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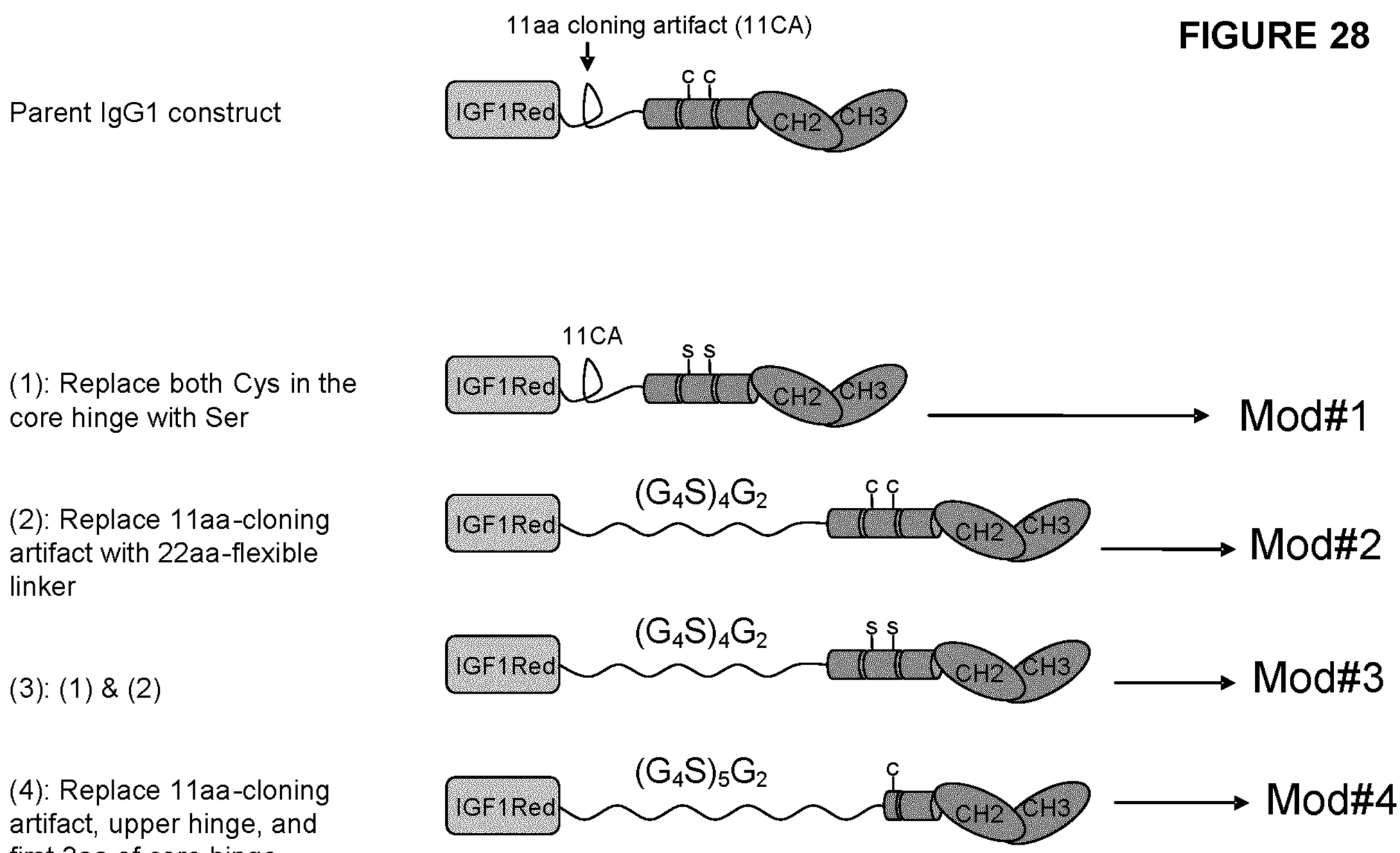
(54) Title: SOLUBLE IGF RECEPTOR FC FUSION PROTEINS AND USES THEREOF

FIGURE 28



(57) Abrégé/Abstract:
There are described herein novel soluble IGF receptor Fc fusion proteins and compositions and methods of use thereof for treating angiogenesis associated disorders and malignant disease, such as cancer and metastasis, wherein the fusion proteins bind specifically to IGF-1 or IGF-2.

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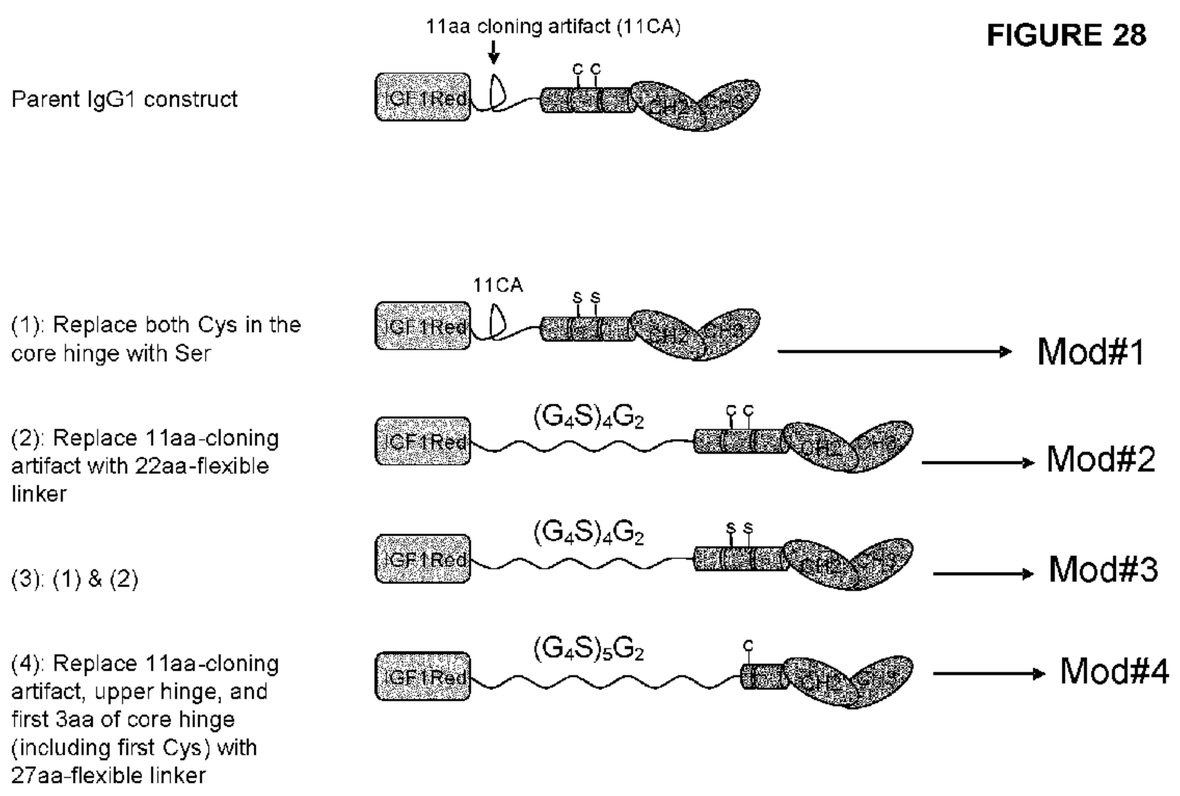

FIGURE 28

(57) Abstract: There are described herein novel soluble IGF receptor Fc fusion proteins and compositions and methods of use thereof for treating angiogenesis associated disorders and malignant disease, such as cancer and metastasis, wherein the fusion proteins bind specifically to IGF-1 or IGF-2.

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SOLUBLE IGF RECEPTOR Fc FUSION PROTEINS AND USES THEREOF

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 61/576,034, filed December 15, 2011, the entire contents of which are hereby incorporated by reference.

TECHNICAL FIELD

[0002] The present invention relates to novel soluble IGF receptor Fc fusion proteins and compositions and methods of use thereof for treating cancer and metastasis.

BACKGROUND OF THE INVENTION

[0003] The receptor for the type I insulin like growth factor (IGF-IR) plays a critical role in progression of malignant disease. Increased expression of IGF-IR and/or its ligands has been documented in many human malignancies and high plasma IGF-I levels were identified as a potential risk factor for malignancies such as breast, prostate and colon carcinomas (Samani et al., 2007, Endocr Rev, 28: 20-47). Recent data have shown that the IGF axis promotes tumor invasion and metastasis through several mechanisms, and it has been identified as a determinant of metastasis to several organ sites, particularly the lymph nodes and the liver (Long et al., 1998, Exp Cell Res, 238: 116-121; Wei, et al., 2006, Ann Surg Oncol, 13: 668-676; Samani et al., 2007, Endocr Rev, 28: 20-47; Reinmuth et al., 2002, Clin Cancer Res, 8: 3259-3269). The IGF receptor can affect metastasis by regulating tumor cell survival and proliferation in secondary sites and also by promoting angiogenesis and lymphangiogenesis either through direct action on the endothelial cells or by transcriptional regulation of vascular endothelial growth factors (VEGF) A and C (reviewed in Li, S. et al., In: Liver metastasis:Biology and Clinical Management 2011; Brodt P., Editor: 233-72)).

[0004] The IGF-IR ligands include three structurally homologous peptides IGF-I, IGF-II and insulin, but the receptor binds IGF-I with the highest affinity. The major site of endocrine production for IGF-I and IGF-II is the liver (Werner & Le Roith, 2000, Cell Mol Life Sci 57: 932-942), but autocrine/paracrine IGF-I production has been documented in extra-hepatic sites such as heart, muscle, fat, spleen and kidney. The physiological activities and bioavailability of IGF-I and IGF-II are modulated through their association with 6 secreted, high-affinity binding proteins (IGFBP1-6).

[0005] IGF-IR has been validated as a target for anti-cancer therapy in various tumor types. A number of IGF-IR inhibitors are in clinical or preclinical development (see, for example, Zha, J. and Lackner, M.R., Clinical Cancer Research 2010; 16: 2512-7; Gualberto, A. and Pollak, M., Oncogene 2009; 28: 3009-21; and Li, S. et al., In: Liver metastasis: Biology and Clinical Management 2011; Brodt P., Editor: 233-72). However, targeting the IGF-I system in vivo poses several challenges: First, due to the high degree of homology between the IGF-I and insulin receptors, drugs that target the IGF axis may also affect the insulin receptor/insulin axis with undesirable effects on glucose and lipid metabolism. Hyperglycemia has, in fact, been observed as one of the undesirable effects of anti-IGF-IR therapy (Karp, D.D. et al., J. Thorac. Oncol. 2009; 4: 1397-403; Bruchim, I., et al., Expert Opinion on Therapeutic Targets 2009; 13: 1179-92; Sachdev, D. and Yee, D., Mol. Cancer Ther. 2007; 6: 1-12; Rodon, J. et al., Mol. Cancer Ther. 2008; 7: 2575-88). Moreover, inhibition of IGF-I signaling may result in altered serum growth hormone levels leading to insulin insensitivity and could potentially cause a reduction in pancreatic insulin production and diabetes (Zha, J. and Lackner, M.R., Clinical Cancer Research 2010; 16: 2512-7). Second, the use of antibody-based therapy may result in ADCC reactions leading to hematological toxicity as observed in some trials (Reidy, D.L., et al., Journal of Clinical Oncology; 28: 4240-6; Zha, J. and Lackner, M.R., Clinical Cancer Research 2010; 16: 2512-7). Furthermore, some tumors also express isoform A of the insulin receptor (IR-A) that can bind IGF-II with high affinity and this may provide an alternate survival mechanism for cancer cells whose IGF-IR has

been neutralized by antibody treatment or kinase inhibitors (Zha, J. and Lackner, M.R., Clinical Cancer Research 2010; 16: 2512-7).

[0006] The use of soluble receptors (decoys) to antagonize the activity of soluble ligands for treatment of malignant disease has been taught as a potential therapeutic treatment and has become an accepted form of therapy for some conditions. Decoy receptors can inhibit the biological activity of the cognate, membrane-bound receptors by binding and decreasing ligand bioavailability for the latter receptor (Rudge, et al., 2007, Proc Natl Acad Sci USA, 104: 18363-18370). Current examples include a soluble TNF receptor (Enbrel) that is in routine clinical use for the treatment of inflammatory conditions (Richard-Miceli, C. and Dougados, M., BioDrugs 2001; 15: 251-9), as well as a VEGF–Trap (Aflibercept) that is in clinical trials for the treatment of cancer and other conditions (Rudge, J.S. et al., Cold Spring Harbor Symposia on Quantitative Biology 2005; 70: 411-8). These reagents are advantageous over antibody-based therapy because they are highly specific, bind to the ligand with high affinity, and bypass some of the undesirable effects of reagents with off-target activity.

[0007] Thus, a soluble IGF-I receptor could potentially overcome some of the shortcomings of current IGF-targeting drugs, such as, for example, cross-reaction with the insulin system, ADCC-related hematological toxicity, and the compensatory effects of insulin receptor isoform A (IR-A).

[0008] It would be highly desirable therefore to be provided with a soluble IGF-1 receptor for treatment of angiogenic-associated disorders and malignant disease, including cancer and metastasis.

SUMMARY OF THE INVENTION

[0009] In accordance with a broad aspect of the invention, there are provided fusion proteins comprising an Fc portion of an antibody and a soluble IGF-IR protein. The Fc portion may be derived from, for example, a human IgG antibody, such as an IgG1 or IgG2 antibody.

