Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3118361 A1 2019/05/09

(21) 3 118 361

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2018/11/01
(87) Date publication PCT/PCT Publication Date: 2019/05/09
(85) Entrée phase nationale/National Entry: 2021/04/28
(86) N° demande PCT/PCT Application No.: EP 2018/079949
(87) N° publication PCT/PCT Publication No.: 2019/086580
(30) Priorité/Priority: 2017/11/01 (EP17199552.5)

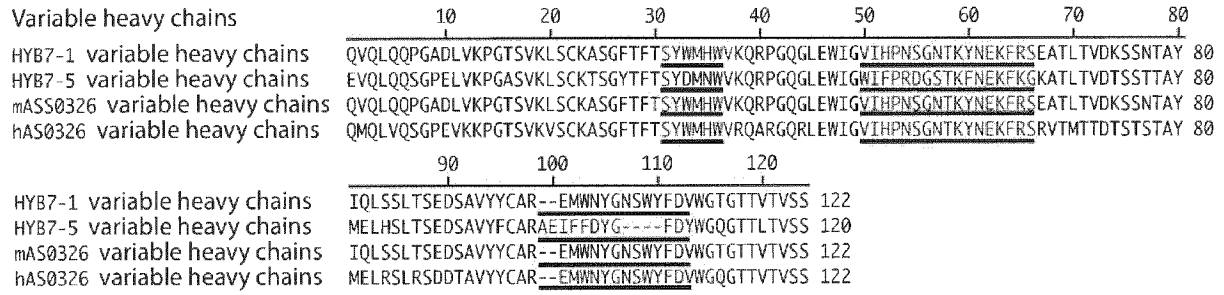
(51) Cl.Int./Int.Cl. *C07K 16/18* (2006.01), *A61K 39/00* (2006.01), *C07K 16/28* (2006.01)

(71) Demandeur/Applicant:
SYDDANSK UNIVERSITET, DK

(72) Inventeurs/Inventors:
LYKKE SORENSEN, GRITH, DK;
SCHLOSSER, ANDERS, DK;
HOLMSKOV, UFFE, DK

(74) Agent: BENOIT & COTE INC.

(54) Titre : ANTICORPS DIRIGES CONTRE MFAP4
(54) Title: ANTIBODIES AGAINST MFAP4



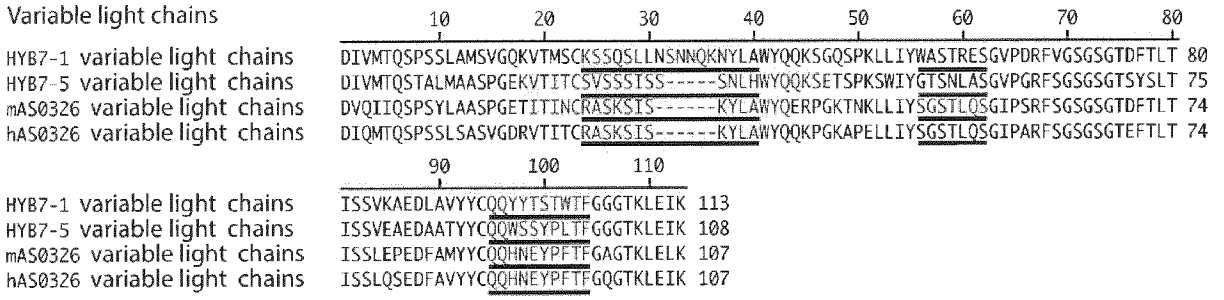




Fig. 1

(57) Abrégé/Abstract:
The present invention relates to antibodies, including humanized antibodies that bind human Microfibrillar-associated protein 4 (MFAP4). The invention also relates to uses of such antibodies.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
09 May 2019 (09.05.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/086580 A1

(51) International Patent Classification:
*C07K 16/18* (2006.01) *A61K 39/00* (2006.01)
*C07K 16/28* (2006.01)

(21) International Application Number:
PCT/EP2018/079949

(22) International Filing Date:
01 November 2018 (01.11.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
17199552.5 01 November 2017 (01.11.2017) EP

(71) Applicant: **SYDDANSK UNIVERSITET** [DK/DK]; Campusvej 55, 5230 Odense M (DK).

(72) Inventors: **LYKKE SØRENSEN, Grith**; Bjørnemosevej 116, 5260 Odense S (DK). **SCHLOSSER, Anders**; Rikkesminde Allé 12, 5250 Odense SV (DK). **HOLMSKOV, Uffe**; Bjørnemosevej 116, 5260 Odense S (DK).

(74) Agent: **PLOUGMANN VINGTOFT A/S**; Strandvejen 70, 2900 Hellerup (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Declarations under Rule 4.17:**
— *of inventorship (Rule 4.17(iv))*

(54) **Title:** ANTIBODIES AGAINST MFAP4

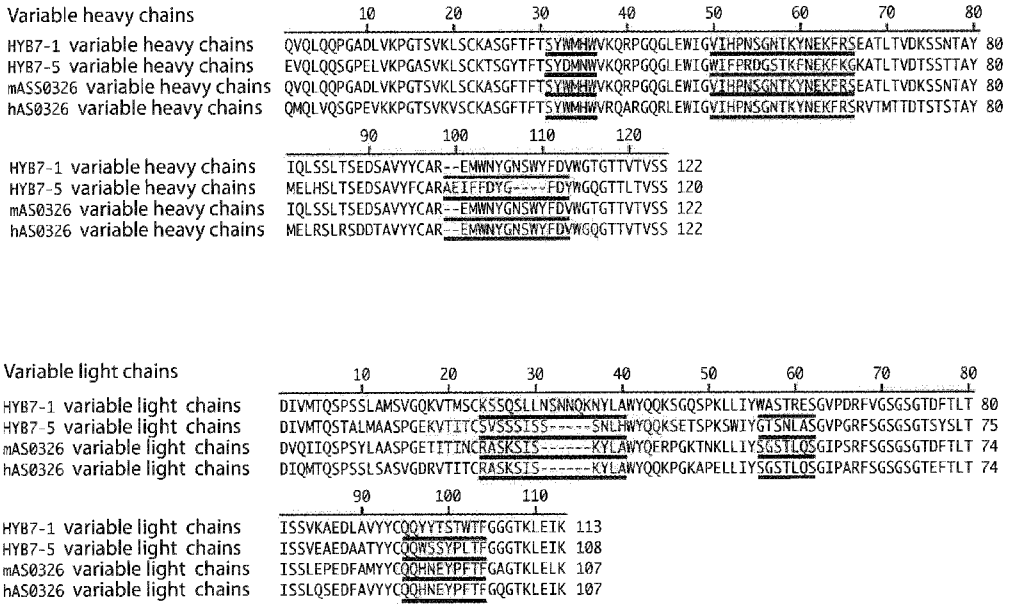



Fig. 1

(57) **Abstract:** The present invention relates to antibodies, including humanized antibodies that bind human Microfibrillar-associated protein 4 (MFAP4). The invention also relates to uses of such antibodies.

*[Continued on next page]*

WO 2019/086580 A1

WO 2019/086580 A1

**Published:**
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

# ANTIBODIES AGAINST MFAP4

## Technical field of the invention

The invention relates generally to medicine and the use of antibodies. The present invention specifically relates to a novel antibody, in particular a humanized monoclonal antibody that binds human Microfibrillar-associated protein 4 (MFAP4).

## Background of the invention

MFAP4 is a 36 kDa glycoprotein composed of a short N-terminal region that contains a potential integrin binding RGD sequence followed by a fibrinogen related domain (FReD). The protein forms a homo-oligomeric structure under native conditions. FReDs are found in a diverse group of human proteins involved in different functions such as coagulation, angiogenesis, tissue growth and remodeling, and innate immunity.

MAGP-36/MFAP4 was first identified as a protein with tenascin resemblance in the amino acid composition and localized to ECM in arteries. MAGP-36 was following demonstrated with direct interaction with ECM fibres including elastin, collagen, or calvasculin. The interaction between MAGP-36 and cellular integrin receptors was demonstrated using inhibition by RGD containing peptides of human aortic smooth muscle cells in attachment to immobilized MAGP-36.

WO 2014/114298 discloses different antibodies (including HG-HYB 7-5), which target MFAP4.

WO 2016/008498 also discloses antibodies, which target MFAP4 (including HG-HYB 7-1).

Hence, improved antibodies against MFAP4 would be advantageous, and in particular a more efficient and/or reliable antibody, which binds to MFAP4 would be advantageous. In addition, new uses of MFAP4 antibodies would be advantageous.

## Summary of the invention

The present invention provides novel antibodies targeting MFAP4, including humanized antibodies. These antibodies have different sequences and different binding properties compared to known antibodies targeting MFAP4.
All RGD dependent integrins may potentially interact with this RGD site, however integrins αVβ3/5 are highly relevant for investigation of vascular remodelling, angiogenesis, vascular leakage and inflammation.

Medical uses of the antibodies according to the invention are described in the example section.

Thus, an object of the present invention relates to the provision of novel ligands (antibodies) targeting MFAP4. In particular, it is an object of the present invention to provide humanized antibodies against MFAP4.

Thus, one aspect of the invention relates to a protein ligand, such as an antibody, which binds to a new epitope of MFAP4.

Another aspect of the present invention relates to a protein ligand, such as an antibody, or the ligand according to the invention, comprising

- a light chain variable region comprising a CDR 1 region according SEQ ID NO: 9, a CDR 2 region according to SEQ ID NO: 10 and a CDR 3 region according to SEQ ID NO: 11; and
- a heavy chain variable region comprising a CDR 1 region according SEQ ID NO: 12, a CDR 2 region according to SEQ ID NO: 13 and a CDR 3 region according to SEQ ID NO: 14.

Yet another aspect of the present invention is to provide a protein ligand, such as an antibody, or the ligand according to the invention, comprising

- a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1 or 3, or sequences having at least 80% sequence identity, such as at least 90% sequence identity, or such as at least 95% sequence identity to SEQ ID NO: 1 or 3.
- a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2 or 4, or sequences having at least 80% sequence identity, such

as at least 90% sequence identity, or such as at least 95% sequence identity to SEQ ID NO: 2 or 4.

Still another aspect of the present invention is to provide a vector encoding the ligand according to the invention.

A further aspect relates to a cell expressing the ligand according to the invention, and/or a cell comprising the vector according to the invention.

Yet a further aspect relates to a composition comprising the ligand according to the invention, and one or more physiologically acceptable carriers, excipients and/or diluents.

An additional aspect relates to ligand according to the invention and/or composition according to the invention, for use as a medicament.

Yet an aspect relates to the ligand according to the invention or the composition according to the invention for use in the prevention or treatment of vascular diseases characterized by pathological proliferation or vascular leakage or inflammation or fibrosis and/or related disorders in a mammal.

**Brief description of the figures**

Figure 1 shows alignment of variable heavy chain (HC) and light chain (LC) sequences for monoclonal antibodies HG Hyb 7-1 (HYB7-1)(LC=SEQ ID NO: 5 and HC=SEQ ID NO: 6), HG Hyb 7-5 (HYB7-5) (LC=SEQ ID NO: 7 and HC=SEQ ID NO: 8), mAS0326 (LC=SEQ ID NO: 1 and HC=SEQ ID NO: 2) and hAS0326 (LC=SEQ ID NO: 3 and HC=SEQ ID NO: 4). CDR sequences are indicated by underlining.

Figure 2 shows epitope mapping of antibodies by competition ELISA. Biotinylated **A)** HG HYB 7-5 (biotinHYB7-5), **B)** hAS0326 (biotinhAS0426) and **C)** mAS0326 (bmAS0326) at a fixed concentration of 0.5 µg/ml were mixed with unlabelled IgG isotype control, HG HYB 7-18 (HYB7-18), HG HYB 7-5 (HYB7-5), hAS0326 and mAS0326 in a 2-fold dilution from 20 µg/ml. The resulting binding patterns demonstrate that the binding of biotinylated monoclonal antibody of interest to

immobilized target recombinant MFAP4 can be inhibited by the same unlabelled Mab. Only HG Hyb 7-5 was capable of inhibiting the HG HYB 7-5 interaction with target. In contrast, mAS0326 was capable of inhibiting hAS0326 binding to target and vice versa.

Figure 3 shows binding of biotinylated monoclonal antibodies (Mab) hAS0326 (biotinhAS0326), mAS0326 (biotinmAS0326) and HG HYB 7-5 (biotinHYB7-5) to **A)** recombinant human MFAP4, **B)** recombinant human MFAP4 with RGD integrin binding motif mutated to AAA and **C)** recombinant mouse MFAP4.

Figure 4 shows binding of HG HYB 7-5, mAS0326 and hAS0326 to mouse serum MFAP4 and human serum MFAP4. Serum from MFAP4-deficient mice (MFAP4 KO) is included as negative control. Microtiter plates coated with **A)** HG HYB 7-5, **B)** mAS0326 and **C)** hAS0326. Biotinylated HG HYB 7-18 was used as detector antibody.

Figure 5 shows antibody aggregation in solution. hAS0326 and mAS0326 were concentrated to the indicated concentrations [7.4 mg/ml – 67 mg/ml] before size exclusion chromatography (SEC) was performed. **A)** SEC chromatogram with mAS0326 concentrated to 44 mg/ml and hAS0326 concentrated to 67 mg/ml. **B)** Zoom of the SEC chromatogram for all tested concentrations. **C)** Test of purity using SDS-PAGE and Commassie staining of 3 µg/lane of hAS0326 and mAS0326 in the reduced state (R) and unreduced state (UR) (M = MW marker).

Figure 6 shows mAS0326-mediated inhibition of pathological ocular angiogenesis, vascular leakage and inflammatory infiltration in the mouse laser-induced CNV model. Intraocular injections of antibodies were performed on day 1 and day 7 after laser burn. There are 4-8 mice per treatment group with up to 4 lesions per eye. Each data point represents one lesion. Fundus fluorescein angiography at **A)** day 7 and **B)** day 14 shows a decrease in lesion size (combined angiogenesis/vascular leakage) in eyes treated with either mAS0326 or anti-VEGF. Scale bar = 100µm. Intraocular injection of mAS0326 also reduces angiogenesis per se (as measured by endothelial marker Lectin IB4 positive volume of the choroid) **C)** and CD45 (inflammatory cell) positive cells infiltrating the choroids **D).** Scale bar = 50µm. Data in C and D are obtained by confocal

imaging of excised choroidal tissue 14 days after laser induced choroidal neovascularization. Statistics shown are for comparison between treatment groups only (scatterplots with median and interquartile range). * $p < 0.05$, *** $p < 0.001$ (Kruskall-Wallis test and Dunn's multiple comparisons test).

Figure 7 shows hAS0326-mediated inhibition of pathological ocular angiogenesis, vascular leakage and inflammatory infiltration in the mouse laser-induced CNV model.

Intraocular injections of antibodies were performed on day 1 and day 7 after laser burn. There are 6-8 mice per treatment group with up to 4 lesions per eye. Each data point represents one lesion. Fundus fluorescein angiography at **A)** day 7 and **B)** day 14 shows a decrease in lesion size (combined angiogenesis/vascular leakage) in eyes treated with hAS0326 compared to anti-VEGF treated eyes. Intraocular injection of hAS0326 also reduces angiogenesis per se (as measured by endothelial marker Lectin IB4 positive volume of the choroid) **C)** and CD45 (inflammatory cell) positive cells infiltrating the choroids. The combinatorial treatment with hAS0326 and anti-VEGF enhances this effect further **D)**. Data in C and D are obtained by confocal imaging of excised choroidal tissue 14 days after laser induced choroidal neovascularization. Statistics shown are for comparison between treatment groups only (scatterplots with median and interquartile range). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Kruskall-Wallis test and Dunn's multiple comparisons test). Scale bar = 100µm.

Figure 8 shows hAS0326-mediated inhibition of pathological ocular leakage in streptozotozin (STZ)-induced rat diabetes model. STZ (50 mg/kg, i.p.) was used to induce diabetes in Sprague–Dawley rats (n = 8). Diabetic rats were treated with intraocular injections of antibodies on day 1 and day 7 after onset of STZ treatment. Vascular area was calculated as the mean from three parts of the retina in each animal using Imaris software and measured as area occupied by Evans Blue dye positive vasculature in a 2D plane. Statistics shown are for comparison between treatment groups only (scatterplots with median and interquartile range). * $p < 0.05$ (Kruskall-Wallis test and Dunn's multiple comparisons test). **A)** Day 0; **B)** Day 21.

