Selecting antagonistic antibodies that control differentiation through inducible expression in embryonic stem cells

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Antibodies that modulate receptor function have great untapped potential in the control of stem cell differentiation. In contrast to many natural ligands, antibodies are stable, exquisitely specific, and are unaffected by the regulatory mechanisms that act on natural ligands. Here we describe an innovative system for identifying such antibodies by introducing and expressing antibody gene populations in ES cells. Following induced antibody expression and secretion, changes in differentiation outcomes of individual antibody-expressing ES clones are monitored using lineage-specific gene expression to identify clones that encode and express signal-modifying antibodies. This in-cell expression and reporting system was exemplified by generating blocking antibodies to FGFR4 and its receptor FGFR1c, identified through delayed onset of ES cell differentiation. Functionality of the selected antibody-expressingReporter cell lines was confirmed by addition of exogenous antibodies to three different ES reporter cell lines, where retained expression of pluripotency markers Oct4, Nanog, and Rex1 was observed. This work demonstrates the potential for discovery and utility of functional antibodies in stem cell differentiation. This work is also unique in constituting an example of ES cells carrying an inducible antibody that causes a functional protein “knock-down” and allows temporal control of stable signaling components at the protein level.

Cellular communication mediated through surface receptors plays a critical role in development and in maintaining homeostasis in adulthood. Antibodies, such as Humira and Avastin, which activate or block receptor function, have proven value in therapeutic applications (1, 2). Functional antibodies also have great potential in stem cell biology, but the realization of this has been limited because only a fraction of antibodies affect receptor function and their identification is laborious. Functional antibodies are required to recognize the native conformation of the target receptor or ligand and to bind an appropriate epitope with high affinity. Identifying such antibodies therefore requires ELISA screening of large numbers of candidates to identify binding clones, followed by expression, purification, and assessment of individual antibodies using target-specific reporter cell assays. The required antibody diversity can be accessed by phage or yeast display, which can generate hundreds of antibodies to a single target (3, 4). The ready availability of the antibody gene from display technologies permits reformatting and production in mammalian cells to generate antibody products for cell-based screening. Importantly, access to the antibody gene also creates the potential for direct expression within mammalian reporter cells, thereby permitting antibody production and functional screening in one cell. Alterations in the characteristics of the antibody-expressing reporter cell could then identify clones encoding functional antibodies. This potential has recently been exemplified through the lentiviral infection of a TF1 reporter cell line with an antibody population, leading to the identification of secreted antibodies, which activate the erythropoietin receptor (5). In an alternative approach, antibodies were retained at the cell surface of BaF3 reporter cells, leading to the identification of an agonistic antibody to the granulocyte colony-stimulating receptor (6).

Pluripotent embryonic stem cells (ES cells) represent an ideal reporter cell system for identifying functional antibodies because they are poised to differentiate into many different cell types in vitro (7). ES cell fate decisions are influenced by a wide range of cell-surface receptors (7–9), creating the potential to target many different classes of ligands and receptors (e.g., receptor tyrosine kinases, G protein-coupled receptors, ion channels, integrins, and cadherins) with antibodies. Irrespective of the signaling pathway involved, the differentiation status of antibody-producing ES clones can be conveniently monitored using lineage-specific promoters driving fluorescent reporter genes or immunostaining with appropriate antibodies. Thus, measuring perturbations in ES cell differentiation represents a sensitive and flexible approach to identify antibodies modifying receptor function.

Results

Establishing an In-Cell Expression and Reporting System Allowing Inducible Antibody Expression and Secretion in ES Cells. An in-cell expression and reporting system (ICER system) was developed

Significance

The ability to control and direct differentiation of stem cells for research or therapeutic applications relies on the availability of ligands that control specific signaling pathways. Natural ligands exhibit promiscuous interactions and have limited availability because of their poor expression/stability profiles. In contrast, antibodies exhibit exquisite specificity and have optimal expression properties. Antibodies that block or activate receptor signaling have great potential for controlling differentiation, but their identification is laborious. Herein we describe an innovative system for identifying functional antibodies by introducing antibody gene populations into ES cells. Antibody-expressing ES clones with altered differentiation outcomes can be readily identified using lineage-specific gene-expression markers. The antibody gene can then be recovered for antibody production and use.

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using ES cells to efficiently identify functional antibodies within a population of target-specific binders. Mouse ES cells are readily modified through gene targeting, allowing insertion of individual antibody genes into a single locus in each cell. This process normalizes expression within the cell population and facilitates the identification of functional antibody genes. In the work presented here, homologous recombination was used to target antibody gene populations into the ubiquitously expressed reverse orientation splice acceptor betago line 26 (Rosa-26) locus. An antibody expression cassette was introduced within a 10-kb region of the Rosa-26 gene, which has previously been used for targeting the mouse genome (10). Within the resulting targeting vector (pROSA-ic) (Fig. 1A), expression of the antibody gene was controlled by a doxycycline-responsive promoter allowing control of antibody expression.

The capacity of the system to achieve gene targeting and controlled antibody expression was initially tested using control anti-Notch antibodies (11). Following transfection, stable puromycin-resistant clones were selected, genomic DNA prepared, and correct integration of the targeting vectors was confirmed by PCR. Western blot analysis (Fig. 1B) confirmed that doxycycline-dependent antibody secretion occurred within the resultant clones. ELISA was used to measure antibody concentration (1–2 μg/mL after 4-d induction) and to confirm binding to antigen.