[0010] In an aspect, fusion proteins provided herein bind specifically to IGF-1 and IGF-2. In some embodiments, fusion proteins bind to IGF-1 and IGF-2 with at least about the same affinity. In some embodiments, the affinity of the fusion proteins for insulin is at least about 1000-fold lower than for IGF-1 or IGF-2. In some embodiments the fusion proteins do not bind detectably to insulin.

[0011] In some embodiments, the Fc portion of a fusion protein of the invention comprises a modified Fc portion. In one embodiment, a fusion protein comprises an Fc domain modified to remove one or more Cys residues, e.g., to replace one or more Cys residues with Ser residues. In another embodiment, a fusion protein comprises an Fc domain modified to replace an 11 aa linker with a longer, more flexible linker, e.g., a 22aa or a 37aa flexible GS linker. In an embodiment, a fusion protein comprises an Fc domain modified both to remove one or more Cys residues, e.g., to replace one or more Cys residues with Ser residues, and to replace an 11 aa linker with a longer, more flexible linker, e.g., a 22aa or a 37aa flexible GS linker. In some embodiments, fusion proteins having modified Fc domains do not produce HMW species or produce a reduced amount of HMW species compared to unmodified Fc domains.

[0012] In some embodiments, a soluble IGF-IR protein comprises or consists of the extracellular domain of IGF-IR having the amino acid sequence of SEQ ID NO: 1 or 6, or a biologically active fragment or analog thereof. In other embodiments, a soluble IGF-IR protein comprises or consists of the amino acid sequence of the extracellular domain of full-length IGF-IR having the amino acid sequence of SEQ ID NO: 4, or a biologically active fragment or analog thereof. A soluble IGF-IR protein may form the tetrameric structure of SEQ ID NO: 1, 4, or 6.

[0013] In some embodiments, a fusion protein comprises or consists of the sequence set forth in SEQ ID NO: 8 (Fc-sIGFIR, IgG1) or SEQ ID NO: 10 (Fc-sIGFIR, IgG2), or a biologically active fragment or analog thereof. The biologically active fragment or analog of the fusion protein may have, for example, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98%

sequence identity to the fusion protein. The biologically active fragment or analog may also retain the binding specificity of the fusion protein.

[0014] In some embodiments, a fusion protein comprises or consists of the sequence set forth in SEQ ID NO: 12 (sIGF1R-hFc-IgG1 Mod#1), SEQ ID NO: 14 (sIGF1R-hFc-IgG1 Mod#2), SEQ ID NO: 16 (sIGF1R-hFc-IgG1 Mod#3), SEQ ID NO: 18 (sIGF1R-hFc-IgG1 Mod#4), or a biologically active fragment or analog thereof. The biologically active fragment or analog of the fusion protein may have, for example, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to the fusion protein. The biologically active fragment or analog may also retain the binding specificity of the fusion protein.

[0015] Nucleic acids encoding the fusion proteins or biologically active fragments or analogs thereof are also provided. For example, the fusion proteins or biologically active fragments or analogs thereof may be encoded by a nucleic acid having the sequence set forth in SEQ ID NO: 5, 7, or 9, or a degenerate variant thereof. In an embodiment, fusion proteins are encoded by a nucleic acid having the sequence set forth in SEQ ID NO: 11, 13, 15, or 17, or a degenerate variant thereof. In an embodiment, nucleic acids having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to the sequence set forth in SEQ ID NO: 5, 7, 9, 11, 13, 15, or 17 are provided herein. Vectors comprising nucleic acids described herein are also provided.

[0016] In other aspects, pharmaceutical compositions comprising fusion proteins or biologically active fragments or analogs thereof, and a pharmaceutically acceptable carrier, are provided.

[0017] In yet other aspects, there are provided uses of fusion proteins or biologically active fragments or analogs thereof, or compositions thereof, for treating an angiogenic associated disorder or a malignant disease, such as cancer or metastasis, in a subject. For example, fusion proteins or compositions of the invention may be used to treat tumor metastasis, colorectal carcinoma, lung carcinoma, breast cancer, liver cancer, bladder cancer, lung cancer, pancreatic cancer, multiple myeloma, glioblastoma multiforme, or liver

metastasis. Methods of inhibiting angiogenesis in a subject having an angiogenic associated disorder, such as tumor metastasis, colorectal carcinoma, lung carcinoma, breast cancer, liver cancer, bladder cancer, lung cancer, pancreatic cancer, multiple myeloma, glioblastoma multiforme, or liver metastasis, are also provided herein. Methods and compositions for preventing or treating cancer or tumor metastasis are provided herein as well.

[0018] In further aspects, there are provided methods of inhibiting angiogenesis in a subject having an angiogenic associated disorder comprising administering to said subject an autologous cell, e.g., a dendritic cell, a hepatocyte, or a stromal cell, genetically modified to express fusion proteins or biologically active fragments or analog thereofs. The autologous cell may be, e.g., a stromal cell, e.g., a bone marrow derived mesenchymal stromal cell.

[0019] In a still further aspect, the methods provided herein further comprise administering a fusion protein or biologically active fragment or analog thereof, or compositions thereof, in combination with another angiogenesis inhibitor and/or in combination with one or more other anti-cancer agents. The two or more agents may be administered concomitantly or sequentially.

[0020] In yet another aspect, fusion proteins or biologically active fragments or analogs, or compositions thereof, are administered via injection, e.g., intravenous or intraperitoneal injection. In another aspect, fusion proteins or biologically active fragments or analogs, or compositions thereof, are administered orally.

[0021] In an embodiment, there is provided herein a fusion protein comprising an Fc portion of an antibody and a soluble IGF-IR protein. In one embodiment, the fusion protein comprises an antibody, which is a human IgG antibody. In an embodiment, the antibody is an IgG1 or an IgG2 antibody. In an embodiment, the fusion protein binds specifically to IGF-1 and IGF-2. In one embodiment, the fusion protein binds to IGF-1 and IGF-2 with at least about the same affinity. In another embodiment, the affinity of the fusion protein for IGF-2 is higher than the affinity of the fusion protein for IGF-1. In yet another embodiment, the affinity of the fusion protein for insulin is at least about 1000-

fold lower than the fusion protein's affinity for IGF-1 or IGF-2. In an embodiment, the fusion protein does not bind detectably to insulin.

[0022] In one embodiment, a fusion protein comprises a soluble IGF-IR protein comprising the extracellular domain of IGF-IR having the amino acid sequence of SEQ ID NO: 1 or 6, or a biologically active fragment or analog thereof. In an embodiment, a soluble IGF-IR protein forms the tetrameric structure of SEQ ID NO: 1 or 6. In another embodiment, a soluble IGF-IR protein consists of SEQ ID NO: 1 or 6 or a biologically active fragment or analog thereof. In yet another embodiment, a soluble IGF-IR protein comprises the extracellular domain of IGF-IR having the amino acid sequence of SEQ ID NO: 4, or a biologically active fragment or analog thereof.

[0023] In one embodiment, a fusion protein comprises an Fc portion of an antibody and a soluble IGF-IR protein, wherein the soluble IGF-IR protein consists of SEQ ID NO: 1 or 6 or a biologically active fragment or analog thereof.

[0024] In an embodiment, a fusion protein comprises the sequence set forth in SEQ ID NO: 8 or SEQ ID NO: 10. In another embodiment, a fusion protein comprises the sequence set forth in SEQ ID NO: 12, 14, 16 or 18. In yet another embodiment, there is provided herein a fusion protein consisting of the sequence set forth in SEQ ID NO: 8, 10, 12, 14, 16 or 18. In a further embodiment, there is provided herein a fusion protein comprising the amino acid sequence encoded by the nucleic acid set forth in SEQ ID NO: 7, 9, 11, 13, 15 or 17, or a degenerate variant thereof. In a still further embodiment, there is provided herein a fusion protein consisting of the amino acid sequence encoded by the nucleic acid set forth in SEQ ID NO: 7, 9, 11, 13, 15 or 17, or a degenerate variant thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Having thus generally described the nature of the invention, reference will now be made to the accompanying drawings, showing by way of illustration, a preferred embodiment thereof, and in which:

[0026] Figure 1 shows subcloning of CHO pools of stably-transduced cell lines to identify best producers of sIGF1R (Trap D) and Fc-sIGF1R (Trap E). Three subclones of CHO cell pools were isolated: CHO-Cum2-CR5-IGF1R-9-33-1-6; CHO-Cum2-CR5-IGF1R-10-48-2-5; and CHO-Cum2-CR5-IGF1R-hFc-16-13-1-6. For each subclone, 600,000 cells/ml were cultured for 2 days at 37°C and 7 days at 300°C. Samples analyzed by denaturing, non-reducing SDS-PAGE, 12µl/lane, Novex® Tris-Glycine 10% TG 1.5. The lanes shown are as follows: 1: IGF1R-9-33-1-6 pool; 2: IGF1R-9-33-1-6 clone #5; 3: IGF1R-9-33-1-6 clone #6; 4: IGF1R-9-33-1-6 clone #10; 5: IGF1R-10-48-2-5 pool; 6: IGF1R-10-48-2-5 clone #5; 7: IGF1R-10-48-2-5 clone #8; 8: IGF1R-10-48-2-5 clone #12; 9: IGF1R-hFc-16-13-1-6 pool; 10: IGF1R-hFc-16-13-1-6 clone #4; 11: IGF1R-hFc-16-13-1-6 clone #5; 12: IGF1R-hFc-16-13-1-6 clone #7.

[0027] Figure 2 shows purification of sIGF1R (Trap D) using a calcium hydroxyapatite (CHT) column followed by gel filtration. For the hydroxyapatite column, 170 ml of 400-fold concentrated & diafiltrated sIGF1R was loaded onto 25 ml of CHT column. Samples were analyzed by denaturing, non-reducing SDS-PAGE, Novex® Tris-Glycine 10% TG 1.5. SDS-PAGE is shown in (A). Samples in lanes 1-9 are from the CHT column and in lanes 10-17 are from the gel filtration column, runs # 3 to 4 as indicated. The lanes shown are as follows: 1: Feed (non-concentrated), 5µg/lane; 2: Permeate; 3: Feed (concentrated); 4: Flow-through, 0 to 115 ml; 5: Flow-through + chase; 6: Pool A2-A7, 15% B1; 7: Pool A3-A5, 15% B1; 8: Pool A10-B1, 20% B1; 9: Pool B3-B7, 100% B2 (CIP); 10: High Molecular Weight markers (details are shown in part B of the figure); 11: Run#3 A6 (5µg); 12: Run#3 A7 (5µg); 13: Run#3 A10 (out of range); 14: Run#4 A6 (5µg); 15: Run#4 A7 (5 µg); 16: Run#4 A11 (out of range); 17: Purified IGF1R-CHT-GF, 2.6µg. Molecular weight markers are shown in detail in (B). Letters and numbers (A2-A7, B1, A3-A5, A10-B1, B3-B7, B2) refer to fractions collected from columns; letters and numbers indicate position of tube on rack of fraction collector.

[0028] Figure 3 shows purification of Fc(IgG1)-sIGF1R (Trap E) using a calcium hydroxyapatite (CHT) column followed by gel filtration. Samples were analyzed by denaturing, non-reducing SDS-PAGE, Novex® Tris-Glycine 10%

TG 1.5. SDS-PAGE is shown in (A). Samples in lanes 1-5 are from the CHT column and in lanes 6-15 are from the gel filtration column. The lanes shown are as follows: 1: A9-A12; 2: B1-B6; B7-C1; 4: C10-D3; 5: E5-E8; 6: Feed (5µl); 7: Feed (2µl); 8: A9-A10; 9: A11- A12; 10: B1-B3; 11: B6; 12: B8-B9; 13: B10-B11; 14: B12-C1; and 15: Purified IGF1R-CHT-hFc-GF, 2.6 µg. The Red arrow indicates the expected position of the Fc-sIGFIR tetramer; HMW: High molecular weight markers. Molecular weight markers are shown in detail in (B). Letters and numbers (A9-A12, B1-B6, B7-C1, C10-D3, E5-E8, etc.) refer to fractions collected from columns; letters and numbers indicate position of tube on rack of fraction collector.

[0029] Figure 4 shows purification of Fc(IgG1)-sIGF1R (Traps F and G) using protein A chromatography. Samples were analyzed by denaturing, non-reducing SDS-PAGE, Novex® Tris-Glycine 4-20% TG 1.5. SDS-PAGE is shown in (A). Samples in lanes 1 to 4 are from purification of Trap F (eluted at pH 4), lane 1: 2 µl; lane 2: 1 µl; lane 3: 0.5 µl; lane 4: 0.25 µl/lane; lane HMW: High molecular weight markers. Samples in lanes 5 to 8 are from purification of Trap G (eluted at pH 3.5), lane 5: 1 µl; lane 6: 0.5 µl; lane 7: 0.25 µl; lane 8: 0.125 µl/lane. Samples in lanes 9 to 14 show IgG2 (purchased from Sigma), lane 9: 3 µg; lane 10: 2 µg; lane 11: 1 µg; lane 12: 0.5 µg; lane 13: 0.25 µg; lane 14: 0.125 µg. The Red arrow indicates the expected position of the Fc-sIGFIR tetramer; the Black arrows indicate high molecular weight (HMW) species.