Figure 9 shows the ability of hAS0326 and variants thereof to inhibit MFAP4-induced cellular activation as assessed by retinal endothelial migration assay. Retinal endothelial migration towards VEGF was assayed using transwell filters. **A)** Full-length hAS0326- versus Fab-mediated inhibition of endothelial migration and **B)** Full-length hAS0326- versus $F(ab')_2$-mediated inhibition of endothelial migration.

The present invention will now be described in more detail in the following.

**Detailed description of the invention**

Definitions

Prior to discussing the present invention in further details, the following terms and conventions will first be defined:

The ligands (such as antibodies and antigen binding domains) of the invention bind selectively to MFAP4 that is they bind preferentially to MFAP4 with a greater binding affinity than to other antigens. The antibodies may bind selectively to human MFAP4, but also bind detectably to non-human MFAP4, such as murine MFAP4. Alternatively, the antibodies may bind exclusively to human MFAP4, with no detectable binding to non-human MFAP4.

The term "protein ligand" refers to ligands constituted mainly of amino acids, such as antibodies or fragments thereof. As also disclosed in here, the protein ligands may comprise moieties such as detectable labels or a substance having toxic or therapeutic activities.

The term "monoclonal antibody" refers to an antibody obtained from a population of substantially homogeneous antibodies, wherein each monoclonal antibody will typically recognize a single epitope on the antigen. The term "monoclonal" is not limited to any particular method for making the antibody. For example, monoclonal antibodies of the invention may be made by the hybridoma method as described in Kohler et al. Nature 256, 495 (1975) or may be isolated from phage libraries using the techniques as described herein.

The term "antigen binding domain" or "antigen binding region" or "fragment or

derivative thereof" refers to that portion of the selective binding agent (such as an antibody molecule), which contains the amino acid residues that interact with an antigen and confer on the binding agent its specificity and affinity for the antigen. Preferably, the antigen binding region will be of human origin. In other embodiments, the antigen binding region can be derived from other animal species, in particular domestic animal and rodents such as rabbit, rat or hamster. The terms "effective amount" and "therapeutically effective amount" when used in relation to an antibody or antigen binding domain, fragment or derivative thereof, immunoreactive with a MFAP4 peptide, refer to an amount of a selective binding agent that is useful or necessary to support an observable change in the level of one or more biological activities of MFAP4, wherein said change may be either an increase or decrease in the level of MFAP4 activity.

In the context of the present invention, the term "sequence identity" or "homologue" indicates a quantitative measure of the degree of homology between two amino acid sequences or between two nucleic acid sequences. If the two sequences to be compared are not of equal length, they must be aligned to give the best possible fit, allowing the insertion of gaps or, alternatively, truncation at the ends of the polypeptide sequences or nucleotide sequences. The sequence identity can be calculated as $\frac{(N_{ref}-N_{dif})100}{N_{ref}}$, wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC (Ndif=2 and Nref=8). A gap is counted as non-identity of the specific residue(s), i.e. the DNA sequence AGTGTC will have a sequence identity of 75% with the DNA sequence AGTCAGTC (Ndif=2 and Nref=8).

With respect to all embodiments of the invention relating to amino acid sequences or nucleotide sequences, the percentage of sequence identity between one or more sequences may also be based on alignments using the clustalW software (http:/www.ebi.ac.uk/clustalW/index.html) with default settings. For nucleotide sequence alignments these settings are: Alignment=3Dfull, Gap Open 10.00, Gap Ext. 0.20, Gap separation Dist. 4, DNA weight matrix: identity (IUB). For amino acid sequence alignments the settings are as follows: Alignment=3Dfull, Gap

Open 10.00, Gap Ext. 0.20, Gap separation Dist. 4, Protein weight matrix: Gonnet.
Alternatively, nucleotide sequences may be analysed using programme DNASIS Max and the comparison of the sequences may be done at http://www.paralign.org/. This service is based on the two comparison algorithms called Smith-Waterman (SW) and ParAlign. The first algorithm was published by Smith and Waterman (1981) and is a well-established method that finds the optimal local alignment of two sequences. The other algorithm, ParAlign, is a heuristic method for sequence alignment; details on the method are published in Rognes (2001). Default settings for score matrix and Gap penalties as well as E-values were used.

In the present context, the terms "$K_D$" or "$K_D$ value" refer to the equilibrium dissociation constant between the antibody (ligand) and its antigen. The $K_D$ value relates to the concentration of antibody (the amount of antibody needed for a particular experiment) and so the lower the $K_D$ value (lower concentration) and thus the higher the affinity of the antibody. In the present context, $K_D$ is measured by Biacore T200.

In the context of the present invention, the definition "AA-yy referring to SEQ ID NO: X" is to be understood as AA=amino acid; -yy is the position of the amino acid in the SEQ ID NO: X. Thus, for example "Trp-33 referring to SEQ ID NO: 2" is to be understood as Trp at position 33 in SEQ ID NO: 2.

In the context of the present invention, the definition "at the most five amino acids" is to be understood as no more than five amino acids i.e. five amino acids, four amino acids, three amino acids, two amino acids or one amino acid.

In the context of the present invention, the definition "a sequence where at the most xx amino acids differ from the SEQ ID NO: X" is to be understood as the sequence being identical to the SEQ ID NO: X except for xx amino acids, which may be different i.e. a different amino acid than the one listed in the sequence. Thus, if at the most two amino acids differ from the SEQ ID NO: 9 this is to be understood as a sequence which differs from the SEQ ID NO: 9 by two, one or none amino acids. "X" is to be understood as any of the sequence listings SEQ ID

NO: 1-14 as listed herein. Alternatively, "X" is to be understood as any of the sequence listings SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14. "xx" is to be understood as any of the numbers five, four, three, two or one.

**Ligand**

As mentioned above, in here is disclosed novel antibodies targeting MFAP4, including humanized antibodies. These antibodies have different sequences and different binding properties compared to known antibodies targeting MFAP4. Medical uses of the antibodies are described in the example section. Thus, in a first aspect, the invention relates to a protein ligand, such as an antibody, which binds to a novel epitope of MFAP4.

The present inventors have identified that the antibodies according to the invention have different CDR sequences compared to known anti-MFAP4 antibodies. Thus, in yet an aspect, the invention relates to a protein ligand, such as an antibody, or a ligand according to the invention, comprising

- a light chain variable region comprising a CDR 1 region according SEQ ID NO: 9, a CDR 2 region according to SEQ ID NO: 10 and a CDR 3 region according to SEQ ID NO: 11; and
- a heavy chain variable region comprising a CDR 1 region according SEQ ID NO: 12, a CDR 2 region according to SEQ ID NO: 13 and a CDR 3 region according to SEQ ID NO: 14.

By means of X-ray crystallography of the paratope of the antibody according to this invention and the epitope of MFAP4 and performed as known to the skilled person in the art, the present inventors have further identified amino acids in the CDRs of the light chain variable region and heavy chain variable region, which strongly interact with the epitope of MFAP4 and thus are important for the binding of the paratope of antibody with the epitope of MFAP4. Thus, in one embodiment, the invention relates to a protein ligand, such as an antibody, or a ligand according to the invention, comprising

- a light chain variable region comprising
    - a CDR 1 region according to SEQ ID NO: 9 or according to a sequence where at the most five amino acids differ, such as at the

most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 9 with the proviso that the amino acid at position 9 is a Tyr;

- a CDR 2 region according to SEQ ID NO: 10 or according to a sequence where at the most five amino acids differ, such as at the most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 10; and
- a CDR 3 region according to SEQ ID NO: 11 or according to a sequence where at the most five amino acids differ, such as at the most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 11 with the proviso that the amino acid at position 6 is a Tyr; and

- a heavy chain variable region comprising
  - a CDR 1 region according SEQ ID NO: 12 or according to a sequence where at the most five amino acids differ, such as at the most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 12 with the proviso that the amino acid at position 3 is a Trp;
  - a CDR 2 region according to SEQ ID NO: 13 or according to a sequence where at the most five amino acids differ, such as at the most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 13; and
  - a CDR 3 region according to SEQ ID NO: 14 or according to a sequence where at the most five amino acids differ, such as at the most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 14 with the proviso that the amino acid at position 1 is a Glu and the amino acid at position 9 is a Trp.

Also, by means of X-ray crystallography performed as known to the skilled person in the art, the present inventors have further identified amino acids in the CDRs of the heavy chain variable region, which are strongly involved in the packaging of the antibody. Thus, in a still further embodiment, the invention relates to a protein ligand, such as an antibody, or a ligand according to the invention, comprising

- a light chain variable region comprising
  - a CDR 1 region according SEQ ID NO: 9 or according to a sequence where at the most five amino acids differ, such as at the most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 9 with the proviso that the amino acid at position 9 is a Tyr;
  - a CDR 2 region according to SEQ ID NO: 10 or according to a sequence where at the most five amino acids differ, such as at the most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 10; and
  - a CDR 3 region according to SEQ ID NO: 11 or according to a sequence where at the most five amino acids differ, such as at the most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 11 with the proviso that the amino acid at position 6 is a Tyr; and
- a heavy chain variable region comprising
  - a CDR 1 region according to SEQ ID NO: 12 or according to a sequence where at the most five amino acids differ, such as at the most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 12 with the proviso that the amino acid at position 3 is a Trp and the amino acid at position 2 is a Met;
  - a CDR 2 region according to SEQ ID NO: 13 or according to a sequence where at the most five amino acids differ, such as at the

most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 13 with the proviso that the amino acid at position 4 is a Pro; and

- a CDR 3 region according to SEQ ID NO: 14 or according to a sequence where at the most five amino acids differ, such as at the most four amino acids differ, like at the most three amino acids differ, such as at the most two amino acids differ, like at the most one amino acid differ from SEQ ID NO: 14 with the proviso that the amino acid at position 1 is a Glu and the amino acid at position 9 is a Trp.

Thus, according to the above embodiments, the CDR regions may differ from the sequences as defined by the SEQ ID NO: 9-14 except for the particular amino acids demonstrated to be important for the binding to the epitope (see example 12).

As shown in the example section, novel antibodies against MFAP4 have been produced (see examples 3 and 4 + corresponding figures). These antibodies have different binding properties compared to other MFAP4 antibodies (see example 5). The antibodies show several advantages compared to other antibodies against MFAP4:

- The humanized version may be more stable in solution, by showing less aggregation (see example 6 and example 10).
- The antibodies show beneficial effects against e.g. pathological ocular angiogenesis, vascular leakage and inflammation (see examples 7-9)

The inventors have also identified the light chain and heavy chain of the ligands according to the invention. Thus, in an embodiment, the ligand comprises:

- a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1 or 3, or sequences having at least 80% sequence identity, such as at least 90% sequence identity, or such as at least 95% sequence identity to SEQ ID NO: 1 or 3.

- a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2 or 4, or sequences having at least 80% sequence identity, such as at least 90% sequence identity, or such as at least 95% sequence identity to SEQ ID NO: 2 or 4.

SEQ ID NO's: 1 and 2 are the light chain and heavy chain from mAS0326 whereas SEQ ID NO's: 3 and 4 are the light chain and heavy chain from hAS0326.

In a further embodiment, the ligand comprises

- a light chain variable region comprising the amino acid sequence of SEQ ID NO: 3; and
- a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 4.

Thus, in this embodiment, the ligand comprises the light chain and heavy chain from hAS0326.

In a further embodiment, the ligand comprises:

- a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1 or 3, or sequences having at least 80% sequence identity, such as at least at least 90% sequence identity, like at least 95% sequence identity, such as 98% sequence identity, or like 99% sequence identity to SEQ ID NO: 1 or 3 with the proviso that the amino acid at position 32 is a Tyr and the amino acid at position 94 is a Tyr;
- a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2 or 4, or sequences having at least 80% sequence identity, such as at least 90% sequence identity, like at least 95% sequence identity, such as at least 98% sequence identity or like 99% sequence identity to SEQ ID NO: 2 or 4 with the proviso that the amino acid at position 33 is a Trp, the amino acid at position 99 is a Glu and the amino acid at position 107 is a Trp.

In a further embodiment, the ligand comprises:

- a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1 or 3, or sequences having at least 80% sequence identity, such as at least at least 90% sequence identity, like at least 95% sequence identity,

such as 98% sequence identity, or like 99% sequence identity to SEQ ID NO: 1 or 3 with the proviso that the amino acid at position 32 is a Tyr and the amino acid at position 94 is a Tyr;

- a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2 or 4, or sequences having at least 80% sequence identity, such as at least 90% sequence identity, like at least 95% sequence identity, such as at least 98% sequence identity or like 99% sequence identity to SEQ ID NO: 2 or 4 with the proviso that the amino acid at position 33 is a Trp, the amino acid at position 34 is a Met, the amino acid at position 53 is a Pro, the amino acid at position 99 is a Glu and the amino acid at position 107 is a Trp.

Thus, according to the above embodiments, the regions may differ from the sequences as described by SEQ ID NO: 1-4 by varying degrees of sequence identity except for the particular amino acids demonstrated to be important for the binding to the epitope (see example 12).

The ligand may be produced in different forms. Thus, in an embodiment, the ligand is selected from the group consisting of a polyclonal antibody, a monoclonal antibody, an antibody, wherein the heavy chain and the light chain are connected by a flexible linker, an Fv molecule, an antigen binding fragment, a Fab fragment, a $F(ab')_2$ molecule, a fully human antibody, a humanized antibody, and a chimeric antibody. In one embodiment, the ligand is a $F(ab')_2$ molecule. In yet a preferred embodiment, the ligand is selected from the group consisting of a monoclonal antibody, a Fab fragment and a humanized monoclonal antibody. In yet another preferred embodiment, the antibody is humanized and/or monoclonal, preferably a humanized monoclonal antibody.

It may also be advantageous to couple different moieties to the ligand according to the invention. Thus, in an embodiment, the ligand is coupled to a detectable label or a substance having toxic or therapeutic activity.

It is of course important that the ligand is able to bind to the target with a sufficient binding efficiency. Thus, in another embodiment, the ligand has $K_D$ value to rhMFAP4 below $1*10^{-7}$, such as below $1*10^{-8}$, or such as below $1*10^{-9}$ M, or

such as in the range $1*10^{-7}$ to $1*10^{-12}$ M, or such as in the range $1*10^{-7}$ to $1*10^{-10}$ M. Example 2 shows $K_D$ values for antibodies according to the invention.

For ligands according to the invention to perform as medicaments, they have to be sufficiently soluble and not form aggregates, which may precipitate. Thus, in yet an embodiment, the ligand according to the invention is primarily in a monomeric form (measured in PBS, pH 7.4). "Primarily" is to be understood as at least 90% of the ligands are in monomeric form, such as at least 95%.

The binding specificity of the ligand according to the invention is different from other known antibodies binding to MFAP4. Thus, in yet another embodiment, the ligand does not bind (directly) to the RGD-integrin interaction sequence in rhMFAP4, but still block MFAP4-mediated activity suggested by steric hindrance of integrin ligation. Binding data for antibodies according to the invention are provided in example 5.

The RGD-integrin interaction domain is located in a short N-terminal region preceding the fibrinogen related domain (FReD) (position 26-28 (when including the signal peptide) and in position 6-8 (when the signal peptide is not included).

**Vector**

The ligands according to the invention may be expressed by one or more vectors. Thus, in an aspect the invention relates to a vector (or vectors) encoding the ligand according to the invention. It is to be understood that e.g. the light chain or heavy chain may be expressed from two different vectors. In an embodiment, the vector is a plasmid.

**Cell**

The vectors may be expressing the ligand in a cell. Thus, yet an aspect of the invention relates to a cell expressing the ligand according to the invention, and/or a cell comprising the vector according to the invention. In an embodiment, the cells are CHO cells.

**Composition**

The ligand according to the invention may of course be in a composition (such as a pharmaceutical composition). Thus, in a further aspect, the invention relates to a composition comprising the ligand according to the invention, and one or more physiologically acceptable carriers, excipients and/or diluents. In an embodiment, said composition comprising one or more stabilizing agents and/or one or more buffering agents. In yet an embodiment, the stabilizing agent is a surfactant.

In yet a further embodiment, the composition further comprises a VEGF-A (including spliceforms hereof) or VEGF-receptor (VEGFR1/VEGFR2) antagonists or anti-VEGF drug, such as an anti-VEGF antibody. As shown in the example section, in certain instances a beneficial effect is seen with such combination.

**Medicament**

The ligand and/or composition according to the invention may be used as a medicament. Thus, yet a further aspect of the invention relates to the ligand and/or composition according to the invention for use as a medicament.

In an embodiment, the ligand or the composition is for use in the prevention or treatment of vascular proliferative diseases and/or related disorders in a mammal. In a preferred embodiment, the mammal is a human.