FGF4 Signaling as a Target for Antibody-Mediated Control of Differentiation. The potential of the ICER system for selection of functional antibodies was exemplified in ES cells (ES-ICER) by the generation of novel antagonists of FGF4 (fibroblast growth factor 4) signaling. In mouse ES cells, autocrine FGF4 activates FGF receptors (FGFRs), providing a permissive signal via extracellular-signal-regulated kinases (ERKs) that allows cells to respond to differentiation cues (12–14). This FGF-mediated differentiation capacity can be blocked by selective small-molecule inhibitors of either FGFR tyrosine kinases or ERK kinase (12). In mouse ES cells, FGF4 is the major activator of ERK. It would therefore be anticipated that ES cells expressing blocking antibodies to FGF4 activity would be shielded from the FGF4-mediated response to differentiation cues, resulting in retained expression of “pluripotency genes,” such as Oct4 (15), Rex1 (16), and Nanog (17), even under differentiation culture conditions.

Fig. 2 summarizes the experimental strategy used. Antibody populations were derived from phage display by selection on target antigen (although the ICER system could equally be applied to antibody gene populations derived from other display methods or from immunized animals). Phage display selections were carried out on FGF4, FGFR1β, and FGFR2β [the IIIc splice variants of FGFR (18)] and generation of binding antibodies was confirmed by ELISA (Fig. S1). Selected antibody populations were cloned into pROSA-ic before targeting into the Rosa-26 locus of mouse Oct4-ΔPE-GFP ES reporter cells (19). In these cells, green fluorescent protein (GFP) is expressed under the control of the distal enhancer of the Oct4 gene, which specifically reports the ES cell state. Following selection of puromycin-resistant colonies, culture medium was changed from self-renewal medium [serum/Leukemia inhibitory factor (LIF)] to medium inducing differentiation (ES-Cult/N227). Expression and secretion of the introduced antibodies was induced by addition of doxycycline. To enable screening of the population en masse, it was necessary to retain the antibodies in the vicinity of the producer cell to prevent “cross-talk” between clones, and this was achieved through growth in semisolid medium. This process has the added benefit of allowing accumulation of higher antibody concentrations in the vicinity of the producer colony compared with liquid culture.

ES Clones Expressing FGF4-Blocking Antibodies Resist Differentiation. The potential for antibody-induced retention of Oct4-GFP expression was initially assessed using flow cytometry of Oct4-Δ

**Fig. 1.** Construction of the pROSA-ic vector for targeting antibody expression cassettes into the ubiquitously expressed Rosa-26 locus of mouse ES cells. (A) Antibody genes (in the form of single-chain Fvs) were subcloned into the Ncol and NotI sites of the pBIOCAM5-GW entry vector to create a fusion with a human IgG1 Fc gene. LR Gateway recombination was used to facilitate the introduction of this scFv-Fc fusion gene into the large (14 kb) pGATOR-DTA targeting vector. The resultant pROSA-ic construct encompasses an antibody gene expression cassette within a 10-kb region of the Rosa-26 locus of mouse Oct4-ΔPE-GFP ES reporter cells. (B) Doxycycline-inducible antibody expression in E14 ES cells. An anti-Notch antibody N1_E7 (11) was subcloned into pROSA-ic and the resultant targeting vector was used to transfect E14 ES cells. Correctly targeted puromycin-resistant colonies were selected and grown under self-renewal conditions with 0, 0.1, or 2 μg/mL doxycycline (lanes a–c, respectively). After 4 d expressed antibody was affinity-purified using anti-FLAG agarose. Samples were run on SDS/PAGE, blotted, and the expressed scFv-Fc was detected with an anti-FLAG mouse antibody and a secondary anti-mouse IR680-labeled antibody (Lico).
PE-GFP ES transfected with the anti-FGF4 antibody population (Fig. S2). After 3–4 d differentiation in the absence of antibody induction, only 76.7% of transfected Oct4–ΔPE-GFP cells retained GFP expression. In contrast, addition of the ERK kinase inhibitor PD0325901 (PD03), which blocks FGFR-mediated signaling (14), inhibited differentiation and caused retention of GFP expression in 98.8% of cells. Induction of antibody expression by addition of doxycycline also maintained GFP expression in 95.2% of cells, suggesting the existence of a proportion of blocking antibodies within the population. Maintenance of the undifferentiated state in the resultant population was independently confirmed through quantification of alkaline phosphatase-positive colony formation following a return of the cells to self-renewal conditions (Fig. S3). This result showed increased clonogenicity of the cells grown in the presence of doxycycline during earlier exposure to differentiation conditions.