[0030] Figure 5 shows purification of endotoxin-free Fc(IgG1)-sIGF1R (Traps H and I) using protein A chromatography. Samples were analyzed by denaturing, non-reducing SDS-PAGE, Novex® Tris-Glycine 4-20% TG 1.5. SDS-PAGE is shown in (A). 14 µl/lane was loaded. Samples in lanes 5 to 8 are from purification of Trap H (eluted at pH 4). Samples in lanes 9 to 12 are from purification of Trap I (eluted at pH 3.5). Lane 1: Feed; lane X: nothing loaded; lane 2: Flow-through (F.T.); lane 3: A1-A2; lane 4: A3-A4; lane 5: A6-A7; lane 6: A8-A10; lane 7: A11-A12; lane 8: B1-B2; lane 9: B3-B4; lane 10: B5-B6; lane 11: B7-B10; lane 12: B11-B12. The Red arrow indicates the expected position of the Fc-sIGFIR tetramer. Letters and numbers (A1-A2, A3-A4, A6-A7, A8-A10,

A11-A12, B1-B2, etc.) refer to fractions collected from columns; letters and numbers indicate position of tube on rack of fraction collector.

[0031] Figure 6 shows a schematic representation of vectors used to make Trap proteins of the invention. The sIGF1R sequence was inserted into the pMPG-CR5 vector as shown in (A), and the sIGF1R sequence fused to either the human IgG1 Fc or IgG2 Fc was inserted into the pMPG-CR5 vector as shown in (B) and (C), respectively. These vectors were used for transient or stable expression of Trap proteins in CHO cells.

[0032] Figure 7 shows a comparison of the most predominant glycopeptides of sIGF1R and sIGF1R-hFc by mass spectrometry. In (A), relative percentage refers to the types of sugars attached at each glycosylation site; sites 4, 5, 7, 8, 12, 15 and 16 are glycosylation sites in the peptides; solid bars represent sIGF-IR (Trap D); cross-hatched bars represent sIGF-IR-hFc (Trap E); and the colors indicate the nature of the glycosylation, as indicated in the legend shown in (B).

[0033] Figure 8 shows that Traps D and E inhibit tumor cell proliferation in response to hIGF-I equally. (A) shows a plot of OD vs. time where 10 ng/mL IGF-I was used; (B) shows a plot of OD vs. time where 50 ng/mL IGF-I was used. ♦ indicates IGFI; ■ indicates sIGF-IR (Trap D) + IGFI; ▲ indicates sIGF-IR-hFc (Trap E) + IGFI and **** indicates $p < 0.001$ at all time points tested.

[0034] Figure 9 shows a dose-dependent increase in anoikis (detachment-induced apoptosis) in the presence of Trap D. FBS: Fetal Bovine Serum; SF: Serum-free; "IGF-I: Trap D" is the molar ratio of IGF-I to Trap D, which is 2:1, 1:1 or 1:2 as indicated; * indicates $p<0.05$; ** indicates $p<0.01$; and **** indicates $p<0.001$.

[0035] Figure 10 shows a dose-dependent increase in anoikis (detachment-induced apoptosis) in the presence of Traps D and E and a comparison between Traps D and E. FBS: Fetal Bovine Serum; SF: Serum-free; Ratios are IGF-I:sIGFIR molar ratios (2:1, 1:1 or 1:2 as indicated); * indicates $p<0.05$; ** indicates $p<0.01$; and **** indicates $p<0.001$. The data illustrate superior performance of Trap E (Fc-sIGFIR).

[0036] Figure 11 shows increased anoikis in the presence of the IGF-Traps E, F and G, illustrating the effect of protein A purification. FBS: Fetal Bovine Serum; SF: Serum-free; Molar ratios of IGF-I:Trap protein are as indicated; **** indicates $p<0.001$.

[0037] Figure 12 shows reduced anchorage-independent growth in the presence of Traps D and E, and a comparison between Traps D and E. In (A) it is shown that the number of colonies was significantly reduced in the presence of the Traps; *indicates $p< 0.05$; p was < 0.01 under all conditions tested. Colors indicate the proteins tested, as indicated in the legend shown in (B). The data illustrate superior performance of the Fc fusion protein.

[0038] Figure 13 shows a time course analysis indicating the effect of Traps D and E on tumor cell invasion and a comparison between Traps D and E. Blue line (♦) represents baseline (no IGF-I); Pink line (■) indicates invasion with IGF-I; Green line (▲) indicates Trap D; and Red line (●) indicates Trap E.

[0039] Figure 14 shows in (A), the effect of Traps D, E, F and G on tumor cell invasion at 48 hours; **** indicates $p<0.0005$. (B) shows a time course analysis for the effect of Traps D, E, F and G on tumor cell invasion: blue line (♦) is IGF-I; green line (■) is baseline (no IGF-I); light brown line (♦) is Trap D; dark green line (▲) is Trap E; red line (■) is Trap F; light blue line (●) is Trap G.

[0040] Figure 15 shows in (A), the effect of Traps E, H and I on tumor cell invasion at 48 hours, illustrating a comparison of Trap E before and after protein A purification; **** indicates $p< 0.001$. (B) shows a time course analysis for the effect of Traps E, H and I on tumor cell invasion: blue line (♦) is IGF-I; pink line (■) is Trap E; green line (■) is Trap H; red line (■) is Trap I; and orange line (■) is baseline (no IGF-I).

[0041] Figure 16 shows curve fitting for a multi-cycle SPR titration. There is shown a representative analysis of experimental data (solid colored lines) to the "1:1 kinetic" model (global fit, dashed black lines) for hIGF-I (0 – 66 nM; 2-fold dilution series) binding to amine-coupled Trap B (9500 RU).

[0042] Figure 17 shows curve fitting for a single-cycle SPR titration. There is shown a representative analysis of experimental data (solid colored lines, 0 – 530 nM, 2-fold dilution series) to the "1:1 titration" model (local fits, dashed black lines) for mIGF-I (green), hIGF-I (red) and hIGF-II (blue) binding to amine-coupled Trap E (6400 RU).

[0043] Figure 18 shows a pharmacokinetic analysis of Traps D and E, indicating a greater than 2-fold increase in the half-life of Fc-sIGF1R (Trap E) compared to sIGF1R (Trap D). Trap D is shown in (A); Trap E is shown in (B); red circles represent observed values; and the blue line shows predicted values.

[0044] Figure 19 shows a pharmacokinetic analysis of Traps D, E, H and I, indicating inferior in vivo performance of Protein A- purified Fc-sIGFIR enriched for HMW species. Trap D is shown in (A); Trap E is shown in (B); Trap H (pH 4.0) is shown in (C); and Trap I (pH 3.5) is shown in (D). Red circles represent observed values, and the blue line shows predicted values.

[0045] Figure 20 shows reduced tumor volume in mice inoculated with colon carcinoma MC-38 cells and treated with IGF-Trap H. Representative H&E stained formalin fixed paraffin embedded sections of livers derived from colon carcinoma MC-38-injected mice 19 days post tumor injection are shown. Top row: livers from mice not treated with IGF-Trap H (Non-treated); bottom row: livers from mice treated with IGF-Trap H (Trap-treated); L indicates liver; T indicates tumor; Mag-x20-50, inset – x400. The far right panel in the top row shows an expanded view (X400) of the indicated metastasis.

[0046] Figure 21 shows reduced IGF-IR phosphorylation in micrometastases. C57BL6 female mice were injected intrasplenically with 10^5 GFP-tagged H-59 cells followed by injection of 5mg/kg IGF-Trap H (Trap-treated) or vehicle only (Non-treated) on days 1 and 3 post tumor injection (3 mice per group). Mice were sacrificed on day 6, livers removed and snap frozen and 10 µM cryostat sections prepared and immunostained with a rabbit polyclonal anti-mouse pIGF1R antibody followed by a goat anti-rabbit Alexa Fluor 647 (far-red) antibody. Sections were washed and mounted with the

GOLD anti-fade reagent and analyzed with a Carl Zeiss LSM 510 Meta, confocal microscope. In (A), there are shown representative merged confocal images, as follows: A. sections from non-treated mice; B. sections from Trap-treated mice; Green fluorescent protein (GFP) is shown in green; DAPI staining is shown in blue; pIGF1R is shown in white; Images were taken at Mag. X200. In (B), there is shown the calculated means of percent of pIGF-IR$^+$ green fluorescent tumor cells in each group (Non-treated, or Trap- treated at 5 mg/Kg, as indicated); P<0.001.

[0047] Figure 22 shows increased tumor cell apoptosis in IGF-Trap H treated mice. Liver cryostat sections were obtained as described above for Fig. 21. Sections were incubated first with a rabbit polyclonal anti-mouse cleaved caspase-3 antibody (ab4501-Abcam) and then with a goat anti-rabbit Alexa Fluor 647 antibody. In (A), representative merged confocal images are shown, as follows: a. sections from non-treated mice (Non-treated); b. sections from Trap-treated mice (IGF-Trap -treated); Green fluorescent protein (GFP) is shown in green; DAPI staining is shown in blue; Cleaved Caspase 3$^+$ cells are shown in red; Images were taken at Mag. X200. In (B), there is shown the calculated means of percent of cleaved-caspase 3$^+$ green fluorescent tumor cells in each group (Non-treated, or Trap- treated at 5 mg/Kg, as indicated); P<0.001.

[0048] Figure 23 shows decreased tumor cell proliferation in IGF-Trap H treated mice. Liver cryostat sections were obtained as described above for Fig. 21. Sections were incubated first with a rabbit polyclonal anti-mouse Ki67 antibody and then with a goat anti-rabbit Alexa Fluor 647 antibody. The percentage of GFP$^+$ tumor cells that were Ki67 positive (a marker of proliferation) was calculated. In (A), representative merged confocal images are shown, as follows: left panel: sections from non-treated mice (Non-treated); right panel: sections from Trap-treated mice (IGF-Trap -treated); Green fluorescent protein (GFP) is shown in green; Ki67 positive cells are shown in red; Images were taken at Mag. X200. In (B), there is shown the calculated means of percent of Ki67$^+$ green fluorescent tumor cells in each group (Non-treated, or Trap –treated at 5 mg/Kg, as indicated); p=0.0012.

[0049] Figure 24 shows decreased vessel count (angiogenesis) in IGF-Trap H injected mice. Liver cryostat sections were obtained as described above for Fig. 21. Sections were incubated first with a rat monoclonal anti-mouse CD31 antibody and then with a goat anti-rat Alexa Fluor 568 (orange-red) antibody. The number of $CD31^+$ endothelial cells within tumor micrometastases per field (20X objective) was counted in 16 sections per treatment group and the mean number was calculated. In (A), representative merged confocal images are shown, as follows: A. sections from non-treated mice (Non-treated); B. sections from Trap-treated mice (IGF-Trap -treated); Green fluorescent protein (GFP) is shown in green; DAPI staining is shown in blue; $CD31^+$ cells are shown in red; Images were taken at Mag. X200. In (B), there is shown the calculated means of $CD31^+$ cells per field in each group (Non-treated, or IGF-Trap –treated at 5 mg/Kg, as indicated); p=0.0057.

[0050] Figure 25 shows tumor growth reduction and increase in animal survival in an orthotopic murine mammary carcinoma (4T1) model. Balb/c female mice were injected into the mammary fatpad (MFP) with 10^5 mouse mammary carcinoma 4T1 cells. Four hours and 3 days later the treatment group received an i.v. injection of 10mg/kg of IGF-Trap H followed by 2 injections of 5 mg/kg on days 6 and 10 post tumor inoculation (indicated by arrows in part (A)).Tumors were measured three times weekly using a caliper and the tumor volumes calculated using the formula 1/2(length x $width^2$). In (A), there is shown a graph of Tumor volume (mm^3) vs. Days post tumor inoculation for mice non-treated (Control) or treated with IGF-Trap (IGF-Trap), as indicated. In (B), there is shown a plot of mouse survival vs. Days post tumor inoculation for control or IGF-Trap treated, as indicated; $p<0.01$ using both Mantel-Cox and Gehan-Breslow-Wilcoxon tests.

[0051] Figure 26 shows tumor growth inhibition in IGF-Trap-treated mice orthotopically implanted with human breast cancer cells. One million MD-MBA-231 human breast cancer cells were orthotopically implanted with Matrigel in the mammary fatpads of nu/nu mice. Tumors were measured three times weekly using a caliper and the tumor volumes calculated using the formula 1/2(length x $width^2$). When tumors were established (50-100mm^3) (day 11,

indicated by an arrow in part (A)), the animals were randomized and treated with 5mg/kg of IGF-Trap H or vehicle (i.v.) twice weekly up to day 33. Mice in the control group were all moribund by day 44 (indicated by a dashed line in part (A)). In (A), there is shown a graph of Tumor volume (mm^3) vs. Days post tumor inoculation for non-treated mice (Control) or mice treated with IGF-Trap (IGF-Trap treated), as indicated. In (B), longitudinal bioluminescence imaging is shown; this was used to monitor tumors. The color scale for bioluminescence is shown at the left side of panel (B), and mice at the indicated day post tumor inoculation are shown; left panel shows non-treated mice and right panel shows Trap-treated mice. The bioluminescence was quantitated and is shown in (C) for control (Non-treated; black line) and Trap-treated (red line) mice. The unit of measurement p/sec/cm^2/sr stands for photons per second per cm^2/steradian.

[0052] Figure 27 shows molecular models serving as templates for the design of modified sIGF1R-ed-Fc constructs. Crystal structures for IR-ed, and for Fc complexes with FcgRIII-ed were retrieved from PDB (codes given in parentheses). The image on the left side shows that 22aa flexible linkers (white lines) utilized in the constructs Mod#2 and Mod#3 are sufficiently long to allow intra-molecular pairing of Fc fragments (cyan/green ribbons) and further allow binding to the FcgRIII-ed (surface rendering). The image on the right side illustrates the same concept for the 27aa linkers of the Mod#4 modified variant protein that uses a hinge-truncated version of the Fc.

