Novel antibodies directed against the human erythropoietin receptor: creating a basis for clinical implementation

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Summary

Recombinant human erythropoietin (rHuEPO) is an effective treatment for anaemia but concerns that it causes disease progression in cancer patients by activation of EPO receptors (EPOR) in tumour tissue have been controversial and have restricted its clinical use. Initial clinical studies were flawed because they used polyclonal antibodies, later shown to lack specificity for EPOR. Moreover, multiple isoforms of EPOR caused by differential splicing have been reported in cancer cell lines at the mRNA level but investigations of these variants and their potential impact on tumour progression, have been hampered by lack of suitable antibodies. The EpoCan consortium seeks to promote improved pathological testing of EPOR, leading to safer clinical use of rHuEPO, by producing well characterized EPOR antibodies. Using novel genetic and traditional peptide immunization protocols, we have produced mouse and rat monoclonal antibodies, and show that several of these specifically recognize EPOR by Western blot, immunoprecipitation, immunofluorescence, flow cytometry and immunohistochemistry in cell lines and clinical material. Widespread availability of these antibodies should enable the research community to gain a better understanding of the role of EPOR in cancer, and eventually to distinguish patients who can be treated safely by rHuEPO from those at increased risk from treatment.

Keywords: cancer anaemia, recombinant erythropoietin, erythropoietin receptor, antibody, risk assessment.

Anaemia is an independent prognostic factor for poor survival in cancer patients (Caro *et al*, 2001), but the use of recombinant human erythropoietin (rHuEPO) to treat these patients is controversial due to concerns about patient safety arising from Phase III clinical trials showing more rapid cancer progression and reduced survival in subjects randomized to rHuEPO (Henke *et al*, 2003; Leyland-Jones *et al*, 2005; Miller *et al*, 2009). Consequently there has been a marked decline in the use of rHuEPO since 2007 (Hill *et al*, 2012). Clearly, the benefits of EPO treatment must be carefully balanced against the risk of enhanced cancer progression for each patient.

Erythropoietin functions by binding to its receptor (EPOR) on the surface membrane of erythroid progenitors and activating JAK2/STAT5 signalling pathways (Jelkmann, 2010; Wenger & Kurtz, 2011). There is now evidence that growth factor receptor-mediated cell signalling pathways can overlap in cancer cells. In a landmark study, Liang *et al* (2010) found that EPOR is co-expressed with human epidermal growth factor receptor-2 (HER2, also known as ERBB2) in breast cancer cell lines and tumour specimens. rHuEPO administered to patients antagonized trastuzumab treatment and resulted in shorter progression-free and overall survival in patients with HER2-positive metastatic breast cancer (Liang *et al*, 2010).

Erythropoietin has pleiotropic actions and EPOR is expressed outside the haematopoietic compartment (Lappin *et al*, 2002; Ghezzi *et al*, 2010; Vogel & Gassmann, 2011). EPOR is functionally active in endothelial cells (Anagnostou *et al*, 1994) and endothelial progenitor cells promoting vascular repair and endothelial regeneration (Trincavelli *et al*, 2013). Moreover, EPO stimulates angiogenesis both *in vitro* and *in vivo* (Trincavelli *et al*, 2013). Positive effects of EPO treatment on the immune system have been documented (Mittelman *et al*, 2004; Prutchi-Sagiv *et al*, 2006, 2008; Katz *et al*, 2007; Lifshitz *et al*, 2010; Mausberg *et al*, 2011; Nairz *et al*, 2012; Oster *et al*, 2013). EPO has thrombotic effects, at least *in vitro*, as it increases plasminogen activator inhibitor-1 release in HUVEC culture (Stasko *et al*, 2002). Patients with end-stage renal disease on high doses of rHuEPO have high haemoglobin levels, are associated with increased risk of cardiovascular and thrombotic events, and have reduced survival (Smith *et al*, 2003; Provatopoulou & Ziroyiannis, 2011). These observations support the hypothesis that supraphysiological levels of circulating EPO could lead to enhanced tumour growth, neovascularization and thrombosis in some cancer patients. Recommendations for the use of erythropoiesis-stimulating agents (ESAs) in patients with cancer have been published (Rizzo *et al*, 2010).

To understand the increased risk of administering rHuEPO to patients with cancer, it is imperative to examine its effects in tumour tissue. Angiogenic factors, such as vascular endothelial growth factor A (VEGFA), platelet-derived growth factors (PDGFs), fibroblast growth factors (FGFs) and angiopoietins are often elevated in the tumour environment. PDGFBB targets perivascular cells to nascent vascular networks (Abramsson *et al*, 2003) and modulates tumour angiogenesis by increasing EPO production in stromal cells, leading to the induction of endothelial cell proliferation, migration, sprouting and tube formation (Xue *et al*, 2012).

Contentious inferences were drawn from clinical studies that were based on immunohistochemistry (IHC) using antibodies, but later shown to lack specificity for EPOR (Elliott *et al*, 2006; Brown *et al*, 2007). Thus, investigation of EPOR expression and function relies heavily on the availability of specific anti-EPOR antibodies.

The EU-based EpoCan project addresses safety concerns related to EPO treatment and is investigating the risks of EPO treatment using mouse models, human clinical samples and patient databases. The studies focus on the long-term effects of EPO treatment on tumour growth and incidence, the role of EPO in angiogenesis and its association with thromboembolic events in cancer and cardiovascular disease. An important initial objective was to prepare a range of highly specific monoclonal antibodies against human EPOR. To this end, a cohort of 15 EPOR mouse and rat monoclonal antibodies were produced and evaluated for different applications. Here we report on the characterization of four of these antibodies, which have proved suitable for a range of applications.

Materials and methods

Cell lines

The human cell lines used were: megakaryoblastic leukaemia cells, UT-7; acute lymphocytic leukaemia cells, REH; pre-B cells, NALM6; breast cancer cells, MDA-MB-231; human embryonic kidney cells HEK293T, and its derivative, BOSC23; and lung cancer cells, A549.

Tissue sections

Formalin-Fixed Paraffin wax-Embedded (FFPE) tissue sections and bone marrow aspirates were obtained under agreement with the Northern Ireland Biobank (Ethical approval reference NIB12-0044).

Monoclonal antibody generation by genetic immunization

Monoclonal antibodies were generated using cDNA constructs encoding the extracellular domain (ECD) of hEPOR cloned into proprietary immunization and screening vectors. Anti-tag antibodies were used to confirm expression after transient transfection of the cDNA constructs into mammalian cells, *in vitro*. The immunization constructs were adsorbed onto the surface of gold particles and introduced intradermally into mice and rats using a BioRad gene gun (Bio-Rad GmbH Mu¨nchen, Germany), with several cDNA boosts, following proprietary protocols. This cDNA was

taken up and translated by skin cells, whereby the protein was brought to the cell surface and finally secreted to allow an optimal immune response against the EPOR ECD. The sera were tested against both the ECD and full-length EPOR construct, the cDNA of which had been transiently transfected into mammalian cells.

Monoclonal antibody generation using synthetic peptides

The amino acid sequence of hEPOR was analysed using hydrophilicity/hydrophobicity software to identify peptide regions representing potential epitopes. In total, six peptides (h1–h6) were chosen for peptide synthesis of which five were located in the cytoplasmic domain (h2–h6) as shown for hEPOR (Table I). The peptides were conjugated with keyhole limpet haemocyanin for immunization and with ovalbumin for screening in peptide enzyme-linked immunosorbent assays (ELISA). Immunization was carried out intraperitoneally with one group of six peptides (h1–h6) into two cohorts of three rats. The peptides were mixed with complete Freund's adjuvant for the initial immunization, followed by several boosts with incomplete Freund's adjuvant. Following immunization of three animals per cohort, sera were tested for positivity in a peptide ELISA against ovalbumin-peptide conjugate mixes, corresponding to their immunogen mixtures. After lymphocyte fusion, the resulting hybridoma supernatants were screened against each individual ovalbumin-peptide conjugate in the same ELISA assay to identify antibodies against specific peptides.