In another embodiment, the vascular proliferative diseases and/or related disorders are caused by hyperplasia or remodeling in blood vessels.

In a further embodiment, the vascular proliferative diseases and/or related disorders are caused by pathological neovascularization or present with vascular leakage or inflammation or fibrosis and/or related disorders in a mammal.

In yet a further embodiment, the diseases and/or related disorders are bronchiolar hyperplasia or eosinophilic inflammation in allergic asthma.

In a further embodiment, the vascular proliferative diseases and/or related disorders is characterized by pathological neovascularization in the eye. In a related embodiment, the disorders characterized by pathological

neovascularization in the eye is selected from the group consisting of age related macular degeneration (AMD), including geographic athropy and proliferative AMD, retinal vein occlusion, retinopathy, hypertensive retinopathy, vitreomacular traction, and diabetic retinopathy (DR), including proliferative DR and diabetic macular edema.

In an embodiment, the vascular proliferative diseases and/or related disorders are cancers or other malignancies. In a related embodiment, the cancer or malignancy is selected from the group consisting of glioblastoma, head, neck and lung cancer.

The ligand or composition may be administered by different routes. Thus, in an embodiment, said ligand or composition is administered intravenously, ocularly (to the eye) or subcutaneously.

**Method of prevention or treatment of vascular proliferative diseases**

In yet another aspect, the invention relates to a method of prevention or treatment of vascular proliferative diseases and/or related disorders in a mammal, said method comprising administering the ligand or the composition according to the invention to a mammal (in need thereof). In a preferred embodiment, said mammal is a human.

In an embodiment, the method is for prevention or treatment of the vascular proliferative diseases and/or related disorders caused by pathological neovascularization.

In yet an embodiment, the method is for prevention or treatment of bronchiolar hyperplasia and eosinophilic inflammation in allergic asthma.

In another embodiment, the method is for prevention or treatment of disorders characterized by pathological neovascularization in the eye. In a related embodiment, the disorders characterized by pathological neovascularization in the eye is selected from the group consisting of age related macular degeneration (AMD), including geographic athropy and proliferative AMD, retinal vein occlusion,

retinopathy, hypertensive retinopathy, vitreomacular traction, and diabetic retinopathy (DR), including proliferative DR and diabetic macular edema.

In an embodiment, the vascular proliferative diseases and/or related disorders are cancers or other malignancies. In a related embodiment, the cancer or malignancy is selected the group consisting of from glioblastoma, head, neck and lung cancer.

The ligand or composition may be administered by different routes. Thus, in an embodiment, said ligand or composition is administered intravenously, to the eye, ocularly or subcutaneously.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting examples.

## Examples

### Example 1 - Materials and methods

In this example the materials and methods used in the following examples are described

Buffers

Tris-buffered saline (TBS): 140 mM NaCl, 10 mM Tris-HCl, 0.02% (w/v) $NaN_2$, pH 7.4; TBS/Tw: TBS containing 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate, MERCK-Schuchardt); phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.4; substrate buffer: 35 mM citric acid, 67 mM $Na_2HPO_4$, pH 5.0.

Generation of mouse monoclonal antibodies

hrMFAP4 used for immunization was produced as previously described (Sækmose et al. PLoS One. 2013 Dec 4;8(12)).

Monoclonal antibodies with affinity for hrMFAP4 were produced using standard hybridoma technique and MFAP4 deficient mice.

This procedure resulted in a series of monoclonal antibodies including:

- HG Hyb 7-1 murine monoclonal antibody raised against human recombinant MFAP4 (hrMFAP4) (described e.g. in WO 2016/008498)
- HG Hyb 7-5 murine monoclonal antibody raised against human recombinant MFAP4 (hrMFAP4) (described e.g. in WO 2014/114298)
- mAS0326 murine monoclonal antibody raised against human recombinant MFAP4 (hrMFAP4) and chosen antibody for humanization.

All antibodies were amino acid sequenced using standard techniques.

Antibody humanization

mAS0326 antibody was humanized by Genscript (www.Genscript.com), using proprietary technology. Variable domain sequences were blasted against human germline and several framework regions FR1, FR2, and FR3 were selected independently from human FRs, which share the highest identity with the mouse antibody. Selected FRs were assembled with mAS0326 CDRs using overlapping PCR and phage display library was constructed for expression of Fab fragments. High MFAP4 protein-binding phages were selected after three rounds of panning using Genscript's proprietary FASEBA screening methodology. Selected Fab genes were amplified from phage DNA. Genes encoding Fab were fused with genes encoding the appropriate constant regions of human IgG1 in order to generate whole IgG. hAS0326 was selected as the best recombinant human MFAP4 binder in this series using Biacore T200 and the resulting light chain and heavy chain constructs were cloned into the mammalian expression vectors pcDNA3.1 plus.

Expression of humanized antibody and recombinant forms of MFAP4

ExpiCHO-S cells were transfected with human MFAP4 expression plasmid (pcDNA 3.4-hMFAP4 TOPO® TA), mouse MFAP4 expression plasmid (pcDNA5/FRT/V5-His TOPO® TA), human MFAP4 RGD-AAA mutation (pcDNA 3.1 plus) and the hAS0326 light and heavy chain expression plasmids (pcDNA3.1 plus- hAS0326 light chain) and (pcDNA3.1 plus- hAS0326 heavy chain) using ExpiFectamine according to the manufacturer's protocol (hAS0326 light chain and hAS0326 heavy chain were co-transfected in a molar 1:1 ratio). After adding ExpiFectamine

CHO Enhancer and ExpiCHO Feed, the cells were incubated at 37 °C in 8% $CO_2$ for 10 days with shaking.

Purification of recombinant forms of MFAP4

The recombinant MFAP4 proteins released into the medium of the ExpiCHO-S cells were purified were affinity purified as described by Lausen et al. (J Biol Chem (1999) 274(45):32234-40) followed by anion ion-exchange chromatography on a Resource Q column *(GE Healthcare Life Sciences)* on a Äkta FPLC apparatus (Amersham Pharmacia Biotech). The purity of the proteins was tested by SDS-PAGE followed by Coomassie staining using SimplyBlue™ SafeStain (Invitrogen).

Purification of antibodies

The antibodies were purified on an Äkta FPLC apparatus (Amersham Pharmacia Biotech) using standard Protein G purification. The culture supernatant was applied to the column and the column was washed in PBS (0.5 M NaCl) and antibodies eluted by increasing the concentration of citric acid. Antibody containing fractions were neutralized immediately with $Na_2CO_3$ and pooled. The pool of purified antibody was dialysed against PBS.

Biotinylation

The antibodies were dialyzed against phosphate-buffered saline adjusted to pH 8.5 with 3% (w/v) $Na_2CO_3$ and biotin-*N*-hydroxysuccinimide ester (Sigma H-1759, 40 mg/ml in dimethyl sulfoxide) was added at 0.17 mg/mg protein. The mixture was incubated O.N at 4°C and dialyzed against PBS.

Epitope mapping of antibodies by competition ELISA

96-well plates (Nunc-MaxiSorp) were coated with 0.5 µg recombinant human MFAP4 in PBS O.N at 4°C followed by washing and then blocking in TBS/Tw O.N at 4°C . Biotinylated Mab (0.5 µg/ml) and unlabeled MAbs (diluted 2-fold from 20 µg/ml to 156 ng/ml) were premixed in a separate microtiterplate in TBS/Tw and then added to the MFAP4 coated microtiter plate. The plates were following

incubated for 2 hours at R.T. The plates were washed three times in TBS/Tw and incubated for 20 min with Strepavidin Horseradish Peroxidase (HRP) conjugate (Invitrogen) diluted 1:2000 in TBS/Tw. After three final washes, the amount of bound enzyme was estimated by adding o-phenylenediamine (OPD, 0.8 mg/ml, Kementec, Taastrup, Denmark) dissolved in substrate buffer (0.03% freshly prepared $H_2O_2$) and allowed to react for 15 minutes in the dark at RT. Colour development was stopped by the addition of 100 µl 1 M $H_2SO_4$, and the plates were read at $OD_{492}$ nm with $OD_{600}$ nm as reference.

Test of antibody binding to recombinant mouse MFAP4, recombinant human MFAP4 and recombinant human MFAP4 with RGD-sequence mutated to AAA-sequence

96-well plates (Nunc-MaxiSorp) ( Nunc™) were coated with 0.5 µg recombinant mouse MFAP4, recombinant human MFAP4 and recombinant human MFAP4 with the RGD-sequence mutated to AAA in PBS O.N at 4°C. Coating was followed by washing and then blocking in TBS/Tw O.N at 4°C. Blocking was followed by addition of biotinylated antibodies diluted 2-fold from an initial concentration of 100 ng/ml. The plates were then incubated for 2 hours at R.T. The plates were washed three times in TBS/Tw and incubated for 20 minutes with Strepavidin Horseradish Peroxidase (HRP) conjugate (Invitrogen) diluted 1:2000 in TBS/Tw. After final three washes, the amount of bound enzyme was estimated by adding OPD (0.8 mg/ml, Kementec, Taastrup, Denmark) dissolved in in substrate buffer (0.03% freshly prepared $H_2O_2$) and allowed to react for 15 minutes in the dark at RT. Colour development was stopped by the addition of 100 µl 1 M $H_2SO_4$, and the plates were read at $OD_{492}$ nm with $OD_{600}$ nm as reference.

Sandwich ELISA assays

In Sandwich ELISA assays, various anti-MFAP4 antibodies were immobilized at 1 µg/ml in PBS in the wells of 96-well plates (Nunc-MaxiSorp) O.N at 4°C. Washed three times in TBS/Tw followed by blocking with TBS/Tw O.N at 4°C. The plates were incubated with twofold dilutions of serum from human, mouse and from MFAP4-deficient mouse from an initial 1:50 dilution O.N at 4°C. The plates were washed three times in TBS/Tw followed by incubation with biotinylated antibodies (0.5 µg /ml in TBS/Tw) for 2 h at R.T. The plates were then washed 3

times in TBS/Tw and incubated for 20 min with Strepavidin Horseradish Peroxidase (HRP) conjugate (Invitrogen) diluted 1:2000 in TBS/Tw. After final 3 washes, the amount of bound enzyme was estimated by adding OPD (0.8 mg/ml, Kementec, Taastrup, Denmark) dissolved in in substrate buffer (0.03% $H_2O_2$ added immediately before use) and allowed to react for 15 minutes in the dark at RT. Colour development was stopped by the addition of 100 µl 1 M $H_2SO_4$, and the plates were read at $OD_{492}$ nm with $OD_{600}$ nm as reference.

Concentrating antibodies for aggregation studies

The Protein G purified antibodies were concentrated using Vivaspin 2 centrifugal concentrators (Viva products) followed by incubation at 4°C for 2 hours before performing size exclusion chromatography. The purity of the concentrated antibodies was tested by SDS-PAGE followed by Coomassie staining using SimplyBlue™ SafeStain (Invitrogen). The antibody concentration was estimated using optical density determination (OD280).

Size exclusion chromatography

Size exclusion chromatography was performed using 50 µl of the concentrated antibodies. The antibody sample was applied to an analytical Superose 6 column connected to an Äkta FPLC system (Amersham Pharmacia Biotech) using PBS, pH 7.4, containing and 0.05% emulphogene as eluant at a flow rate of 0.4 ml/min.

Animals ethics

Mice and rats were treated in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, *at the University of Nottingham Biological Services Unit, under a UK Home Office license.*

Mouse choroidal neovascularization (CNV) model

Female C57/BL6J mice were used for this study. Animals were anaesthetised with an intraperitoneal (IP) injection of 50mg/kg Ketaset (ketamine hydrochloride, Zoetis), and recovered with 0.5mg/kg Sedastop (Atipamezole hydrochloride, Animalcare), IP. Pupils were dilated with topical applications of 5% phenylephrine hydrochloride (Bausch & Lomb) and 0.8% tropicamide (Bausch & Lomb), and eyes were coated with Lubrithal (*Dechra)* to prevent dehydration.

Lesions were produced using a Meridian Merilas 532α Green Laser Photocoagulator to penetrate the Bruch's membrane at 4 points per eye, in both eyes. The presence of a sub-retinal bubble was used to determine the successful rupture of the Bruch's membrane. Laser settings were maintained at 450mW for 130ms for each photocoagulation lesion.

Mice received an intraocular injection of 1µg m/hAS0326, 5µg m/hAS0326, 1µg mouse IgG (DAKO) or saline as control, or 1µg anti-VEGF-A (Biolegend). Antibodies were diluted to 0.5µg/µl (2.5µg/µl for 5µg injections) in sterile PBS and 2µl administered with a 36 gauge Hamilton needle (World Precision Instruments) with fine forceps used to stabilize the eye. Antibodies were administered on day 0 (post lesion), and day 7.

To visualize the vasculature and lesion development *in vivo* (day 7 and day 14), 200µl of 100mg/ml sodium fluorescein (Sigma-Aldrich) in saline was injected IP and allowed to circulate before imaging with a Micron IV Retinal Imaging Microscope (Phoenix Research Labs). Development of cataracts meant that some eyes were excluded from the consecutive fundus flourescein angiography (FFA).

After animal termination and ocular dissection (day 14), choroids were flatmounted and blocked in serum (5% Goat Serum, 3% Triton X-100, 1% BSA) and stained with Isolectin-B4 (IB4) (Sigma Aldrich, biotin conjugated) 5µg/ml and CD45 (Abcam) 5µg/ml overnight at 4°C. Streptavidin conjugated Alexafluor 488 2µg/ml and donkey anti-rabbit Alexafluor 555 4µg/ml were used to detect IB4 and CD45 staining respectively. Coverslips were mounted with Fluoroshield with DAPI. Images were obtained using a Leica TCS SPE confocal microscope, and all settings were maintained between images. Lesion and inflammatory cell areas in $\mu m^2$ were measured directly by Imaris (Bitplane, UK). Any lesions that had merged, or animals in which the contralateral eye had burns measuring greater than 2 standard deviations from the mean were excluded from analysis.

Rat Streptozotocin (STZ)-induced diabetes model

To induce diabetes, male Norway Brown rats (250–300g, Envigo, US) were given a single IP injection of streptozotocin (STZ, 50 mg/kg, Sigma–Aldrich). Control rats were injected with 300 µl saline IP. A third of an insulin pellet (LinShin) was implanted subcutaneously to maintain body weight over the following 4 weeks. On day 4 post-induction, blood was taken from the tail vein and blood glucose levels

measured. Rats with blood glucose >15 mmol/l and were deemed diabetic. STZ-injected rats that did not become hyperglycaemic on day 4 were re-injected with STZ the following morning.

Rats were anaesthetized with 3-5% isoflurane (IsoFlo, Abbott Laboratories), pupils dilated with topical applications of 5% phenylephrine hydrochloride (Bausch & Lomb) and 0.8% tropicamide (Bausch & Lomb), and eyes were coated with Lubrithal (Dechra) to prevent dehydration. Animals received an introcular injection of 1µg m/hAS0326, 5µg m/hAS0326, saline as control, or 1µg anti-VEGF-A (Biolegend). Antibodies were diluted to 0.5µg/µl (2.5µg/µl for 5µg injections) in sterile PBS and 2µl administered with a 36 gauge Hamilton needle (World Precision Instruments) with fine forceps used to stabilize the eye. Antibodies were administered on day 0 (pre diabetic), and day 7 (post diabetic). To visualize the vasculature and lesion development *in vivo* (day 0, 7, 14 and 21), 200µl of 100mg/ml sodium fluorescein (Sigma-Aldrich) in saline was injected IP and allowed to circulate before imaging with a Micron IV Retinal Imaging Microscope (Phoenix Research Labs). Development of cataracts meant that some eyes were excluded from the consecutive fundus flourescein angiography (FFA).

Evans Blue dye preparation, administration and the consecutive monitoring of ocular vascular permeability by dissolving is previously described in Ved N, Clin Sci (Lond) 2017.

Statistics

All statistics and graphs were produced in GraphPad Prism 6. Statistics shown are for comparison between treatment groups. Comparisons were performed using Kruskall-Wallis test and Dunn's multiple comparisons test. Significant differences were indicated on graphs as asterisks, where: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

## Example 2 – $K_D$ estimates for the interaction between hAS0326 and mAS0326 to rhMFAP4

*Aim*

To estimate $K_D$ values for the interaction between hAS0326 and mAS0326 to rhMFAP4.

*Materials and methods*

Binding interactions between antibody of interest and rhMFAP4 was performed using Biacore T200 at GenScript.

