCCAC-3’ were used as primers for C_{H1}, 5’-GAAATTAAACGTACGGTGGCTGC-3’ and 5’-TCCTCTAGATGCATGCTTATCAGCACTCGCCAC-3’ for C_{\kappa} and 5’-GCGGCACGAAGTTAACCGTTC-3’ and 5’-TCCTCTAGATGCATGCTTATCAGCTGCACTCAGTCGG-3’ for C_{\lambda}.

PCR-products were gel-purified and digested with BlpI/EcoRI (C_{H1}), BsmI/SphI (C_{\kappa}) and HpaI/SphI (C_{\lambda}). Digests were separated on agarose gels and bands corresponding to the
final products purified with the QIAquick Gel Extraction Kit (Qiagen). FabCys vectors containing the cysteine codon at the 3'-end of C\textsubscript{H1}, C\textsubscript{\kappa} or C\textsubscript{\lambda}, respectively, were constructed as follows: In case of \kappa-Fabs, the Bs\textsc{N}I/EcoRI-digested vector-fragment and in case of \lambda-Fabs, the Hpa\textsc{I}/EcoRI vector-fragment of pMORPH\textsuperscript{®}X7\_FS were ligated with the fragments encoding either C\textsubscript{\kappa} (Bs\textsc{N}I/Sph\textsc{I}) or C\textsubscript{\lambda} (Hpa\textsc{I}/Sph\textsc{I}), C\textsubscript{H1} (Blp\textsc{I}/EcoRI) and a fragment corresponding to the intergenic region, the PhoA signal sequence and VH (Sph\textsc{I}/Blp\textsc{I}) in a four-fragment-ligation. \textit{E. coli} JM83 (54) was subsequently transformed with the ligation products. The final expression vectors were designated pMORPH\textsuperscript{®}X7\_FabCys\_FS.

5. Cloning and expression of disulfide-linked and non-covalently linked Fab-fragments

Control \kappa-scFv and \lambda-scFvs were converted into the Fab format by cloning the VL and VH fragments into the expression vectors for disulfide-linked Fabs pMORPH\textsuperscript{®}X7\_FabCys\_FS and the vectors for non-covalently linked Fabs pMORPH\textsuperscript{®}X7\_Fab\_FS. The cloning procedure and expression in JM83 (54) were performed as described (22). Purification from crude periplasmic extracts being generated by osmotic shock (55) was performed by StrepTactin affinity chromatography (IBA, Göttingen, Germany) (56).

6. Thermal stability of disulfide-linked and non-covalently linked Fab-fragments

To analyze the heat stability of disulfide-linked and unlinked Fab-fragments, 1 \textmu M solutions of purified proteins in PBS were incubated at temperatures of 4, 30, 40, 50, 60 and 70°C for 30 min. Samples were centrifuged and supernatants were tested for binding activity using the Biacore instrument. Biacore analysis was performed at 22°C as described (22) under mass transport limited conditions. The binding signal at the end of the injection was measured. The binding signal of the sample incubated at 4°C was set to 100% and the
binding signals of the samples incubated at the other temperatures were normalized to that value.

7. Comparison of the OmpA and StII signal sequence for light chain expression

The StII signal sequence for light chain expression in pMORPH®X7_Fab _FS (22) was replaced by the OmpA signal sequence from a derivative of pIG10 (57) via XbaI/EcoRV. The new expression vector was designated pMORPH®X9_Fab _FS.

Heavy and light chains of the control κ-Fab and λ-Fab were excised from pMORPH®X7_Fab_FS and cloned into pMORPH®X9_Fab_FS by EcoRV/EcoRI. Expression yields of these Fabs containing either the StII or the OmpA signal sequence were measured as follows: after expression in JM83 (54, 22), crude periplasmic extracts were generated by osmotic shock (55) and Fab protein was analyzed by ELISA: 500 ng of the corresponding antigens were coated on Maxisorp plates (Nunc, Rochester, NY, USA) and after blocking with 5% non-fat milk powder in TBS, 0.05% Tween20 (Sigma, St. Louis, MO, USA), periplasmic extracts were added in 2.5% non-fat milk powder in TBS, 0.05% Tween20 (Sigma, St. Louis, MO, USA). Anti-FLAG M2 antibody, anti-mouse IgG-horseradish peroxidase antibody and BM Blue POD substrate, soluble (Roche Diagnostics, Grenzach-Wyhlen, Germany) were used for ELISA-development (OD at 370 nm).

8. Generation of HuCAL®-Fab 1

The phagemid used for cloning of the HuCAL®-Fab 1 library was designated pMORPH®18 (Fig. 1). Briefly, the vector contains a non-covalently linked λ- or κ-Fab with the PhoA signal sequence for heavy chain and the OmpA leader sequence for light chain expression. The C_H1 gene is directly followed by a truncated version of gene III with the first 249 codons deleted.
The whole VH-chain (MunI/StyI-fragment) was replaced by a 1205 bp dummy fragment containing the β-lactamase transcription unit (bla) in order to facilitate subsequent steps for vector fragment preparation and to allow for selection of complete VH removal. The bla cassette was amplified from pMORPH®1_bla_MCS_C1 by PCR. After VH-replacement, VLλ was removed by EcoRV/DraIII and VLκ by EcoRV/BsNI, and replaced with bacterial alkaline phosphatase (bap) gene fragment. The BAP fragment was generated from pMORPH®X7_AS_5D22 by PCR. The final library vectors were pMORPH®18_Fab_κ-binder_VH-bla_VL-BAP and pMORPH®18_Fab_λ-binder_VH-bla_VL-BAP.

As the variability of the light chains is lower than that of the heavy chains, cloning was started with the light chain libraries. The VLλ and VLκ light chain libraries diversified in L-CDR3, which were generated for the HuCAL®-scFv library (1), were also used for cloning of HuCAL®-Fab 1: pMORPH®4_scH3λ1-TRIM-library, pMORPH®4_scH3λ2-TRIM-library, pMORPH®4_scH3λ3-TRIM-library and pMORPH®4_scH3κ1-TRIM-library, pMORPH®4_scH3κ2-TRIM-library, pMORPH®4_scH3κ3-TRIM-library and pMORPH®4_scH3κ4-TRIM-library. In case of λ, they consisted of the λ1-, λ2- and λ3-HuCAL®-framework and had variabilities of 1.5 x 10^5, 5.5 x 10^6 and 7.5 x 10^4, respectively. VLλ fragments were amplified from these libraries by 15 PCR cycles (Pwo-polymerase) with primers 5'-GTGGTGGTTCCGATATC-3´ and 5´-AGCGTCACACTCGGTGCGGCTTTCGGCTGGCCAAGAACGGTTA-3´. PCR-products were digested with EcoRV/DraIII, gel-purified and mixed according to the variability of the sub-libraries.

The AP-dummy was removed by EcoRV/DraIII from the library vector. 2 µg of gel-purified vector were ligated with a 3-fold molar excess of VLλ-chains for 16 h at 16°C, and the ligation mixtures were electroporated in 800 µl E. coli/TOP10F cells (Invitrogen), yielding

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1 Pack, unpublished results
2 Söhlemann, unpublished results
altogether $4.1 \times 10^8$ independent colonies. The transformants were amplified in 2 x YT/1% glucose/34 µg/ml chloramphenicol/100 µg/ml ampicillin, harvested and stored in 20% (w/v) glycerol at –80°C.