Flow cytometry

Flow cytometry was performed essentially as described (Lifshitz *et al*, 2010). Cell suspensions were analysed on a FACSort flow cytometer (Becton Dickinson, Oxford, UK) and the results were analysed using WinMDI software (J.Trotter free download, http://winmdi.software.informer.com/2.8/). Anti-HA antibody (MMS-101R) was from Covance (Princeton, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated AffinityPure Goat Anti-Mouse IgG and FITC-conjugated Affinity Pure Goat Anti-Rat IgG were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

Table I. Choice of human EPOR peptides for immunization.

Human EPO-R peptides	Location	Exon
h1: PPPNLPDPKFES	Extracellular domain	1
h2: KIWPGIPSPESEFEG LFTTHKGN	Intracytoplasmic domain	7
h3: VEPGTDDEGPL	Intracytoplasmic domain	8
h4: LPRNPPSEDLPGPG	Intracytoplasmic domain	8
h5: PSSQLLRPWTLC	Intracytoplasmic domain	8
h6: GDSQGAQGGLSDGPYSN	Intracytoplasmic domain	8

Immunofluorescence

Cells seeded on glass cover slips (A549, MDA-MB-231, COS7 and HEK293T) or collected in a 1·5 ml tube (UT-7) were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature (RT) for 30 min. After incubation in quenching buffer (0·1% Triton, 5% fetal bovine serum, and 2% bovine serum albumin) at RT for 1 h, cells were incubated with primary antibodies diluted in quenching buffer at RT for 1 h. After washing with PBS, cells were incubated with secondary antibodies for 1 h at RT. Nuclei were stained with 2·5 lmol/l DRAQ5(ab108410; Abcam, Cambridge, MA, USA) for 30 min at RT. Confocal fluorescent images were obtained by a TCS SP5 II confocal microscope (Leica, Wetzlar, Germany) with a 63x/1.4NA objective or a CSU10 spinning disc unit coupled to a Zeiss Axiovert 200M microscope with a 100x/1.3NA oil immersion objective. For some experiments, fusion proteins of enhanced cyan fluorescent protein (ECFP) and EPOR were made by cloning cDNA encoding for the hEPOR into the pECFP-N1 vector (Clontech, Mountain View, CA, USA) between HindIII and BamHI. A construct specific for the intracytoplasmic domain (ICD) of EPOR was generated by replacing the extracellular and transmembrane domain in the described construct by that of the epithelial growth factor receptor (EGFR) (Gross *et al*, 2014). For transient transfections plasmid DNA was mixed with the transfection reagent Turbofect (Thermo Scientific, Waltham, MA, USA) according to the supplier's recommendations. The cells were incubated for 24 h with the transfection mix and subjected to immunofluorescence staining as described.

Generation of EPOR-silenced cells

Human EPOR (sc-37092-V) and control (sc-108080) shRNA lentiviral particles (Santa Cruz Biotechnology, Dallas, TX, USA) were used to generate stable transfectants in MDA-MB-231 breast cancer cells and A549 lung carcinoma cells. After lentiviral infection, infected cells were selected with puromycin (1 lg/ml) to finally generate MDA-MB-231-shEPOR, A549-shEPOR cells and their corresponding MDA-MB-231-shSCR and A549-shSCR control cells. Following puromycin selection for 11–18 d, cells were lysed in Laemmli buffer and subjected to Western blot analysis using the GM1201 antibody (see Table II).

Western blot analysis

Cells were lysed in Laemmli buffer. Western blots were performed using 10% sodium dodecyl sulphate (SDS)-polyacrylamide gels. Membranes were incubated with a 1:1000 dilution in Tris-buffered saline (TBS)-Tween 20 (TBS-T) buffer of the indicated anti-EPOR antibody at 4°C overnight. Membranes were incubated with the corresponding secondary antibody at RT for 1 h and, finally, washed three times

Table II. Characteristics of the four selected EPOR monoclonal antibodies.

Immunogen	Subclone name	Isotype	Epitope location	Applications
Peptide h6	GM1201	rIgG2b	Intracytoplasmic domain	WB, IP, IF, IHC
Genetic immunization	GM1202	mIgG1	Extracellular domain	IP, IF, FACS
Genetic immunization	GM1203	mIgG1	Extracellular domain	IP, IF, FACS
Genetic immunization	GM1204	rIgG2a	Extracellular domain	FACS

WB, Western blotting; IP, immunoprecipitation; IF, immunoflourescence, IHC, immunohistochemistry; FACS, fluorescence-activated cell sorting.

with TBS-T buffer for 10 min. Immunolabeling was detected by enhanced chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Scientific) and visualized with a digital luminescent image analyser (Image Quant LAS400 mini; GE Healthcare Life Sciences, Buckinghamshire, UK).

Coupling of antibodies for immunoprecipitation

For immunoprecipitation (IP), anti-EPOR antibodies or anti-HA antibodies (clone 12CA5, Abcam) were covalently coupled to protein-A agarose (Immunosorb A, Medicago, Uppsala, Sweden). Antibody (2 lg) was incubated with 5 ll Protein-A bead slurry in PBS for 1 h at RT. Beads were washed with sodium borate (0·2 mol/l, pH 9·0) and the antibody was cross-linked to protein-A upon addition of 40 mmol/l dimethylpimelimidate.

Cell transfection, protein extraction and IP

Plasmid encoding the N-terminal HA-tagged hEPOR was transiently transfected into HEK-293T cells with calcium-phosphate (Graham & van der Eb, 1973) and harvested 48 h later. Cell extracts were prepared in IP buffer with 1% Triton X-100. Protein extracts were cleared by centrifugation (10 000 g; 20 min), protein concentration determined by BioRad-DC assay and IP performed using 40 lg extract from transfected HEK-293T cells, 750 lg of UT-7 cells or 1·5 mg extract from A549 and MDA-MB231 cells. Proteins were diluted with IP buffer and IP was performed in the presence of 0·5% Triton X-100 for 2 h at 4°C. After 3 washes with 0·5% Triton X-100 IP buffer, EPOR protein was eluted by boiling in Laemmli sample buffer (pH 6·8) for 5 min, loaded on a SDS-polyacrylamide gel and analysed by Western blot using GM1201 antibody.

Mass spectrometry – Nano-high performance liquid chromatography – MS/MS

Four milligram of UT-7 cell extract was immunoprecipitated with GM1202 and GM1203 (see Table II) agarose-coupled antibodies (mixture of 1:1, 6 lg antibodies each). After extensive washing, the precipitated proteins were separated by a 10% SDS-polyacrylamide gel electophoresis and visualized with Coomassie Blue. Protein digests of the gel piece covering the 65 kD region were analysed using an UltiMate 3000 nano-HPLC system (Dionex, Germering, Germany) coupled to an LTQ Orbitrap XL mass spectrometer. An in-house fritless fused-silica microcapillary column (75 lm i.d., 280 lm o.d.) packed with 10 cm of 3-lm reversed-phase C18 material (Reprosil, Dr Maish GmbH, Ammerbuch- Entringen, Germany) was used. The gradient (solvent A, 0·1% formic acid; solvent B, 0·1% formic acid in 85% acetonitrile) started at 4% B. The concentration of solvent B was increased linearly from 4 to 50% over 50 min and from 50 to 100% over 5 min. A flow rate of 250 nl/min was applied. Data analysis was performed using Proteome Discoverer 1.3 (ThermoScientific) with search engine Sequest (http://fields.scripps.edu/sequest/). Precursor mass tolerance was set to 10 ppm, fragment mass tolerance was 0·8 Da. Raw data obtained by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) were searched against the *Homo sapiens* protein database extracted from the NCBInr database using false discovery rate (FDR) for peptide evaluation. Only peptides with a significance threshold of 0·01 (99% confidence) or less were used for protein identification.

Immunohistochemistry

To test the suitability of antibodies for clinical applications in FFPE material, we used UT-7, REH and NALM-6 cell lines, as they have differential endogenous EPOR expression. They were grown to confluence in 29 T75 flasks, removed and fixed in 10% formal saline (BCS Biosciences Ltd, Cambridge, UK) overnight and processed to paraffin wax. Sections were prepared and stained by all antibodies at concentrations of 2– 10 lg/ml following pressure cooking antigen retrieval, and using anti-rabbit/-mouse Envision (Dako, Cambridge, UK), or peroxidase-conjugated anti-rat immunoglobulin (Sigma-Aldrich, Gillingham, UK) localization. Sections were prepared from bone marrow samples and stained at 4 lg/ml concentration using automated IHC (Discovery XT; Roche Tissue Diagnostics, Burgess Hill, UK) and CC1 conditioning.