[0053] Figure 28 shows schematic depictions of the designed sIGF1R-ed-Fc modified variant proteins. On the basis of sequence modeling of Insulin growth hormone fused to human IgG Fc fragment, we designed and generated 4 new constructs with different modifications in the junction of the sIGF1R and IgG1 sequences. The modifications are as follows: (1): Both cysteines in the core hinge were substituted with serines (referred to as sIGF1R-hFc-IgG1-Mod#1); (2): The 11aa-cloning artifact was replaced with a 22aa-flexible linker (referred to as sIGF1R-hFc-IgG1-Mod#2); (3): A combination of 1 & 2 (referred to as sIGF1R-hFc-IgG1-Mod#3); and (4): The 11aa-cloning artifact, upper hinge, and first 3aa of core hinge (including first Cysteine) were replaced with a 27aa-flexible linker (referred to as sIGF1R-hFc-IgG1-Mod#4).

[0054] Figure 29 shows SDS-PAGE analysis of fusion proteins. Five µg (lanes 1 to 6) and 10µg (lanes 8 to 13) of each parental and modified sIGF1R-hFc-IgG1 protein were separated with SDS-PAGE under denaturing and non-reducing conditions. Lanes 1 & 8: sIGF1R-hFc-IgG1 (parent construct, Trap H) purified by Hydroxyapatite chromatography follow with gel filtration; lanes 2 & 9: sIGF1R-hFc-IgG1 (parent construct, Trap H) purified by protein A; Lanes 3 & 10: sIGF1R-hFc-IgG-Mod#1 purified by protein A; Lanes 4 & 11: sIGF1R-hFc-IgG1-Mod#2 purified by protein A; Lanes 5 & 12: sIGF1R-hFc-IgG1-Mod#3 purified by protein A; Lanes 6 & 13: sIGF1R-hFc-IgG1-Mod#4 purified by protein A; Lane 7: Hi-Mark Unstained HMW protein standard (InVitrogen); Lane 14: Precision Plus Protein™ Unstained Standards (BioRad).

[0055] Figure 30 shows Western blot analysis of designed modified sIGF1R-hFc-IgG1 proteins expressed in cells. Twenty ml of supernatant of CHO-BRI-rcTA-IGF1R-hFc-IgG1-Mod#1 (lanes 2, 7 & 12), Mod#2 (lanes 3, 8 & 13), Mod#3 (lanes 4, 9 & 14) and Mod#4 (lanes 5, 10 & 15) were separated on SDS-PAGE under denaturating and non-reducing conditions. The membrane blot was probed with anti-α chain (lanes 1-5), anti-β chain (lanes 6-10) or anti-Fc (lanes 11-15) antibodies. Lanes 1, 6 & 11: Ez-Run Prestained Rec protein ladder (Fisher). It is noted that β+Fc is about 80-90kD; Fc+β+α is about 210-220kD (monomer); and Fc+β+α+α+β+Fc is about 420-440kD (homodimer).

[0056] Figure 31 shows Western blot analysis of fusion proteins. Non-purified or purified parental fusion protein (Trap H) or purified modified sIGF1R-hFc-IgG1 were the subject of SDS-PAGE under denaturing and non-reducing (lanes 1-7 & 9-15) or reducing (lanes 16-22) conditions. Membranes were probed with anti-α (lanes 1-7) and anti-Fc antibodies (lanes 9-22). The lanes shown are as follows: lanes 1, 9 & 16: supernatant of non-purified parental sIGF1R-hFc-IgG1; lanes 2, 10 & 17: parental construct purified by Hydroxyapatite chromatography followed by gel filtration; lanes 3, 11 & 18: parental construct purified by protein A; lanes 4, 12 & 19: purified sIGF1R-hFc-IgG1-Mod#1; lanes 5, 13 & 20: purified sIGF1R-hFc-IgG1-Mod#2; lanes 6, 14 & 21: purified IGF1R-hFc-IgG1-Mod#3; lanes7, 15 & 22: purified IGF1R-hFc-IgG1-Mod#4; lane 8: EZ-Run* Prestained *Rec* Protein Ladder (Fisher).

[0057] Figure 32 shows stability testing for 9 sub-clones of CHO-Cum2-CR5-sIGF1R-hFc-IgG1 (non modified (parent) trap protein). Nine sub-clones of CHO-Cum2-CR5-sIGF1R-hFc-IgG1 were kept in culture for 2 months. At time zero, 1 month and 2 months, 7 ml of 1.5X 10^6 cells/ml of each sub-clone in Power-CHO medium was cultured in presence of cumate for 1 day at 37°C and 7 days at 30°C. 14 ml of supernatant of each was loaded on SDS-PAGE under denaturing, non-reducing conditions.

[0058] Figure 33 shows representative single-cycle surface plasmon resonance (SPR) for the indicated ligands (hIGF-1, hIGF-2, mIGF-1, h-insulin, maltose binding protein (MBP); 3-fold serial dilutions) binding to the indicated amine-coupled sIGF1R-hFc-IgG1 proteins (Mod#1, Mod#2, Mod#3, Mod#4, Trap H; 25 µL/min x 5 min association + 1-10 min dissociation).

[0059] Figure 34 shows representative multi-cycle SPR for the indicated ligands (hIGF-1, hIGF-2, mIGF-1, h-insulin, and control MBP; 3-fold serial dilutions) binding to the indicated amine-coupled sIGF1R-hFc-IgG1 proteins (Mod#3, Mod#4, Trap H; 25 µL/min x 5 min association + 10 min dissociation).

[0060] Figure 35 shows representative multi-cycle SPR for the indicated ligands (hIGF-1, hIGF-2; 2-fold serial dilutions) binding to the indicated amine-coupled sIGF1R-hFc-IgG1 proteins (Mod#3, Mod#4, Trap H; 25 µL/min x 5 min association + 10 min dissociation).

DETAILED DESCRIPTION

[0061] The present invention provides novel soluble IGF receptor Fc fusion proteins (Fc-sIGFR) and compositions and methods of use thereof for treating angiogenic-associated disorders and malignant disease, including cancer and metastasis.

[0062] We have previously described a 933 amino acid soluble form of the IGF-IR that exhibits a potent anti-tumorigenic/anti-metastatic activity against three different tumor types as well as anti-angiogenic properties (Wang, N., et

al., Mol. Ther. 2009; 17: 1241-9; WO 2010/012088). Here, we report a novel recombinant fusion protein including the 933 amino acid soluble form of IGF-IR and the Fc portion of a human IgG antibody (Fc-sIGF-IR fusion protein).

[0063] We report also the finding that the Fc-sIGF-IR fusion proteins described herein may bind, in some cases, with high specificity and affinity to both IGF-1 and IGF-2. In some cases, the affinity of the sIGFIR-Fc fusion for IGF-2 may be unexpectedly about the same as its affinity for IGF-1. In some cases, the sIGFIR-Fc fusion may unexpectedly have higher affinity for IGF-2 than IGF-1. In some cases, the affinity of the sIGFIR-Fc fusion for IGF-1 is also increased compared to the affinity of the soluble sIGF-IR alone. Thus, we report the finding that Fc-sIGF-IR fusion proteins may, in some embodiments, bind with high affinity and with at least about the same affinity to both IGF-1 and IGF-2, in contrast to reports in the literature that IGF-IR binds IGF-2 with about 6-10 fold lower affinity than it binds IGF-1 (see, for example, Surinya et al JBC, 2008, 283: 5355-5363; Forbes, B.E., et al., Eur. J. Biochem. 2002; 269: 961-8; and Jansson, M., et al., J. Biol. Chem. 1997; 272: 8189-97). In some embodiments, however, Fc-sIGF-IR fusion proteins bind with high affinity to IGF-1 and, as expected based on reports in the literature, bind to IGF-2 with an affinity approx. 6-7 fold lower than affinity for IGF-1.

[0064] In addition, we report herein that Fc-sIGF-IR fusion proteins bind, in some embodiments, with unexpectedly high specificity to IGF-1 and IGF-2 as compared to insulin. As reported herein, sIGFIR-Fc fusion's binding affinity, as determined using surface plasmon resonance, is about 1-2000 fold lower for insulin than for the IGF-1 and IGF-2 ligands.

[0065] The Fc-sIGF-IR proteins provided herein also have an in vivo stability (half-life) in mice of between 35 and 48 hours, which would be expected to provide a half-life in humans that is amply sufficient for therapeutic applications.

[0066] It is further reported herein that the Fc-sIGF-IR proteins show enhanced potency in vitro, compared to the sIGF-IR protein, in assays for anti-cancer effects, and this in vitro activity was improved with purification. Although an increase in stability in vivo is expected with addition of the Fc portion, it was

not expected that this would lead also to increased activity in vitro in anti-cancer assays.

[0067] The Fc-sIGF-IR proteins of the invention may therefore present significant therapeutic advantages compared to the sIGF-IR protein alone. Unexpectedly, the Fc portion increased the affinity of the protein for ligand (i.e., IGF-1 and IGF-2). Not only is the binding affinity of Fc-sIGF-IR for IGF-2 significantly higher than expected in some embodiments (e.g., similar to or higher than binding affinity to IGF-1, in some embodiments), but in addition the binding affinity of Fc-sIGF-IR for IGF-1 is in some cases about 2-fold higher than that of native sIGFIR alone. Without wishing to be bound by theory, it is believed that the high affinity of Fc-sIGF-IR protein to both ligands (IGF-1 and IGF-2) in some embodiments will provide significant therapeutic benefit. For example, it has been reported that tumors can develop resistance to monoclonal antibodies against IGFIR by increasing expression of IGF-1, IGF-2 and IR-A (see, for example, BioCentury, The Bernstein Report on BioBusiness, April 11, 2011, page A5). Similarly, if an agent binds and inhibits only one of IGF-1 and IGF-2, then tumors can develop resistance. Higher binding specificity would also be expected to increase therapeutic benefit by limiting off-target effects. Finally, the high specificity of binding of some Fc-sIGF-IR proteins to ligand (IGF-1/2) compared to insulin may eliminate or reduce many of the unwanted side effects of other agents (e.g., antibodies, kinase inhibitors), such as undesirable effects on glucose and lipid metabolism through interaction with insulin. Further, fusion proteins having modified Fc domains may present further advantages, as discussed herein.

[0068] As used herein, the term "angiogenesis" means the proliferation of new blood vessels that penetrate into tissues or organs or into cancerous growths. Under normal physiological conditions, humans or animals undergo angiogenesis only in very restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonic development and formation of the corpus luteum, endometrium and placenta.

[0069] Pathological angiogenesis occurs in a number of disease states, for example, tumor metastasis and abnormal growth by endothelial cells, and supports the pathological damages seen in these conditions. The diverse pathological disease states in which abnormal angiogenesis is present have been grouped together as "angiogenic dependent" or "angiogenic associated" disorders.

[0070] Angiogenesis is tightly regulated by both positive and negative signals. Angiogenic stimulators, such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), are potent mitogens for endothelial cell proliferation and strong chemoattractants for endothelial cell migration. These positive regulators can promote neovascularization to sustain the expansion of both primary and metastatic tumors. Among the negative regulators described to date, angiostatin ranks as one of the most effective endogenous inhibitors of angiogenesis.

[0071] The receptor for the type 1 insulin-like growth factor (IGF-IR) has been identified as a target for anti-cancer therapy. IGF-IR is a heterotetrameric receptor tyrosine kinase (RTK) consisting of two 130-135 kDa α and two 90-95 kDa β chains, with several α-α and α-β disulfide bridges. It is synthesized as a polypeptide chain of 1367 amino acids that is glycosylated and proteolytically cleaved into α- and β- subunits that dimerize to form a tetramer. The ligand binding domain is on the extracellular α subunit, while the β subunit consists of an extracellular portion linked to the α subunit through disulfide bonds, a transmembrane domain and a cytoplasmic portion with a kinase domain and several critical tyrosines and serine involved in transmission of ligand-induced signals (Samani et al., 2004, Cancer Research, 64: 3380-3385).

[0072] The ability of cancer cells to detach from the primary tumor and establish metastases in secondary organ sites remains the greatest challenge to the management of malignant disease. The liver is a major site of metastasis for some of the most prevalent human malignancies, particularly carcinomas of the upper and lower gastrointestinal (GI) tract. IGF-IR expression and function are critical for liver metastases formation in different tumor types. Tumor cells

engineered to express a soluble form of IGF-IR (sIGFIR) lost the ability to metastasize to the liver (Samani *et al.*, 2004, Cancer Res, 64: 3380-3385).

[0073] An effective strategy for blocking the action of cellular receptor tyrosine kinases (RTKs) is the use of soluble variants of these receptors that can bind and reduce ligand bioavailability to the cognate receptor in a highly specific manner (Kong & Crystal, 1998, J Natl Cancer Inst, 90: 273-286; Tseng *et al.*, 2002, Surgery, 132: 857-865; Trieu *et al.*, 2004, Cancer Res, 64: 3271-3275). One example for successful application of this strategy is the production of the VEGFR1/VEGFR2-Fc decoy receptor (the VEGF Trap) that is currently in clinical trials as a new type of anti-angiogenic, anti-cancer drug (Rudge *et al.*, 2005, Cold Spring Harb Symp Quant Biol, 70: 411-418).