*Results*

| **Antibody** | **$K_D$** |
|---|---|
| hAS0326 | $1.14*10^{-9}$ M. |
| Chimeric antibody (mAS0326 with human Fc, CHO expression) | $5.04*10^{-10}$ M |
| mAS0326 | $1.4^{-10}$ M |

*Conclusion*

These results indicate that hAS0326 is a high-affinity antibody, with a $K_D$ in the nanomolar range. hAS0326 had lower rhMFAP4 affinity compared to mAS0326, but retained high affinity.

**Example 3 - Alignment of CDR sequences in the produced monoclonal antibodies**

The CDR homologies between the produced antibodies HG Hyb 7-5, HG Hyb 7-1, mAS0326 and hAS0326 are depicted in **Figure 1**.

- 100% homology between heavy chain CDR sequences for HG Hyb 7-1, mAS0326 and hAS0326.
- <50% homology between HG Hyb 7-5 and hAS0326 heavy chain CDR.
- 100% homology between light chain CDR sequences for mAS0326 and hAS0326.
- <50% homology between HG Hyb 7-1 or HG Hyb 7-5 and hAS0326 light chain CDR.

**Example 4 - Heavy and light chain variable domain homologies between monoclonal antibodies**

hAS0326 heavy chain variable domain has the following homologies to the produced mouse monoclonal antibodies

- HG Hyb 7-1: 78.7%
- HG Hyb 7-5: 66.1%
- mAS0326: 78.7%

hAS0326 light chain variable domain has the following homologies to the produced mouse monoclonal antibodies:

- HG Hyb 7-1: 66.4%
- HG Hyb 7-5: 61.7%
- mAS0326: 79.4%

*Conclusion*

All homologies were <80%, which indicates that hAS0326 amino acid sequence is markedly dissimilar relative to the other tested antibodies, including antibodies presented in previous patent applications.

**Example 5 - MFAP4 binding properties of hAS0326/mAS0326 in comparison to HG HYB7-5**

*Aim*

To evaluate MFAP4 binding properties of hAS0326/mAS0326 in comparison to HG HYB7-5.

*Results*

It is demonstrated that HG HYB7-5 has a different epitope compared to hAS0326 and mAS0326 using a competitive ELISA setup (**Figure 2**). In a direct binding assay using purified recombinant mouse MFAP4, recombinant human MFAP4 and recombinant human MFAP4 with the RGD integrin binding motif mutated to AAA it was demonstrated that the RGD motif is an essential part of the HG HYB7-5 epitope, whereas it is not a part of the hAS0326/mAS0326 epitope (**Figure 3A and 3B**). Furthermore it is demonstrated that whereas hAS0326/mAS0326 binds equally well to human and mouse MFAP4, HYB7-5 does not bind mouse MFAP4 **(Figure 3C)**.

Using serum as a source of native mouse and human MFAP4, **Figure 4** demonstrates that HG HYB 7-5 does not bind mouse MFAP4 whereas hAS0326/mAS0326 does.

*Conclusion*

Studies of sequence homology and epitope overlap revealed that HG Hyb 7-5 and mAS0326 (and hAS0236) had low CDR/variable chain homology and that they recognize different recombinant MFAP4 epitopes. Further, it is demonstrated that HG Hyb 7-5 recognizes the RGD-integrin interaction domain in rhMFAP4 whereas mAS0326 (and hAS0236) does not react with the RGD-integrin interaction domain in recombinant human MFAP4. Furthermore, it is demonstrated that AS0326 has interaction with murine MFAP4 whereas HG HYB 7-5 has not.

**Example 6 - Antibody aggregation**

*Aim*

To compare the aggregation properties of hAS0326 and mAS0326

*Results*

After concentrating antibody solutions, it was demonstrated that aggregation properties of hAS0326 and mAS0326 are different (**Figure 5**). hAS0326 essentially stays in a monomeric form. In contrast, mAS0326 forms aggregates.

*Conclusion*

hAS0326 was more stable in solution than mAS0326, which formed aggregates shortly after preparation and thus was not suitable for clinical use. The lack of aggregation of hAS0326 suggests that this antibody may be suitable for clinical use in the present form.

**Example 7 – Ability of mAS0326 to reduce ocular angiogenesis, vascular leakage and inflammation in the mouse laser-induced choroidal**

**neovascularization (CNV) model of wet age-induced macular degeneration (wet AMD)**

*Aim*

To evaluate the ability of mAS0326 to reduce ocular angiogenesis, vascular leakage or inflammation in the mouse laser-induced choroidal neovascularization (CNV) model of wet age-induced macular degeneration (wet AMD).

*Results*

Seven and 14 days after laser-induced CNV, injection with sodium fluorescein highlighted the vasculature within the eye, and showed laser burns and regions of leakage. At day 7, there was no significant difference between the IgG negative control and the anti-VEGF positive control **(Figure 6A)**. Treatment with 5 µg mAS326 significantly reduced the lesion size compared to anti-VEGF positive control ($P<0.05$).

At day 14, further fluorescein angiograms were taken immediately before culling and tissue collection. As seen at day 7, 5µg mAS0326 was able to significantly reduce lesion size compared to IgG controls ($p<0.01$, not shown in figure) **(Figure 6B)**. However, no significant differences were found between treatment groups.

Staining of vasculature with the endothelial marker IB4 showed no significant differences in burn area between any of the treatment groups **(Fig 6C)** in line with observations in Figure 6B.

Choroids were immunostained for CD45 expression to detect infiltration of inflammatory cells into the laser burn area **(Fig 6D)**. 1 µg anti-VEGF as well as 5 µg mAS0326 treatment both significantly reduced inflammatory cell infiltration relative to the IgG negative control ($p<0.01$ and $p < 0.0001$, respectively, staistics not shown in figure). Moreover, 5 µg mAS0326 treatment significantly reduced inflammatory cell infiltration relative to the 1 µg anti-VEGF positive control ($p < 0.01$).

*Conclusion*

mAS0326 efficacy study in the mouse CNV for wet AMD showed proof-of-concept for beneficial effect against pathological ocular angiogenesis, vascular leakage and

inflammation. The observed efficacy was similar and/or superior to the efficacy of standard treatment anti-VEGF.

**Example 8 – Ability of hAS0326 to reduce ocular angiogenesis, vascular leakage and inflammation in the mouse laser-induced CNV model of wet AMD**

*Aim*

To evaluate the Ability of hAS0326 to reduce ocular angiogenesis, vascular leakage or inflammation in the mouse laser-induced CNV model of wet AMD.

*Results*

In contrast to the mAS0326 CNV trial described above (example 7), the separation from control treatments was clear in this hAS0326 CNV trial where saline was used as negative control. At day 7, all treatments provided significantly reduced lesions sizes compared to the saline negative control ($p < 0.0001$ - $p < 0.05$ statistics not shown) **(Figure 7A)**. Treatment with 5µg hAS326 significantly reduced the lesion size compared to anti-VEGF positive control ($p < 0.001$ and $<0.0001$, respectively) **(Figure 7B)**. No significant differences in lesion size were found between hAS0326 treatment groups and there was no significant effect of combining hAS0326 and anti-VEGF treatment.

Staining of vasculature with the endothelial marker IB4 was significantly reduced by all treatments compared to saline control treatment (statistics not shown) **(Figure 7C)**. No significant differences in IB4 defined burn area were observed between any of the treatment groups **(Fig 7C)**.

Choroidal immunostaining for CD45 expression showed that all treatments significantly reduced inflammatory cell infiltration relative to the IgG negative control (statistics not shown in figure). The combinatorial treatment significantly reduced the inflammatory infiltration compared to all other treatment groups **(Fig 7D)**.

*Conclusion*

hAS0326 efficacy study in the mouse CNV for wet AMD showed proof-of-concept for beneficial effect against pathological ocular angiogenesis, vascular leakage and inflammation. mAS0326 appeared with the same qualitative response as hAS0326. In contrast to hAS0326, the ability of mAS0326 treatment to reduce

ocular lesion sizes in the CNV model were not significantly different from treatment with anti-VEGF. The observed hAS0326 efficacy was similar and/or superior to the efficacy of standard treatment anti-VEGF in the CNV model and combinatorial treatment with anti-VEGF and hAS0326 was superior to treatment with either compound alone in reduction of inflammation.

**Example 9 – Ability of hAS0326 to reduce ocular angiogenesis in the rat streptozotocin (STZ)-model of diabetic retinopathy.**

*Aim*

To evaluate the ability of hAS0326 to reduce ocular angiogenesis in the rat streptozotocin (STZ)-model of diabetic retinopathy.

*Results*

There was no statistical difference between the vascular areas in treatment groups before onset of STZ-model **(Figure 8A).**

Twenty-one days after onset of STZ-treatment with induction of hyperglycemia (data not shown), treatment with 5µg hAS0326 alone or combinatorial treatment with 1 µg anti-VEGF and 5 µg hAS0326 both significantly had reduced the lesion size compared to saline negative control ($p < 0.001$ and $p < 0.0001$, respectively, statistics not shown) **(Figure 8B)**. When comparing the efficacy of treatments, the combinatorial treatment with 1 µg anti-VEGF and 5 µg hAS0326 significantly reduced the lesion size compared to the anti-VEGF positive control ($p < 0.05$) **(Figure 8B)**.

*Conclusion*

hAS0326 efficacy study in reduction of vascular leakage in the rat streptozotocin (STZ)-model of diabetic retinopathy showed proof-of-concept for beneficial effect at par with anti-VEGF.

**Example 10 - Low pH and thermal stress induces aggregation of monoclonal antibodies HG HYB 7-5, HG HYB 7-14, mAS0326 and hAS0326 and show superior stability of hAS0326.**

*Aim*

In order to evaluate and compare the stability of HG Hyb 7-5 (HYB7-5), HG Hyb 7-14 (HYB7-14), mAS0326 and hAS0326, we subjected the monoclonal antibodies (Mabs) to thermal stress. The formation of high molecular forms (aggregates) of the Mabs was induces by subjecting the Mabs to low pH and high temperatures and soluble aggregates were analyzed by size exclusion chromatography (SEC). The amounts of insoluble aggregates were tested using centrifugation followed by measurement of the protein concentration.

*Material and methods*

Size exclusion chromatography

The HG Hyb 7-5, HG Hyb 7-14, mAS0326 and hAS0326 were purified using a 5 ml HiTrap column protein A (GE Healthcare) and following washed in 25 mM Tris, 25 mM NaCl pH 7.2 and then eluted in 100 mM citric acid pH 3.5. As a viral inactivation step the Mabs were incubated for 30 min in the elution buffer before adjustment to pH 5 using 1 M tris pH 8.6. After 5 and 10 days of incubation at 50 °C the samples were analyzed by size exclusion chromatography. 30 µl of of the supernatant each stressed sample prepared at 6 mg/ml was injected onto a column (MabPac SEC-1 from Thermo Scientific) operated at 25 °C, and the absorbance at 280 nm was recorded. The flow rate was 0.76 ml/min with a total elution time of 30 min. The mobile phase contained 50 mm sodium phosphate, pH 6.8, and 300 mm sodium chloride. High molecular weight (HMW) peaks included everything in the range between the excluded volume and the start of the anti-MFAP4 (monomer) peak. The integrated areas were taken as a percentage of the total integrated area.

Aggregate concentration

To estimate the percent of original protein present as aggregates, stressed samples after 10 days at 50 °C were centrifuged at 12,000 rpm to pellet the insoluble material, and the protein concentration before and after centrifugation was determined by measuring the $A_{280}$ on a NanoDrop ONE UV-visible spectrophotometer (Thermo Scientific).

*Results*

Three high molecular weight forms of the Mabs were observed by SEC and their retention volumes are shown in **Table 1** with reduced retention volumes compared to the monomeric Mab hAS0326. Thus, **Table 1** shows an overview of high molecular weight (HMW) forms (aggregates) observed by size exclusion chromotography (MabPac SEC-1 column from Thermo Scientific) collectively for all experiments and with their respective retention volumes.

**Table 1**

| Type | Approximate volume (ml) |
|---|---|
| HMW1 | 6.1 |
| HMW2 | 7.7 |
| HMW3 | 8.1 |
| Anti-MFAP4 hAS0326 (monomer) | 10.1 |

HMW = High Molecular Weight: 1 HMW1> HMW2 >HMV3> anti-MFAP4 hAS0326 (monomer)

Low pH stress test

The Mabs were purified in parallel on a protein A column followed by a viral inactivation step at pH 3.5 for 30 min. **Table 2** shows the distribution of the anti-MFAP4 Mabs High Molecular Weight (HMV) forms after the low pH viral inactivation step with only HG Hyb 7-14 and hAS0326 displaying a monodisperse distribution. Thus, Table 2 shows percentages of anti-MFAP4 and HMWs observed by size exclusion chromatography (soluble HMW forms) after protein A purification and viral inactivation for 30 min at pH 3.5.

**Table 2**

| Sample | %anti-MFAP4 (monomer) | %HMW1 | %HMW2 | %HMW3 |
|---|---|---|---|---|
| HG Hyb 7-5 | 97.7% | 0.0% | 0.0% | 2.3% |
| HG Hyb 7-14 | 100.0% | 0.0% | 0.0% | 0.0% |
| mAS0326 | 96.8% | 0.0% | 0.0% | 3.2% |
| hAS0326 | 100.0% | 0.0% | 0.0% | 0.0% |

Thermal stress test with to time intervals

Thermal stress tests of the Mabs were performed and the samples analyzed at day 5 and 10 using SEC in order to assess the amount of soluble HMW forms. As shown in **Tables 3 and 4**, hAS0326 displayed the lowest amount of HMW forms in both conditions. Thus, **Table 3** shows percentages of anti-MFAP4 and HMW forms observed by size exclusion chromatography after thermal stress at 50 degrees Celcius for 5 days and **Table 4** shows percentages of anti-MFAP4 and HMW forms observed by size exclusion chromatography after thermal stress at 50 degrees Celsius for 10 days.

**Table 3**

| Sample | %anti-MFAP4 (monomer) | %HMW1 | %HMW2 | %HMW3 |
|---|---|---|---|---|
| HG Hyb 7-5 | 91.0% | 7.2% | 0.0% | 1.8% |
| HG Hyb 7-14 | 96.6% | 3.4% | 0.0% | 0.0% |
| mAS0326 | 91.3% | 7.7% | 0.0% | 1.1% |
| hAS0326 | 99.4% | 0.0% | 0.0% | 0.6% |

**Table 4**

| Sample | % anti-MFAP4 (monomer) | %HMW1 | %HMW2 | %HMW3 |
|---|---|---|---|---|
| HG Hyb 7-5 | 84.6% | 13.8% | 0.0% | 1.6% |
| HG Hyb 7-14 | 90.8% | 8.9% | 0.0% | 0.3% |
| mAS0326 | 91.7% | 8.2% | 0.0% | 0.1% |
| hAS0326 | 96.4% | 0.0% | 2.5% | 2.1% |

To estimate the percentage of original protein present as insoluble aggregates, stressed samples (thermal stress at 50 degrees Celcius) were centrifuged at 12,000 rpm for 30 minutes to pellet the insoluble material, and the protein concentration before and after centrifugation was determined by measuring the $A_{280}$ on a NanoDrop ONE (Thermo Scientific). The results obtained hereby is shown in **Table 5** illustrating that unlike mAS0326 and HG Hyb 7-1, hAS0326 did not form any insoluble aggregates after 10 days at 50 °C.

**Table 5**

| Sample | Before* | After** |
|---|---|---|
| HG Hyb 7-5 | 100% | 100% |
| HG Hyb 7-14 | 100% | 71% |
| mAS0326 | 100% | 51% |
| hAS0326 | 100% | 100% |

*Soluble protein before thermal stress

**Soluble protein after thermal stress (10 days)

*Conclusion*

The aggregate formation after low pH and thermal stress was markedly lower in hAS0326 compared to the mAS0326, HG Hyb 7-5 and HG Hyb 7-14 showing that the stability of hAS0326 was markedly higher.

**Example 11 – Inhibition of MFAP4-induced migration of retinal endothelial cells – *in vitro* study**

*Aim*

To study the effect of hAS0326 and variants thereof on blocking MFAP4-induced cellular activation, a retinal endothelial cell migration assay was performed. Full-length hAS0326, Fragment antigen-binding (Fab), and $F(ab')_2$ (including two Fabs) were tested.

*Material and methods*

Expression of full-length antibody and antibody variants

Full-length and antibody fragments were expressed in EXPI CHO cells (Thermo Scientific) as recommended by the manufacturer.