Bone marrow aspirate smears and cultured erythroid progenitor cells were mounted on glass slides and fixed overnight in 95% methylated ethanol. After washing in water, slides were flooded with PBS pH 7·0 and primary antibodies incubated overnight at 4°C (rat anti-EPOR GM1201 Aldevron, Freiburg, Germany) and rabbit anti-ferritin H (ab75972,

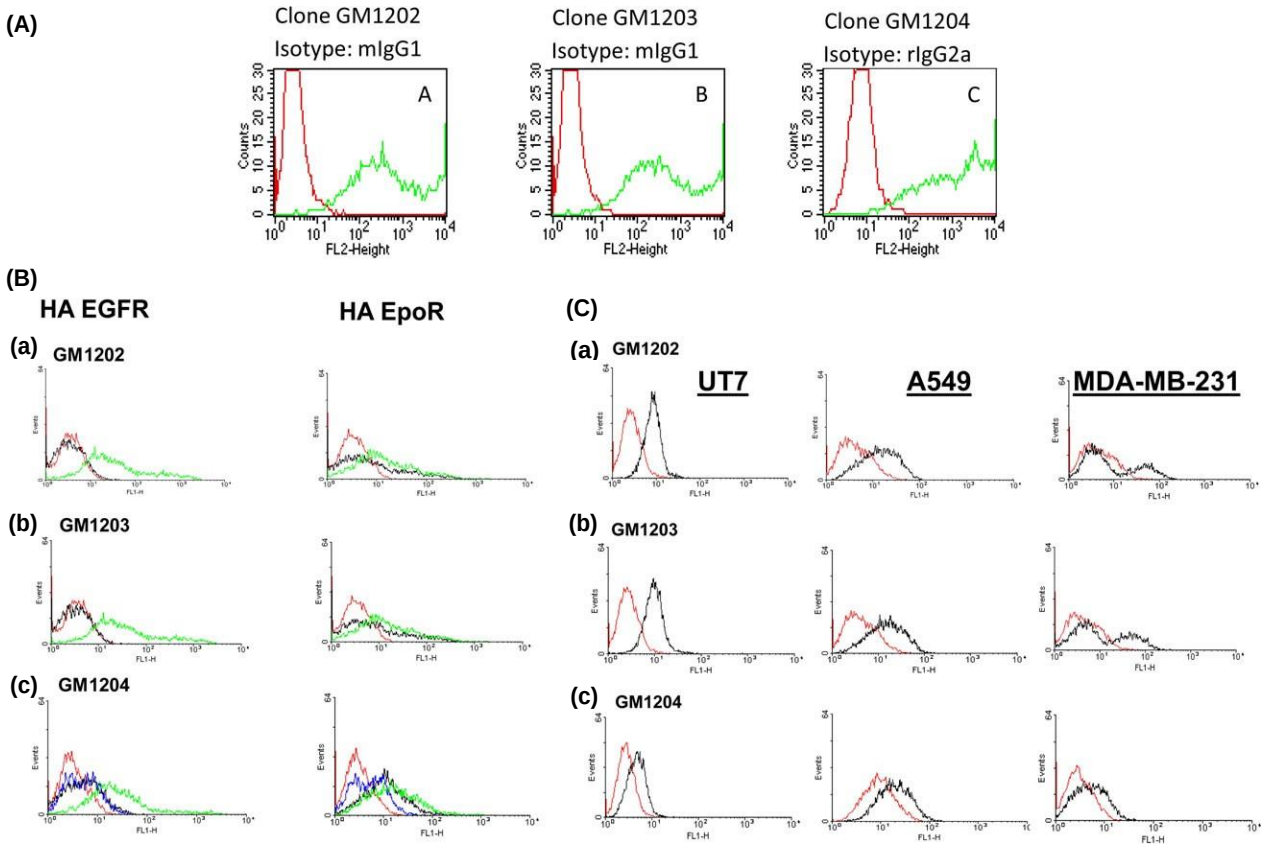


Fig 1. Specificity of individual anti-EPOR hybridoma clones. (A) Hybridoma supernatants were analysed by flow cytometry on cells transiently transfected with the human EPOR extracellular domain (ECD) cDNA cloned into a proprietary Aldevron test vector (green curves). Depending on the antibody source, a goat anti-mouse or anti-rat IgG R-phycoerythrin (R-PE) conjugate were used as secondary antibodies. Negative controls: Mammalian cells, transfected with an irrelevant control cDNA cloned in the corresponding expression vector (red curves). (B) BOSC23 cells expressing HA-tagged hEPOR or EGFR were incubated at 4°C with primary antibodies (10 lg/ml) followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Black line: GM1202, GM1203 and GM1204; Green line: anti-HA antibody. Red and blue lines: secondary antibody only. Depending on the antibody source, a goat anti-mouse IgG R- conjugate (#1030-09, Southern Biotech, Birmingham, AL, USA), or a goat anti-rat IgG R-PE conjugate (#3030, Southern Biotech) were used as secondary antibodies at 10 lg/ml. As negative control BOSC23 cells, transfected with an irrelevant control cDNA cloned into the corresponding expression vector, were incubated with each monoclonal antibody and detected with the secondary antibody described above (black curves). (C) Cells were incubated with primary antibodies (4 lg/ml for UT-7 cells; 10 lg/ml for A549 and MDA-MB-231 cells), followed by FITC-conjugated secondary antibodies (15 lg/ml). Black line: GM1202, GM1203 and GM1204; Red line: secondary antibody only. EPOR, erythropoietin receptor; EGFR, epithelial growth factor receptor.

Abcam, Cambridge, UK) or rabbit anti-glycophorin C (ab108619, Abcam) at 4 lg/ml. FITC anti-rat (Millipore, Billerica, MA, USA) and Alexa Fluor 568 anti-rabbit (Life Technologies, Paisley, UK) were used to localize immunoreactivity for 2 h at 37°C. Cell preparations were mounted in aqueous mounting medium containing 4^0 ,6-diamidino- 2-phenylindole (DAPI, Aquilant Scientific, Belfast, UK).

Results

Monoclonal antibodies generated by genetic immunization against hEPOR-ECD

Four murine and five rat mother hybridomas were preselect-ed for their ability to recognize the hEPOR ECD constructs in flow cytometry. Purified monoclonal antibodies were tested by flow cytometry at 1 lg/ml (Fig 1A–C).

Monoclonal antibody generation using synthetic peptides

Supernatants from the stable mother clones were pre-tested for the selection of the best mother clones for each assay (Western blots, IP, IHC, immunofluorescence and flow cytometry) with respect to signal strength and specificity. The chosen mother clones were then subcloned by serial dilution, expanded, isotyped and antibodies purified using protein G columns and resuspended in PBS to a given concentration (1–2 mg/ml). The results of the chosen subclones that revealed the most unequivocal results in the various assays were generated against human peptide 6, which is located close to the C-terminal region of EPOR (Table I).

Systematic nomenclature of monoclonal antibodies

Of 15 monoclonal antibody mother clones, four were selected for subcloning and additional testing (Table II).

Flow cytometry analysis of EPOR

The GM1202, GM1203 and GM1204 antibodies, raised against the ECD, recognized hEPOR by fluorescence-activated cell sorting (FACS) analysis, on Bosc23 cells transfected with HA tagged hEPOR or EGFR cDNA constructs. The three antibodies showed similar levels of hEPOR expression as compared to anti-HA antibody (positive control). Isotype controls (data not shown) and secondary antibodies were used as negative controls. The specificity of the antibodies was verified by the lack of reactivity in HA-EGFR transfected cells. Using FACS analysis, the same antibodies also detected endogenous hEPOR in UT-7, A549 and MDA-MB-231 cells. hEPOR was detected with higher sensitivity in UT-7 cells (4 lg/ml antibody concentration) than in A549 and MDA-MB-231 (10 lg/ml).