[0074] Such soluble variants of cellular receptor tyrosine kinases that bind and reduce ligand bioavailability to the cognate receptor in a highly specific manner are referred to herein as “decoy” receptors or “Trap” proteins (because they “trap” the ligand). The terms “decoy receptor”, “Trap protein” (or simply “Trap”) and “soluble receptor” are used interchangeably herein.

[0075] U.S. patent No. 6,084,085 discloses the use of soluble IGF-IR proteins for inducing apoptosis and inhibiting tumorigenesis. The soluble IGF-IR proteins disclosed in U.S. patent No. 6,084,085 comprise up to about 800 amino acids of the N-terminus of IGF-IR, such that the C-terminus transmembrane domain is completely deleted or is present to the extent that the protein comprising a portion of the transmembrane domain is not able to be anchored in the cell membrane. U.S. patent No. 6,084,085 disclosed the preferred use of a protein comprising the N-terminal 486 amino acids of IGF-IR without a signal peptide (amino acids 1 to 486), or comprising 516 amino acids with a signal peptide (amino acids -30 to 486). The proteins disclosed in U.S. patent No. 6,084,085 do not include the regions of the IGF-IR required for dimerization and multimerization.

[0076] International patent application No. WO/2010/012088 describes a 933 amino acid soluble form of the IGF-IR that exhibits a potent anti-tumorigenic/anti-metastatic activity against three different tumor types, both in a

gene therapy setting and when injected directly into mice (see also Wang, N., et al., Mol. Ther. 2009; 17: 1241-9). This 933 amino acid soluble form of the IGF-IR is referred to herein as soluble IGF-IR, sIGFIR, sIGF-IR, sIGFIR933 or sIGFR; these terms are used interchangeably throughout. It was shown previously that sIGFR forms a complex with circulating mouse IGF-I; that bone marrow stromal cells producing a soluble IGF-I receptor inhibit the development of experimental hepatic metastases and associated angiogenesis and apoptosis; and that liver metastasis is reduced in sIGFIR injected mice. These experiments represented the first demonstration that administration of a purified sIGFR reduced metastasis and induced apoptosis of tumor cells.

[0077] However, it should be noted that in studies described previously, the treatment was *prophylactic* only, as sIGFIR was injected *before* tumor cell injection. In contrast, we report herein for the first time a therapeutic use of fusion proteins of the invention. As reported herein, fusion proteins of the invention, e.g., Fc-sIGFIR proteins, can be used therapeutically to treat tumors. For the first time, fusion proteins injected *after* tumor cell injection are shown to have a therapeutic effect.

[0078] We also report herein for the first time that a fusion protein including a soluble IGF-IR receptor and the Fc portion of a human IgG antibody has high binding specifity for ligand (e.g., IGF-1, IGF-2) compared to insulin, and therefore has significant potential therapeutic advantages compared to soluble IGF-IR receptor alone.

[0079] In addition, we report herein for the first time novel Fc fusion proteins having modified Fc domains. In order to avoid production of undesirable high molecular weight species (HMW) of Fc fusion proteins, novel Fc-modified fusion proteins (also referred to herein as variant proteins) were designed and produced. For example, in some modified Fc domains, cysteines in the hinge region of the Fc were replaced with serine residues. In other modified Fc domains, an 11aa linker was replaced with a 22aa flexible (GS) linker. In some modified Fc domains, both of these approaches (mutation of Fc hinge Cys residues, and utilization of a longer flexible linker) were combined. In further

modified Fc domains, the Fc hinge region was truncated to retain only the lower Cys residue and the length of the flexible linker was increased to 27aa. As reported herein, these novel Fc domains reduce HMW species in fusion proteins of the invention. Further, in some embodiments, modified Fc linkers and fusion proteins may have the advantage of being sufficiently long and flexible to allow not only binding to the FcRn receptor for improved pharmacokinetic properties (half-life), but also to allow simultaneous binding of the Fc portions to the FcRγIII receptor ectodomain that may confer other beneficial properties (e.g., complement function). Our results indicate that hinge Cys residues are involved in promoting inter-molecular oligomerization, and that in some cases, a longer linker promotes intra-molecular dimerization, which may protect a Fc fragment from proteolytic degradation. In some embodiments, Fc fusion proteins of the inventions have some or all of these advantages.

[0080] Thus, in some embodiments there are provided herein fusion proteins including a soluble IGF-IR receptor and the Fc portion of a human IgG antibody, wherein the Fc portion is modified. For example, the Fc portion may be modified to remove one or more Cys residues, e.g., to replace one or more Cys residues with Ser residues, and/or to replace an 11 aa linker with a longer, more flexible linker, e.g., a 22aa or a 37aa flexible GS linker. In an embodiment, fusion proteins having a modified Fc portion do not produce HMW species or produce reduced HMW species compared to fusion proteins having an unmodified Fc portion.

[0081] Accordingly, there are provided herein Fc-sIGF-IR fusion proteins having anti-tumorigenic, anti-metastatic and/or anti-angiogenic properties.

[0082] Soluble IGF-IR receptor is referred to herein as sIGFIR, sIGF-IR, soluble IGFIR, soluble IGF-IR, sIGFR, or sIGFIR933 and these terms are used interchangeably. The fusion protein including the soluble IGF-IR receptor is referred to herein as Fc-sIGFIR, Fc-sIGF-IR, soluble Fc-IGFIR, soluble Fc-IGF-IR, Fc-sIGFR, sIGFIR-Fc, sIGFR-Fc, Fc-sIGFIR933, etc.; these terms are used interchangeably herein.

[0083] In some embodiments, the term “about the same” as in, e.g., “about the same binding affinity”, refers to two values that are approximately the same within the limits of error of experimental measurement or determination. For example, two values which are about 5%, about 10%, about 15%, about 20%, about 25%, or about 30% apart from each other, after correcting for standard error, are considered to be “about the same”. Two values that are “about the same” may also be referred to as “similar” herein, as in, e.g., two proteins having similar binding affinity. In one embodiment, “about the same” or “similar” binding affinity refers to binding affinities where one affinity is not more than 2- or 3-fold greater than the other. In another embodiment, a difference in binding affinity of at least about 6-fold or at least about 10-fold means that the two binding affinities are not “about the same” or “similar”.

[0084] The term "genetically-engineered stromal cell" or "transgenic stromal cells" as used herein is intended to mean a stromal cell into which an exogenous gene has been introduced by retroviral infection or other means well known to those of ordinary skill in the art. The term "genetically-engineered" may also be intended to mean transfected, transformed, transgenic, infected, or transduced. Other autologous cells may also be genetically-engineered or transgenic, e.g., dendritic cells or hepatocytes may also be used in methods and compositions of the invention.

[0085] The term "ex vivo gene therapy" is intended to mean the *in vitro* transfection or retroviral infection of cells, e.g., stromal cells, to form transfected cells, e.g., transfected stromal cells, prior to implantation into a mammal.

[0086] The expression "transduction of bone marrow stromal cells" refers to the process of transferring nucleic acid into a cell using a DNA or RNA virus. A RNA virus (i.e., a retrovirus) for transferring a nucleic acid into a cell is referred to herein as a transducing chimeric retrovirus. Exogenous genetic material contained within the retrovirus is incorporated into the genome of the transduced bone marrow stromal cell. A bone marrow stromal cell that has been transduced with a chimeric DNA virus (e.g., an adenovirus carrying a cDNA encoding a therapeutic agent), will not have the exogenous genetic material

incorporated into its genome but will be capable of expressing the exogenous genetic material that is retained extrachromosomally within the cell.

[0087] The term "stromal cells" as used herein is intended to mean marrow-derived fibroblast-like cells defined by their ability to adhere and proliferate in tissue-culture treated petri dishes with or without other cells and/or elements found in loose connective tissue, including but not limited to, endothelial cells, pericytes, macrophages, monocytes, plasma cells, mast cells, adipocytes, etc. Other cell types, e.g., dendritic cells, hepatocytes, may also be used in methods and compositions of the invention, and are intended to be encompassed herein. The term “autologous cells” is used herein to refer to such cells and includes, for example, stromal cells, dendritic cells, and hepatocytes.

[0088] The use of autologous cells that have a regenerative capacity and can be genetically engineered to produce effective concentrations of the desired protein is a promising therapeutic strategy (Buckley, 2000, Nat Med, 6: 623-624; Cavazzana-Calvo *et al.*, 2000, Science, 288: 669-672; Dobson, 2000, Bmj, 320: 1225; Stephenson, 2000, Jama, 283: 589-590). Bone marrow derived mesenchymal stromal cells (BMSC) have been used to this end and have several advantages as delivery vehicles: they are abundant and available in humans of all age groups, can be harvested with minimal morbidity and discomfort, have a proliferative capacity, can be genetically engineered with reasonable efficiency and are easy to re-implant in the donor without “toxic” conditioning regimen such as radiotherapy, chemotherapy or immunosuppression. BMSCs have been validated as an efficient autologous cellular vehicle for the secretion of various beneficial proteins *in vivo* in both immunodeficient and immunocompetent hosts and could become an effective tool for protein delivery in clinical practice (Stagg & Galipeau, 2007, Handb Exp Pharmacol, 45-66). Thus, BMSCs autologous cells can be used as vehicles for the secretion of Fc-sIGFIR933. Any other vehicle for expressing protein known in the art is also encompassed herein, and thus BMSCs represent one embodiment of the present invention, which is not restricted to BMSCs.

[0089] We have previously shown that genetically altered stromal cells produced and secreted high levels of the soluble receptor that were detectable in the serum for up to several weeks post implantation (WO10/012088). In mice implanted with these cells, but not with control stromal cells, marked reductions in the number of hepatic metastases were seen following the injection of murine colorectal carcinoma MC-38 (up to 82% reduced) and lung carcinoma H-59 (up to 95%) cells, as well as human colorectal carcinoma KM12SM cells (up to 64%) that were inoculated into athymic nude mice. These results identified sIGFIR as a potent anti-angiogenic agent and also as a therapeutic, anti-metastatic agent.

[0090] Also encompassed within the scope of the present invention are Fc-sIGFIR933 variations and fragments, including biologically active fragments, and biologically active analogs involving amino acid deletions, additions and/or substitutions. "Biologically active fragment" includes fragments of Fc-sIGFIR933 that maintain essentially the same biological activity of the Fc-sIGFIR933 from which the fragment is derived. "Biologically active analogs" includes variations of Fc-sIGFIR933 region(s) that do not materially alter the biological activity (i.e., anti-angiogenic or anti-metastatic activity or binding specificity) of the Fc-sIGFIR933 from which the analog is derived. Included within the scope of the invention are changes made to the Fc-sIGFIR933 and Fc-sIGFIR933 fragment(s) that increase anti-angiogenic activity and/or anti-metastatic activity and/or binding specificity.

[0091] In one embodiment, an Fc-sIGFIR fusion protein of the invention includes a biologically active fragment of sIGFIR, which retains the ability to form α-α and α-β disulfide bridges. Particularly, a biologically active fragment of sIGFIR may comprise α- and β- subunits that dimerize to form a tetramer. In another embodiment, the invention encompasses a Fc-sIGFIR fusion protein comprising a biologically active fragment of sIGFIR which retains the disulfide bonds in the extracellular domain of the native (wild-type) receptor and/or mimics the 3D conformation of the native (wild-type) receptor. In another embodiment, a biologically active fragment of Fc-sIGFIR retains high affinity ligand binding specificity. In a further embodiment, a biologically active

fragment of Fc-sIGFIR retains binding specificity for IGF-1 and/or IGF-2 as compare to insulin. For example, in an embodiment, a biologfically active fragment of Fc-sIGFIR binds IGF-1 and/or IGF-2 with an affinity at least about 100-fold or at least about 1000-fold higher than its affinity for binding insulin.

[0092] Some embodiments include analogs that incorporate modifications to the sIGFIR933 region(s) and/or fragment(s). The resulting sequences differ from the wild-type sequence of sIGFIR933 by one or more conservative amino acid substitutions or by one or more non-conservative amino acid substitutions, deletions or insertions, wherein the substitutions, deletions or insertions do not abolish the biological activity of the wild-type sequence. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g., substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Other conservative amino acid substitutions are known in the art and are included herein. Non-conservative substitutions, such as replacing a basic amino acid with a hydrophobic one, are also well-known in the art.

[0093] Other analogs within the invention are those with modifications which increase protein or peptide stability; such analogs may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the protein or peptide sequence. Also included are analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids.

[0094] Fc-sIGFR fusion proteins having a variety of configurations are also included. For example, the N-terminus of sIGFIR may be linked by a polypeptide bond to the C-terminus of the immunoglobulin heavy chain constant region. Alternatively, the C-terminus of sIGFIR may be linked by a polypeptide bond to the N-terminus of the immunoglobulin heavy chain constant region.

[0095] As used herein, the term "immunoglobulin heavy chain constant region" is used interchangeably with the terms “Fc”, "Fc region" and “Fc domain” and is understood to mean the carboxyl-terminal portion of an

immunoglobulin heavy chain constant region, or an analog or portion thereof capable of binding an Fc receptor. As is known, each immunoglobulin heavy chain constant region comprises four or five domains. The domains are named sequentially as follows: CH1-hinge-CH2-CH3(--CH4). CH4 is present in IgM, which has no hinge region. The immunoglobulin heavy chain constant region useful in the fusion proteins of the invention may comprise an immunoglobulin hinge region, a CH2 domain and a CH3 domain. As used herein, the term immunoglobulin "hinge region" is understood to mean an entire immunoglobulin hinge region or at least a portion of the immunoglobulin hinge region sufficient to form one or more disulfide bonds with a second immunoglobulin hinge region.