Endothelial migration assay

The experiment was performed using a modified Boyden migration assay and human retinal microvascular endothelial cells (Neuromics). The lower side of a 8 μm transwell filter (Falcon cat#35097) was coated with recombinant human MFAP4 10 $\mu g/cm^2$ overnight at 4°C and following washed in PBS. 50.000 cells in 0.5 ml endothelial basal medium (PromoCell) with 0.5% FBS were added to the apical side of the filter and 1 ml endothelial basal medium with 0.5% FBS was added to the basal side of the filter. For inhibition of migration various Mabs and

Mab variants were added together with VEGF (25 ng/ml) in the lower chamber. After 3.5 hours, the non-migrated cells were removed by swiping a cotton bud gently on the upper surface followed by wash in PBS and then the filter was stained with Reastain Quick-Diff kit (Gentaur Molecular Products). The cells that had traversed the membrane were counted under bright field microscopy (200x magnification).

*Results*

Full-length and antibody fragments were all able to inhibit endothelial migration but with a different efficiency. Full-length hAS0326 considerably inhibited endothelial migration whereas a recombinant Fab fragment of hAS0326 did not infer the same degree of inhibition even at increased doses (**Figure 9A**).

The F(ab´)$_2$ fragment of hAS0326 inhibited the migration to a similar degree as hAS0326 (**Figure 9B**). It is considered well-established that if efficient F(ab)'s can be produced (which performs well *in vitro* compared to complete antibodies), they may very well perform better than complete antibodies *in vivo*, since the use of immunoglobulin fragments eliminate non-specific binding between the Fc portions of antibodies and the Fc receptor on cells. Thus, the production of an efficient F(ab´)$_2$ fragment of hAS0326 makes it a promising candidate.

*Conclusion*

This *in vitro* assay demonstrates the ability of full-length hAS0326, hAS0326 Fab and hAS0326 F(ab')$_2$ to inhibit endothelial migration even though not at the same efficiency.

**Example 12 – X-ray crystallography of interaction between epitope and paratope**

*Aim*

X-ray crystallography was used to determine the amino acids of the paratope important for the binding to the epitope of the MFAP4.

*Materials and methods*

For Fab generation, anti-MFAP4 was incubated with immobilized papain beads (Thermo scientific) in 20 mM sodium phosphate, 10 mM EDTA and 20 mM L-cysteine pH 7.4 for 4 h at 37 °C. Beads were pelleted by centrifugation and the supernatant was loaded on a 1 ml Mono S column equilibrated in 50 mM sodium acetate pH 5.5. The Fab was eluted with a gradient from 20 to 500 mM NaCl and subsequently purified by size exclusion chromatography on a 24 ml Superdex 200 increase equilibrated in 20 mM Hepes, 150 mM NaCl pH 7.4. Prior to crystallization MAP4 was deglycosylated for 18 hours at 4 °C with in house prepared Endoglycosidase H. Deglycosylated MFAP was mixed with an excess of Fab and the complex was purified on a 24 ml Superdex 200 Increase equilibrated in 20 mM Hepes pH 7.4, 150 mM NaCl. The isolated complex was concentrated to 6 mg/ml and crystallized by vapor diffusion at 19 °C after mixing 0.5 μl protein with 0.5 μl reservoir solution containing 0.14 M Ammonium phosphate dibasic, 14% w/v Polyethylene glycol 3,350. Prior to data collection crystals were soaked in reservoir solution supplemented with 20 % glycerol and flash cooled in liquid nitrogen.

Diffraction data were collected at ESRF ID23-1 and processed and scaled with XDS and XSCALE (Kabsch, W. (2010) *Acta crystallographica. Section D, Biological crystallography* 66, 133-144). The structure was determined by molecular replacement with Phaser (McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) *Journal of applied crystallography* 40, 658-674) using the structure of monomeric FIBCD1 (PDB ID 4M7H) and a germline Fab (PDB ID 4JPI) as search models. In an iterative manner the structure was manually rebuilt in Coot and refined with Phenix.refine (Afonine, P. V. et al. (2012) *Acta Crystallogr D Biol Crystallogr* 68, 352-367) using positional refinement, grouped B-factors and TLS groups and positional non-crystallographic symmetry restraints. Upon completion of the protein part of the model the structure was fitted to the electron density map using molecular dynamics restrained real space fitting as described (Croll, T. I., et al. (2016) *Acta Crystallogr D Struct Biol* 72, 1006-1016). Subsequently $Ca^{2+}$ ions and two coordinating water molecules were inserted into each site, and the calcium-ligand coordination geometry was restrained according to that observed in FIBCD1 (Shrive, A. K. et al. (2014) *J Biol Chem* 289, 2880-2887). During the final refinement cycles a few cycle of individual B-factor refinement were allowed in

addition to positional refinement. The final structure displayed excellent stereochemistry according to Molprobity (Chen, V. B. et al. (2010) *Acta Crystallogr D Biol Crystallogr* 66, 12-21) considering the resolution of the diffraction data. The intermolecular interface was analysed with PyMol 1.8.6 (Schrodinger, LLC. (2015) The PyMOL Molecular Graphics System, Version 1.8.) and PISA (Krissinel, E. et al. (2007) *J Mol Biol* 372, 774-797) and figures prepared in PyMol.

*Results*

The strongly interacting and packaging amino acids of the paratope comprises residues from all three CDRs in the variable heavy chain while only two CDRs of the variable light chain i.e. CDR1 and CDR3 show amino acids strongly interacting with the epitope in the MFAP4

The strongly interacting amino acids in the variable heavy chain are as follows:

In CDR1: Trp-33 referring to SEQ ID NO: 2 and 4; Trp-3 referring to SEQ ID NO: 12;

In CDR3: Glu-99 referring to SEQ ID NO: 2 and 4; Glu-1 referring to SEQ ID NO: 14;

In CDR3: Trp-107 referring to SEQ ID NO: 2 and 4; Trp-9 referring to SEQ ID NO: 14.

The strongly interacting amino acids in the variable light chain are as follows:

In CDR1: Tyr-32 referring to SEQ ID NO: 1 and 3; Tyr-9 referring to SEQ ID NO: 9;

In CDR3: Tyr-94 referring to SEQ ID NO: 1 and 3; Tyr-6 referring to SEQ ID NO:11.

The results further demonstrated that two amino acids in the variable heavy chain are important for packaging i.e. for correct folding of the paratope:

In CDR1: Met-34 referring to SEQ ID NO: 2 and 4; Met-4 referring to SEQ ID NO: 12;

In CDR2: Pro-53 referring to SEQ ID NO: 2 and 4; Pro-4 referring to SEQ ID NO: 13.

*Conclusion*

X-ray crystallography successfully defined the hAS0326 paratope and its binding to the MFAP4 epitope. The analysis shows that five amino acids of the paratope bind strongly to the epitope and that two amino acids are important for the packaging. Thus, it could be hypothesized that site directed mutagenesis of one or more of these amino acids may abolish or weaken the binding of the paratope to the epitope.

## Claims

1. A protein ligand, such as an antibody, comprising
   - a light chain variable region comprising a CDR 1 region according to SEQ ID NO: 9, a CDR 2 region according to SEQ ID NO: 10 and a CDR 3 region according to SEQ ID NO: 11; and
   - a heavy chain variable region comprising a CDR 1 region according to SEQ ID NO: 12, a CDR 2 region according to SEQ ID NO: 13 and a CDR 3 region according to SEQ ID NO: 14.

2. A protein ligand, such as an antibody, or the ligand according to claim 1, comprising
   - a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1 or 3, or sequences having at least 80% sequence identity, such as at least at least 90% sequence identity, or such as at least 95% sequence identity to SEQ ID NO: 1 or 3; and
   - a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2 or 4, or sequences having at least 80% sequence identity, such as at least 90% sequence identity, or such as at least 95% sequence identity to SEQ ID NO: 2 or 4.

3. The ligand according to claim 1 or 2, comprising
   - a light chain variable region comprising the amino acid sequence of SEQ ID NO: 3; and
   - a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 4.

4. The ligand according to any of the preceding claims, wherein the ligand is selected from the group consisting of a polyclonal antibody, a monoclonal antibody, an antibody, wherein the heavy chain and the light chain are connected by a flexible linker, an Fv molecule, an antigen binding fragment, a Fab fragment, a Fab' fragment, a $F(ab')_2$ molecule, a fully human antibody, a humanized antibody, and a chimeric antibody.

5. The ligand according to any of the preceding claims, wherein the ligand is a $F(ab')_2$ molecule.

6. The ligand according to any of the proceeding claims, wherein the antibody is humanized, preferably a humanized monoclonal antibody.

7. The ligand according to any of the proceeding claims, coupled to a detectable label or a substance having toxic or therapeutic activity.

8. The ligand according to any of the proceeding claims, having $K_D$ value to rhMFAP4 below $1*10^{-7}$, such as below $1.*10^{-8}$, or such as below $1*10^{-9}$ M, or such as in the range $1*10^{-7}$ to $1*10^{-12}$ M, such as in the range $1*10^{-7}$ to $1*10^{-10}$ M.

9. The ligand according to any of the proceeding claims, being primarily in a monomeric form.

10. The ligand according to any of the proceeding claims, wherein the ligand does not bind to the RGD-integrin interaction sequence in the rhMFAP4 N-terminal domain.

11. A vector encoding the ligand according to any of the preceding claims.

12. A cell expressing the ligand according to any of claims 1-10, and/or a cell comprising the vector according to claim 11.

13. A composition comprising the ligand according to any of claims 1-10, and one or more physiologically acceptable carriers, excipients and/or diluents.

14. The ligand according to any of claim 1-10 and/or composition according to claim 13, for use as a medicament.

15. The ligand according to any one of claims 1-10 or the composition according to claim 13, for use in the prevention or treatment of vascular proliferative diseases and/or related disorders in a mammal, such as characterized by pathological neovascularization, vascular leakage, inflammation, or fibrosis of the eye.

16. The ligand or composition for use according to claim 15, wherein the disorders characterized by pathological neovascularization in the eye is selected from the group consisting of age related macular degeneration (AMD), including geographic athropy and proliferative AMD, retinal vein occlusion, retinopathy, hypertensive retinopathy, vitreomacular traction, and diabetic retinopathy (DR), including proliferative DR and diabetic macular edema.

17. The ligand or composition for use according to claim 15, wherein the vascular proliferative diseases and/or related disorders are cancers or other malignancies.

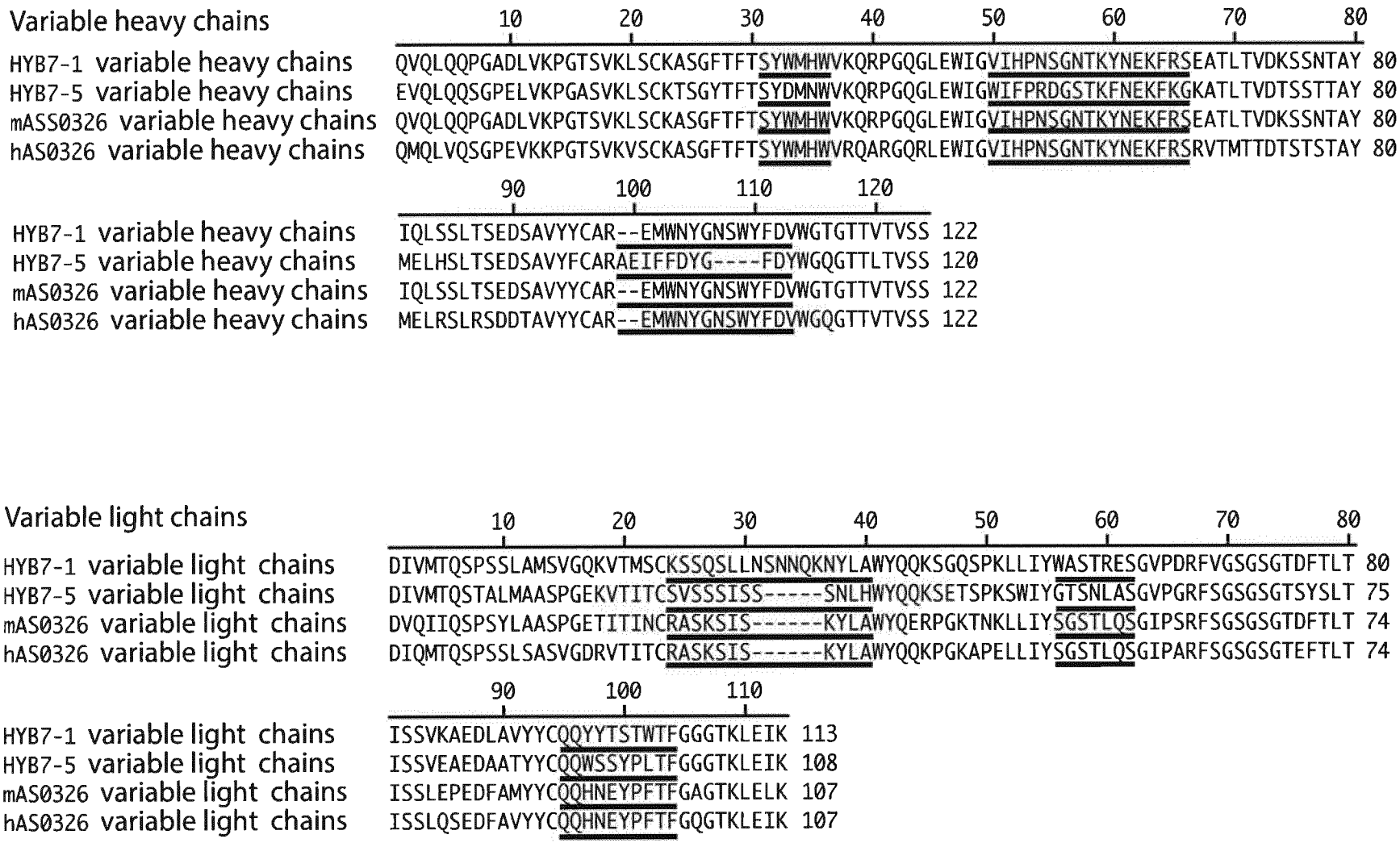

Variable heavy chains
10 20 30 40 50 60 70 80
HYB7-1 variable heavy chains QVQLQQPGADLVKPGTSVKLSCKASGFTFTSYWMHWVKQRPGQGLEWIGVIHPNSGNTKYNEKFRSEATLTVDKSSNTAY 80
HYB7-5 variable heavy chains EVQLQQSGPELVKPGASVKLSCKTSGYTFTSYDMNWVKQRPGQGLEWIGWIFPRDGSTKFNEKFKGKATLTVDTSSTTAY 80
mAS0326 variable heavy chains QVQLQQPGADLVKPGTSVKLSCKASGFTFTSYWMHWVKQRPGQGLEWIGVIHPNSGNTKYNEKFRSEATLTVDKSSNTAY 80
hAS0326 variable heavy chains QMQLVQSGPEVKKPGTSVKVSCKASGFTFTSYWMHWVRQARGQRLEWIGVIHPNSGNTKYNEKFRSRVTMTTDTSTSTAY 80
90 100 110 120
HYB7-1 variable heavy chains IQLSSLTSEDSAVYYCAR--EMWNYGNSWYFDVWGTGTTVTVSS 122
HYB7-5 variable heavy chains MELHSLTSEDSAVYFCARAEIFFDYG----FDYWGQGTTLTVSS 120
mAS0326 variable heavy chains IQLSSLTSEDSAVYYCAR--EMWNYGNSWYFDVWGTGTTVTVSS 122
hAS0326 variable heavy chains MELRSLRSDDTAVYYCAR--EMWNYGNSWYFDVWGQGTTVTVSS 122
Variable light chains
10 20 30 40 50 60 70 80
HYB7-1 variable light chains DIVMTQSPSSLAMSVGQKVTMSCKSSQSLLNSNNQKNYLAWYQQKSGQSPKLLIYWASTRESGVPDRFVGSGSGTDFTLT 80
HYB7-5 variable light chains DIVMTQSTALMAASPGEKVTITCSVSSSISS-----SNLHWYQQKSETSPKSWIYGTSNLASGVPGRFSGSGSGTSYSLT 75
mAS0326 variable light chains DVQIIQSPSYLAASPGETITINCRASKSIS------KYLAWYQERPGKTNKLLIYSGSTLQSGIPSRFSGSGSGTDFTLT 74
hAS0326 variable light chains DIQMTQSPSSLSASVGDRVTITCRASKSIS------KYLAWYQQKPGKAPELLIYSGSTLQSGIPARFSGSGSGTEFTLT 74
90 100 110
HYB7-1 variable light chains ISSVKAEDLAVYYCQQYYTSTWTFGGGTKLEIK 113
HYB7-5 variable light chains ISSVEAEDAATYYCQQWSSYPLTFGGGTKLEIK 108
mAS0326 variable light chains ISSLEPEDFAMYYCQQHNEYPFTFGAGTKLELK 107
hAS0326 variable light chains ISSLQSEDFAVYYCQQHNEYPFTFGQGTKLEIK 107