Direct observation by fluorescent microscopy also demonstrated antibody-directed inhibition of differentiation within individual colonies. In control cultures differentiated for 3 d in the absence of doxycycline, the expected reduction in GFP expression was observed along with a loss of the compact, round colony shape seen in undifferentiated cultures (Fig. S4). PD03 helped retain the compact morphology and fluorescence (Fig. 3B), whereas all ES colonies grown in self-renewal conditions (–dox/2i) retained their compact shape and high levels of GFP expression (Fig. S3A). In the presence of doxycycline, ES colonies grown under differentiation conditions showed a range of phenotypes (Fig. S4). Among these colonies, ~5% retained the compact shape and GFP fluorescence of the undifferentiated colonies observed when grown under self-renewal conditions (Fig. 3D and Fig. S4). This approach therefore allowed the in situ identification of the few select ES colonies maintaining an undifferentiated phenotype. Four colonies (of 217) from the anti-FGF4 population were picked and propagated under self-renewal conditions, where they demonstrated their retained undifferentiated state, despite earlier exposure to differentiation conditions. Furthermore, upon secondary testing in liquid differentiation culture (i.e., in the absence of methylcellulose) for 7 d, a doxycycline-dependent resistance to differentiation was demonstrated in two clones, FGF4_A and FGF4_C (Fig. 4 and Fig. S5).

![Diagram](https://example.com/diagram.png)

**Fig. 2.** Overview of the experimental strategy (numbered 1–6) to identify antibody-expressing ES colonies that resist differentiation through blockade of FGF signaling. Antibody populations binding to FGF4, FGFR1, and FGFR2 were selected from a phage display library and were cloned into the targeting vector pROSA-ic. Homologous recombination was used to direct integration into the ubiquitously expressed Rosa26 genomic locus of individual Oct4–ΔPE-GFP ES cells. Puromycin-resistant colonies were selected, antibody expression was induced, and ES colonies were subjected to differentiation in semisolid medium for 2.5–4.5 d. Clones that resisted differentiation (judged by retention of round morphology and Oct4–ΔPE-GFP expression) were picked and the antibody genes retrieved by PCR for further use.

![Phagocytosis](https://example.com/figure.png)

**Fig. 3.** Colonies obtained following transfection of Oct4–ΔPE-GFP ES cells with an anti-FGF4 antibody population, after 3 d of growth in differentiation medium ES-Cult/N227. (A–C) Typical colonies obtained in the absence of doxycycline under the indicated conditions. (D) Example of colony retaining an undifferentiated phenotype, obtained in the presence of doxycycline (1 μg/mL) (magnification 20x).
different markers of pluripotency, the ES-ICER system was shown to permit the identification of functional antibodies that delayed the onset of ES cell differentiation. Furthermore the selected antibodies were specific to FGF4 as judged by their lack of binding to acidic and basic FGF (FGF1 and FGF2) by ELISA (Fig. S7A, antibody sequences shown in Fig. S7C).

Selection of Anti-FGFR Antibodies, Which Inhibit ES Cell Differentiation.

Using the strategy outlined above, Oct4−ΔPE-GFP ES cells were transfected with antibody populations selected by phage display on FGFR1β and FGFR2β (Fig. 6, and Figs. S8 and S9). For FGFR1β and FGFR2β, 3 of 462 and 4 of 132 colonies, respectively, were found to retain a clear undifferentiated phenotype in semisolid medium (Fig. 6A and Fig. S9B) and could be subsequently propagated under self-renewal conditions. Upon addition of exogenous FGFR1β antibodies, only one clone retained Rex-1 GFP expression when the respective purified antibody (α-FGFR1_A) was added exogenously to Rex1-GFPd2 ES cells grown in differentiation conditions (Fig. 6B and C). To investigate this further, an in vitro biochemical assay was established, which recapitulated the interaction of FGF receptors with immobilized FGF4/heparin. In this assay all three antibodies inhibited the ligand:receptor interaction, although α-FGFR1_A demonstrated the greatest potency (Fig. S8F). In the case of FGFR2β, addition of exogenous antibodies failed to inhibit differentiation of Rex1-GFPd2 cells (Fig. 6C and Fig. S9C), although all showed blocking activity in the biochemical assay (Fig. S9D). All antibodies were shown by ELISA to be specific for the appropriate FGF receptor (Fig. S7B). These results suggest that the primary differentiation assay was sufficiently sensitive to identify specific functional antibodies covering a range of potencies (probably aided by the high local

![Fig. 4. Selected anti-FGF4 clones maintain Oct4-ΔPE-GFP expression in liquid differentiation in the presence of doxycycline. (A) Cells from the αFGF4_A and αFGF4_C clones were analyzed by flow cytometry after 7-d growth in differentiation conditions in the absence or presence of doxycycline (1 μg/mL) or in the presence of 2 μM control Erk kinase inhibitor PD0325901 (PD03). (B) Phase and fluorescent images of Oct4-ΔPE-GFP cells expressing the α-FGF4A antibody after 7 d of differentiation in N227 in the presence or absence of doxycycline. The differentiation experiment was performed two times with similar results (magnification 20x).](image)