[0096] As used herein, in some embodiments “Fc” includes modified Fc domains, e.g., Fc domains which are modified to remove one or more Cys residues, e.g., to replace one or more Cys residues with Ser residues, and/or to replace an 11 aa linker with a longer, more flexible linker, e.g., a 22aa or a 37aa flexible GS linker. In an embodiment, fusion proteins having modified Fc domains do not produce HMW species or produce a reduced amount of HMW species compared to fusion proteins having unmodified Fc domains.

[0097] It is contemplated that suitable immunoglobulin heavy chain constant regions may be derived from antibodies belonging to each of the immunoglobulin classes referred to as IgA, IgD, IgE, IgG, and IgM, however, immunoglobulin heavy chain constant regions from the IgG class are preferred. Furthermore, it is contemplated that immunoglobulin heavy chain constant regions may be derived from any of the IgG antibody subclasses referred to in the art as IgG1, IgG2, IgG3, and IgG4. In one embodiment, an Fc region is derived from IgG1. In another embodiment, an Fc region is derived from IgG2.

[0098] Immunoglobulin heavy chain constant region domains have cross-homology among the immunoglobulin classes. For example, the CH2 domain of IgG is homologous to the CH2 domain of IgA and IgD, and to the CH3 domain of IgM and IgE. Preferred immunoglobulin heavy chain constant regions include protein domains corresponding to a CH2 region and a CH3 region of IgG, or

functional portions or derivatives thereof. The choice of particular immunoglobulin heavy chain constant region sequences from certain immunoglobulin classes and subclasses to achieve a particular result is considered to be within the level of skill in the art. The Fc regions of the present invention may include the constant region such as, for example, an IgG-Fc, IgG-C_H, an Fc or C_H domain from another Ig class, i.e., IgM, IgA, IgE, IgD or a light chain constant domain. Truncations and amino acid variants or substitutions of these domains may also be included.

[0099] A variety of nucleic acid sequences encoding Fc fusion proteins may also be used to make the Fc-sIGFR fusion proteins of the invention. For example, the nucleic acid sequences may encode in a 5' to 3' direction, either the immunoglobulin heavy chain constant region and the sIGFR polypeptide, or the sIGFR polypeptide and the immunoglobulin heavy chain constant region. Furthermore, the nucleic acid sequences optionally may also include a "leader" or "signal" sequence based upon, for example, an immunoglobulin light chain sequence fused directly to a hinge region of the immunoglobulin heavy chain constant region. In a particular embodiment, when the Fc region is based upon IgG sequences, the Fc region encodes in a 5' to 3' direction, at least an immunoglobulin hinge region (i.e., a hinge region containing at least one cysteine amino acid capable of forming a disulfide bond with a second immunoglobulin hinge region sequence), an immunoglobulin CH2 domain and a CH3 domain. Furthermore, a nucleic acid sequence encoding the Fc-sIGFR fusion proteins may also be integrated within a replicable expression vector that may express the Fc fusion protein in, for example, a host cell.

[00100] In one embodiment, the immunoglobulin heavy chain constant region component of the Fc-sIGFIR fusion proteins is non-immunogenic or is weakly immunogenic in the subject. The Fc region is considered non- or weakly immunogenic if the immunoglobulin heavy chain constant region fails to generate a detectable antibody response directed against the immunoglobulin heavy chain constant region. Accordingly, the immunoglobulin heavy chain constant region should be derived from immunoglobulins present, or based on amino acid sequences corresponding to immunoglobulins present in the same

species as the intended recipient of the fusion protein. In some embodiments, human immunoglobulin constant heavy region sequences are used for the Fc-sIGFIR fusion protein, which is to be administered to a human. Nucleotide and amino acid sequences of human Fc IgG are known in the art and are disclosed, for example, in Ellison et al., Nucleic Acids Res. 10:4071-4079 (1982).

[00101] The Fc-sIGFR fusion proteins of the invention may be made using conventional methodologies known in the art. For example, Fc-sIGFIR fusion constructs may be generated at the DNA level using recombinant DNA techniques, and the resulting DNAs integrated into expression vectors, and expressed to produce the Fc-sIGFIR fusion proteins of the invention. As used herein, the term "vector" is understood to mean any nucleic acid comprising a nucleotide sequence competent to be incorporated into a host cell and to be recombined with and integrated into the host cell genome, or to replicate autonomously as an episome. Such vectors include linear nucleic acids, plasmids, phagemids, cosmids, RNA vectors, viral vectors and the like. Non-limiting examples of a viral vector include a retrovirus, an adenovirus and an adeno-associated virus. As used herein, the term "gene expression" or "expression" of an Fc-sIGFIR" fusion protein, is understood to mean the transcription of a DNA sequence, translation of the mRNA transcript, and secretion of an Fc fusion protein product. As an alternative to fusion of proteins by genetic engineering techniques, chemical conjugation using conventional chemical cross-linkers may be used to fuse protein moieties.

[00102] In an embodiment, Fc-sIGFIR fusion proteins of the invention comprise an amino acid sequence comprising the sequence set forth in SEQ ID NO: 8, 10, 12, 14, 16, or 18, and/or are encoded by a nucleic acid comprising the sequence set forth in SEQ ID NO: 5, 7, 9, 11, 13, 15, or 17. In one embodiment, the Fc region is an IgG1 Fc. In another embodiment, the Fc region is an IgG2 Fc. Intron sequences, e.g., introns in the Fc regions, may or may not be included in fusion proteins. Linker sequences between the sIGFIR and the Fc may or may not be included.

[00103] In other embodiments, Fc-sIGFIR fusion proteins of the invention consist of the amino acid sequence set forth in SEQ ID NO: 8 or 10. In other embodiments, Fc-sIGFIR fusion proteins of the invention consist of the amino acid sequence set forth in SEQ ID NO: 12, 14, 16, or 18.

[00104] In one aspect, there is provided herein a therapeutic approach for the prevention and/or treatment of angiogenic dependent or angiogenic associated disorders and/or metastatic disease, e.g. hepatic metastases, based on the sustained *in vivo* delivery of soluble Fc-IGFR fusion protein.

[00105] In an embodiment, compositions comprising the Fc-sIGFIR933 fusion protein described herein, or a biologically active fragment or analog thereof, which are useful to treat angiogenic-dependent or angiogenic-associated disorders and/or metastasis are provided herein. Such compositions may also include a pharmaceutically acceptable carrier, adjuvant or vehicle.

[00106] In an aspect, the compositions and methods of the invention are used to inhibit angiogenesis in a subject in need thereof, e.g. in a subject having an angiogenic dependent or angiogenic associated disorder. In one aspect, the angiogenic associated disorder is tumor metastasis, colorectal carcinoma, lung carcinoma or hepatic cancer or hepatic metastases. In another aspect, the compositions and methods of the invention are used to treat metastasis in a subject in need thereof.

[00107] The present invention includes methods of treating an angiogenic-dependent or angiogenic-associated disorder with an effective amount of a Fc-sIGFIR fusion protein or composition thereof. The present invention also includes methods of treating metastatic disease with an effective amount of a Fc-sIGFIR fusion protein or composition thereof.

[00108] Angiogenic dependent and/or angiogenic associated disorders include, but are not limited to, solid tumors, blood born tumors such as leukemias; tumor metastasis; benign tumors, for example, hemangiomas, acoustic acuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example,

diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation. The compositions of the present invention are useful in treatment of disease of excessive or abnormal stimulation of endothelial cells. These disorders include, but are not limited to, intestinal adhesions, atherosclerosis, scleroderma, and hypertrophic scars, i.e., keloids. The compositions can also be used as birth control agents by preventing vascularization required for embryo implantation.

[00109] Additional embodiments include methods of treating a malignant tumor or a metastasis in a mammal. These methods can include selecting a mammal in need of treatment for a malignant tumor or metastasis; and administering to the mammal a therapeutically effective amount of a Fc-sIGF-IR fusion protein or composition thereof. In some aspects, the animal is human. In some aspects, the fusion protein has the sequence set forth in SEQ ID NO: 8, 10, 12, 14, 16, or 18, or is a biologically active fragment or analog thereof.

[00110] Non-limiting examples of treatable diseases include melanoma, non-small cell lung cancer, glioma, hepatocellular (liver) carcinoma, thyroid tumor, gastric (stomach) cancer, prostrate cancer, breast cancer, ovarian cancer, bladder cancer, lung cancer, glioblastoma, endometrial cancer, kidney cancer, colon cancer, pancreatic cancer, Ewing sarcoma, osteosarcoma, pancreatic carcinoma and epidermoid carcinoma. In an aspect, there are provided methods of treating colon cancer, breast cancer, liver metastasis, glioblastoma multiforme, and/or multiple myeloma comprising administering a Fc-sIGFIR fusion protein or composition thereof to a subject. In another aspect, there are provided methods of treating breast, liver, bladder, lung and/or pancreatic cancer.

[00111] The compositions and methods of the present invention may be used in combination with other compositions, methods and/or procedures for the treatment of angiogenic-dependent or angiogenic-associated disorders and/or metastasis. For example, a tumor may be treated conventionally with surgery,

radiation, chemotherapy, or targeted (biological) therapy (e.g., monoclonal antibody, TKI, etc.), and then compositions comprising a Fc-sIGFIR933 fusion protein as disclosed herein may be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize any residual primary tumor.

[00112] The present invention also provides pharmaceutical (i.e., therapeutic) compositions comprising Fc-sIGFIR, or a biologically active fragment or analog thereof, optionally in combination with at least one additional active compound, and/or any pharmaceutically acceptable carrier, adjuvant or vehicle. "Additional active compounds" encompasses, but is not limited to, an agent or agents such as an immunosuppressant or an anti-cancer agent.

[00113] Non-limiting examples of anti-cancer agents which may be used in combination with compositions and methods of the invention include targeted cancer therapies and treatments, which interfere with specific mechanisms involved in carcinogenesis and tumour growth. Non-limiting examples of targeted cancer therapies include therapies that inhibit tyrosine kinase associated targets (such as Iressa®, Tarceva® and Gleevec®), inhibitors of extracellular receptor binding sites for hormones, cytokines, and growth factors (Herceptin®, Erbitux®), proteasome inhibitors (Velcade®) and stimulators of apoptosis (Genasense®). Such targeted therapies can be achieved, for example, via small molecules, monoclonal antibodies, antisense, siRNA, aptamers, gene therapy and/or cancer vaccines.

[00114] Non-limiting examples of anti-cancer treatments and procedures which may be used in combination with compositions and methods of the invention include surgery, radiology, chemotherapy, or a targeted cancer treatment. More specifically, the targeted cancer treatment is selected from the group consisting of small molecules, monoclonal antibodies, cancer vaccines, antisense, siRNA, aptamers and gene therapy. A subject may also receive a combination of treatments, procedures or therapeutic regimens. Any other treatment, procedure or therapeutic regimen known in the art can be used in the

methods described herein, alone or in combination with other treatments or therapeutic regimens.

[00115] The term "pharmaceutically acceptable carrier, adjuvant or vehicle" refers to a carrier, adjuvant or vehicle that may be administered to a subject, incorporated into a composition of the present invention, and which does not destroy the pharmacological activity thereof. Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of the present invention include, but are not limited to, the following: ion exchangers, alumina, aluminum stearate, lecithin, self-emulsifying drug delivery systems ("SEDDS"), surfactants used in pharmaceutical dosage forms such as Tweens or other similar polymeric delivery matrices, serum proteins such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat. Cyclodextrins such as α-, β- and γ-cyclodextrin, or chemically modified derivatives such as hydroxyalkylcyclodextrins, including 2- and 3-hydroxypropyl-β-cyclodextrins, or other solubilized derivatives may also be used to enhance delivery of the compositions of the present invention.

[00116] The compositions of the present invention may contain other therapeutic agents as described herein and may be formulated, for example, by employing conventional solid or liquid vehicles or diluents, as well as pharmaceutical additives of a type appropriate to the mode of desired administration (for example, excipients, binders, preservatives, stabilizers, flavors, etc.) according to techniques such as those well known in the art of pharmaceutical formulation.

[00117] The compositions of the present invention may be administered by any suitable means, for example, orally, such as in the form of tablets, capsules, granules or powders; sublingually; buccally; parenterally, such as by subcutaneous, intravenous, intramuscular, intraperitoneal or intrastemal injection or infusion techniques (e.g., as sterile injectable aqueous or non-aqueous solutions or suspensions); nasally such as by inhalation spray; topically, such as in the form of a cream or ointment; or rectally such as in the form of suppositories; in dosage unit formulations containing non-toxic, pharmaceutically acceptable vehicles or diluents. The present compositions may, for example, be administered in a form suitable for immediate release or extended release. Immediate release or extended release may be achieved by the use of suitable pharmaceutical compositions, or, particularly in the case of extended release, by the use of devices such as subcutaneous implants or osmotic pumps.

[00118] Exemplary compositions for oral administration include suspensions which may contain, for example, microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners or flavoring agents such as those known in the art; and immediate release tablets which may contain, for example, microcrystalline cellulose, dicalcium phosphate, starch, magnesium stearate and/or lactose and/or other excipients, binders, extenders, disintegrants, diluents and lubricants such as those known in the art. The present compounds may also be delivered through the oral cavity by sublingual and/or buccal administration. Molded tablets, compressed tablets or freeze-dried tablets are exemplary forms which may be used. Exemplary compositions include those formulating the present compositions with fast dissolving diluents such as mannitol, lactose, sucrose and/or cyclodextrins. Also included in such formulations may be high molecular weight excipients such as celluloses (avicel) or polyethylene glycols (PEG). Such formulations may also include an excipient to aid mucosal adhesion such as hydroxy propyl cellulose (HPC), hydroxy propyl methyl cellulose (HPMC), sodium carboxy methyl cellulose (SCMC), maleic anhydride copolymer (e.g., Gantrez), and agents to control

release such as polyacrylic copolymer (e.g., Carbopol 934). Lubricants, glidants, flavors, coloring agents and stabilizers may also be added for ease of fabrication and use.