Fig. 1

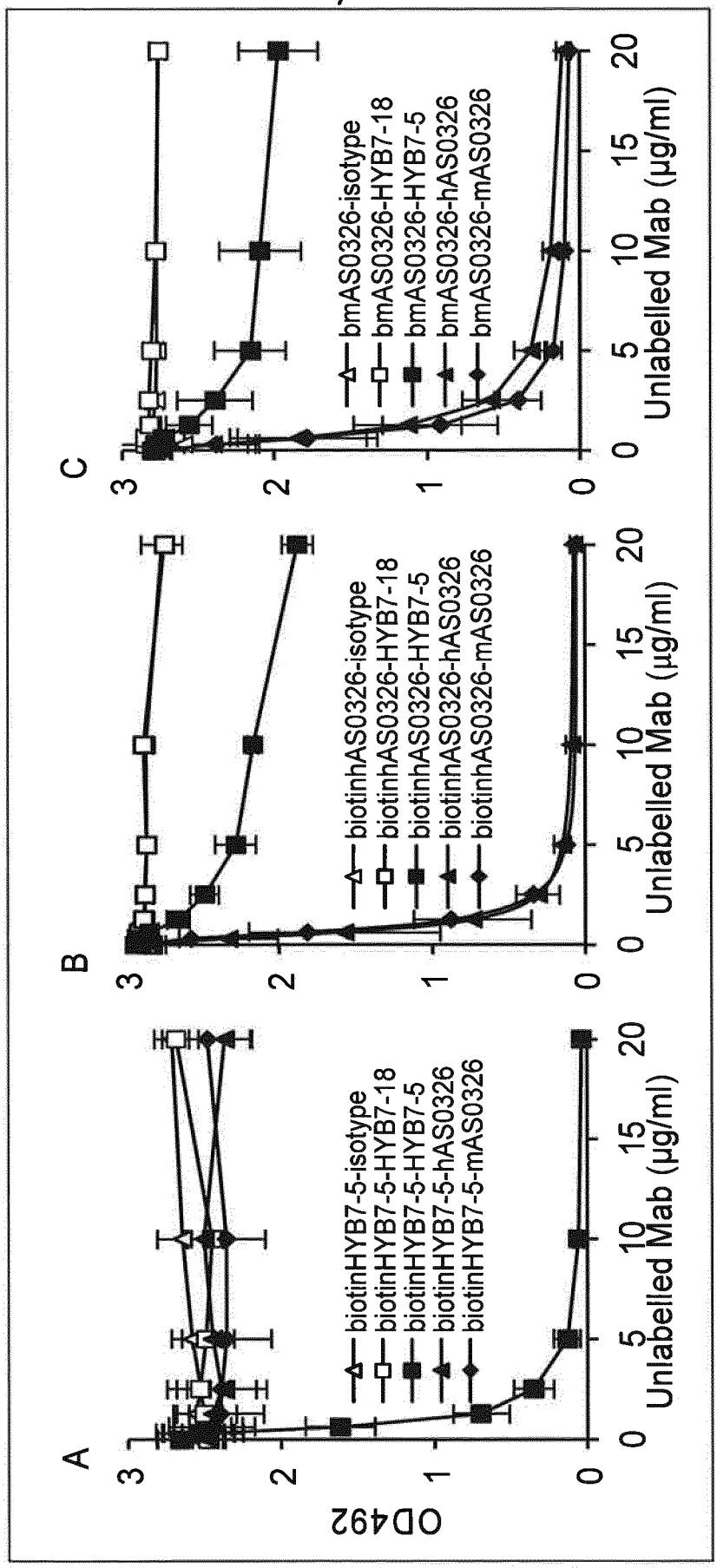

A
B
C
OD492
Unlabelled Mab (µg/ml)
biotinHYB7-5-isotype
biotinHYB7-5-HYB7-18
biotinHYB7-5-HYB7-5
biotinHYB7-5-hAS0326
biotinHYB7-5-mAS0326
biotinAS0326-isotype
biotinAS0326-HYB7-18
biotinAS0326-HYB7-5
biotinAS0326-hAS0326
biotinAS0326-mAS0326
bmAS0326-isotype
bmAS0326-HYB7-18
bmAS0326-HYB7-5
bmAS0326-hAS0326
bmAS0326-mAS0326

Fig. 2

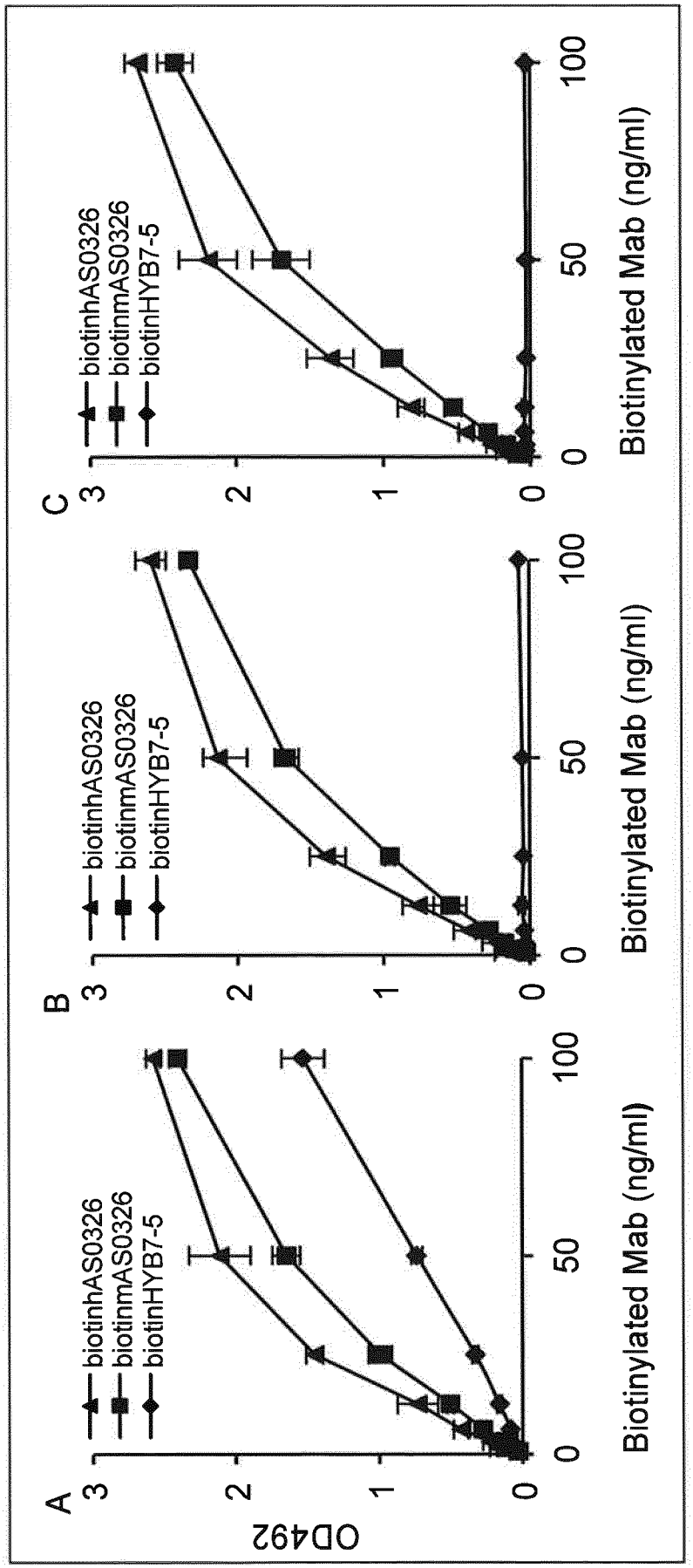

A
B
C
OD492
Biotinylated Mab (ng/ml)
biotinhAS0326
biotinmAS0326
biotinHYB7-5
0
1
2
3
0
50
100

Fig. 3

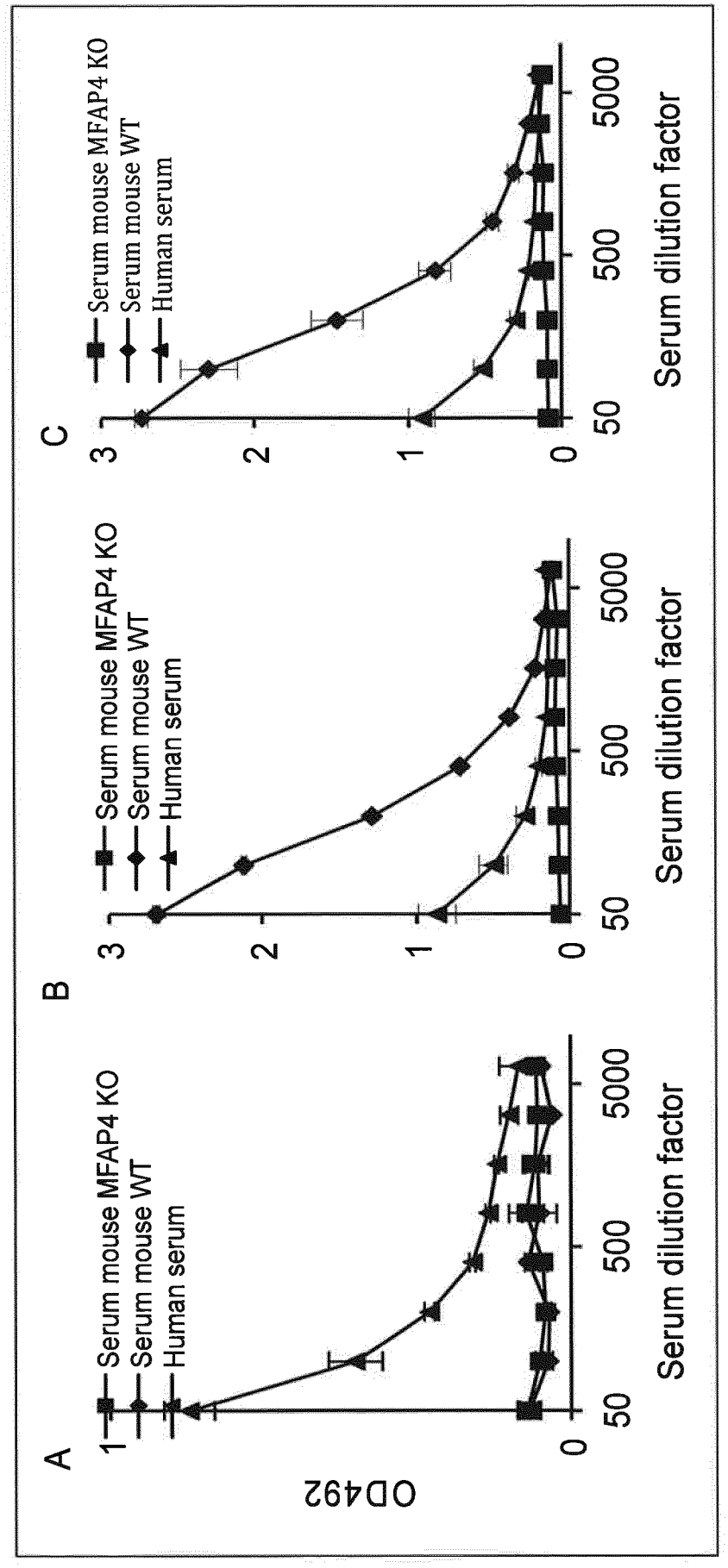

A
B
C
OD492
Serum mouse MFAP4 KO
Serum mouse WT
Human serum
Serum dilution factor
0
50
500
5000
1
2
3

Fig. 4

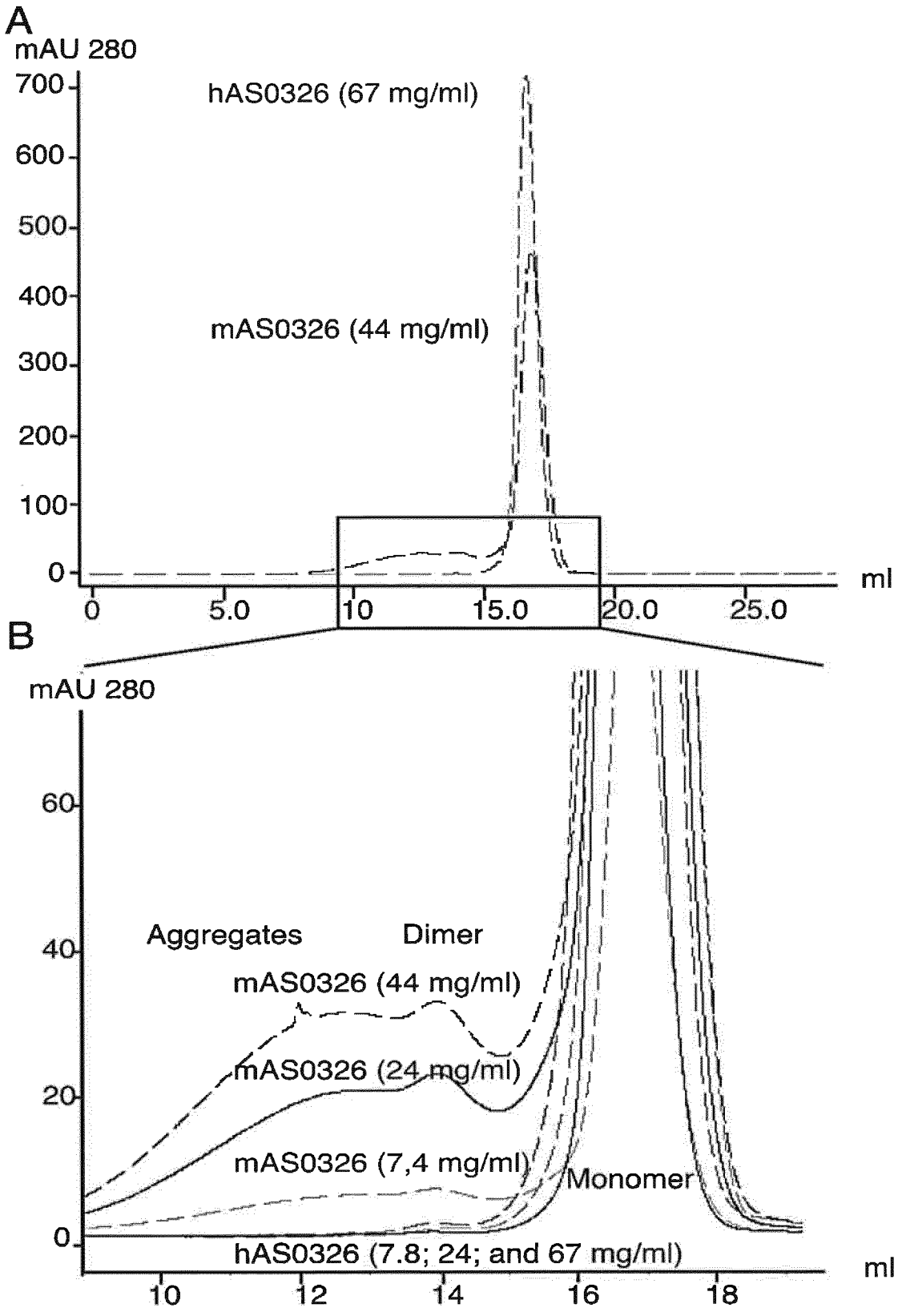

A
mAU 280
700
600
500
400
300
200
100
0
hAS0326 (67 mg/ml)
mAS0326 (44 mg/ml)
0
5.0
10
15.0
20.0
25.0
ml
B
mAU 280
60
40
20
0
Aggregates
Dimer
mAS0326 (44 mg/ml)
mAS0326 (24 mg/ml)
mAS0326 (7,4 mg/ml)
Monomer
hAS0326 (7.8; 24; and 67 mg/ml)
10
12
14
16
18
ml