![Fig. 5. Exogenous anti-FGF4 antibodies cause retained expression of pluripotency markers when added to the three independent ES cell lines. (A) Oct4-ΔPE-GFP ES cells grown in differentiation medium for 7 d in the presence or absence of purified αFGF4_A antibody (20 μg/mL) and compared with PD03 control (magnification 20x). (B) Flow cytometry of Rex1-1GFPd2 ES cells after 3 d of differentiation in the presence of αFGF4_A and compared with PD03 control. (C) Flow cytometry of Nanog-GFP ES cells after 3 d of differentiation in the presence of αFGF4_A and compared with PD03 control. (D) Percentages of cells expressing Rex1-GFP after 3 d of differentiation in the presence or absence of the indicated purified anti-FGF4 antibodies (20 μg/mL), negative (α-desmin and Fc only) and positive (PD03) controls. The bars represent the average of triplicates of one flow cytometry experiment ± SD. The asterisk denotes statistical significance. P = 0.0017 for αFGF4_A vs. antidesmin. In addition, P = 0.004 for αFGF4_C vs. α-desmin.)](image)
antibody concentration in the semisolid medium). Only those antibodies with the greatest potency (demonstrated for FGF4 and FGFR1β) inhibited differentiation when exogenously added to the cells. For less-potent clones, affinity maturation (21) could be used to improve the affinity/potency following their identification from the initial screen.

**Discussion**

In this article we introduce a system for selecting functional antibodies within populations through the expression of secreted antibodies causing perturbation of receptor function in embryonic stem cells (ES cells). Zhang et al. (5) have recently described a similar approach, using secreted antibodies retained in the semisolid medium, to identify agonists of the erythropoietin receptor (using TF1 erythroleukemic reporter cells transfected with the erythropoietin receptor). As an alternative, agonistic antibodies recognizing G-CSF receptor (G-CSFR) were also identified by anchoring the expressed antibodies to the membrane of a G-CSFR–transfected BaF3 cell line (6). Importantly the isolated anti–G-CSFR antibody was shown on subsequent addition to CD34 hematopoietic stem cells to cause transdifferentiation, unlike the natural ligand. In the ES-ICER system described here, antagonistic antibodies were selected. These antibodies were identified using pluripotent ES cells and were targeted to either autocrine ligand (FGF4) or endogenous FGF receptor.

The key elements of the ES-ICER system involve targeting of single antibody genes from preselected populations into the Rosa-26 locus of mouse ES cells to create a combined expression/reporting cell system, allowing autoexpression and detection of an altered phenotype. The approach could be extended to identify functional peptides or alternative binding scaffolds. This method could be used to generate antibodies, both agonistic and antagonistic, to the many ligands/receptors that are expressed during ES differentiation. Our growing knowledge of the role of these molecules in cell fate determination, combined with the development of more efficient protocols for directed stem cell differentiation, can be exploited to extend the versatility of this approach. In this study, Oct4 gene expression was used to monitor differentiation status but phenotypic changes could be detected by other lineage-specific reporters, by immunostaining or through changes in morphology.

The principle use of functional antibodies to date has been as disease-modifying drugs in oncology and immunology, and the presented ICER system will facilitate future drug discovery efforts. With their optimal stability and expression properties, functional antibodies also have great, untapped potential in the control of stem cell fate. In particular, the exquisite specificity of antibodies confers advantages over natural receptor agonists, which often exhibit promiscuous interactions and are subject to regulatory feedback mechanisms. Functional antibodies could therefore transform methods for the derivation and control of human ES/iPS cells lines, creating human cell lines for research and valuable models of embryogenesis. Ultimately, the ability to control differentiation of genetically unmodified human stem cells through the administration of functional antibodies or peptides, could unleash new ex vivo or in vivo approaches to stem cell-based therapeutics.

**Methods**

**Targeting Antibody Genes to the ROSA-26 Locus.** The Rosa-26 targeting vector pGATOR was modified to insert the Dipheria toxin fragment A (DTA) within the vector backbone. Homologous recombination will result in the loss of the DTA gene, whereas random plasmid integration will result in DTA expression, which will cause cell death, thereby reducing the background from random integration. The DTA gene was PCR-amplified from pL3I4- DTA (EUCOMMtools) and cloned into the SfiI site of the pGATOR vector to create pGATOR-DTA (SI Methods). pBIOCAM5-GW was created by PCR amplification from the pBIOCAM plasmid to give a PCR product encoding a single chain Fv (scFv)-Fc fusion flanked by the attB1 and attB2 GATEWAY recombination sites (22), which was then BP recombined with pDONR221 (Invitrogen) to give pBIOCAM5-GW. Selected antibody gene populations (or the previously created anti-Notch clones) (11) were amplified by PCR, as described previously (4), restriction digested, and ligated into the NcoI/NotI sites of pBIOCAM5-GW. Ligated DNA was electrotransferred into Escherichia coli DH5α cells to produce an average library size of 2 × 10^5 clones. DNA was prepared from this pBIOCAM-GW library entry plasmid and was LR recombined with pGATOR-DTA. The resultant plasmid was introduced into chemically competent E. coli DH10B cells, where an average library size of 3,300 clones was created for the anti-FGFR4, anti-FGFR1β, and anti-FGFR2β populations. Plasmid DNA was prepared, linearized with SfiI, extracted with phenol/chloroform, ethanol-precipitated, and dissolved in TE (pH 8.0) to give a concentration of 1–2 μg/μL of linearized targeting vector.