Immunofluorescence

COS7 cells were transfected with HA-hEPOR cDNA and stained with GM1202 and GM1203 directed against the ECD and GM1201 directed against the ICD (Fig 2A) using anti-HA as a positive control. The three antibodies detected hEPOR and specificity was verified using isotype controls (data not shown). HEK293T cells were transfected with DNA encoding a fusion protein of ECFP and EPOR, incubated for 24 h and then stained with GM1201, directed against the ICD, and GM1202 or GM1203, directed against the ECD, of EPOR (Fig 2B). Colour coding was set to equal values for ECFP and Alexa555 (secondary antibody label) channels with the exception listed in the legend to Fig 2B. For the anti-EPOR antibodies GM1202 and GM1203 (rows 2 and 3), perfect co-localization at the membrane of immunofluorescence with ECFP fluorescence was observed, indicating specific recognition of EPOR protein by the antibodies. In contrast, the antibodies did not detect the ECFP-EGFR-EPOR fusion protein, which was sufficiently expressed (as can be seen in the ECFP channel) and therefore served as a negative control (upper row). GM1201 antibody was only able to detect EPOR protein in permeabilized cells, indicating correct orientation of the ECFP-EPOR fusion protein and supporting its specificity for the ICD of EPOR. Interestingly, this antibody also detects the ICD in a fusion protein made from ECFP, the ECD of EGFR and the ICD of EPOR. Reactivity of the GM1201, GM1202 and GM1203 antibodies with hEPOR was also demonstrated in UT-7, A549 and MDA-MB-231 cells, showing significantly higher sensitivity in UT-7 cells.

Endogenous EPOR expression in tumour cell lines

The ability of the antibodies to recognize endogenous EPOR expression in tumour cells was tested by immunofluorescence (Fig 3) and Western blot analysis (Fig 4) using the UT-7 cell line as well as EPOR-silenced breast carcinoma MDA-MB-231 cells and EPOR-silenced lung carcinoma A549 cells (shEPOR) and the corresponding control cells (shSCR). RNA analysis in these cells showed that the *EPOR* mRNA level in UT-7 is 270 ± 8 times higher than in MDA-MB-231 control cells (MDA-MB-231-shSCR) and 56 ± 2 times higher than in A549 control cells (A549-shSCR). Further RNA analysis in EPOR-silenced MDA-MB-231 and A549 cells showed that *EPOR* mRNA expression declined by 82 ± 3% and 70 ± 3% respectively, which validated these cellular models for testing GM1201 antibody by Western blot. In line with these RNA data, Western blotting showed a main signal around 63 kDa in these three cell lines and was negative for EPOR-silenced cells (Fig 4). This signal was much higher in UT-7 than in MDA-MB-231 and A549 control cells, reflecting that Western blot signal parallels *EPOR* mRNA levels in these cells. Moreover, GM1201 has the potential to detect higher molecular weight EPOR forms, which are much weaker than the main 63 kDa form. Collectively, these data indicate that GM1201 reliably detects endogenous EPOR by Western blotting.

Identification of antibodies that specifically immunoprecipitate overexpressed and endogenous human EPOR

Using overexpressed HA-hEPOR as a source, GM1201, GM1202 and GM1203, were identified as antibodies with the highest immunoprecipitating efficiency (Fig 5A). Of note, the rat-derived antibody GM1201 was as efficient as the commercial anti-HA-tag antibody by IP (Fig 5A). The GM1201 antibody was also used to detect untagged immunoprecipitated hEPOR in Western blots. Besides this antibody, two mouse-derived antibodies directed against the ECD of EPOR, GM1202 and GM1203, were able to efficiently recover endogenous hEPOR in immunoprecipitates from UT-7, A569 and MDA-MB-231 cells (Fig 5B–D). In the case of very low endogenous hEPOR expression in A549 or MDA-MB-231 cells, the use of these mouse antibodies prevented the detection of cross-reacting bands, which were precipitated if the same antibody (GM1201) was used in IP and Western blot [A549: Fig 5C (*); MDA-MB-231: data not shown].

In all cases and with all antibodies, IP was able to significantly enrich the EPOR compared to the cell lysate (10% of the IP input). Specificity of the immunoprecipitated bands was confirmed by knockdown of EPOR using shRNAs in A549 cells and MDA-MB-231 cells (Fig 5C, D). The recovery of hEPOR in immunoprecipitates using GM1202 and GM1203 antibodies was further confirmed by Nano-HPLC–MS/MS, where seven EPOR-specific peptides were identified in trypsin-digested samples (Fig 6). Together, these data revealed a specific and highly efficient hEPOR immunoprecipitating capacity of GM1201, GM1202 and GM1203.

Immunohistochemistry

Differential expression of *EPOR* mRNA expression was confirmed in NALM-6, REH and UT-7 cell lines by Q-PCR