The κ libraries comprise the κ1-, κ2-, κ3- and κ4-HuCAL®-master genes with a variability of $1.6 \times 10^6$, $6.1 \times 10^5$, $3.4 \times 10^6$ and $1.1 \times 10^5$, respectively. "VL₅"-chains were obtained by a restriction digest with EcoRV/BsNI, gel-purified and mixed according to the variabilities of the sub-libraries. 2 µg of gel-purified vector were mixed with a 5-fold molar excess of "VL₅"-chains. Ligation and transformation into E. coli TOP10F cells (Invitrogen) were performed as described for "VL₅"-chains, yielding altogether $1.6 \times 10^8$ independent colonies.

DNA of the two light chain libraries was prepared and the bla-dummy was removed by MunI/StyI. Thereby the two vector fragments for insertion of the VH sub-libraries were generated.

The VH sub-libraries of HuCAL®-scFv were used for the generation of HuCAL®-Fab 1. The VH libraries of HuCAL®-scFv consist of the master genes VH1-6 diversified with two VH-CDR3 trinucleotide library cassettes differing in CDR3 length separately, and each sub-library combined with a mixture of the four "VL₅"- and with a mixture of the three "VL₅"-libraries yielding 24 different sub-libraries (1). For the generation of the HuCAL®-Fab 1, DNA from all sub-libraries, except those containing the VH4 master gene was prepared, preserving the original variability. The DNA was digested with MunI/StyI and gel-purified. The two VH-sub-libraries of the same HuCAL® master gene - diversified with the same H-CDR3 cassette, but combined either with the VL₅-mix or the VL₅-mix - were combined according to the variability of the two VH-sub-libraries. A 5-fold molar excess of the VH-chains was ligated with 3 µg of the VL₅-library vector and with 3 µg of the VL₅-library vector for 4 h at 22°C separately for each sub-library. The ligation mixtures were electroporated for each vector in E. coli TOP10F cells (Invitrogen), yielding 20 different sub-libraries with altogether $2.1 \times 10^{10}$ independent
colonies. The transformants were amplified in 2 x YT/1% glucose/34 µg/ml chloramphenicol/10 µg/ml tetracycline, harvested and stored in 20% (w/v) glycerol at -80°C.

For quality control, the light chain and heavy chain of single clones were sequenced with 5’-CAGGAAACAGCTATGAC-3’ and 5’-TACCGTTGCTCTTCACCCC-3’, respectively (SequiServe, Vaterstetten, Germany).

9. Selection of FGFR3 specific phage

Phagemid rescue, phage amplification and purification of HuCAL®-Fab 1 were performed as described (22), with the only exception that expression of Fab-gIII-fusion protein was not induced with IPTG as the basal transcription from the lac-promotor is sufficient for monovalent Fab-display (data not shown). For each panning, three subsequent selection rounds were performed. Each round was performed either on recombinant protein immobilized to 96 well plates (Maxisorp, Nunc, Rochester, NY, USA) or on cells expressing FGFR3. For panning on immobilized protein, either 1 µg/well of purified FGFR323-374TDhis was coated directly, or 1 µg/well of goat anti-human IgG (Fcγ specific) was coated and subsequently incubated with cell culture supernatant containing FGFR3-Fc. Then, wells were blocked with 5% milk powder in TBS (blocking buffer) and incubated with 1x10^{13} HuCAL®-Fab 1 phages pre-blocked with 1 volume of blocking buffer. After 40 minutes at 4°C, wells were washed six times with TBS, 0.05% Tween20 (Sigma, St. Louis, MO, USA) and six times with TBS. Remaining phage were eluted with 100 mM glycine, 0.5 M NaCl, pH 2.2 for 10 minutes at room temperature, before they were incubated with a mid-phase TG-1 culture (Stratagene, OD600 = 0.5) for infection. Subsequently, wells were incubated with mid-phase TG-1 culture as an additional elution step. Phagemids were amplified in TG-1, rescued and purified as described above. Selection on FGFR3 expressing cells was done by blocking 2 x 10^7 cells with blocking buffer, before incubating them with 1.5 - 4 x 10^{12} phage for 2 hours,
followed by several washing steps. Remaining phage were eluted from cells with an acidic step followed by TG-1 elution as described above. The pool of phagemids from the third selection round was amplified in E. coli and subsequently purified. Fab-encoding fragments were excised as a pool and cloned into the expression vector pMORPH®X9_Fab_FS for transformation of TG1-F' (TG-1 depleted for the F pilus). Single clone expression and preparation of periplasmic extract containing HuCAL®-Fabs were described previously (22).

10. Specificity ELISA

Maxisorp (Nunc, Rochester, NY, USA) 96 well plates were coated with goat anti-human IgG (Fcγ specific), blocked with TBS, 10% FCS, and incubated with cell culture supernatant containing FGFR3-Fc, or FGFR1-Fc. After incubation with periplasmic extract (diluted 1:10 in TBS) or purified HuCAL® antibodies (10 µg/ml in TBS), Fabs were detected with a POD-conjugated goat anti-human IgG specific for the F(ab')2 fragment (Jackson 109-035-097) and visualized with BM Blue POD substrate (Roche Diagnostics, Grenzach-Wyhlen, Germany). OD370 was measured with a BIO-RAD (Hercules, CA) Benchmark microplate reader.

11. FACS analysis

2 x 10^5 cells were incubated with periplasmic extract diluted 1:5 with FACS-buffer (PBS, 3% FCS, 0.02% azide) or with purified Fab fragments. HuCAL®-Fabs were detected with a F(ab')2 fragment of goat anti-human IgG conjugated with R-phycoerythrin and analyzed by a FACSCalibur (Becton Dickinson, San Jose, CA). Affinities of HuCAL®-Fab fragments were assessed by indirect labeling using Scatchard analysis (58). Therefore, RCJ-FGFR3 cells (-tet) were incubated with purified HuCAL®-Fabs in FACS buffer, followed by the same secondary PE-labeled antibody as mentioned above. Calculation of affinities was done as described elsewhere (59).
12. Cell proliferation assay

In 96 well cavities, 2 x 10^4 FDCP-1 cells transfected with FGFR3 (FDCP-1-FGFR3) or FGFR1 (FDCP-1-FGFR1) were incubated at 37°C in 40 µl medium supplemented with 10 ng/ml FGF and 10 µl of HuCAL®-Fabs dialyzed to PBS. After two days, 50 µl of XTT reagent (Roche Diagnostics, Mannheim, Germany) were added per well, incubated for 3 hours at 37°C and adsorption was measured at OD415 to determine the relative amount of viable cells per well. IC_{50} value was calculated with the Origin 4.1 program (Microcal Software, Northhampton, MA) applying a sigmoidal curve fit.