[00119] The effective amount of a compound of the present invention may be determined by one of ordinary skill in the art, and includes exemplary dosage amounts for an adult human of from about 0.1 to 500 mg/kg of body weight of active compound per day, which may be administered in a single dose or in the form of individual divided doses, such as from 1 to 5 times per day. It will be understood that the specific dose level and frequency of dosage for any particular subject may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the species, age, body weight, general health, sex and diet of the subject, the mode and time of administration, rate of excretion and clearance, drug combination, and severity of the particular condition. Preferred subjects for treatment include animals, most preferably mammalian species such as humans, and domestic animals such as dogs, cats and the like, subject to angiogenic dependent or angiogenic associated disorders.

[00120] The compositions of the present invention may be employed alone or in combination with other suitable therapeutic agents useful in the treatment of angiogenic dependent or angiogenic associated disorders, such as angiogenesis inhibitors other than those of the present invention.

[00121] The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLES

[00122] Table I shows purified sIGFIR and Fc-sIGFIR Trap proteins, which were prepared and tested as described in the Examples.

Table I. Description of purified Trap proteins.

Trap protein	Description
A	His-tagged Human- (h-) sIGF1R purified from 293 cells[1]
B	His-tagged h-sIGF1R purified from 293 cells[1]
C	His-tagged h-sIGF1R purified from 293 cells[1]
D	h-sIGF1R, purified from CHO cells by calcium hydroxyapatite (CHT) column followed by gel filtration (GF)
E	h-sIGF1R-Fc, purified from CHO cells by CHT and GF[2] (SEQ ID NO: 8)
F	h-sIGF1R-Fc, purified from CHO cells using protein A, pH 4.0 elution[2] (SEQ ID NO: 8)
G	h-sIGF1R-Fc, purified from CHO cells using protein A, pH 3.5 elution[2] (SEQ ID NO: 8)
H	h-sIGF1R-Fc, purified from CHO cells using protein A, pH 4.0 elution, endotoxin-free[2] (SEQ ID NO: 8)
I	h-sIGF1R-Fc, purified from CHO cells using protein A, pH 3.5 elution, endotoxin-free[2] (SEQ ID NO: 8)
Mod#1	Modified Trap H protein, in which the cysteines in the hinge region of the Fc are replaced with serine residues (see Fig. 28; SEQ ID NO: 12)
Mod#2	Modified Trap H protein, in which the 11aa linker is replaced with a 22aa flexible (GS) linker (see Fig. 28; SEQ ID NO: 14)
Mod#3	Modified Trap H protein, in which the cysteines in the hinge region of the Fc are replaced with serine residues, and the 11 aa linker is replaced with a 22aa flexible (GS) linker (see Fig. 28; SEQ ID NO: 16)
Mod#4	Modified Trap H protein, in which the Fc hinge region is truncated to retain only the lower Cys residue, and the length of the flexible linker is increased to 27aa (see Fig. 28; SEQ ID NO: 18)

[1] Traps A-C are different batches of the same trap protein.
[2] Traps E, F, G, H and I are the same trap protein (SEQ ID NO: 8), produced using different purification conditions.

Mod#1, Mod#2, Mod#3, and Mod#4 are modified sIGF1R-hFc-IgG1 proteins (also referred to herein as h-sIGF1R-Fc and h-sIGF1R-Fc IgG1 proteins), created by modifying a parent sIGF1R-hFc-IgG1 protein (Trap H; SEQ ID NO: 8, encoded by the DNA sequence set forth in SEQ ID NO: 7), as described in Table I and in Figs. 27 and 28. The four modified proteins are

encoded by the DNA sequences set forth in SEQ ID NOs: 11, 13, 15 and 17, respectively.

The sequence for an exemplary sIGF1R-hFc-IgG2 protein is set forth in SEQ ID NO: 10, which is encoded by the DNA sequence set forth in SEQ ID NO: 9.

Example 1. Production and purification of Trap proteins.

[00123] We first developed and optimized a purification method for His-tagged sIGFIR. Thirteen liters of 293 cells expressing sIGF1R were produced and concentrated. The His-tagged sIGF1R was purified from the concentrated stock using IMAC-chromatography. The purified protein was used as control for developing an affinity chromatography purification protocol using insulin for sIGF1R capture. After unsuccessful attempts to capture sIGF1R on insulin columns, a new 2-step purification method was developed: a capture step on a hydroxyapatite column followed by gel filtration. Purified protein was obtained ("Traps A, B, C"; Table I) for testing. After developing the method with His-tagged sIGFIR produced in 293 cells, it was validated using tag-free sIGFIR that was produced from pooled CHO cells expressing sIGFIR and Fc-sIGFIR (i.e., without and with Fc, respectively) as described below.

[00124] For purification of sIGFIR from a CHO cell pool, two independent lentivirus vectors expressing sIGF1R were generated by transient transfection of 293-PacLV cells and by producer pools as described and detailed elsewhere (Gaillet, B. et al., Biotechnol. Bioeng.; 106: 203-15). The CHO cell lines were transduced up to 6 times with lentiviruses harboring the sIGFIR gene. The CHO pools of stable cell lines were subcloned to isolate the best producer clone (Fig. 1). Production was scaled up, CHO supernatants were concentrated, and sIGFIR was purified using hydroxyapatite columns followed by gel filtration (as noted above). Purified sIGFIR ("Trap D") was obtained for testing; representative results are shown in Fig. 2.

[00125] For purification of Fc-sIGFIR from a CHO pool, two independent lentivirus vectors expressing Fc-sIGFIR (Fc of human IgG1) were generated by

transient transfection of 293-PacLV cells and by producer clones as described and detailed elsewhere (Gaillet, B. et al., Biotechnol. Bioeng.; 106: 203-15). The CHO cell lines were subsequently transduced up to six times with lentivirus vectors harboring the Fc-sIGFIR gene. The pools of stably transduced CHO cell lines were subcloned to select the best producer clones (Fig. 1). Large-scale production of Fc-sIGFIR was then initiated, CHO supernatants harvested and concentrated, and Fc-sIGFIR purified using hydroxyapatite columns followed by gel filtration. Purified Fc-sIGFIR ("Trap E") was obtained for testing; representative results are shown in Fig. 3.

[00126] A fraction of Fc-sIGF1R was also purified using protein A chromatography. High molecular weight (HMW) species were detected in the crude and purified preparations, but elution at low pH (4.0 – 4.5) partially reduced the HMW protein fraction in the preparations (Figs. 4 & 5). It is noted that by using a pH step elution of IGF1R-hFc bound to protein A, approximately half of the high molecular weigh (HMW) species could be removed.

[00127] Purified Fc-sIGF1R was eluted at pH 4.0 ("Trap F") and pH 3.5 ("Trap G") for testing; representative results are shown in Fig. 4. For purified Trap F (pH 4), the *Bio-Rad* DC *Protein* micro-assay indicated 2.7 mg/ml (2.27ml total); Gel scanning results showed 3 to 3.2 mg/ml with a purity of 100%.

[00128] Endotoxin-free batches of these Fc-sIGF1R preparations were also produced and eluted at pH 4.0 ("Trap H") and pH 3.5 ("Trap I") for additional in vivo studies; representative results are shown in Fig. 5. For endotoxin-free Trap H (pH 4.0) and Trap I (pH 3.5) in Fig. 5, 304 ml of production CHO-cum2-CR5-IGF1R-hFc-(IgG1)-16-13-1-6#7 was loaded into mabSelect SuRe 2.08 ml, 10.75 cm H, lot #10029791. Sanitization was in 0.5M NaOH, A11 to A14, Pump 690, F2, F8 ON; A15 and column, 1h30 with 0.5M NaOH + overnight with 0.1M NaOH; binding buffer was 20mM sodium phosphate pH 7; and elution was with sodium citrate 0.1M, pH 4.5, 4, 3.5 & 2.5. (Letters and numbers, such as A1 to A15, B1 to B15, C1, D1, E1, and so on, refer to fractions collected from columns; letters and numbers indicate position of tube on rack of fraction collector. Two types of fraction collectors were used; for small tubes, the

positions were A1 to A15, B1 to B15 and so on, and for large tubes the positions were A1 to A12, B1 to B12, and so on).

[00129] We also generated an alternative Fc-sIGFIR fusion protein using the Fc region of human IgG_2. The production of HMW species with this fusion protein could be reduced due to increased stability in the hinge region, thereby eliminating concerns regarding potential secondary effects of HMW species.

[00130] It will be appreciated that stable CHO lines capable of industry grade production of Trap proteins can also be produced using standard methods known in the art.

Example 2. Analytical assays for quality control of Trap proteins.

[00131] For characterization of Trap proteins, analytical assays to determine, for example, purity, integrity, aggregation and glycosylation of the proteins, were developed. Both sIGFIR and Fc-sIGFIR proteins appeared to be significantly pure, except for the presence of HMW species in the Fc-sIGFIR preparations, based on gel scanning (See Fig. 2, for which gel scanning indicated purity of 95 to 97% for sIGFR for lane 17; and Fig. 3, for which gel scanning indicated purity of 94% for Fc(IgG_1)-sIGF1R for lane 15). No aggregation of either protein was observed after several months of storage at 4 °C or -70 °C.

[00132] Glycosylation patterns in the two proteins were analyzed by mass spectrometry (Fig. 7). The analysis showed that sIGFIR ("Trap D") and Fc-sIGFIR ("Trap E") have 19 and 20 potential N-linked sites, respectively. Each site is decorated with a variety of glycans differing in size and degree of sialilation. The glycoform distribution varies between sites, but small, bi-antennary glycoforms are most common at most sites. Glycoform distribution and degree of sialilation, but not glycan type, were found to differ between sIGFIR and Fc-sIGFIR. Overall, Fc-sIGF1R was found to contain more complex (larger), less sialilated glycans than sIGFIR.

Example 3. Functional in vitro assays for sIGFR and Fc-sIGFR proteins.

[00133] In order to select the most sensitive and functional in vitro assays for tesing the decoy proteins of the invention, we first used 4 different in vitro assays to measure the effect of purified Trap proteins on tumor cell properties relevant to malignant progression and metastasis (Table I). Namely, we measured the ability of the Trap proteins to block tumor proliferation, cell survival, anchorage independent growth, and invasion in the presence of IGF-I. For all experiments, we used highly metastatic Lewis lung carcinoma subline H-59 cells. After the initial screening, we selected the anoikis and invasion assays for complete analyses of all Trap proteins because of they are: (i) semi-automated, (ii) less subject to user-dependent variability, (iii) have superior reproducibility, and (iv) are considered better in vitro correlates of the metastatic potential of tumor cells. The results of all functional in vitro assays are summarized below.

[00134] Proliferation was measured using the colorimetric (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After preliminary analyses to optimize the assay conditions, the cells were serum starved overnight and then incubated with 10 or 50 ng/ml IGF-I in the presence or absence of purified Traps D and E at a concentration calculated to deliver an IGF-I:Trap molar ratio of 1:1. The results (Fig. 8) showed a complete inhibition of cell proliferation in the presence of either 10 ng/ml or 50 ng/ml IGF-I ($p<0.001$ at all time points).

[00135] Cell survival was analyzed using the anoikis (detachment-induced apoptosis) assay, as previously described (Burnier, J.V., et al., Oncogene, 30: 3766-83, 2011]). Briefly, tumor cells ($2.5x10^5$ / well) were plated in 24-well plates that were pre-coated with 10 mg/ml PolyHEMA (Sigma) to prevent their attachment; they were then incubated at 37^0C for 48 hr in the presence of serum or serum-free medium containing IGF-I, with or without Traps D and E. At the end of the incubation period, apoptosis was analyzed using the In Vivo Cell Death Detection-RED staining kit (Roche Canada) as per the manufacturer's instructions. Results of this analysis clearly identified IGF-I as a survival factor in this assay and showed that dose-dependent increases in anoikis (i.e., blockage of pro-survival/anti-apoptotic effect of IGF-I) with Trap E

were more significant compared to Trap D (Fig. 9; $p< 0.05$). Furthermore, subsequent Trap purification using protein A columns (i.e., Traps F and G) improved somewhat their ability to block the anti-apoptotic effect of IGF-I, especially at the lower Trap: IGF-I ratio of 1:2 (Fig. 10; $p< 0.05$).

[00136] Anchorage independent tumor cell growth was measured using the semi-solid agar clonogenicity assay, as previously described (Brodt, P. et al., J. Biol. Chem. 2001; 276: 33608-15). Briefly, tumor cells in RPMI medium containing the indicated concentration of FCS, with or without IGF-I, were mixed with a 0.8% agarose solution (at 1:1 ratio) and plated onto 35 mm culture dishes ($2x10^4$ cells/dish) on a solidified 1% agarose layer. To the overlay, RPMI medium containing the same concentration of FCS was added and the plates incubated at 37^0C for 14 days, at which time the cells were fixed and colonies exceeding 80 µM in diameter scored using a microscope equipped with an ocular grid. Results of this assay (Fig. 11) showed that Trap D and Trap E significantly reduced the ability of the tumor cells to form colonies in semi-solid agar ($p<0.01$ at all conditions) and there was only a minor difference in the activities of the two Traps under these assay conditions ($p<0.05$ only in the presence of 1% FCS).