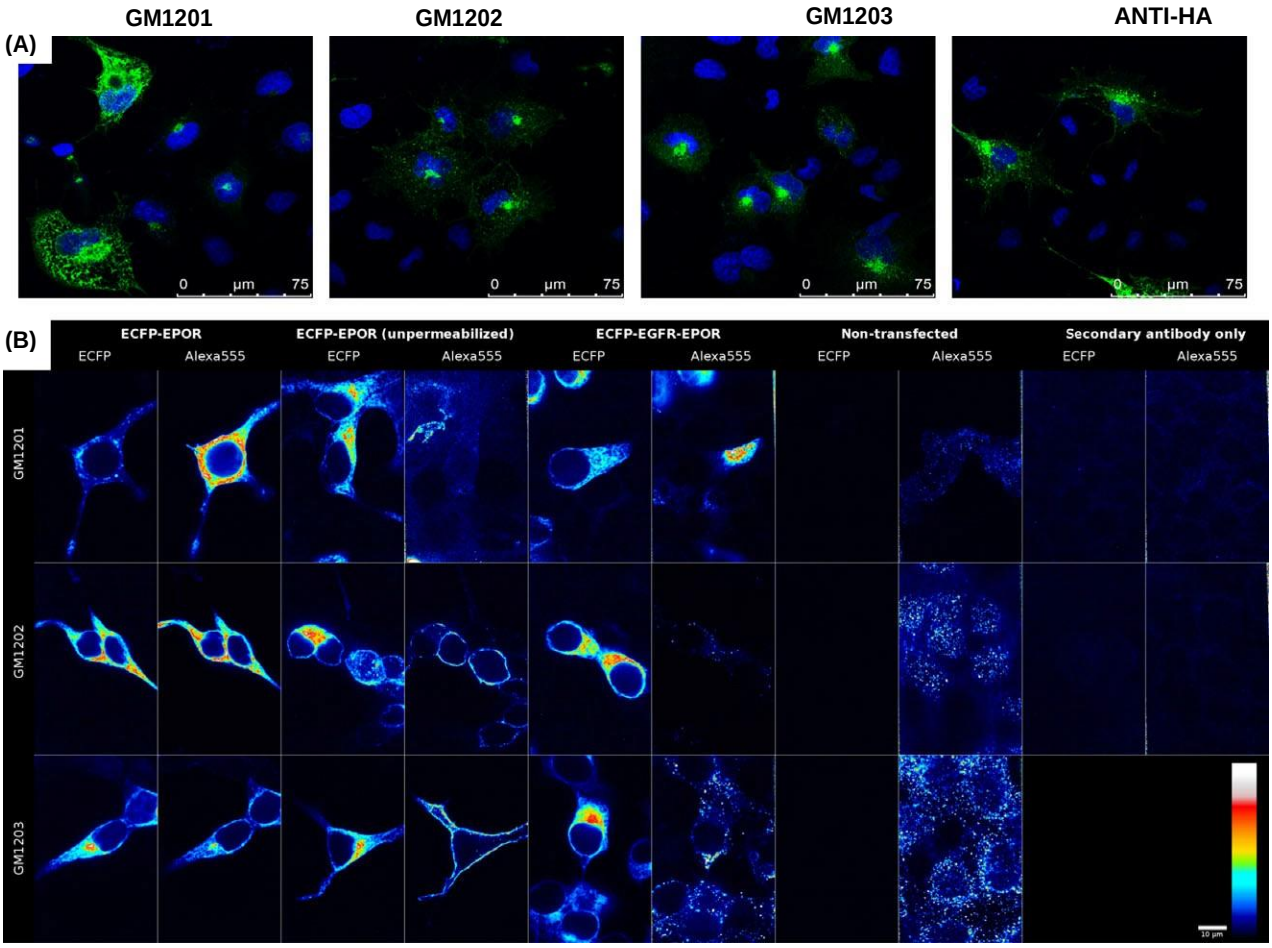


Fig 2. Immunofluorescence of transiently transfected hEPOR. (A) Cos7 cells were seeded on glass coverslips and transfected with HA-tagged hEPOR cDNA. Primary antibodies (and isotype matched controls – data not shown) were used at a 7 lg/ml, and secondary antibodies – at 4 lg/ml. Nuclei were stained with DAPI. Confocal fluorescent images were obtained with a LEICA TCS SP5 II confocal microscope with 63x/1.4NA objective. (B) HEK293T cells were transfected with plasmids encoding the indicated fusion proteins and hEPOR for 24 h, and stained with GM1201 (intracytoplasmic domain; ICD) and GM1202 or GM1203 (extracellular domain; ECD). Colour coding is set to equal values for ECFP and Alexa555 channels except for the following recording: antibody GM1201 in ECFP-EGFR-EPOR transfected cells (antibody channel 8x less sensitive). All antibodies show perfect co-localization with the EPOR-ECFP (enhanced cyan fluorescent protein) chimaeras. Staining disappears when the ECD and the transmembrane domain of EPOR are substituted by that of EGFR except for the GM1201 antibody that has its epitope in the ICD. Localization differences are mostly due to different epitopes of the respective antibodies (GM1201: ICD vs.GM1202 and GM1203: ECD). Confocal fluorescent images were obtained by a Zeiss Axiovert 200M microscope equipped with a CSU10 spinning disc (Yokogawa, Tokyo, Japan) and a 100x/1.3NA oil immersion objective. EPOR, erythropoietin receptor; EGFR, epithelial growth factor receptor; ECFP, enhanced cyan fluorescent protein.

(Fig 7A). The rat antibody GM1201 showed strong staining in UT-7 cells and differential immunoreactivity between FFPE REH (relatively high endogenous *EPOR*) and FFPE NALM-6 (relatively low *EPOR*), see Fig 7B, C. On high magnification, this was seen to be cytoplasmic in distribution.

GM1201 was tested on FFPE non-erythroid cells, non-small cell lung carcinoma A549 and breast cancer MDA-MB-231 cell lines as knockdown models where lentiviral particles generated from three independent shEPOR sequences were used to infect MDA-MB-231 and A549 cells. A decline in EPOR protein was observed. Specificity of the antibodies towards EPOR in these two latter cell lines was ensured by the lack of immunoreactivity with the corresponding EPOR-silenced cells. High magnification confirmed reduced cytoplasmic immunoreactivity of shEpoR cells compared with those derived from cells treated with scrambled sequences (Fig 7D, E). Summary results for GM1201 immunoreactivity, compared with GM1202, GM1203 and GM1204 are presented in Table III.

Immunoreactivity was found in erythroid cells in erythroblastic islands using the rat antibody GM1201, while metamyelocyte and neutrophil band forms were negative (Fig 7F). Immunoreactivity was also observed in cultured erythroid progenitor cells (Fig 7G) and bone marrow aspirate (Fig 7H). Furthermore, GM1201 immunoreactivity co-localized with the erythroid differentiation antigens ferritin H and glycophorin C (Fig 7G, H).

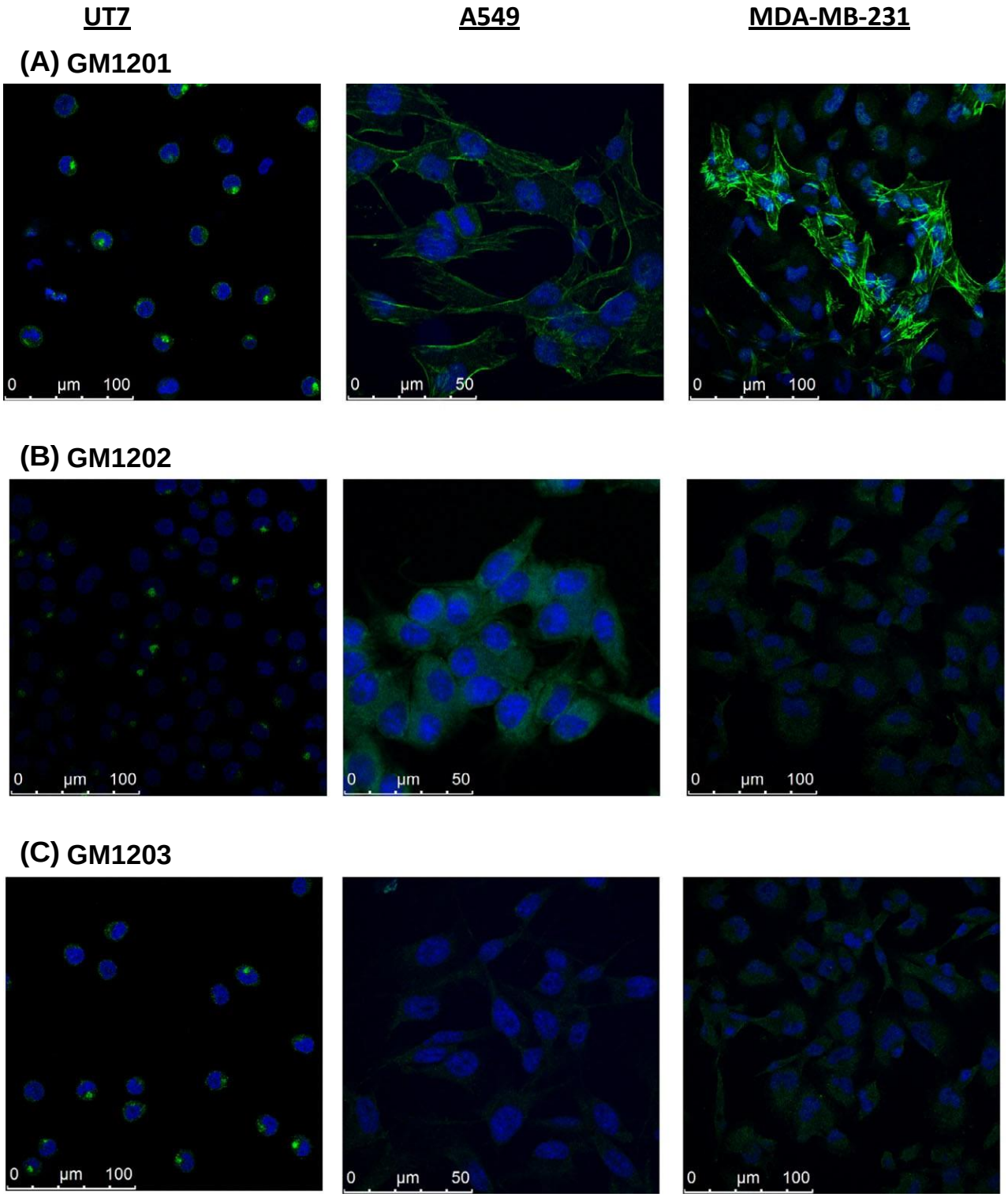


Fig 3. Detection of endogenous hEPOR by immunofluorescence. UT-7 cells were collected and fixed in 3% paraformaldehyde in phosphate-buffered saline at room temperature (RT) for 30 min. Primary antibodies (and isotype controls – not shown) were diluted to 4 lg/ml in quenching buffer. Secondary antibodies [fluorescein isothiocyanate (FITC)-conjugated AffinityPure Goat Anti-Mouse IgG for GM1202 and GM1203 antibodies and FITC-conjugated AffinityPure Goat Anti-Rat IgG for the GM1201 antibody] were diluted to 4 lg/ml. MDA-MB-231 and A549 cells were seeded on glass coverslips. Primary antibodies (and Isotype controls – not shown) were diluted to 20 lg/ml and secondary antibodies were used at 7·5 lg/ml. Nuclei were stained with DRAQ5 (2·5 lmol/l) for 30 min at RT. Confocal fluorescent images were obtained by a LEICA TCS SP5 II confocal microscope with a 63x objective.