13. Surface plasmon resonance (SPR) studies of FGFR3 specific HuCAL®-Fabs

All surface plasmon resonance (SPR) studies of FGFR3 specific HuCAL®-Fabs were conducted in HBS buffer using a Biacore 3000 (Biacore, Uppsala, Sweden). For preparation of FGFR surfaces, goat anti-human IgG antibodies specific for the Fc\gamma fragment were coupled covalently to CM 5 chips (Biacore, Uppsala, Sweden), according to manufacturer’s protocol. Subsequently, FGFR-Fc proteins were captured from cell culture supernatant. After each interaction of HuCAL®-Fab fragments with FGFR-Fc the surface was regenerated with 10 mM HCl, and FGFR-Fc protein was captured again. For affinity determination of purified HuCAL®-Fab fragments, concentrations from 16 to 500 nM were injected. Data analysis was performed as described previously (22).

In order to qualitatively compare binding epitopes of different HuCAL®-Fabs to FGFR3 the Fc fusion protein was captured as described before, followed by injecting 1 µM of the first HuCAL®-Fab until saturation of binding. Then, 1µM of the second HuCAL®-Fab was co-injected. We concluded that epitopes were independent if the signal increased for the second
HuCAL<sup>®</sup>-Fab fragment. No increase of signal, therefore, meant overlapping or similar binding epitopes.

To test antibodies for interference with FGF binding, FGFR3-Fc was captured as described before, followed by injection of 100 µg/ml heparin and 1 µM of FGF9 until saturation level. Then, 1 µM of purified HuCAL<sup>®</sup>-Fab was co-injected. The maximum binding capacity of the free FGFR3 surface was reached with 1 µM HuCAL<sup>®</sup>-Fab. Therefore, this binding signal was set to 100%. The signal resulting from HuCAL<sup>®</sup>-Fab binding to the FGFR3-FGF9 complex in comparison to HuCAL<sup>®</sup>-Fab binding to FGFR3 without FGF9 was calculated as a percentage.

For stability tests of HuCAL<sup>®</sup>-Fab fragments, the Fabs were incubated at 37°C for 0, 4, 8 and 13 days in 10% FCS, at a concentration of 1 µM. The Fabs were then diluted to 100 nM and injected to the FGFR3 surface. The initial slope of the binding was determined for each time point and compared to the slope of the Fab not exposed to 37°C, which was set to 100%.
Results

1. Expression and stability analysis of disulfide-linked and non-covalently linked Fab-fragments

In parallel to constructing the HuCAL®-scFv library, all 7 VH and 7 VL master genes had been fused also to synthetic gene fragments encoding the C\text{H}1 and CL domains, respectively (1). These constant domains had been synthesized without the C-terminal cysteines responsible for inter-molecular disulfide bonding of IgG-type Fab fragments, since it is known that the Fd and the light chain do not dissociate into the component chains even when only non-covalently linked, as seen with mouse IgA or with mildly reduced fragments (60-63). Fig. 1a, b show the arrangement of HuCAL®-Fabs, which are transcribed from a \textit{lac}-promoter as one bicistronic mRNA with two different signal sequences for the heavy and light chain to avoid homologous recombination.

Figure 1

Before starting the construction of a Fab library, we were interested in the expression and stability properties of Fab fragments with and without the inter-molecular disulfide bridge. Therefore, we introduced cysteine codons by site-directed mutagenesis into the gene fragments encoding the constant domains, thereby changing the C-termini of C\text{H}1, C\text{\kappa} and C\text{\lambda} from -EPKSEF to -EPKSC\textsubscript{EF}, from -NRGEA to -NRGEC and from -APTEA to APTEC\textsubscript{S} (introduced cysteines underlined), respectively. The expression vectors encoding those domains were designated pMORPH\textsuperscript{®}x7_FabCys_FS (key features in Fig. 1c).

Three scFv fragments previously selected from the HuCAL®-scFv against different antigens were then converted into either of both Fab formats. Western-Blot analysis under reducing and non-reducing conditions of crude periplasmic extracts (55) from \textit{E. coli} expression cultures clearly demonstrated the formation of the disulfide-bond between heavy
and light chain only for the three Fab fragments cloned into pMORPH®X7_FabCys_FS (data not shown).

For comparison of the expression yields the three disulfide-linked and the three non-covalently linked Fab fragments were expressed in 1 L shaking flask cultures and purified from crude periplasmic extracts by StrepTactin affinity chromatography (53, 56). 0.5-4.4 mg of non-covalently linked and 0.2-2.8 mg of disulfide-linked Fab-fragments were obtained. In all three cases, the expression yield was higher with the non-covalently linked Fab fragments (1.3 to 4 fold). SDS-PAGE and Coomassie Blue staining demonstrated that heavy and light chains were equally represented with a purity of > 90% (data not shown).

Purified Fab fragments of two disulfide-linked and the corresponding unlinked Fab fragments (1 Fab with κ− and 1 Fab with λ− light chain) were incubated at temperatures from 4 to 70°C in order to analyze their thermal stability. Relative binding activities were recorded in Biacore. As shown in Fig. 2, the binding activity stayed unchanged up to an incubation temperature of 60°C for both the non-covalently linked and the disulfide-linked Fab fragments. Only when raising the incubation temperature to 70°C, the binding activity of the unlinked Fab fragments was reduced between 5 and 25 fold, whereas the binding activity of disulfide-linked Fab fragments was reduced less than 2 fold. Long-term storage at 37°C in buffer containing 10% FCS showed no difference between both antibody formats (data not shown).

Taken together, the expression and stability analysis of disulfide-linked and non-covalently linked Fab fragments show that unlinked Fab fragments constantly reach better expression yields than disulfide-linked Fab fragments and are sufficiently stable for standard applications. Therefore, we decided to use the non-covalently linked Fab format for library construction. Figure 2
2. Influence of the signal sequence on light chain expression

For a further improvement of the expression rate of the non-covalently linked Fab fragments, we analyzed the influence of the signal sequence for VL. The initial Fab expression vector, pMORPH®X7_Fab_FS, contained the StII signal sequence for the light chain (64, 65), and preliminary experiments indicated that the amount of light chain might limit expression of Fab fragments (data not shown). As the OmpA signal peptide (66) is an often used leader for protein expression in bacteria (67), the StII signal sequence was replaced by the OmpA leader thus creating the expression vector pMORPH®X9_Fab_FS (see Fig. 1 c).

Two Fab genes (one with κ- and one with λ-light chain) were excised from pMORPH®X7_Fab_FS and cloned into pMORPH®X9_Fab_FS by EcoRV/EcoRI without transfer of the light chain signal sequence. Expression yields of the StII-Fab fragments and their OmpA counterparts were compared by measuring the binding activity of crude extracts in ELISA on the corresponding specific antigens (Fig. 3). The signal for the Fab fragments originating from the pMORPH®X9_Fab_FS vector was about 2 fold higher than for the Fab fragments originating from the pMORPH®X7_Fab_FS vector, which can only be attributed to a superior expression rate of Fab fragments equipped with the OmpA signal sequence. Quantitative Western-Blot analysis of these crude extracts confirmed this result (data not shown). Therefore, replacement of the StII leader by the OmpA signal peptide for the light chain allows for a further improvement of Fab expression.