**ES Cell Lines and Culture.** E14tg2a ES cells were described previously (22). Oct4–ΔPE-JPF-GFP ES cells were from gain of function (GOF) mice, which in GFP is specifically expressed in the preimplantation embryo and primordial germ cells but not the postimplantation epiblast (19). In culture, GFP is expressed in the ES cells but not the epiblast stem cell (EpSCb). Both E14tg2a and Oct4–ΔPE-JPF-GFP ES cells were maintained on tissue-culture dishes coated with gelatin (0.1% swine skin), in GMEM (Sigma) supplemented with: recombinant LIF (100 U/mL), 15% (v/v) FCS (Sigma selected batch), 1 mM sodium pyruvate (Invitrogen), 2 mM l-glutamine (Invitrogen), 1 μM MEM nonessential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen). For experiments involving doxycycline-induced antibody secretion in the medium, 1.5 × 10^5 utility in the exploration of receptor function both in vitro and in vivo. These clones, encoding an inducible functional antibody, will permit exquisite temporal control of signaling components at the protein level, enabling protein knockdown in diverse developmental contexts.

Although the approach was exemplified by blockade of autocrine FGF4-mediated ES cell differentiation, the basic strategy could be adapted to generate antibodies, both agonistic and antagonistic, to the many ligands/receptors that are expressed during ES differentiation. Our growing knowledge of the role of these molecules in cell fate determination, combined with the development of more efficient protocols for directed stem cell differentiation, can be exploited to extend the versatility of this approach. In this study, Oct4 gene expression was used to monitor differentiation status but phenotypic changes could be detected by other lineage-specific reporters, by immunostaining or through changes in morphology.
cells/cm² were plated and cultured in ES medium in the presence or absence of doxycycline (1 μg/mL). For colony-forming assays, 0.5 × 10⁵ cells/cm² were plated. Nanog-GFP and Rex-GFP/+ ES cells were grown in ND11-N227 (previously ND11-N2287) Stem Cells containing 1 μM of CHIR99021 (a GSK-3 inhibitor) and 2 μM P0325901 (an ERK kinase inhibitor; Stratagene) plus Lif (2 μLIF) (14).

**Differentiation of ES Colonies in Semisolid, Serum-Free Medium (ES-Cult/N227).** After 4 d of puromycin selection and 36 h before differentiation, doxycycline was added, where appropriate, to initiate antibody expression before the autoculture production of FGF4. At the fifth day of selection, the ES medium was replaced with a semisolid differentiation medium, consisting of 60% (vol/ vol) N227 and 40% (vol/vol) ES-Cult M3120 (StemCell Technologies) plus puromycin (1 μg/mL) and doxycycline (1 μg/mL), where appropriate. Differentiation progress was monitored daily by observing the morphology and the fluorescence of the colonies. The maximum time the colonies could be sustained in semisolid medium without detaching from the dish was around 4 d. This timing was sufficient to allow for discrimination between colonies retaining and those down-regulating Oct4-GFP fluorescence.

**Recovery of Antibody Genes from ES Cell Clones.** PCR reactions were carried out using 100 ng of genomic DNA from selected clones as template in 20 μL of KOD buffer with 100 μM dNTPs, 1.5 mM MgSO₄, 5% (vol/vol) DMSO, 0.4 units KOD Hot Start Polymerase (EMD Millipore) using 0.25 μM primers: 2546 ′c-CTTCCTCACAGGCGGCATGG-3′ and 2545 ′G-TGTTGGCTTCTGGTCGC-3′. Cycling conditions were 95 °C for 2 min followed by 40 cycles of 95 °C, 20 s; 57 °C, 10 s; 70 °C, 40 s. PCR products were purified with GeneJet PCR purification kit (Thermo Scientific), digested with Ncol and NotI and purified by electrophoresis on a 1% agarose-TBE gel, followed by excision of the band TTCTCTCCACAGGCGCCATGG-3.

**Development and Use of In Vitro Biochemical Assay Replicating Interaction of FGFRI or FGF2 Binding to FGF4.** scFv gene inserts from stem cell genomic DNA of selected clones were subcloned into the Ncol/NotI-digested expression vector pBIOCMS and scFv-Fc expressed by transient transfection of HEK293F suspension cells (11). Affinity purification by Ni-NTA Sepharose was carried out as described previously (11). Black Maxisorp 96-well plates (A37111; Thermo Scientific) were coated with heparin sulfate proteoglycan (3 μg/mL in PBS; Sigma) overnight at 4 °C and blocked with PBS-M (2% w/vol) milk powder in PBS) for 1 h at room temperature. Dilutions of scFv-Fcs were preincubated with FGFRI-Fc or FGFRII-Fc in TBS-BSA (TBS, 0.2 mg/mL BSA) for 1 h at room temperature in a total volume of 112.5 μL in a 96-well polystyrene plate. FGF4 (37.5 μL in TBS-BSA) was then added and two separate 50-μL aliquots (for duplicate assays), transferred to separate wells of the heparin sulfate proteoglycan-coated plate, and washed with TBS. Final concentrations of the FGFRI-Fg and FGFRII-Fg receptor/filaggrin pairs were 158 ng/mL and 2 μg/mL, respectively. After 1-h incubation, plates were washed three times each with TBS-T and BSA. Anti-Fc-biotin (0.5 μg/mL in TBS-BSA, 109–065-006, Jackson Immunoresearch) was added, plates incubated for 1 h, washed as above and DELFIA Eu-N1 Streptavidin (50 μL, 1:100 dilution in TBS-BSA; Perkin-Elmer) incubated for 30 min, washed as above and DELFIA Dissociation-enhancement (50 μL) added before time-resolved fluorescence measurement (φmax = 320 nm at 615 nm) with a Fusion plate reader (Perkin-Elmer).