Fig. 5A-B

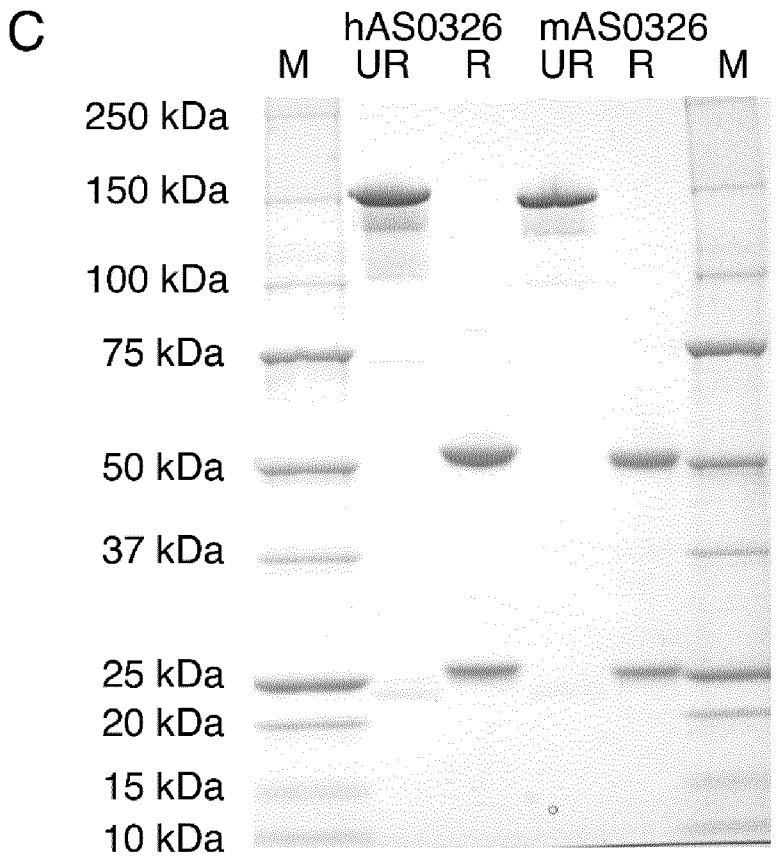

C
hAS0326
mAS0326
M
UR
R
UR
R
M
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa

Fig. 5C

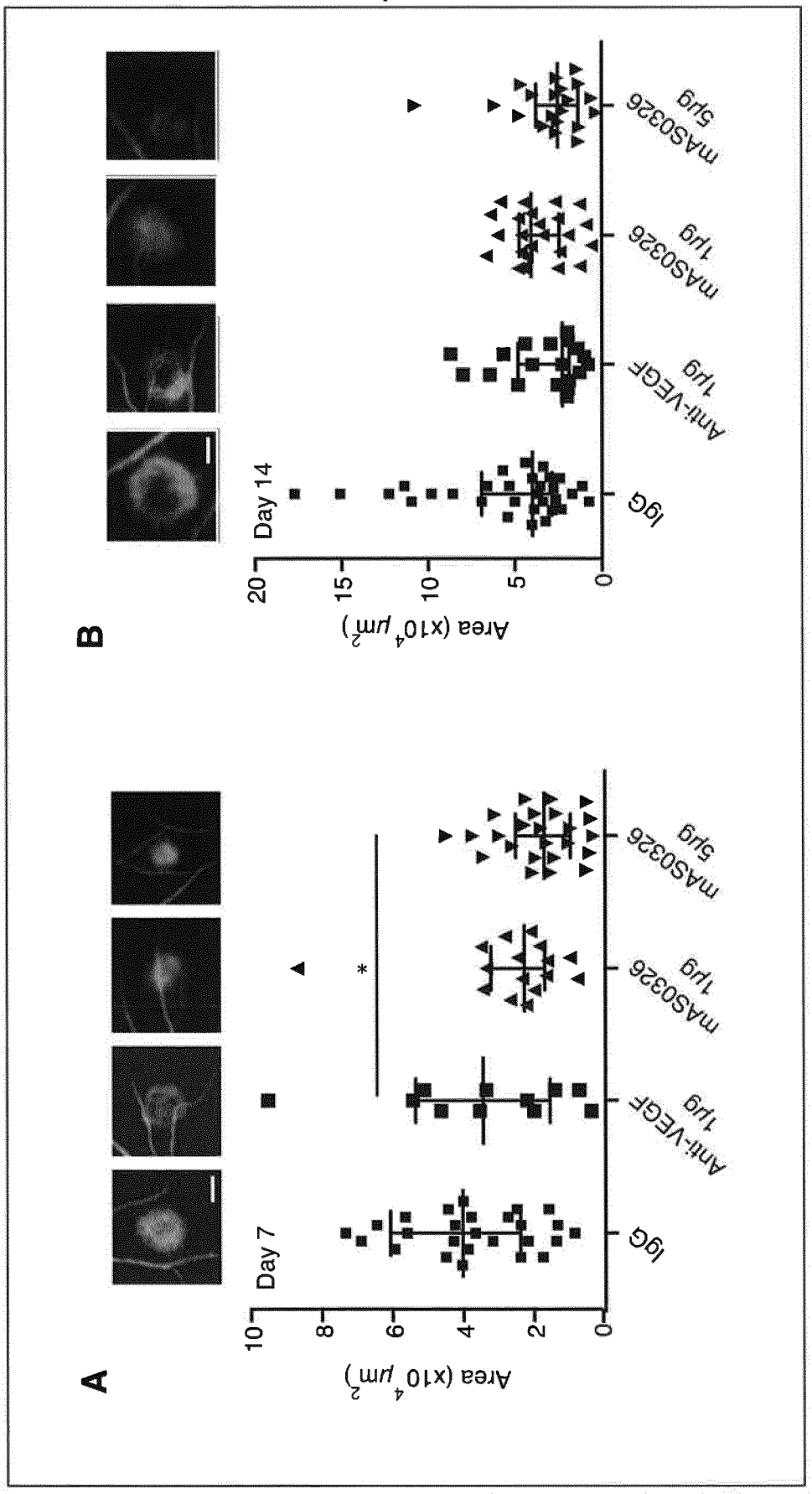

A
Day 7
Area (x10$^4$ $\mu m^2$)
10
8
6
4
2
0
*
IgG
Anti-VEGF 1µg
mAS0326 1µg
mAS0326 5µg
B
Day 14
Area (x10$^4$ $\mu m^2$)
20
15
10
5
0
IgG
Anti-VEGF 1µg
mAS0326 1µg
mAS0326 5µg

Fig. 6A and 6B

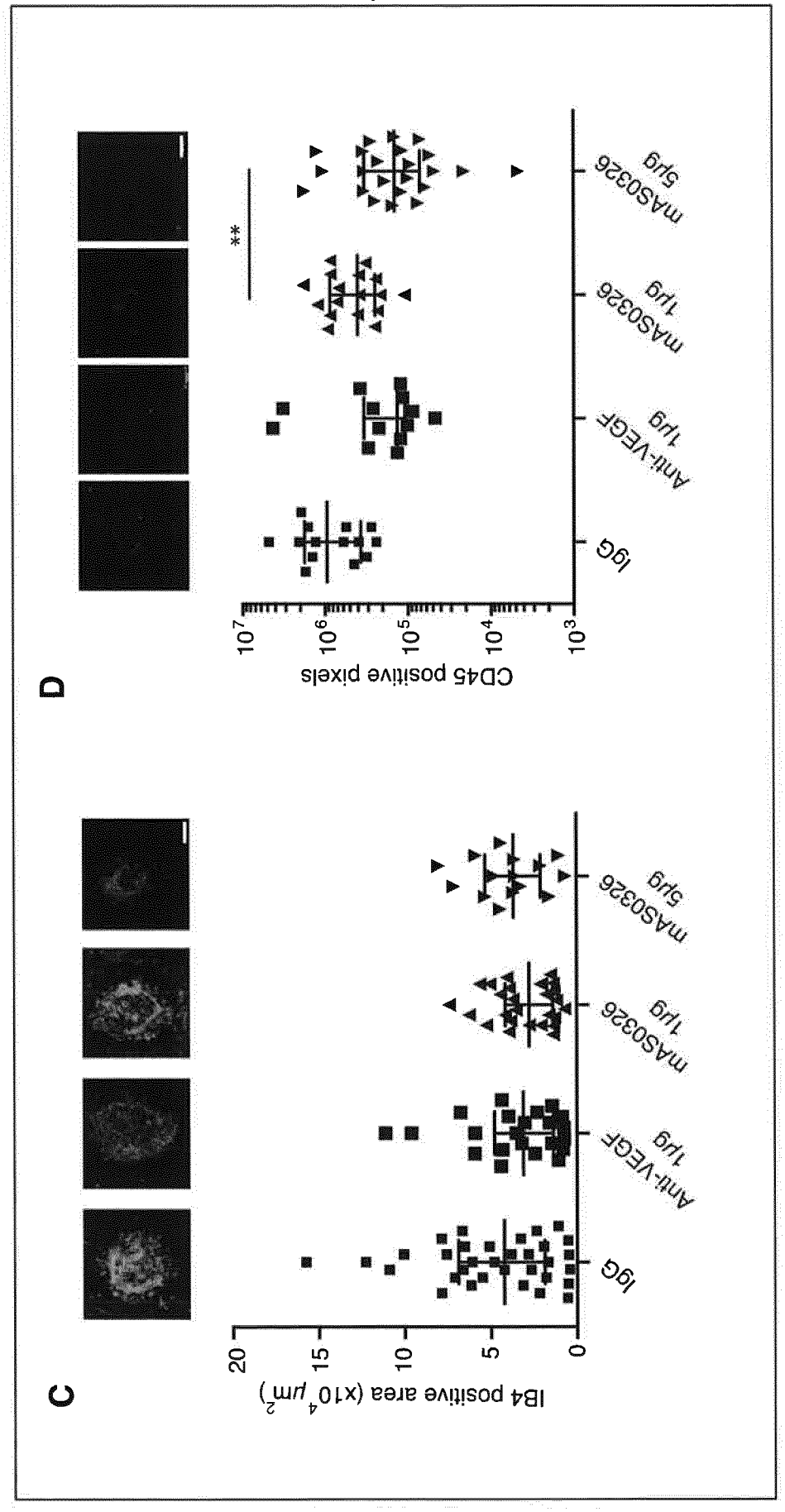

C
IB4 positive area (x10$^4$ $\mu m^2$)
20
15
10
5
0
IgG
Anti-VEGF 1µg
mAS0326 1µg
mAS0326 5µg
D
CD45 positive pixels
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
**
IgG
Anti-VEGF 1µg
mAS0326 1µg
mAS0326 5µg

Fig. 6C and 6D

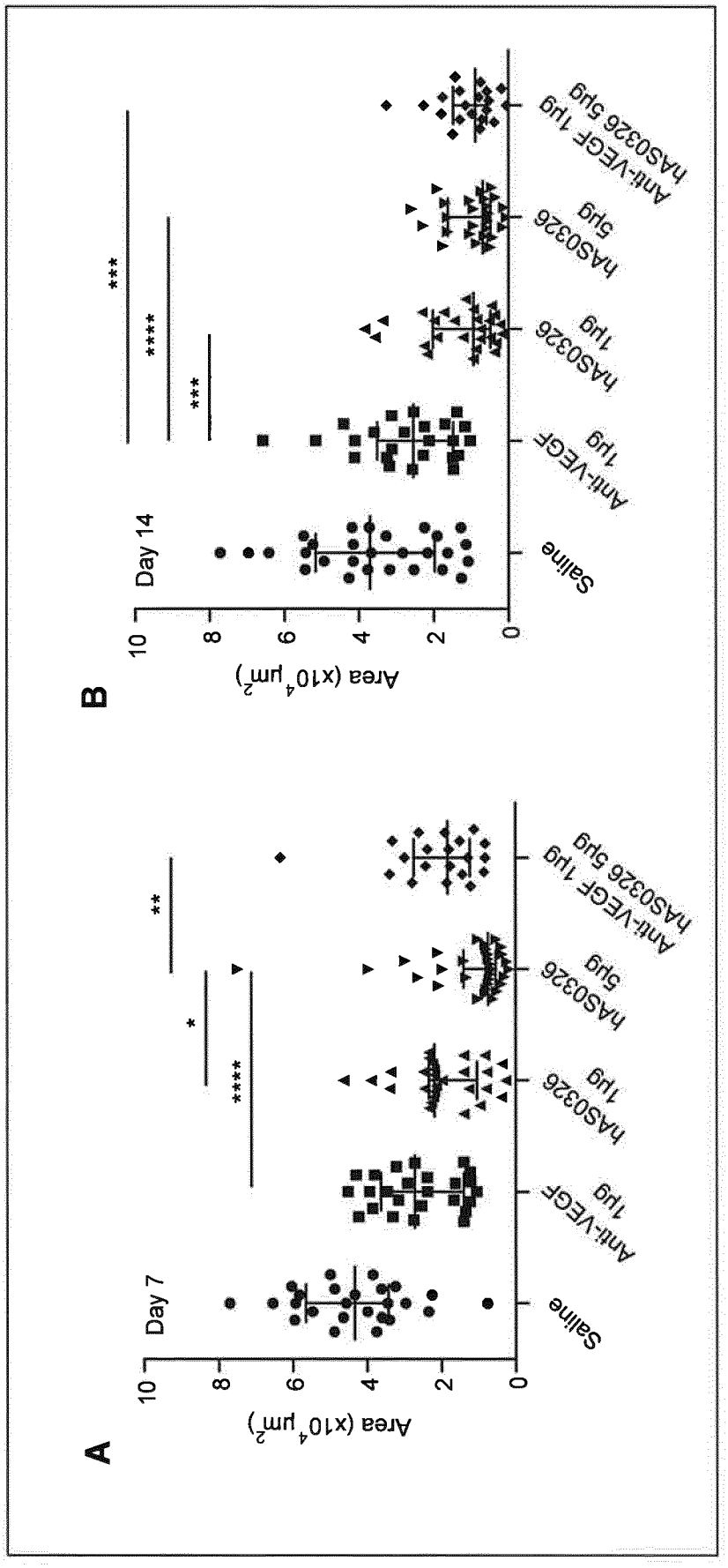

A
Day 7
Area (x10⁴ µm²)
0
2
4
6
8
10
Saline
Anti-VEGF 1µg
hAS0326 1µg
hAS0326 5µg
Anti-VEGF 1µg hAS0326 5µg
*
**
****
B
Day 14
Area (x10⁴ µm²)
0
2
4
6
8
10
Saline
Anti-VEGF 1µg
hAS0326 1µg
hAS0326 5µg
Anti-VEGF 1µg hAS0326 5µg
***
****
***

Fig. 7A and 7B

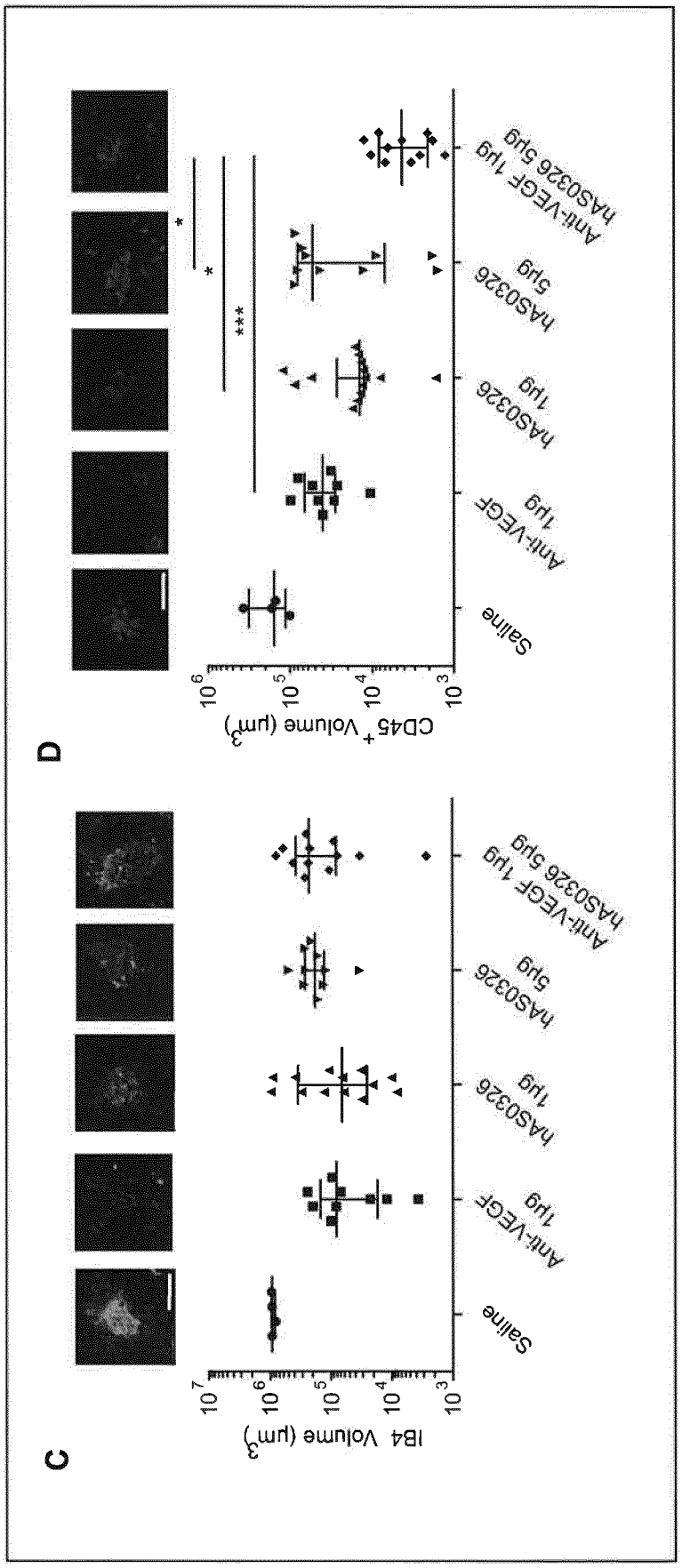

C
D
IB4 Volume (µm³)
CD45⁺ Volume (µm³)
Saline
Anti-VEGF 1µg
hAS0326 1µg
hAS0326 5µg
Anti-VEGF 1µg
hAS0326 5µg
*
*
***