This demonstrates that the non-covalently linked HuCAL®-Fab format using the OmpA signal sequence for light chain and the PhoA signal sequence for heavy chain expression guarantees good expression and stability, thus being ideal features for generating a Fab-library.
3. Generation of the HuCAL®-Fab 1 library

The expression vectors pMORPH®X9_Fab_FS, having an OmpA signal peptide for VL expression and lacking the cysteine for the VL-VH linkage, were used as starting material. A fragment (codon 250 to 406) of the mature gene III protein of bacteriophage M13 was used to replace the FLAG- and Strep-tag directly fused to the heavy chain to generate the display vector pMORPH®18. To create vectors for library cloning, for which preparation of the vector fragment is facilitated and in which VH- and VL-chains of parental binders are completely absent, we removed the VH gene and inserted the complete β-lactamase transcription unit (bla) as a dummy sequence. A fragment of the bacterial alkaline phosphatase gene (bap) served as a cloning stuffer for VLλ and VLκ.

HuCAL®-λ1, λ2 and λ3 diversified in L-CDR3 were amplified by PCR from precursor sub-libraries of the HuCAL®-scFv library (1) and mixed according to their variability. VLκ-chains of κ1, κ2, κ3 and κ4 also diversified in L-CDR3 were obtained from precursor sub-libraries of the HuCAL®-scFv library (1) by restriction digest and also mixed according to their variability. VLλ and VLκ gene segment libraries were inserted into the Fab phage display vector, yielding 4.1 x 10⁸ independent transformants for the VLλ and 1.6 x 10⁸ independent transformants for the VLκ sub-library. Thus, in each case library size was at least 30-fold larger than in the corresponding scFv library, ensuring full coverage of the initial diversity. Quality control by sequencing of 54 clones revealed more than 90% correct clones.

The HuCAL® VH-gene segment libraries diversified in the H-CDR3 region were prepared by restriction digest of all six HuCAL® VH master gene sub-libraries except VH4, which was omitted because only very few binders could be selected from this VH family during various pannings (1). During the construction of the HuCAL®-scFv library, each VH-
gene segment had been diversified separately with two different H-CDR3 library cassettes
and combined with either the VLκ or the VLλ libraries, generating 20 different sub-libraries
(1).

From all these 20 sub-libraries, we prepared VH gene segments, combined VH chains
from VLκ and VLλ sub-libraries diversified by the same H-CDR3 cassette and ligated the
generated 10 different VH-pools separately with the VLλ- and the VLκ-libraries obtained
above (for details see Material and Methods). Thus, 20 different sub-libraries were generated
with in total 2.1 x 10¹⁰ different Fab fragments. This final library was designated HuCAL®-Fab 1.

VH and VL regions of 207 clones were sequenced in order to analyze the library
quality. 138 clones (= 67%) were fully correct.

4. Selection of FGFR3 specific antibodies from HuCAL®-Fab 1

HuCAL®-Fab 1 was used to select specific Fab fragments against the receptor tyrosine
kinase FGFR3 from two separate panning strategies. In panning A, three selection rounds
were performed. Phage were incubated with immobilized FGFR3²³-³⁷⁴TDhis in the first and
third round and antigen expressing 293T cells were used in the second round. 293T cells
express endogenous human FGFR3 on the cell surface. In panning B, the first and third
selection rounds were performed on FGFR3-Fc captured by an anti-Fc antibody alternating
with a selection round on RCJ-FGFR3G³⁸⁰R cells (-tet) expressing high amounts of
recombinant human FGFR3G³⁸⁰R on the cell surface. Individual Fab antibodies were produced
in E. coli and periplasmic preparations were tested in FACS for FGFR3 binding on RCJ-FGFR3
cells. In these cells, the regulated expression of recombinant FGFR3 is induced in the
absence of tetracycline (-tet), whereas it is suppressed in the presence of tetracycline (+
tet). Positive clones were subsequently tested on RCJ-FGFR3 cells cultured with tetracycline
(+tet) to confirm FGFR3 specificity. In total, 1076 clones of panning A were tested in FACS and 210 (19.5%) bound specifically to RCJ-FGFR3(-tet) (Table 1). The VH-gene segment of 69 FACS positive clones was sequenced, yielding 15 unique binders (clone #1-15). In panning B, 1632 clones were screened for FGFR3 binding in FACS. Out of these clones 521 binders were FGFR3-specific (31.9%). Then, 120 clones were selected and sequenced revealing another 34 unique binders. In total, 49 FGFR3 specific HuCAL®-Fab fragments were obtained, of which 37 were chosen for detailed analysis (see below). The unique clones from panning A and B represented all VH families of HuCAL®-Fab 1, with a bias on VH1, from which 36 unique clones were selected. Furthermore, two clones with VH2, five clones with VH3, three clones with VH5 and three clones with VH6 frameworks were isolated. Additionally, a high variety in length distribution of the H-CDR3, ranging from 8 to 20 amino acids was found.

Table 1

5. Characterization of primary hits

In order to test the specificity of the binders for FGFR3, purified Fab fragments were tested in ELISA (Fig. 4). We used FGFR1 as a representative of the FGFR family in order to analyze the FGFR3 binding clones for their antigen selectivity, as we could not exclude having generated clones that cross-react with FGFR1 because of an overall homology of 62% of both family members. All anti-FGFR3 Fab fragments in general showed strong binding to FGFR3, but differed in their cross-reactivity with FGFR1. E.g. MSPRO2, 12, 21, 24, and 26 exclusively bound to FGFR3; MSPRO11 additionally showed weak binding to FGFR1; and MSPRO28 and 29 strongly cross-reacted with FGFR3-Fc and FGFR1-Fc in ELISA.

This difference in cross-reactivity to FGFR1 and 3 could similarly be shown in FACS analyses using RCJ cells expressing FGFR1 or FGFR3, respectively. Examples for a specific
(MSPRO24) and a cross-reactive (MSPRO28) HuCAL \textsuperscript{®}-Fab are given in Fig. 5. Overall, we found that all FGFR3 specific Fab fragments that were cross-reactive with FGFR1-Fc in ELISA also bound to FGFR1 expressing RCJ cells. Surprisingly, some Fab fragments that bound only weakly to FGFR1-Fc in ELISA now caused a clear shift on RCJ-FGFR1 (-tet) cells in FACS, indicating that under our conditions FACS analysis is the more sensitive assay. Based on the FACS analysis, 10 Fab fragments (MSPRO11, 22, 26, 27, 28, 29, 51, 52, 53, 54) out of 37 selected clones (MSPRO1-15; MSPRO20-33; MSPRO52-59) were cross-reactive for FGFR3 and FGFR1 and 27 antibodies were specific for FGFR3.

In parallel, purified Fab fragments were tested in FACS analysis on RCJ cells expressing the mutated form of FGFR3, FGFR3\textsuperscript{G380R}. FGFR3-specific Fab fragments bound to RCJ-FGFR3\textsuperscript{G380R} (-tet) expressing the mutant FGFR3\textsuperscript{G380R} but did not bind the RCJ-FGFR3\textsuperscript{G380R} (+tet) cells in which expression of FGFR3\textsuperscript{G380R} was suppressed. In each case, the Fab antibody bound equally well to RCJ cells expressing FGFR3 (wt) and to RCJ cells expressing FGFR3\textsuperscript{G380R} (data not shown).