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**Supporting Information**

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**SI Methods**

**Creation of Antibody Populations for In-Cell Expression in ES Cells.**

Antibody populations for in-cell expression could be derived from display methods, such as phage display, yeast display, or ribosome display. Alternatively, antibody populations could potentially be generated from PCR amplification of V gene populations from the B cells of immunized animals. Expression of a diversity of surface receptors have been reported on ES cells providing potential targets for functional perturbation (1, 2). In this study, the “McCaffrey” antibody phage-display library (3) was used to select antibodies to recombinant mouse FGF4, FGFRIβ (IIIe), and FGFR2β (IIIe) (4) Fc chimeras (R&D Systems; 5846-F4, 661-FR, and 716-MF, respectively). Antibodies are in the form of single-chain Fvs (scFvs). After two rounds of selection, the polyclonal scFv population was subcloned into the pSANG10-3F expression vector (5) and used to transform Escherichia coli BL21 (DE3) V2R pRARE2 (a gift of Doug Cossar, Structural Genomics Consortium, Toronto, Canada). The 96–384 colonies were picked and screened by ELISA, which indicated 48%, 32%, 42% and of clones bound their target antigen for FGF4, FGFRIβ, and FGFR2β, respectively (Fig. S1). The same populations were subcloned into an entry plasmid (pBIOCAM5-GW) that encoded a signal peptide to allow antibody secretion in mammalian cells, a C-terminal human Fc domain for antibody dimerization, a tri-FLAG tag for detection, and a hexahistidine tag. The scFv-Fc libraries were then Gateway LR-recombined (6) with the Rosa-26 GATEWAY targeting vector pGATOR-DTA (Fig. L4) to give the antibody targeting vector pROSA-ic.

**Targeting Vector Construction.** The Diphtheria toxin fragment A (DTA) gene, including the phosphoglycerate kinase I promoter and bovine growth hormone polyadenylation site (bpA), were PCR-amplified from pL3L4 DTA (EUCOMTools) with primers 2478 (5′-TTTTTTGCGGAGCCGAGCCGAGTTGGCCGAATTCTACC-GGGTAGGAGAGGAGGC-3′) and 2480 (5′-TTTTTTCACTACG-TGTTCCTTTCCGGCCTCAGAAGCCATAGAG-3′). A fragment of diphtheria toxin (DtxL) was digested with DraIII and SfiI, and ligated with SfiI cut pGATOR vector to create pGATOR-DTA. pBIOCAM5-GW was created by PCR amplification from the pBIOCAM5 plasmid (in this case harboring an anti-MHC class I scFv 72.1A10 (7) using the primers 2346 (5′-GGGAAAATGGGTTGTACAAAAAACAGGGACTCCGCCGACCAATGAGTGCTGTA-TCATC) and 2347 (5′-GGGACCACTTTGGAACAGAAAGCTGGTTATTAGTCCTTGCTTGATGGTGATG-ATGTC) to give a PCR product encoding a scFv-Fc fusion flanked by the attB1 and attB2 GATEWAY recombination sites (6). This result was then BP-recombined with pDONR221 (Invitrogen) to give pBIOCAM5-GW.

**Transformation of Mouse ES Cells.** E14 and Oct4-GFP mouse ES cells were transfected with linearized DNA by nucleofection using the Mouse ES Cell Nucleofector kit (Lonza) and program A-023 of Nucleofector II (Amaza Biosystems). Linearized DNA (3.8 μg) was used to transfect 4.5 × 10⁵ ES cells and the transfected cells were plated into two 100-mm dishes containing ES cell medium. After 24 h, puromycin was administered (1 μg/mL) and positive selection of puromycin ES colonies took place for at least 4 d. Following selection, single ES colonies were either picked and propagated under self-renewal conditions for further analysis/storage (anti-Notch ES clones), or subjected to differentiation conditions (anti-FGF4, anti-FGFR1β, and anti-FGFR2β ES clones).

**Serum-Free ES Differentiation.** Serum-free neural differentiation has previously been described (8). Briefly, ES cells were plated at a density of 0.5 × 10⁵/cm² in ES medium. The next day, ES cells were washed to remove all traces of serum and the ES medium was replaced with NDiff-N227. Medium was changed every day for up to 7 d.

**Flow Cytometry.** Flow cytometry was performed using a Becton-Dickinson (FACSCalibur) flow cytometer. Live cell gating was based on forward scatter vs. side scatter and the exclusion of the 7-AAD (Biolegend) stain. Cells were analyzed from three separate dishes and the experiments were repeated three times, unless stated otherwise. Analysis of the data was performed using Summit 4.3 software (DAKO).

**Statistical Analysis.** The Two-Sample t-Test analysis tool within Microsoft Excel were used to test for equality of the population means within each sample set of the flow cytometry data. P values were calculated using a paired two-sample Student’s t-test assuming a two-tailed distribution for the average of three separate differentiation experiments.