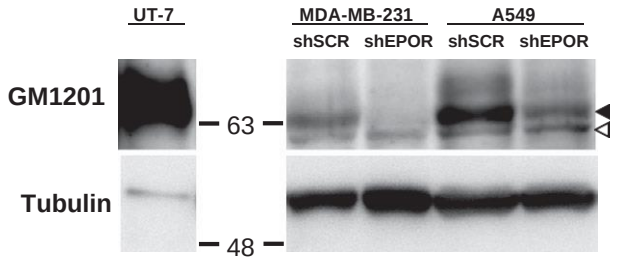


Fig 4. EPOR Western blot with GM1201 antibody. MDA-MB-231 and A549 cells were seeded in 60-mm plates at subconfluency and maintained for 72 h. UT-7 cells were grown in T-25 flasks for 48–72 h. Then MDA-MB-231 and A549 plates as well as UT-7 pellets were lysed in 350 µl of Laemmli buffer 1×. Cell lysates of UT-7 cells (0·2 µl) and EPOR-silenced MDA-MB-231 and A549 cells (shEPOR) (15 µl) as well as their corresponding controls (shSCR) (15 µl) were subjected to Western blot analysis with GM1201 antibody. Tubulin was used as loading control. The black arrowhead indicates the main specific EPOR Western blot signal (which specifically declines in EPOR-silenced cells), and the white arrowhead indicates the non-specific signal (does not decline in EPOR-silenced cells).

Discussion

A spectrum of strongly held views concerning EPOR function in tumours is evident in the literature, ranging from claims that malignant cells are devoid of functional EPOR-mediated signalling pathways to assertions that there is an overlap of cell signalling pathways that has pathogenic significance (Ghezzi *et al*, 2010).

Previous immunohistochemical studies on tumour tissue have drawn controversial conclusions about the expression of EPOR, based on antibodies that were later shown to cross-react with other cellular proteins (Elliott *et al*, 2006; Brown *et al*, 2007). The ideal antibody for IHC would be monoclonal rather than polyclonal, possess immunoreactivity against a defined EPOR domain and lack cross-reactivity with other tissue constituents. Unfortunately, most studies reported to date have used polyclonal antibodies or monoclonal antibodies of undefined specificity. Undoubtedly, the resulting discrepancies have masked the important debate about the safety of treating anaemic cancer patients with rHuEPO. Two other related factors that have received scant consideration are the occurrence of EPOR splice variants (Arcasoy *et al*, 2003) and the possible involvement of a heterodimeric form of the receptor comprising one EPOR and one common β chain component (Broxmeyer, 2013). Whereas homodimeric EPOR has been extensively studied, the existence of the heterodimeric complex is debated and requires further study.

The aim of the EpoCan consortium is to produce, characterize and validate a panel of EPOR monoclonal antibodies that would be readily available to the research community. These include antibodies raised against the ECD of EPOR for FACS analysis; those that recognize the denatured EPOR

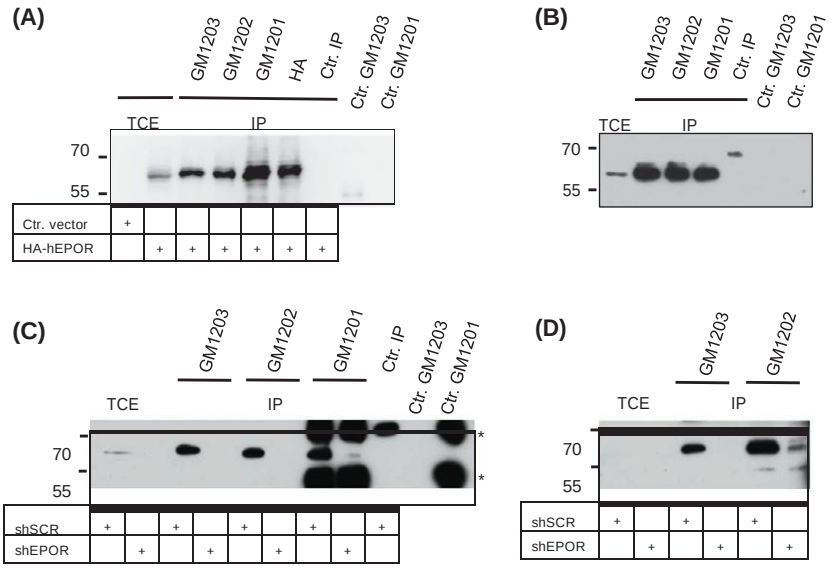
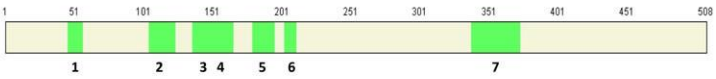


Fig 5. Antibodies that immunoprecipitate hEPOR. (A) Human HA-EPOR expressing plasmids (HA-hEPOR) or the empty control vector (Ctr. vector) were transfected in HEK293T cells. Precipitated EPOR was detected using the GM1201 antibody. 1/10 of the extract (4 µg) was loaded as total cell extract (TCE). Mouse anti-HA antibody 12CA5, (HA), was used as a positive IP control, mouse IgG was used as a negative control (Ctr. IP). GM1201, GM1203: GM1201 antibodies or GM1203 antibodies coupled to Protein-A agarose were incubated in the absence of cell extract and loaded. (B) IP of EPOR from UT-7 cells. Ctr. IP: IP with normal mouse antibodies. Ctr. GM1203, Ctr. GM1201: Antibodies coupled to Protein-A agarose were incubated in the absence of cell extract. TCE: 10% of the input for the IP (75 µg) was loaded. (C) IP of hEPOR from A549 lung carcinoma cells expressing control (shSCR) and three EPOR-specific small hairpin RNA (shEPOR). GM1201 was used for Western blot detection. Cross-reacting bands (*) were detected when GM1201 was used in IP and WB. Ctr. IP: IP with normal mouse antibodies; Ctr GM1203, Ctr. GM1201: antibodies coupled to Protein-A agarose incubated in the absence of cell extract. TCE: 10% of the protein input for the IP (150 µg) was loaded. (D) IP of hEPOR from MDA-MB-231 cells transfected with shSCR or shEPOR. TCE: 10% of the protein input for the IP (150 µg) was loaded. TCE, total cell extract; IP, immunoprecipitation; Ctr., control.

(A) **P19235** (EpoR_HUMAN) UniProtKB/Swiss-Prot
Coverage:23·82%



MDHLGASLWPQVGSLCLLLAGAAWAPPPNLPDPKFESKAALLAAR**GPEELLCFTER**LE
DLVCFWEEAASAGVGPGNYSFSYQLEDEPWKLCRLHQAPTARGAVR**FWCSLPTADTS**
SFVPLELRVTAASGAPRYHR**VIHINEVVLLDAPVGLVARLADESGHVVLR**WLPPPETP
MTSHIR**YEVDVSAGNGAGSVQR**VEILEGR**TECVLSNLR**GRTRYTFAVRARMAEPSFG
GFWSAWSEPVSLLTPSDLDPLILTLSLILVVILVLLTVLALLSHRRALKQKIWPGIPSPESEFE
GLFTTHKGNFQLWLYQNDGCLWWSPCTPFTEDPPASLEVLSER**CWGTMQAVEPGT**
DDEGPLLEPVGSEHAQDTYLVLDKWLLPRNPPSEDLPGPGGSVDIVAMDEGSEASSC
SSALASKPSPEGASAASFEYTILDPSSQLLRPWTLCPELPPTPPHLKYLYLVVSDSGISTDY
SSGDSQGAQGGLSDGPYSNPYENSLIPAAEPLPPSYVACS

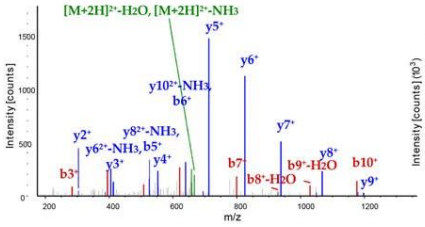
(B)

	Sequence	*Mol. mass [M+H⁺]*	*Charge*	*Xcorr*
(1)	GPEELLcFTER	1350·6379 Da	+2	2·58
(2)	FWcSLPTADTSSFVPLELR	2226·0945 Da	+2	4·76
(3)	VIHINEVVLLDAPVGLVAR	2027·2025 Da	+3	3·40
(4)	LADESGHVVLR	1195·6438 Da	+2	3·69
(5)	YEVDVSAGNGAGSVQR	1608·7631 Da	+2	3·97
(6)	TEcVLSNLR	1091·5516 Da	+2	2·37
(7)	cWGTmQAVEPGTDDEGPLLEPVGSEHAQDTYLVLDK	3973·8163 Da	+3	6·17