Figure 4, 5

6. Inhibition of FGFR3 mediated cell signaling

In order to analyze the inhibitory potential of FGFR3 specific HuCAL \textsuperscript{®}-Fab fragments, they were tested in the FDCP-1-FGFR3 proliferation assay. Cell survival and growth of FDCP-1 cells normally depend on the presence of IL3 in the culture media. Yet, IL3 can also be substituted by RTK ligands in FDCP-1 cells expressing the corresponding recombinant RTK (68). FDCP-1 cells expressing FGFR3 were grown in the presence of the FGF9 ligand. HuCAL \textsuperscript{®}-Fab fragments were added to these cells for 48 h. Then, cells were stained with XTT to analyze the amount of living cells in a microtiter plate reader. In total, 25 of the 37 analyzed HuCAL \textsuperscript{®}-Fab fragments inhibited FDCP-1-FGFR3 proliferation in the presence of
None of the HuCAL®-Fab fragments affected FDCP-1-FGFR3 proliferation in the presence of IL3 (data not shown), demonstrating that the inhibitory effect of HuCAL®-Fab fragments is not mediated by unspecific cytotoxic effects. IC₅₀ values were calculated to compare the activity of individual HuCAL®-Fab fragments. Inhibition curves of two different purified HuCAL®-Fab fragments are shown in Fig. 6. A summary of those ten HuCAL®-Fab fragments showing the best binding and inhibition characteristics is given in Table 2. Eight of ten HuCAL®-Fab fragments show an IC₅₀ value below 100 nM, ranging from 19 nM for the best clone (MSPRO59) to 70 nM (MSPRO24).

Figure 6

7. Determination of affinity to FGFR3

The affinity of the Fab fragments was analyzed in Biacore on captured FGFR3-Fc fusion protein. Overall, relative affinities ranged from 1.5 nM to 1.5 µM. The data for 10 HuCAL®-anti-FGFR3 Fab fragments with relative affinities below 50 nM are shown (Tab. 2). The monovalent affinities of 8 HuCAL®-Fab fragments measured in Biacore were 10 nM or below. For 9 of these 10 HuCAL®-Fabs, we also calculated the affinities in FACS using indirect labelling and Scatchard analysis (58). In Scatchard analysis the amount of bound antibody to antigen expressed on cells is measured via indirect fluorescence labeling. In Biacore analysis, however, the association and dissociation rate constants for the antibody interaction with immobilized antigen are determined. Taking into account that the principles of the two binding assays are completely different, the obtained affinities of six of these Fab fragments are in good agreement differing by a factor ranging from 1 to maximally 3 (Tab. 2). However, for the three anti-FGFR3 Fab fragments MSPRO21, 28, and 29 the FACS experiments resulted in significantly lower values of the monovalent Kᵦ below 1 nM. We presume that these Fabs bind to epitopes that undergo conformational changes upon
coating of the recombinant protein to the Biacore chip in contrast to the native protein expressed on the cell surface as it is detected in FACS analysis. Therefore, we consider the FACS data to be more relevant.

Comparing the IC₅₀ values with the apparent Kᵦ of the selected 10 HuCAL®-Fab fragments showed that the IC₅₀ values were not as good as the apparent Kᵦ. The difference ranged from a factor of 3.3 (MSPRO29) to even a factor of 55 (MSPRO11). This might reflect the fact that the HuCAL®-Fab fragments block different epitopes on FGFR3. Therefore, a characterization of the binding epitopes was performed.

Table 2

8. Characterization of the binding epitope on FGFR3

In a first set of experiments, FGFR3 specific HuCAL®-Fab fragments were tested for identical (or overlapping) binding epitopes on FGFR3. FGFR3-Fc was captured via an anti-Fc antibody to the BIAcore chip surface and saturated with the first anti-FGFR3 HuCAL®-Fab fragment. Then, a second anti-FGFR3 HuCAL®-Fab fragment was added. An additional increase in the binding signal could only occur if the epitope on FGFR3 of the second Fab fragment was not already blocked by the first Fab fragment. In contrast, no increase in RU was seen if binding of the first Fab fragment interfered with that of the second Fab fragment. Fig. 7 shows the experiment for MSPRO11 in comparison to MSPRO2, 12 and 21. MSPRO11 did not block binding of MSPRO2 and 12 whereas binding of MSPRO11 interfered with MSPRO21. All of the previously characterized inhibitory FGFR3 specific HuCAL®-Fab fragments were similarly tested. Finally, we identified two groups of inhibitory clones (Table 3). Binding of the non-inhibitory FGFR3 specific Fab fragments did not interfere with binding of any of the inhibitory Fab fragments (data not shown).

Figure 7, Table 3
In a second set of BIAcore experiments, competition of HuCAL®-Fab fragments and FGF9 for binding to FGFR3 was tested. An example with MSPRO11 and a control Fab fragment is shown in Fig. 8. HuCAL®-Fab fragments were applied to the FGFR3-Fc coated surface (bold line) or FGFR3-Fc surface previously saturated with FGF9/heparin (dotted line). If FGF9/heparin interfered with binding of the Fab fragment to FGFR3 a smaller increase of RU compared to binding to the free receptor was measured as is shown for MSPRO11 (Fig. 8A). All of the 10 selected inhibitory HuCAL®-Fab fragments showed a significantly reduced binding to the receptor if FGFR3 was occupied with the ligand. MSPRO6 and other FGFR3-specific binders that did not inhibit FDCP-1-FGFR3 proliferation did not show reduced binding capacity to the FGFR3/FGF9/heparin complex (Fig. 8B).

Figure 8

9. Stability of HuCAL®-Fab fragments

The stability of anti-FGFR3 HuCAL®-Fab fragments in vitro was analyzed. Fab fragments at a concentration of 1 µM were incubated with 10% FCS at 37°C up to thirteen days. The remaining relative binding activities of the antibodies MSPRO2, 11, 12, 21, 26, 28 and 29 were evaluated in Biacore studies and compared to freshly purified Fabs, where the initial slope of binding was set to 100% (Fig. 9). The remaining inhibitory potential was also tested in FDCP-1-FGFR3 proliferation assays (data not shown). None of the tested HuCAL®-Fab fragments showed a significant loss of activity after 4 days at 37°C either in Biacore experiments or in FDCP-1-FGFR3 proliferation assays. After 8 days, all tested Fabs retained more than 80% activity, as determined by Biacore. After 13 days of incubation, the remaining biochemical activity measured in Biacore ranged from 58 to 88%. These results were confirmed in the proliferation assays, which revealed only a weak decrease of FGFR3 neutralizing activity after 13 days at 37°C. These data confirm the high stability of non-
covalently linked Fab fragments as already determined in the pre-experiments with some control HuCAL®-Fab fragments.

Figure 9
Discussion

The HuCAL® concept is based on covering the essential features of the human antibody repertoire with a minimal number of different sequences, which are designed to facilitate extensive engineering with standard techniques. A fully synthetic human antibody library in the scFv format, with a diversity of $2 \times 10^9$, has recently been constructed, and it could be demonstrated that antibodies with high affinity and specificity against any target can easily be isolated from this library using phage display (1) or ribosomal display methods (69).