**Isolation of Genomic DNA from ES Cell Clones.** Confluent cell cultures on 96-well plates were washed once with 100 μL of PBS, trypsinized, and transferred into the wells of another 96-well plate containing 100 μL of lysis buffer [10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% (wt/vol) SDS, 1 mM EDTA and Protease K (0.5 mg/mL)]. The plates were sealed with nylon film and incubated overnight at 37 °C. The next day iso-propanol (100 μL) was added directly to the lysates and the 96-well plate was sealed with thermo–pierce lids (Merck). The plates were inverted several times until DNA strands were visible. Plates were centrifuged at 2,000 × g for 30 min, after which the DNA formed a visible pellet. Pellets were washed in 70% (vol/vol) ethanol, liquid removed, and the plates were then left at room temperature to dry completely. DNA was resuspended in 50 μL of TE, pH 8.0, and stored at 4 °C.

**Expression and Affinity Purification of Recombinant Antibody for Functional Verification.** DNA encoding antibody scFv genes from selected ES cell clones was amplified from genomic DNA and subcloned into NcoI/NotI digested pBIOCAM5 (9) to create an expression construct for expression of scFv-Fc fusion. Expression plasmids were used to transfect HEK293F suspension cells (Invitrogen), as described previously (9). scFv-Fc fusions were affinity-purified by immobilized metal affinity chromatography using Ni-NTA agarose (Qiagen), dialyzed against PBS, and filter-sterilized with a 0.2-μm Spin-X column (Costar). Expression yields ranged from 5 to 30 mg/L.


Fig. S1. Primary ELISA screen of antibodies selected against FGF4, FGFR1β, and FGFR2β. Two rounds of phage–antibody selection against FGF4 were performed as described previously (1). Populations were subcloned into the bacterial expression vector pSANG10 (2), and 288 anti-FGF4 clones (A), 96 anti-FGFR1β clones (B), and 384 anti-FGFR2β clones (C) were picked and screened by ELISA. Binding of the scFvs to the immobilized antigen was quantified using europium-labeled anti-FLAG secondary antibody. Graphs plot the time-resolved fluorescence signal in intensity units (y axis) for each scFv (x axis). One-hundred thirty-nine (48%) anti-FGF4, 31 (32%) anti-FGFR1β, and 163 (42%) anti-FGFR2β antibody clones were positive as judged by a fluorescent signal above 100. FGF4, FGFR1β, and FGFR2β were from R&D Systems (catalog nos. 5846-F4, 661-FR, and 716-MF, respectively).


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Fig. S2. Maintenance of Oct4–ΔPE-GFP expression in ES cells by expression of a polyclonal population of anti-FGF4 antibodies. (A–C) Flow cytometry analysis after 3 d of ES-Cult/N2B27 differentiation of the Oct4–ΔPE-GFP cells (primary differentiation assay) following transfection with the anti-FGF4 library and puromycin selection. Differentiation was performed (A) in the absence of doxycycline (−dox), (B) in the absence of doxycycline and the presence of 2 μM of PDO3 (−dox/PDO3), and (C) in the presence of 1 μg/mL doxycycline (+dox). Plots are GFP fluorescence intensity (FL1-H) vs. forward side scatter (FSC-H).