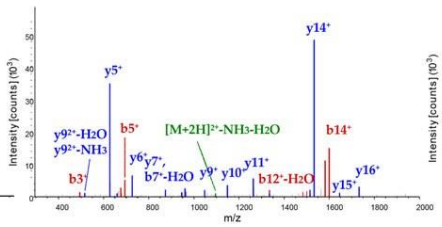
c*:* Carbamidomethylated cysteine; m: Oxidized methione

(C)

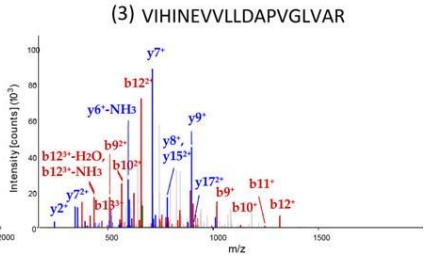
(1) GPEELLcFTER



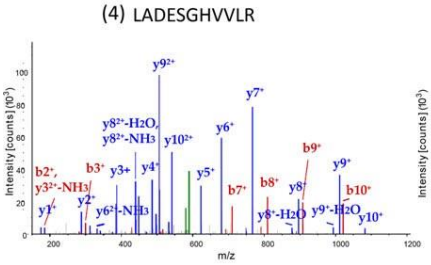
(2) FWcSLPTADTSSFVPLELR



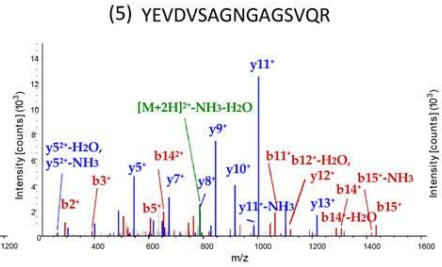
(3) VIHINEVVLLDAPVGLVAR



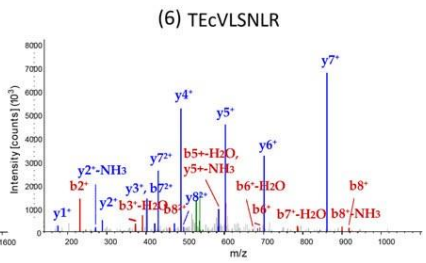
(4) LADESGHVVLR



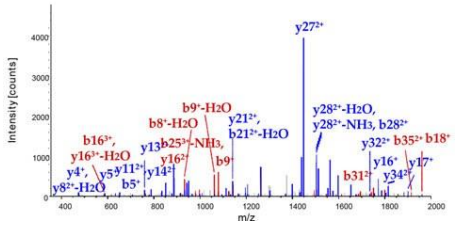
(5) YEVDVSAGNGAGSVQR



(6) TEcVLSNLR



(7) cWGTmQAVEPGTDDEGPLLEPVGSEHAQDTYLVLDK



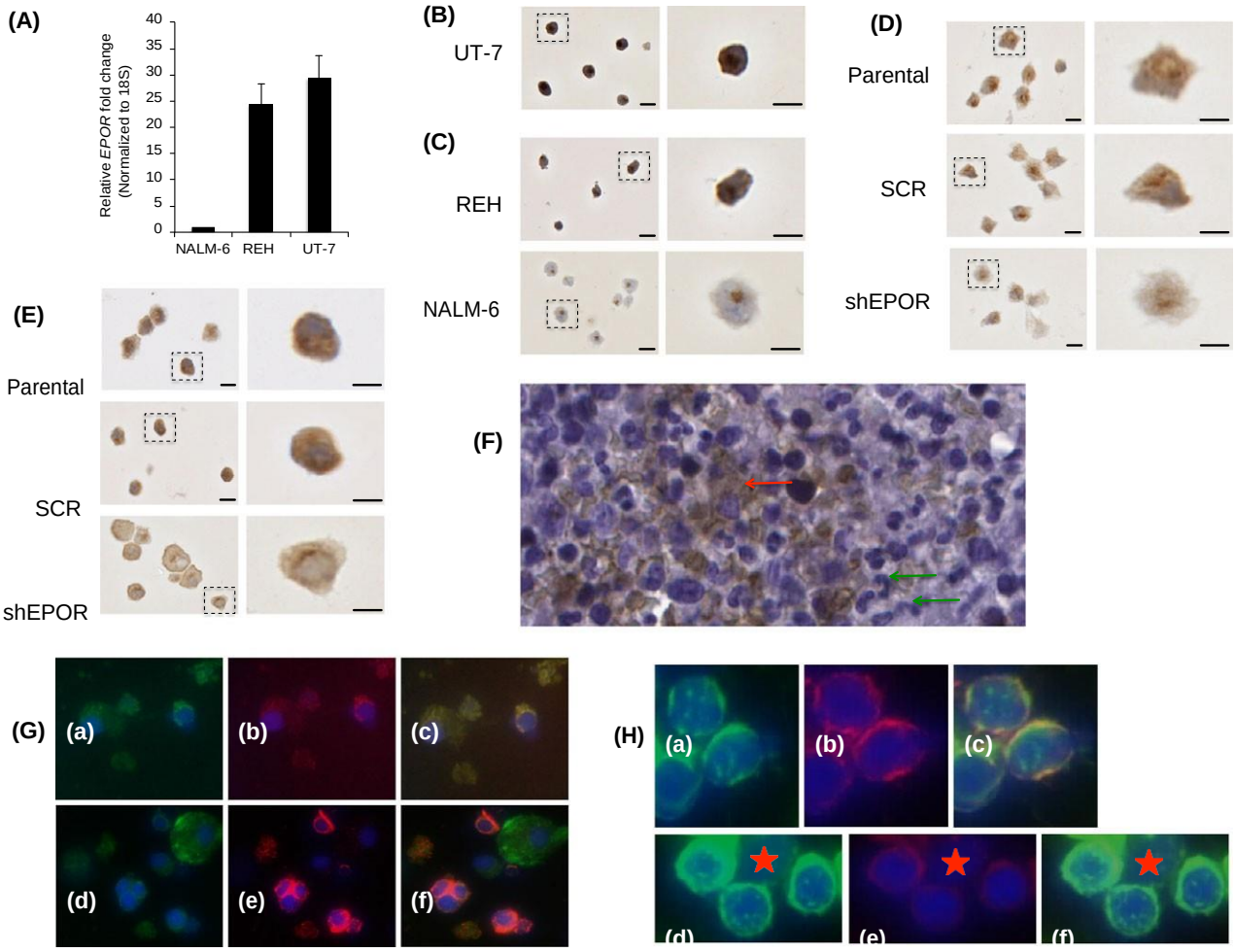


Fig 7. Immunocytochemical analysis of anti-EPOR antibodies in FFPE cancer cell lines and normal tissues. (A) Relative mRNA expression of *EPOR* in NALM-6, REH and UT-7 cell lines depicted as fold-change relative to NALM-6; $n = 3$, error bars indicate the standard error. (B) EPOR immunocytochemistry using GM1201 (4 lg/ml) in FFPE UT-7 cells. (C) EPOR immunoreactivity using GM1201 (4 lg/ml) in FFPE REH human pre-B ALL cells compared with FFPE NALM-6 human non-T/non-B ALL cells. (D) EPOR immunocytochemical analysis using GM1201 (4 lg/ml) in FFPE MDA-MB-231 cells. shEPOR cells represent parental cells transfected with three *EPOR*-specific shRNA seuences. (E) EPOR immunocytochemical analysis using GM1201 (4 lg/ml) in FFPE A549 cells. shEPOR cells represent parental cells transfected with three *EPOR*-specific shRNA sequences. (F) Immunohistochemical staining of bone marrow for EPOR. Bone marrow aspirate from a patient with polycythaemia vera stained with rat anti-EPOR (GM1201). Red arrow denotes an erythroblastic island consisting of a central macrophage surrounded by erythroblasts (brown staining). Nuclei were counterstained with haematoxylin (blue). Green arrows denote metamyelocytes and neutrophil band forms, which are clearly negative for EPOR staining. Final magnification 9750. (G) Immunohistochemical dual staining of erythroid progenitor cells. To illustrate erythroblast differentiation, cryopreserved bone marrow mononuclear cells were cultured for 14 d in MethoCult™ H4034 Optimum and dual stained with rat anti-EPOR (GM1201) and either rabbit anti-ferritin H or rabbit anti-glycophorin C. Co-immunoreactivity of EPOR (a, green) and ferritin heavy chain (b, red) is evident during erythroid differentiation (c, merge). EPOR is expressed more prominently in an early erythroblast (green; d and f) and to a lesser degree in later stages as demonstrated by cells with glycophorin C expression (red; e and f). Nuclei were counterstained with DAPI (blue). Cultures were obtained from the Stem Cell Technologies Human Bone Marrow Proficiency Testing Program and used with permission. Final magnification 9630. (H) Immunohistochemical dual staining of bone marrow. Bone marrow aspirate from a patient with erythroid hyperplasia dual stained with rat anti-EPOR (GM1201) and either rabbit anti-ferritin H or rabbit anti-glycophorin C showing co-immunoreactivity of EPOR (a, green) and ferritin H (b, red) in panel c (merged). EPOR (d, green) and glycophorin C (e, red) show similar cellular localization (merged images, f). The red star in (d–f) indicates a differentiating erythroblast with low immunoreactivity for both EPOR and glycophorin C. Nuclei were counterstained with DAPI (blue). Final magnification 9630. All slides were scanned using the Hamamatsu Nanozoomer 2.0 HT scanner, C9600 series. Each specimen was scanned at brightfield 409 magnification using nine layers and 3·0 lm spacing, with an off set of either 0 or +3. Scale bars, 10 lm.