However, scFv fragments in general have been shown to tend to multimerize in a clone-dependent and unpredictable way (25-27), which often complicates affinity determination due to avidity effects. In addition, phage display of multimeric scFv fragments may lead to avid binding during the panning process, and, therefore, may lead to selection for apparent instead of intrinsic affinity (13). In contrast, Fab fragments do not form multimers (70). Moreover, conversion from the scFv to the Fab format, i.e. removal of the linker sequence between VH and VL and addition of the CH and CL domains, sometimes yields antibodies with reduced or even completely lost binding properties (22), indicating that the linker peptide may sometimes be involved in antigen binding or VH / VL domain orientation. Thus we decided to construct a HuCAL®-Fab library.

Fab fragments derived from IgG1 isotype containing two domains in addition to those of scFv fragments, are built from two genes instead of one, and typically harbor an additional inter-molecular disulfide bridge at the C-terminus. Hence, the functional E. coli expression of Fab fragments usually gives lower yields than that of scFv fragments (71, 72, 22), and this fact is probably one of the major reason why scFv libraries are more widespread than Fab libraries. Before constructing the HuCAL®-Fab 1 library, we therefore
tried to optimize Fab expression using a set of Fab fragments converted from scFv previously selected from the HuCAL®-scFv library.

The Fd chain and the light chain of Fab fragments created by papain digestion of IgG1 are covalently linked by an disulfide bridge located at the beginning of the hinge region, and consequently antibody Fab libraries constructed so far encode this disulfide bridge (70, 20, 73, 13). It is known, however, that the two chains of the Fab fragment self-associate and also interact non-covalently with high affinity (60-62). We rationalized that removal of the two cysteines might improve expression yield, but decrease stability. Indeed, we found that for all three antibodies tested, final yields after purification were up to four-fold higher without the inter-molecular disulfide bond. However, the stability of purified, non-covalently linked Fab fragments against thermal denaturation was reduced only above 60°C under the experimental conditions. Incubation of Fab fragments at 37°C in 10% FCS for up to 13 days, did not lead to any significant reduction of binding activity, regardless of the presence of the C-terminal disulfide bridge. We, therefore, decided to construct the HuCAL®-Fab 1 library without the C-terminal cysteines in the constant domains.

During phage display of Fab fragments only the Fd chain is fused to the phage coat protein, while the light chain gets attached to Fd after folding in the E. coli periplasm by non-covalent interactions only, if the C-terminal cysteines are missing. Therefore, it cannot be excluded that some light chains might get exchanged between different phages in a phage preparation, which would abolish the genotype-phenotype linkage. The non-covalent Fd-light chain interaction was found to be extremely strong (74), however, and extensive usage of the HuCAL®-Fab library proved that it is a reliable source for the isolation of binders against a variety of targets.

The HuCAL® master genes had been constructed originally in both the scFv and the Fab format (see (1)). For the Fab format, the StII signal sequence derived from the E. coli
heat-stable enterotoxin II (65) had been used to direct secretion of the light chain to the periplasm, mainly because StII has already been successfully used for functional Fab secretion (75). Preliminary expression experiments had shown, however, that light chains equipped with StII are less well expressed than light chains preceded by the OmpA signal sequence (66). We found that the replacement of StII by OmpA leads to about two-fold higher expression yields and therefore, decided to use the OmpA signal sequence for all Fab light chains. The HuCAL®-Fab 1 library was finally constructed by inserting the diversified variable regions, transferred from the HuCAL®-scFv libraries, into the phage display vector pMORPH®18 containing the HuCAL® master genes in the Fab configuration. We omitted the VH4 master gene, since the extensive usage of the HuCAL®-scFv library has shown that this particular gene is only very rarely obtained after selection (1). A detailed analysis (76) has shown that the VH4 domain is less stable and not as efficiently expressed as other domains. It seems possible, however, to improve the VH4 properties by changing crucial amino acid residues. Respective experiments were performed already, and the selection frequency of VH4 could be increased. After re-cloning, the original diversity of the HuCAL®-scFv library (2 \( \times \) 10^9 independent members) was increased 10-fold, since 2.1 \( \times \) 10^{10} colonies could be obtained after electroporation, and hence about 10 times more H / L combinations have been created. The library quality was analyzed by sequencing, and almost 70% of all clones were found to be entirely correct.

The facts that HuCAL®-Fab 1 is a fully human antibody library and that, due to the modularity of HuCAL®, conversion into full immunoglobulins as well as antibody optimization is rapidly feasible render this new library ideally suited for the selection of antibodies for therapeutic application.

We, therefore, tested HuCAL®-Fab 1 against FGFR3, a receptor tyrosine kinase involved in skeletal disorders like achondroplasia and in tumor genesis. A potential
therapeutic antibody candidate should be fully human, display high affinity and specificity to its target and efficiently block FGFR3 activity.

Several antibodies to FGFR3 had been described in the past. Monoclonal antibodies were derived from an in vitro immunization approach and used for immunohistochemistry (77). Otherwise, mainly polyclonal antibody sera were raised by immunizing rabbits with peptides or domains of the human FGFR3 (78, 79). A blocking antibody to human FGFR3, which inhibits its signaling activity, had not been described so far. Difficulties in making monoclonal antibodies in mice could be due to the high homology of the mouse and human FGFR3 amino acid sequence. Human FGFR3 has an overall sequence homology of 92% to the mouse equivalent (80). We overcame homology problems and succeeded in rapidly isolating a plethora of specific antibodies against FGFR3 using the synthetic yet fully human HuCAL®-Fab library.

The members of the FGFR family (FGFR1 – 4) show an overall amino acid identity of 48% to 69% and some sub-regions are up to 100% identical (31, 32, 81). Additionally, FGFR1 and FGFR3 have similar ligand-binding pockets, and some (but not all) FGF ligands can activate both receptors (81). Thus, generation of antibodies specific to FGFR3 could be severely hampered as seen with other anti-FGFR antibodies cross-reacting with at least one other member of the family (77, 82). We also generated a variety of FGFR1/FGFR3 cross-reactive binders, but additionally, Fab antibodies discriminating between FGFR1 and FGFR3 were identified.

Sequence analysis of H-CDR3 revealed a length variation from 8 to 20 amino acids, covering a broad range of naturally occurring CDR3 lengths (83). FGFR3 antibodies were found from all VH families present in HuCAL®-Fab 1, although the VH1 family was the dominant source for FGFR3 binders providing 73% of the selected Fabs. We have seen such a prevalence of certain VH families over the others already in the HuCAL®-scFv library (22)
and also in applying HuCAL®-Fab 1 to other antigens. The dominating VH family varies and is dependent on the antigen. As a result, we conclude that it is advantageous for a library to contain a set of different VH family frameworks to ensure successful selections against a variety of antigens.

With a unique, differential whole cell panning approach (22), we succeeded in isolating 25 of 37 analyzed antibodies from HuCAL®-Fab 1 that blocked FGFR3 mediated cell proliferation. Of these 25 inhibitory clones, 13 clones had an affinity below 50 nM, and of these 13 clones, 6 Fabs blocked only FGFR3 activity and not FGFR1.