Fig. S3. Expression of polyclonal anti-FGF4 antibodies in the primary differentiation assay increase clonogenicity of ES cells. Oct4–ΔPE-GFP cells were transfected with an anti-FGF4 antibody population and selected with puromycin for 4 d. ES colonies were subjected to differentiation (in ES-Cult-N2B27) in the presence or absence of doxycycline. PDO3 and 2i-positive controls were included. In two independent experiments, cells were trypsinized after 3.5 and 4.5 d, respectively, plated at 5,000 cells per condition in ES medium (serum/LIF), and left to grow for 7 d. Colonies were stained with alkaline phosphatase (SIGMAFAST BCIP/NBT tablets; Sigma) and counted in duplicate wells. The numbers represent the average of duplicates.
Morphology of typical colonies obtained after 3 d of differentiation in the presence of doxycycline. At 3 d of differentiation in ES-Cult/N227 plus doxycycline, there were roughly four types of colonies. The majority showed obvious signs of differentiation (colony types 3 and 4), much fewer colonies showed milder signs of differentiation (colony type 2), and a minority (∼5%) showed a phenotype (colony type 1), comparable to that of an ES colony growing under self-renewal conditions (see Fig. 3C). The colonies that were picked were mostly of the type 1 and some of the type 2 (magnification 20×).
Fig. S5. Maintenance of Oct4–ΔPE-GFP expression in ES cells by endogenous expression of monoclonal anti-FGF4 antibodies in liquid differentiation. Flow cytometry profiles of Oct4–ΔPE-GFP anti-FGF4 clones subjected to a 7-d differentiation period in N227 medium in the presence or absence of doxycycline (secondary differentiation assay). Clones A and C show the most promising result, maintaining Oct4–ΔPE-GFP fluorescence to high levels, comparable to the −dox/PDO3 controls. Clones B and D performed less optimally in this assay. Histograms are representative (Center value) of triplicates from one (but not the same) differentiation experiment. The position of the bar (M1) was determined with the use of Oct4–ΔPE-GFP cells grown under self renewal conditions (LIF, 2i). For each clone, the differentiation/flow cytometry experiment was performed at least two times with similar results.
Fig. S6. Time-course analysis of the effect of α-FGF4_A antibody in maintaining Rex1 and Nanog expression under differentiation conditions. Flow cytometry profile of Rex1-GFPd2 and Nanog-GFP ES cells after 2, 3, and 4 d of differentiation in N2B27 in the presence or absence of antibody α-FGF4_A. This experiment determined day 3 as the time point when α-FGF4_A showed maximum inhibitory effect on differentiation, as judged by the proportion of GFP⁺ cells. The experiment also showed a more sensitive response of Rex1-GFPd2 cells to the addition of the antibody and PDO3 than Nanog-GFP ES cells, which may be explained by the fact that GFP is destabilized in Rex1-GFPd2 cells. The position of the bar (M1) was determined with the use of ES cells grown under self-renewing conditions (LIF, 2i, not shown).
Fig. S7. Specificity and amino acid sequences of selected scFvs. (A) scFv genes were PCR-amplified from ES cell genomic DNA, subcloned into the expression plasmid pBIOCAM5 and expressed in HEK293 cells, as described previously (1). scFv-Fcs were purified by Ni-NTA affinity chromatography and tested for their ability to bind immobilized antigen coated at a concentration of 5 μg/mL. Detection was by anti-FLAG Europium-labeled antibody, as described previously (1), and ELISA signal was plotted on a logarithmic scale. This result demonstrates that the antibodies were specific for FGF4, binding murine FGF4 but not murine FGF1 or FGF2 (acidic and basic FGF, respectively). The antibodies, which were generated against mouse FGF4, failed to bind human FGF4 (hFGF4). (B) The binding specificity of the FGF4 and FGFR antibodies was determined by ELISA using antibodies expressed as scFv in E. coli and purified as described previously (2). All antibodies were shown to be specific to their selected target. (C) Amino acid sequences of the variable heavy (VH) and variable light (VL) sequences of antibodies recognizing FGF4 (αFGF4_A and αFGF4_C), FGFR1 (αFGFR1_A), and FGFR2 (αFGFR2_A). Positions for complementarity determining regions (CDRs) and framework regions (FW) are indicated.

Fig. S8. Identification of Oct4–ΔPE-GFP clones expressing anti-FGFR1 antibodies. (A–C) Flow cytometry analysis of the Oct4–ΔPE-GFP cells transfected with the phage display-derived anti-FGFR1 antibody population. Differentiation was performed for 4.5 d in ES-Cult/N2B27 differentiation medium (A) in the absence of doxycycline (−dox), (B) in the absence of doxycycline and the presence of 2 μM of PDO3 (−dox/PDO3), and (C) in the presence of 1 μg/mL doxycycline (+dox). The figure shows retention of Oct4-GFP within the population in the presence of doxycycline. (D) Expression of polyclonal anti-FGFR1 antibodies in the primary differentiation assay increased the clonogenicity of ES cells in a subsequent self-renewal assay (colony-forming assay, as described in Fig. S3). (E) Phase and fluorescent images of Oct4–ΔPE-GFP cells expressing the α-FGFR1_A antibody after 7 d of differentiation in N2B27 in the presence or absence of doxycycline (magnification 20×). (F) Anti-FGFR1 antibodies inhibited binding of FGFR1-Fc to immobilized FGF4/heparin sulfate in the presence of heparin sulfate (an essential cofactor for the interaction). Bound receptor was detected with an anti-Fc Europium labeled antibody. α-FGFR1_D1 and α-FGFR1_D2 were two different antibodies derived from the same ES cell clone.
Fig. S9. Identification of Oct4–ΔPE-GFP clones expressing anti-FGFR2 antibodies. (A) Expression of polyclonal anti-FGFR2β antibodies in the primary differentiation assay increase clonogenicity of ES cells in a subsequent self-renewal assay (colony-forming assay as described in Fig. S5). (B) Example of a colony maintaining Oct4-GFP fluorescence and ES-like morphology after 4 d of differentiation in ES-Cult/N227 following transfection with the phage display-derived anti-FGFR2 antibody population. Four colonies were picked of 132 puro-resistant colonies (magnification 20×). (C) Histogram representing FACS analysis of the Rex1-GFPd2 cells after 3 d of differentiation in N227 in the presence or absence of the purified anti-FGFR2β antibodies, negative (anti-desmin and Fc only) and positive (PdO3) controls (α-FGFR2_D1 and α-FGFR2_D2 were two different antibodies derived from the same ES cell clone). The bars show the percentage of cells expressing Rex-GFP and are the average of triplicates of a single experiment ± SD. The experiment was performed twice with similar results. (D) Selected antireceptor antibodies can block FGFR2-Fc binding to FGF4 in an in vitro assay. scFv-Fcs were preincubated with FGFR2-Fc (158 ng/mL) for 1 h, FGF4 (158 ng/mL) added, and the mix transferred to a heparin sulfate proteoglycan coated Nunc plate. Bound receptor was detected with anti–Fc-biotin/streptavidin–europium. Plots show fluorescence intensity plotted against scFv concentration.