Fig. 7C and 7D

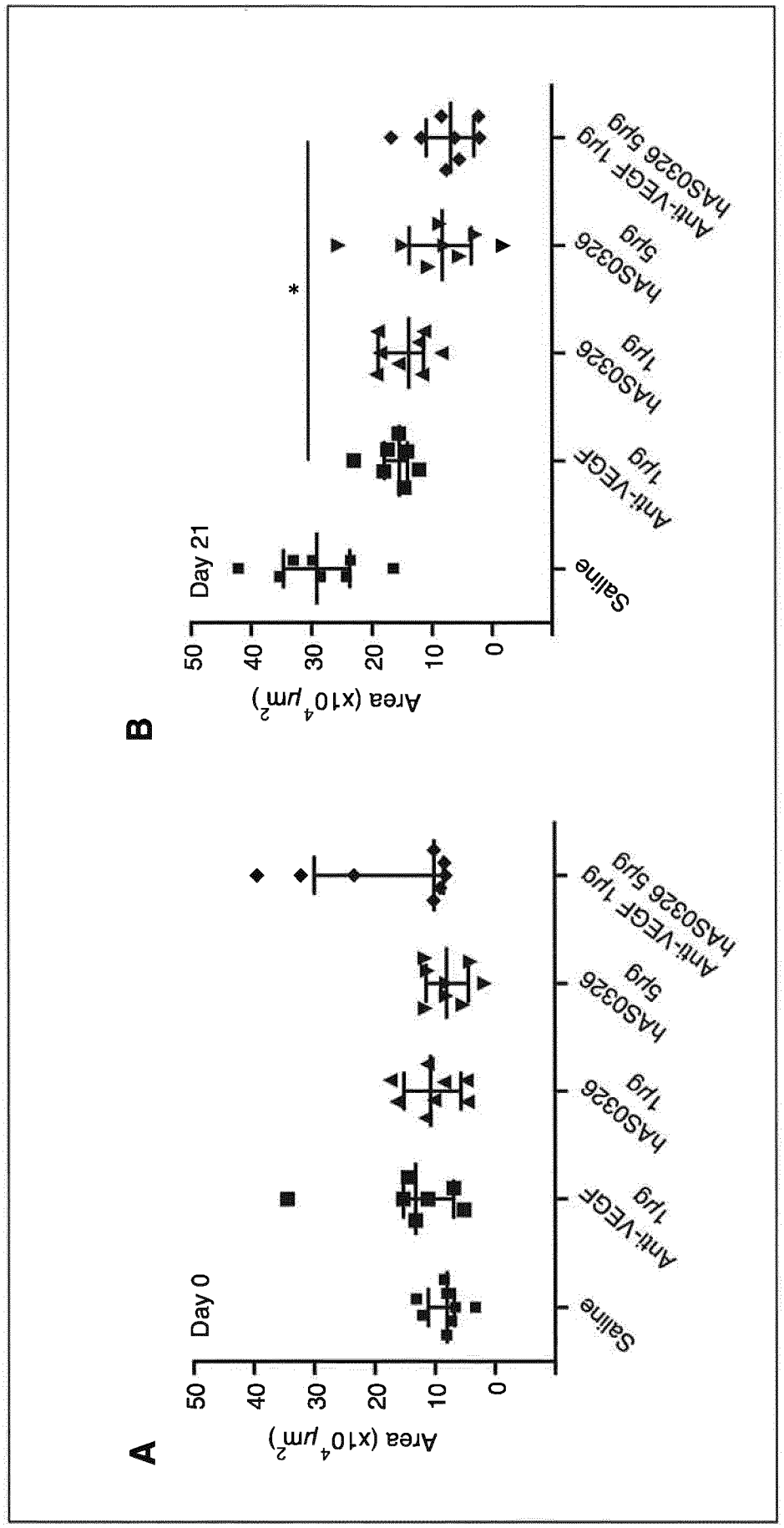

A
Day 0
Area (x10$^4$ $\mu m^2$)
50
40
30
20
10
0
Saline
Anti-VEGF 1µg
hAS0326 1µg
hAS0326 5µg
Anti-VEGF 1µg hAS0326 5µg
B
Day 21
Area (x10$^4$ $\mu m^2$)
*

Fig. 8

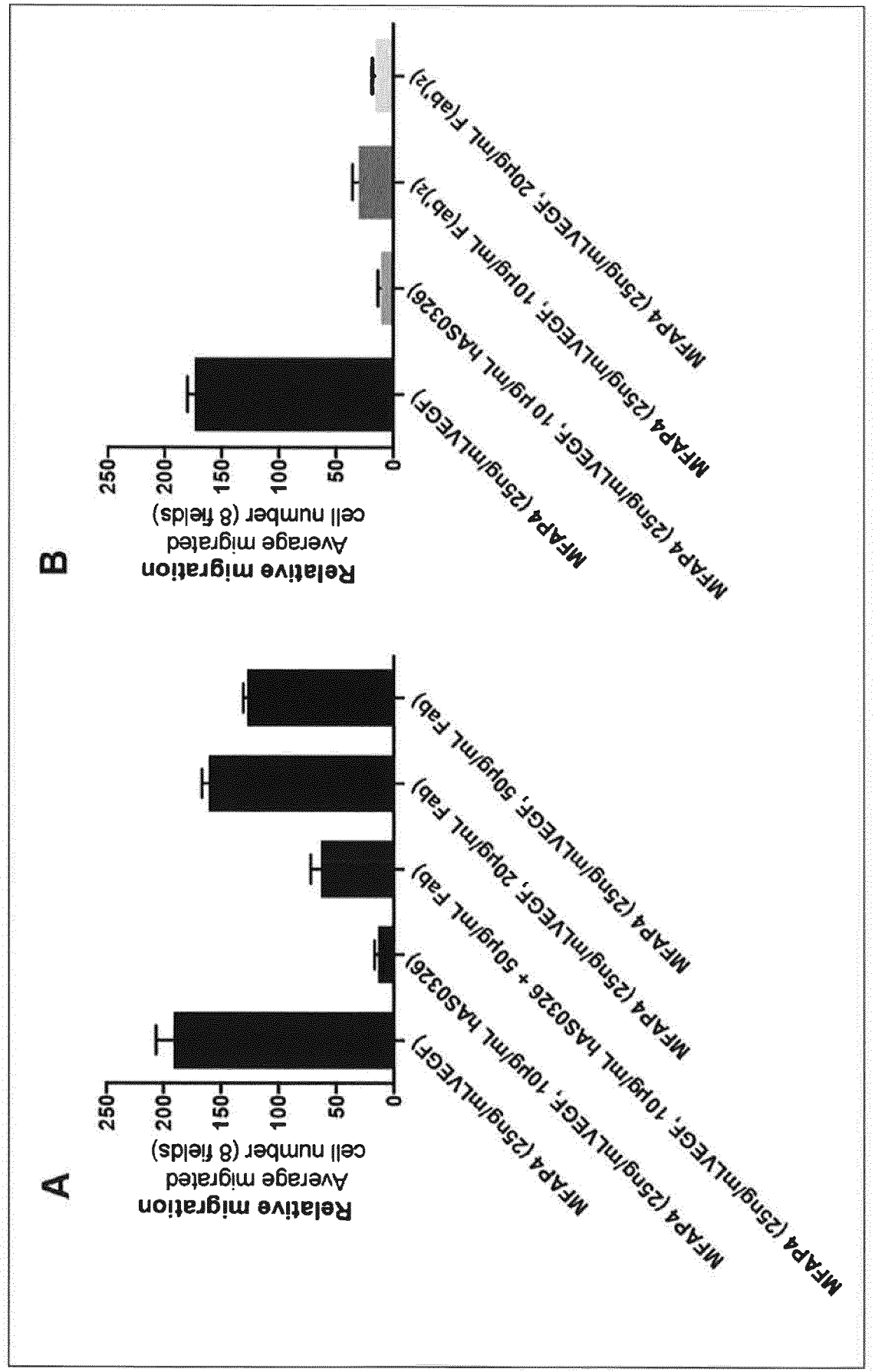

A
Relative migration
Average migrated cell number (8 fields)
250
200
150
100
50
0
MFAP4 (25ng/mL VEGF)
MFAP4 (25ng/mL VEGF, 10µg/mL hAS0326)
MFAP4 (25ng/mL VEGF, 10µg/mL hAS0326 + 50µg/mL Fab)
MFAP4 (25ng/mL VEGF, 20µg/mL Fab)
MFAP4 (25ng/mL VEGF, 50µg/mL Fab)
B
Relative migration
Average migrated cell number (8 fields)
250
200
150
100
50
0
MFAP4 (25ng/mL VEGF)
MFAP4 (25ng/mL VEGF, 10 µg/mL hAS0326)
MFAP4 (25ng/mL VEGF, 10µg/mL F(ab')2)
MFAP4 (25ng/mL VEGF, 20µg/mL F(ab')2)

Fig. 9

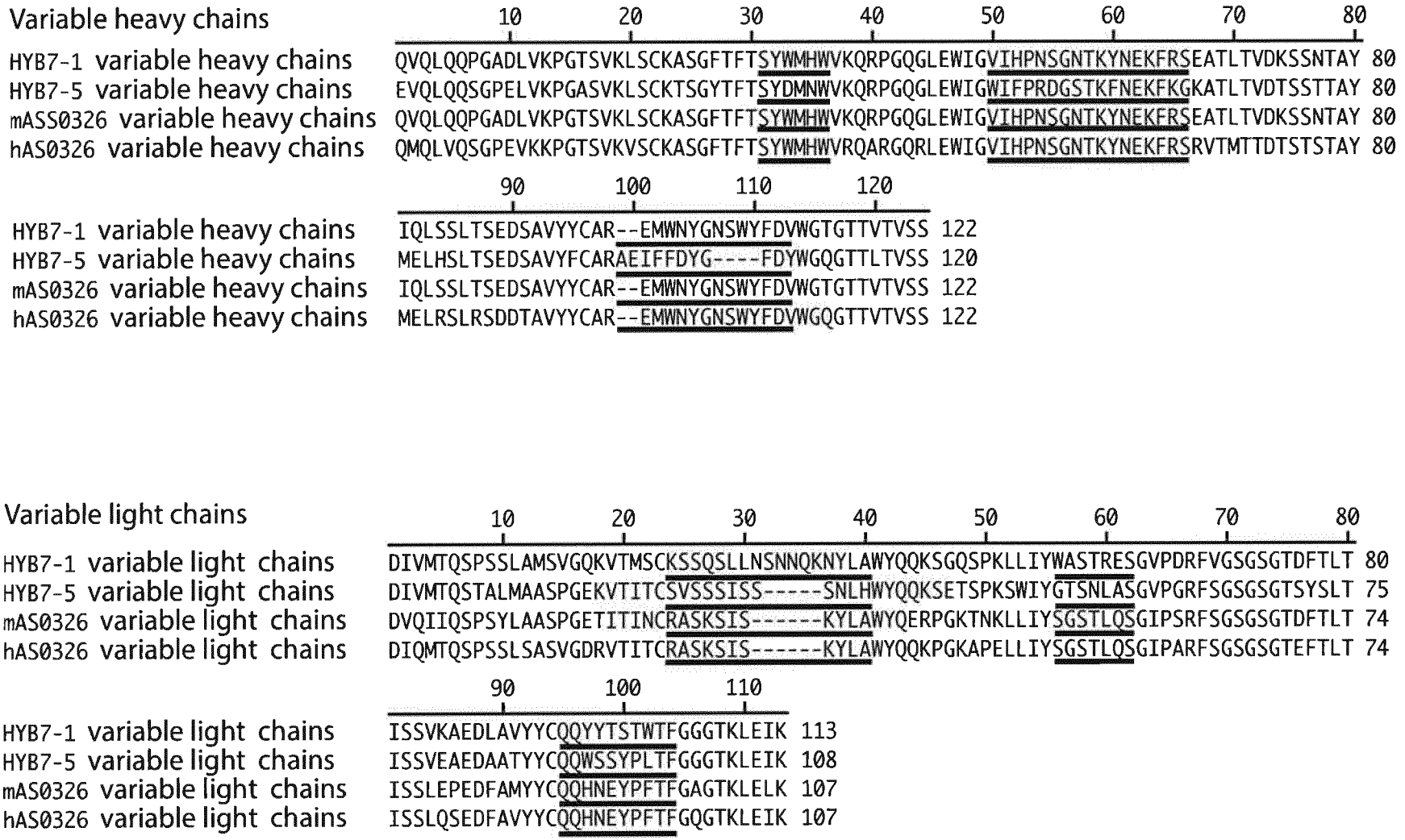

Variable heavy chains
10 20 30 40 50 60 70 80
HYB7-1 variable heavy chains QVQLQQPGADLVKPGTSVKLSCKASGFTFTSYWMHWVKQRPGQGLEWIGVIHPNSGNTKYNEKFRSEATLTVDKSSNTAY 80
HYB7-5 variable heavy chains EVQLQQSGPELVKPGASVKLSCKTSGYTFTSYDMNWVKQRPGQGLEWIGWIFPRDGSTKFNEKFKGKATLTVDTSSTTAY 80
mAS0326 variable heavy chains QVQLQQPGADLVKPGTSVKLSCKASGFTFTSYWMHWVKQRPGQGLEWIGVIHPNSGNTKYNEKFRSEATLTVDKSSNTAY 80
hAS0326 variable heavy chains QMQLVQSGPEVKKPGTSVKVSCKASGFTFTSYWMHWVRQARGQRLEWIGVIHPNSGNTKYNEKFRSRVTMTTDTSTSTAY 80
90 100 110 120
HYB7-1 variable heavy chains IQLSSLTSEDSAVYYCAR--EMWNYGNSWYFDVWGTGTTVTVSS 122
HYB7-5 variable heavy chains MELHSLTSEDSAVYFCARAEIFFDYG----FDYWGQGTTLTVSS 120
mAS0326 variable heavy chains IQLSSLTSEDSAVYYCAR--EMWNYGNSWYFDVWGTGTTVTVSS 122
hAS0326 variable heavy chains MELRSLRSDDTAVYYCAR--EMWNYGNSWYFDVWGQGTTVTVSS 122
Variable light chains
10 20 30 40 50 60 70 80
HYB7-1 variable light chains DIVMTQSPSSLAMSVGQKVTMSCKSSQSLLNSNNQKNYLAWYQQKSGQSPKLLIYWASTRESGVPDRFVGSGSGTDFTLT 80
HYB7-5 variable light chains DIVMTQSTALMAASPGEKVTITCSVSSSISS-----SNLHWYQQKSETSPKSWIYGTSNLASGVPGRFSGSGSGTSYSLT 75
mAS0326 variable light chains DVQIIQSPSYLAASPGETITINCRASKSIS------KYLAWYQERPGKTNKLLIYSGSTLQSGIPSRFSGSGSGTDFTLT 74
hAS0326 variable light chains DIQMTQSPSSLSASVGDRVTITCRASKSIS------KYLAWYQQKPGKAPELLIYSGSTLQSGIPARFSGSGSGTEFTLT 74
90 100 110
HYB7-1 variable light chains ISSVKAEDLAVYYCQQYYTSTWTFGGGTKLEIK 113
HYB7-5 variable light chains ISSVEAEDAATYYCQQWSSYPLTFGGGTKLEIK 108
mAS0326 variable light chains ISSLEPEDFAMYYCQQHNEYPFTFGAGTKLELK 107
hAS0326 variable light chains ISSLQSEDFAVYYCQQHNEYPFTFGQGTKLEIK 107

Fig. 1