Competition experiments revealed, that we identified two groups of FGFR3 neutralizing antibodies binding to different regions on FGFR3, yet, both efficiently blocking the binding of the ligand FGF9 to FGFR3. These antibodies will be useful for further research and could be developed for e.g. therapy of achondroplasia caused by the G380R mutation of FGFR, since this activated receptor isoform was described to be still predominantly ligand dependent (51, 84). Additionally, it was speculated that disregulation of the wildtype FGFR3 in multiple myeloma supports tumor growth (42, 44). The inhibitory anti-FGFR3 antibodies could thus also represent a new treatment for this blood borne malignancy.

For FGFR3, some mutations are described, leading to ligand independent activation of FGFR receptors e.g. the constitutively active receptor mutant FGFR3S249C (85, 45), which is present in e.g. about 30% of bladder carcinomas (45-47). Antibodies, inhibiting such ligand-independent signalling of constitutively active mutated FGFR3 receptor forms, would be of special interest for cancer treatment. Therefore, experiments have been initiated, to evaluate whether the HuCAL® anti-FGFR3 Fabs inhibit different activated receptor forms as well.

We identified several HuCAL®-Fabs that may become promising candidates for a future therapeutic development program. To the best of our knowledge, these are the first
human monoclonal antibodies that are able to specifically block FGFR3 activity. They display a high selectivity for FGFR3, without cross-reactivity to FGFR1, and have an affinity in the sub-nanomolar and an IC₅₀ in the low nanomolar range. The monomeric affinities (0.7 - 0.9 nM) of these non-optimized HuCAL®-Fabs are similar to affinities of other therapeutic antibodies against receptor tyrosine kinases, e.g. VEGFR2, c-erbB2 or EGFR which are in the range of 50 pM to 1 nM for bivalently binding IgGs (86-89).

At least two different inhibitory binding regions were defined. It will have to be tested which group will be more efficient in blocking FGFR3 activity in vivo. In the future, the efficacy of the HuCAL® anti-FGFR3 Fabs will be tested in different in vitro and in vivo settings in order to characterize their therapeutic potential for achondroplasia and tumor treatment. As HuCAL® enables the rapid generation of different antibody formats via one step cloning procedures (22), it will be a fast process to define the best format for each indication.

Here, we report the generation of HuCAL®- Fab1, a fully human phage display library, applying the HuCAL®-concept on the Fab-format. HuCAL®-Fab1 was challenged against the tyrosine kinase receptor FGFR3 playing a crucial role in achondroplasia and tumor genesis. For the first time FGFR3 specific and inhibiting antibodies have been isolated with the potential for further pre-clinical and clinical development.

In the meantime we have further developed our HuCAL®-Fab technology by constructing the HuCAL®-GOLD library. HuCAL®-GOLD is a Fab library, based on the CysDisplay™ technology (4, 90), with all 6 CDRs being diversified according to the natural composition of antibody CDR regions.
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Figure Legends:

Figure 1: Arrangement of HuCAL®κ (a)- and λ (b)-Fabs and key features of the vectors (c). Some of the unique restriction sites common to all master genes are shown.

Figure 2: Thermal stability of non-covalently (Fab) and disulfide-linked (FabCys) Fab-fragments. Two different binders (Fab1 has a λ light chain, Fab2 a κ-chain) with and without intermolecular disulfide bond were incubated at the indicated temperatures for 30 min, aggregates removed and supernatants tested for binding activity in Biacore. Resonance units at 4°C were set to 100%.

Figure 3: Comparison of the StII and OmpA signal sequence for VL in respect of Fab expression. Two control Fabs (Fab1 has a λ light chain, Fab2 a κ-chain) with StII and OmpA signal sequence for VL were expressed and equal amounts of crude periplasmic extracts were analysed for antigen binding by ELISA. ELISA signals reflecting expression differences of functional Fab, were recorded at 370 nm.

Figure 4: Specificity of selected HuCAL®-Fab fragments was tested in ELISA. FGFR1-Fc ( ), FGFR3-Fc (■), or human IgG as a control (□) was captured by a goat anti-human IgG (Fcγ specific), and incubated with the indicated HuCAL®-Fabs. Specific binding was detected by peroxidase staining.

Figure 5: Example of FGFR3 specificity and FGFR3/FGFR1 cross-reactivity of selected HuCAL®-Fab fragments was tested in FACS analysis. RCJ-FGFR3 (-tet), RCJ-FGFR1 (-tet) and RCJ-FGFR3 (+tet) were incubated with the FGFR3 specific MSPRO24 and
the FGFR1 and FGFR3 crossreactive MSPRO28. FGFR3 or FGFR1 expression, respectively, was detected by staining with the secondary goat anti-human IgG conjugated with R-phycoerythrin in a FACS setting (black line). Grey histograms indicate staining with the secondary antibody only.

**Figure 6: Inhibitory effect of selected HuCAL®-Fabs on FGFR3 mediated cell proliferation.** FDCP-1-FGFR3 cells were grown in the presence of FGF9. Increasing amounts of the indicated HuCAL®-Fabs were added. After two days proliferation was determined by adding XTT reagent (OD415). Inhibition of proliferation (IC₅₀ values) was calculated by a sigmoidal curve fit of the data.

**Figure 7: Example of competition for FGFR3 binding epitopes by HuCAL®-Fabs in Biacore.** First, HuCAL®-Fab MSPRO11 bound to FGFR3 until saturation was reached. Then a second HuCAL®-Fab (either MSPRO12 ----, MSPRO2 ·····, MSPRO21--- ·, or buffer - - - - ) was injected (indicated by an arrow). Additional increase in response units resulted from binding to different, non-overlapping epitopes (e.g. MSPRO11 and MSPRO2). No increase in response units (RU) meant that the two Fabs recognized the same or overlapping epitopes on FGFR3 (e.g. MSPRO11 and MSPRO12).

**Figure 8: Example of competition of ligand and HuCAL®-Fab binding to FGFR3.** Binding of selected HuCAL®-Fabs was tested in Biacore either to FGFR3-Fc alone (---) or to FGFR3-Fc/FGF9 complex (FGFR3-Fc saturated with 1 μM FGF9 (······)). Timepoint of Fab injection is indicated by an arrow. A smaller increase of response units on the FGFR3/FGF9 complex compared to FGFR3-Fc alone is seen when Fabs compete for the
binding epitope on FGFR3-Fc with FGF9, e.g. MSPRO11 (A). In contrast binding intensity of
the non-inhibitory MSPRO6 is not affected by binding of FGF9 to FGFR3-Fc (B).

**Figure 9: Stability of selected HuCAL®-Fab fragments.** 7 different anti-FGFR3 HuCAL®-
Fabs were incubated for 0, 4, 8 and 13 days at 37°C. Activity of MSPRO2(♦), MSPRO11(■),
MSPRO12(▲), MSPRO21(◆), MSPRO26(□), MSPRO28(●) and MSPRO29(∆) was analyzed
in Biacore. Activity means initial slope of binding normalized to the freshly purified Fab.