Fig 6. Identification of hEPOR by Nano-LC-ESI-MS/MS of GM1202 and GM1203 immunoprecipitates. (A) Illustration of identified hEPOR specific peptides in the amino acid sequence of EPOR Precursor P19235. Identified specific peptides are schematically illustrated as green boxes and highlighted in red in the amino acid sequence. (B) The table represents the amino acid sequences of the identified peptides (1–7), their singly protonated molecular ions [$M+H^+$], charge states and cross-correlation Xcorr values. (C) Tandem mass spectroscopy ion trap collision-induced dissociation (CID) spectra of peptides (1–7). Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3.

Table III. Specificity of EPOR antibodies in FFPE cell line models.

	Immunogen	Antibody	Isotype	Epitope location	UT-7	REH/NALM-6 model			shEPOR isogenic models – reduced expression in shEPOR cells?		Lung tissue – effective?
						REH	NALM-6	Difference?	MDA-MB-231	A549	
1	Peptide	GM1201	r-IgG2b	h-EPOR cytoplasmic domain	2+	3+	1+	Yes	Yes	Yes	Yes
2	Genetic	GM1202	m-IgG1a	h-EPOR ECD	3+	3+	2+	Yes	No	No	No
3	Immunization	GM1203	m-IgG1a	h-EPOR ECD	3+	2+	1+	Yes	Yes	No	No
4		GM1204	r-IgG2a	h-EPOR ECD	–	N/E	N/E	N/A	N/E	N/E	N/E

FFPE, formalin-fixed paraffin wax-embedded; h-EPOR, human erythropoietin receptor; ECD, extracellular domain; N/E, not evaluated; N/A, not applicable.
Shaded areas are meant to distinguish between peptide immunization and genetic immunization.

and/or potential EPOR isoforms by Western blot; and those that would be useful for IHC studies of clinical specimens.

In the current study, peptide immunization was used to generate antibodies for assays on denatured proteins, such as Western blotting and IHC, whereas novel genetic immunization protocols were used to generate antibodies that recognize EPOR in its native conformation for flow cytometry, immunofluorescence and IP assays. In total, 15 monoclonal antibody mother clones, were pretested in different assays for human EPOR. They comprised six rat monoclonal antibodies generated by immunization of synthetic peptides based on the cytoplasmic domain of the hEPOR, as well as four murine and five rat monoclonal antibodies generated against the hEPOR ECD domain by genetic immunization.

The specificity of the EPOR antibodies has been validated by the use of EPOR-silenced cells. For example, we show that an endogenous Western blot signal using the GM1201 antibody is specifically down-regulated in EPOR-silenced cells, strongly indicating that this antibody recognizes endogenous EPOR in these cells. Although the GM1201 antibody may also detect non-specific signals in the A549 cell line (Fig 3) the EPOR-silenced cells clearly permit discrimination between a true EPOR-dependent signal and the non-specific signal. The number of monoclonal antibodies was reduced to a panel of four for Western blotting, IHC, IP, immunofluorescence and flow cytometry.

For simplicity, the monoclonal antibodies have been systematically named, based on an in-house nomenclature system at Aldevron Freiburg (see Table I). GM1201 is a rat monoclonal antibody raised against one of six synthetic peptides used to immunize rats, which is located in the cytoplasmic domain of hEPOR (Table I). This antibody reveals specific down-regulation of EPOR in EPOR-silenced MDA-MB-231 and A549 cells, confirming that it also recognizes endogenous EPOR in these cells. Importantly, GM1201 was sensitive and specific in immunohistochemical studies of both cultured erythroid cells and bone marrow aspirates. For example using FFPE sections of bone marrow and bone marrow aspirates, it was possible to visualize erythroblastic islands in a patient with polycythaemia vera, and to demonstrate co-localization of EPOR with either ferritin H or glycophorin C in differentiating erythroid progenitors in a patient with erythroid hyperplasia.

GM1202 and GM1203 are mouse monoclonal antibodies, raised by genetic immunization against the ECD of hEPOR. By co-localization of an ECFP-tagged EPOR and the signal obtained with our new antibodies GM1202 and GM1203 we show that GM1202 and GM1203 detect EPOR when it is expressed on the membrane (Fig 4). Furthermore, GM1202 and GM1203 have proved useful in immunopreciptation experiments in combination with GM1201 (Fig 7). GM1204 is a rat monoclonal antibody raised by genetic immunization of the ECD of hEPOR which detects both human and murine EPOR by FACS (mouse data not shown).

In a thorough, often overlooked study, Arcasoy *et al* (2003) isolated and characterized several novel cDNAs for EPOR splice variants expressed in cancer cells. Predicted amino acid sequences of these cDNAs indicated splice variants encoding soluble EPOR, variants containing insertions from intron 6 or intron 7, and membrane-bound EPOR peptides with intracytoplasmic truncations. These multiple EPOR isoforms in human cancer cells may modulate the cellular effects of recombinant EPO. Recently, Elliott *et al* (2013) reported on differences in detection of EPOR in primary human tumour tissue samples using different antibodies. Using an anti-hEPOR monoclonal antibody, they could not detect EPOR protein in normal human and matching cancer tissues from breast, lung, colon, ovary or skin. Detection of EPOR in breast cancer tissues using a polyclonal antibody was interpreted as cross-reactivity. However, it cannot currently be ruled out that the epitope recognized by the specific monoclonal antibody could be missing in EPOR isoforms found in tumour tissues.

The availability of monoclonal antibodies directed against specific exons, as those described herein, will enable, for the first time, the investigation of the resulting EPOR protein isoforms in different tissues. Thus, one antibody may recognize an epitope common to many EPOR isoforms, indicating

a broader EPOR expression pattern compared to other antibodies whose epitope might only be present in fewer EPOR isoforms, with a more limited expression pattern.

The new antibodies will enable many interesting topics in EPOR biology to be explored. These include resolution of the major clinical question of which patients can be treated safely with EPO and its derivatives, dissection of the signalling mechanisms in non-erythroid cells and investigation of cancer cell:stromal cell interactions in tumours.

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Authorship contributions

John Thompson and Fritz Grunert generated the antibodies and performed initial screenings. Perry Maxwell, Florinda Melendez, Kyle B. Matchett, Heidelinde Jaekel, Herbert Lindner, Nathalie Ben-Califa, Andre Bernardini, Ulf Brockmeier, Markus Thiersch, Edith Schneider-Gasser, Alexandra Irvine and Anne Jordan planned and performed experiments, as well as analysed data. Perry Maxwell, Kyle B. Matchett, Julian Aragones, Ludger Hengst, Joachim Fandrey, Howard Oster, Moshe Mittelman, Robert Cuthbert, Mohamed El-Tanani, Max Gassmann, David Dangoor, Terence Lappin and Drorit Neumann planned experiments, analysed data and wrote the manuscript.

Competing interests

The authors have no competing interests.

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