**Acknowledgement**

We thank Dr. Margit Urban, Dr. Bernhard Virnekaes and Dr. Thomas von Rüden for critical
reading of the manuscript; Dr. Herbert Weich (GBF Braunschweig, Germany) for providing
recombinant FGF9, Dr. Josef Schlessinger (Department of Pharmacology, New York
University School of Medicine) for providing the FGFR1 cDNA and the entire team of
technicians at MorphoSys AG for the excellent technical assistance.
**TABLE1**

*Selection of FGFR3 specific HuCAL®-Fabs from two independent pannings.*

<table>
<thead>
<tr>
<th>Panning</th>
<th>Screened clones</th>
<th>Primary hits (%)</th>
<th>Sequenced clones</th>
<th>Unique sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1076</td>
<td>210 (19.5%)</td>
<td>69</td>
<td>15</td>
</tr>
<tr>
<td>B</td>
<td>1632</td>
<td>521 (31.9%)</td>
<td>120</td>
<td>34</td>
</tr>
<tr>
<td>Total</td>
<td>2708</td>
<td>731 (26.9%)</td>
<td>189</td>
<td>49 *</td>
</tr>
</tbody>
</table>

* 37 of these unique clones were analyzed in more detail.
**TABLE 2**

*Inhibition of FGFR3 dependent cell proliferation (IC$_{50}$) by selected HuCAL$^\text{®}$-Fabs and affinity to FGFR3 (K$_d$)*

<table>
<thead>
<tr>
<th>HuCAL$^\text{®}$-Fab</th>
<th>IC$_{50}$ (nM)</th>
<th>Kd in Biacore (nM)</th>
<th>Kd in FACS (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSPRO2</td>
<td>360</td>
<td>37</td>
<td>43*</td>
</tr>
<tr>
<td>MSPRO11</td>
<td>220</td>
<td>4</td>
<td>4*</td>
</tr>
<tr>
<td>MSPRO12</td>
<td>58</td>
<td>14</td>
<td>6.5*</td>
</tr>
<tr>
<td>MSPRO21</td>
<td>50</td>
<td>9</td>
<td>1.1</td>
</tr>
<tr>
<td>MSPRO24</td>
<td>70</td>
<td>10</td>
<td>n.d.</td>
</tr>
<tr>
<td>MSPRO26</td>
<td>50</td>
<td>4</td>
<td>1.4*</td>
</tr>
<tr>
<td>MSPRO28</td>
<td>40</td>
<td>9</td>
<td>0.7</td>
</tr>
<tr>
<td>MSPRO29</td>
<td>20</td>
<td>6</td>
<td>0.9</td>
</tr>
<tr>
<td>MSPRO54</td>
<td>45</td>
<td>3.7</td>
<td>5*</td>
</tr>
<tr>
<td>MSPRO59</td>
<td>19</td>
<td>1.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

All values are calculated from three independent experiments except the numbers marked by * which are derived from single measurements.
TABLE3

*Competition of selected inhibitory HuCAL<sup>®</sup>-Fabs for the binding epitope on FGFR3*

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSPRO11</td>
<td>MSPRO2</td>
</tr>
<tr>
<td>MSPRO21</td>
<td>MSPRO12</td>
</tr>
<tr>
<td>MSPRO24</td>
<td>MSPRO59</td>
</tr>
<tr>
<td>MSPRO26</td>
<td></td>
</tr>
<tr>
<td>MSPRO28</td>
<td></td>
</tr>
<tr>
<td>MSPRO29</td>
<td></td>
</tr>
<tr>
<td>MSPRO54</td>
<td></td>
</tr>
</tbody>
</table>

*HuCAL<sup>®</sup>-Fabs inhibiting FGFR3 dependent cell proliferation can be divided in two groups. Binders of the different groups do not compete for the same or overlapping epitopes on FGFR3 in BIAcore analysis.*
Figure 1

<table>
<thead>
<tr>
<th>Vector</th>
<th>Fab</th>
<th>Function</th>
<th>VL Signal sequence</th>
<th>EcoRI/HindIII-fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMORPH®X7_Fab_FS</td>
<td>non-covalently linked</td>
<td>expression</td>
<td>StII</td>
<td>FLAG (M2)-tag</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>StreptagII</td>
</tr>
<tr>
<td>pMORPH®X7_FabCys_FS</td>
<td>disulfide-linked</td>
<td>expression</td>
<td>StII</td>
<td>FLAG (M2)-tag</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>StreptagII</td>
</tr>
<tr>
<td>pMORPH®X9_Fab_FS</td>
<td>non-covalently linked</td>
<td>expression</td>
<td>OmpA</td>
<td>FLAG (M2)-tag</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>StreptagII</td>
</tr>
<tr>
<td>pMORPH®18_Fab</td>
<td>non-covalently linked</td>
<td>phage display</td>
<td>OmpA</td>
<td>gene III, first 249 codons deleted</td>
</tr>
</tbody>
</table>

**Legend:**
- **a** and **b** depict the structural diagrams of vectors with different expression systems.
- **Fab** refers to the fragment antigen-binding domain.
- **Function** indicates whether the expression is for antibody or phage display.
- **VL Signal sequence** refers to the signal sequence for VL domain.
- **EcoRI/HindIII-fragment** specifies the tagged regions for each vector.

**Notes:**
- The diagrams illustrate the gene constructs, showing the signal sequences, the frame of the Fab domain, and the tag sequences.
- The expression systems include non-covalent linking and disulfide bonding.
- The phage display system utilizes an OmpA gene for display on the bacterial cell surface.

**References:**
- The vectors are part of the pMORPH® series provided by the company.
- The constructs are designed for various cellular expression platforms.
Figure 2.

**Fab1/FabCys1**

**Fab2/FabCys2**

Resonance units in %

°C
Figure 3

OD at 370 nm

Fab1

Fab2

StII

OmpA

blank
Figure 4

The graph shows the Optical Density (OD) at 370 nm for different Fab fragments. The x-axis represents the Fab fragments with controls labeled as PBS, Fab, MSPRO2, MSPRO11, MSPRO12, MSPRO21, MSPRO24, MSPRO26, MSPRO28, and MSPRO29. The y-axis represents the OD ranging from 0.0 to 1.0.

- **FGFR1-Fc** is represented by light grey bars.
- **FGFR3-Fc** is represented by dark grey bars.
- **hIgG** is represented by white bars.

The graph indicates variations in OD across different Fab fragments, with some showing higher OD values than others.
Figure 5

MSPRO24

MSPRO28

Cells

RCJ-FGFR3 (-tet)

RCJ-FGFR1 (-tet)

RCJ-FGFR3 (+tet)
Figure 6

**MSPRO29; IC$_{50}$=0.019 µM**

**MSPRO21; IC$_{50}$ = 0.068 µM**
Figure 8

A

MSPRO11

B

MSPRO6
Figure 9

Stability of HuCAL®- Fabs

Incubation at 37°C (days)

activity (%) - BIAcore

- MSPRO2
- MSPRO11
- MSPRO12
- MSPRO21
- MSPRO26
- MSPRO28
- MSPRO29
Human combinatorial Fab Library yielding specific and functional antibodies against the human fibroblast growth factor receptor 3

J. Biol. Chem. published online July 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303164200

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