[00137] Tumor cell invasion was measured using a real-time, electrical-impedance-based technique using the new, automated xCELLligence™ system (Roche). The xCELLigence™ instrument measures changes in electrical impedance at an electrode/cell interphase, as a population of (malignant) cells invades a Matrigel layer and migrates to a lower chamber of a Boyden-chamber system. The impedance is displayed as a dimensionless parameter termed cell-index (or cellular unit), which is directly proportional to the total area of tissue-culture well that is covered by cells, as described and demonstrated by others (Ungefroren, H. et al., Int. J. Oncol.; 38:797-805; Rahim, S. and Uren, A., J. Vis. Exp., 50: 1-4, 2011). Tumor H-59 cells (in the upper chamber) were plated in wells ($5x10^4$ cells/well) that were pre-coated with the extracellular matrix mixture MatrigelTM (BD Biosciences) at a concentration pre-determined to allow optimal invasion. They were then placed on top of a lower chamber containing 50 ng/ml IGF-I to which the indicated IGF-Traps were added (or not) at different

(approximate) IGF: Trap molar ratios. When the inhibitory effects of Trap D (sIGF1R) and Trap E (Fc-sIGF1R) on cell invasion were compared at a Trap:IGF-I molar ratio of 1:1 (Fig 13), they demonstrated an apparent increased activity for Trap E ($p<0.05$ at 36 hr). Following protein A purification without (i.e. Traps F and G; Fig. 12) or with (Traps H and I; Fig. 13) endotoxin removal, the enhanced activity of all these preparations indicated that the significant inhibition seen was not related to non-specific effects of endotoxin. It should be noted that in the invasion assays, fractions eluted at pH 3.5 (i.e. enriched for high molecular weight species, see Fig. 5 and 6) appeared more active than those depleted of high molecular weight species ($p<0.01$), suggesting that the high molecular weight proteins retained an IGF-I "trapping" ability.

[00138] It can be seen from the results presented here that, surprisingly, the Fc-sIGFR protein demonstrated increased potency in vitro in these anti-cancer assays compared to the sIGFR protein, and this enhanced potency of the Fc-sIGFR protein was improved with purification.

Example 4. Binding specificity and affinity of sIGFR vs. Fc-sIGFR.

[00139] Binding between purified Trap receptors ("A" to "I") and IGF-IR ligands (mIGF-1, hIGF-1, hIGF-2, and human insulin) was measured using label-free, real-time Surface Plasmon Resonance (SPR). Experiments were performed at 25 °C using BIACORE™ 3000 instrumentation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as described by others (Forbes, B.E., et al., Eur. J. Biochem. 2002; 269: 961-8; Jansson, M., et al., J. Biol. Chem. 1997; 272: 8189-97; Surinya, K.H., et al., J. Biol. Chem. 2008; 283: 5355-63). Initially, the ligands were immobilized (~125 RU; Biacore Amine Coupling Kit) to dextran-coated sensor chips and the receptors were titrated over reference (i.e., no ligand) and ligand surfaces in tandem. In reciprocal experiments, the ligands were titrated over immobilized Trap surfaces (~8000 RU). Mass transport-independent data were double-referenced (Myszka DG. Improving biosensor analysis. J Mol Recognit 1999; 12: 279-84) and were representative of duplicate injections acquired from two independent trials. For the multi-cycle titrations, equilibrium dissociation constants (K_D) were determined by global fitting of the

data to a "1:1 kinetic" model (BIAevaluation v4.1 software) or the "steady-state affinity" model (for human insulin titrations only). For the single-cycle titrations, K_D values were determined by local fitting of the data to a "1:1 titration" model (Karlsson, R., et al., Anal. Biochem. 2006; 349: 136-47).

[00140] His-tagged sIGFIR variants ("Traps A, B, C") were initially tested and used to standardize assay conditions for Trap binding to amine-coupled ligand surfaces in preparation for subsequent analysis of Trap proteins. Over nanomolar titration ranges, Trap B exhibited the best overall activity and its binding to immobilized mouse or human IGF-I was significant compared to little or no response with human insulin (specificity control; micromolar affinity) or maltose-binding protein (negative control; no affinity). In reciprocal experiments, hIGF-I was titrated and bound to HEK293-purified Trap B and CHO-purified Trap D and E surfaces with nanomolar affinity, whereas human insulin bound with weaker, micromolar affinity in all cases (Table II). On average, Traps B, D, and E exhibited similar association and dissociation rate constants (k_a ~2.6 x 10^5 $M^{-1}s^{-1}$ and k_d ~2 x 10^{-3} s^{-1}, respectively) in these multi-cycle trials. The results confirmed that Traps D and E could specifically bind hIGF-I ligand with high affinity; the interaction of Trap E with hIGF-I was modestly stronger compared to Trap D.

Table II. Results of initial SPR screening using multi-cycle analyses (n=4). Shown are calculated equilibrium dissociation constants (K_D +/- SE) for binding between Trap proteins and ligands. The results clearly demonstrate Trap specificity for hIGF-I compared to insulin. The Fc-sIGF-IR fusion showed an affinity for hIGF-1 approximately 1000-2000 times higher than its affinity for human insulin.

Trap	hIGF-1	Human insulin
B (control)	**8 +/- 0.1 nM**	**10 +/- 2 μM**
D	**13 +/- 0.2 nM**	**16 +/- 4 μM**
E	**6 +/- 0.2 nM**	**14 +/- 8 μM**

[00141] Protein A-purified Traps F and G (with D as control) were flowed over ligand-immobilized surfaces and exhibited low nanomolar affinities for hIGF-I as well as mIGF-I and hIGF-II (Table III). It was also noted in these multi-cycle trials that Trap F had a slower dissociation rate constant (k_d ~4.3 x 10^{-4} s^{-1}) compared to Trap D (k_d ~8 x 10^{-4} s^{-1}), and Trap G was even slower to dissociate (k_d ~1.5 x 10^{-4} s^{-1}) compared to Trap F. Finally, endotoxin-free versions of Traps F and G (i.e. Traps H and I, respectively, with E as control) were immobilized for SPR analysis. While Traps E (Fig. 14), H, and I shared similar association and dissociation kinetics in these single-cycle trials, the nanomolar KD values estimated for Trap I were quite different than those of Traps E and H (Table III). This finding was likely due to the increased sample complexity (i.e. HMW species) of the Trap I preparation, and the very low amount of the desired species in the Trap I preparation (see Fig. 5, lanes 9 to 12; the red arrow indicates the desired species).

[00142] In general, it is noted that Traps I and E were contaminated by high molecular weight species. It is believed that this contamination accounts for the differences seen between Traps I and E and Trap H (e.g., in Table III, and elsewhere), and for much of the variability in the results reported herein. In an embodiment, therefore, Trap H represents the preferred preparation.

Table III. Affinity of Traps D, E, H and I for IGF-IR ligands.
Shown are the calculated equilibrium dissociation constants (K_D +/- SE) for binding between purified Traps and IGF-IR ligands. ***n=4 in multi-cycle SPR.

Trap	mIGF-1	hIGF-1	hIGF-2
D ***	10 +/- 0.1 nM	11 +/- 0.1 nM	16 +/- 0.1 nM
E (control)	14 +/- 0.5 nM	4 +/- 0.1 nM	26 +/- 0.9 nM
H	18 +/- 0.8 nM	10 +/- 0.5 nM	8 +/- 0.4 nM
I	71 +/- 3 nM	53 +/- 2 nM	127 +/- 56 nM

[00143] In summary, the SPR results successfully demonstrated binding between the purified Trap proteins and IGF-IR ligands using two different

coupling orientations. Despite variable constructs and purification protocols employed to generate different Trap preparations (i.e. "A" – "I"; see Table I), the traps exhibited mostly similar association and dissociation kinetics. However, the protein A- purified preparations containing the enriched, native tetrameric protein (e.g., Traps F and H, eluted at pH 4.0) generated better quality SPR fits as compared to preparations containing a higher relative proportion of the high molecular weight species (e.g., Traps G and I eluted at pH 3.5).

[00144] Overall, the affinity constants for Traps A – I were in agreement with similar published SPR data in which ligand binding to immobilized hIGF-IR have been reported: for example, Forbes et al. (Forbes, B.E., et al., Eur. J. Biochem. 2002; 269: 961-8) reported KD (hIGF-I → hIGF-IR) = 4.5 nM and KD (hIGF-II → hIGF-IR) = 23 nM; Jansson et al. (Jansson, M., et al., J. Biol. Chem. 1997; 272: 8189-97) reported KD (hIGF-I → hIGF-IR) = 3.5 nM and KD (hIGF-II → hIGF-IR) = 20 nM. However, surprisingly Traps E, F, H and I demonstrated similar binding affinities for both the IGF-1 and IGF-2 ligands, or in some cases, even higher affinity for IGF-2 than IGF-1. In addition, in some cases the affinity of the Trap Fc-fusion proteins for IGF-1 was higher than that of the soluble sIGFIR alone. It is noted that Trap E did not show similar affinities for both ligands as Traps H and F did; this is likely due to the purification protocol used.

Example 5. In vitro stability and pharmacokinetic properties of SIGFR vs. Fc-sIGFR.

[00145] As indicated above, no aggregation of either protein was observed after several months of storage at 4°C or -70°C. However, we noted that functional activity of these proteins was optimal within the first 3-6 months of storage at -70°C. This may explain the reduced half life of Traps D and E observed in latter analyses (e.g., after 9 month storage, see Table V) compared to earlier ones (e.g., after 3 months storage, see Table IV).

Table IV. Final pharmacokinetic parameters for Traps D and E.
(N/A: not applicable)

Final Parameters	Units	Trap D	Trap E
Corr_XY	N/A	-0.9955	-0.9819
Tmax	hr	0.0830	0.0830
Cmax	µg/mL	55.4600	28.5830
C0	µg/mL	65.6995	35.5274
Tlast	hr	240.0000	240.0000
Clast	µg/mL	0.0020	0.0050
AUCall	hr* µg/mL	405.8128	98.0906
AUCINF_obs	hr* µg/mL	405.8649	98.2187
MRTINF_obs (Half-Life)	hr	21.8848	47.5156

Table V. Final pharmacokinetic parameters for Traps D, E, I, and H.
(N/A: not applicable)

Final Parameters	Units	Trap D	Trap E	Trap I	Trap H
Corr_XY	N/A	-0.99	-0.98	-0.96	-1.00
Tmax	hr	0.08	0.08	0.08	0.08
Cmax	µg/mL	50.83	28.58	23.98	67.14
C0	µg/mL	57.05	35.53	28.21	77.26
Tlast	hr	240	312	288	312.0
Clast	µg/mL	0	0	0	0
AUCall	hr*µg/mL	452.38	107.37	74.86	541.45
AUCINF_obs	hr*µg/mL	452.40	107.42	74.89	541.49
MRTINF_obs (Half-Life)	hr	20.88	39.94	10.92	35.15

[00146] Mice were injected intravenously with 10 mg/kg of each of the tested Trap proteins. The mice were divided into several groups of 3 mice each and

blood was collected from alternate groups beginning at 5 minutes post injection and continuing at 0.33, 1, 3 ,6, 12, 16 and 24 hr and daily thereafter for up to 14 days. Plasma was prepared and soluble IGF-IR levels analyzed using ELISA (R&D Systems). Data for each group of mice bled at the same interval were pooled.

[00147] The results (Fig. 15) showed a superior in vivo stability for CHO cell - produced Trap proteins D and E as compared to His-tagged Trap protein (293 cell-produced Trap A). They also showed distinct clearance and in vivo stability profiles for Traps D and E. Pharmacokinetic analysis subsequently performed on these data showed a greater than 2-fold difference in half-life, with Trap E showing superior stability in vivo (47.5 hr as compared to Trap D at 21.8 hr; Table IV). These data confirmed that the addition of the Fc-IgG_1 fragment increased the in vivo stability of the Trap proteins. When endotoxin-free, protein A-purified, Fc-sIGFIR proteins (Traps H and I) were then analyzed in a similar manner, we found that the fraction eluted at pH 4.0 (Trap H, high molecular weight species depleted) had superior pharmacokinetics performance (3.5-fold increase in half life, Table V) to that eluted at pH 3.5 (Trap I, high molecular weight species-enriched) (Fig. 16).

[00148] These results show that the addition of the Fc fragment to the soluble IGF-IR significantly improved both binding affinity and the pharmacokinetic properties of the Trap protein. In vitro, Fc-sIGFIR had increased activity as compared to native sIGF-IR and the activity of Fc-sIGFIR was increased following protein A purification. Protein A purification was not effective in separating the single tetrameric Trap protein from high molecular weight species. However, elution at pH 4.0 was effective in reducing their relative proportion in the preparations. Finally, while the presence of high molecular weight species did not markedly affect the IGF-trapping activity of the proteins in vitro (with a possible slight advantage to high molecular weight proteins), they had a markedly reduced pharmacokinetic profile, with half-life values of 10 hr constituting the lowest observed for all Trap proteins tested.

Example 6. Reduction of liver metastases in a mouse model.

[00149] Mice were injected with $5X10^4$ lung carcinoma H-59 or colon carcinoma MC-38 cells via the intrasplenic/portal route to generate experimental liver metastases. On the following day they received the first i.v. injection of 5mg/kg Trap H (or vehicle for control) followed by a second injection of the same dose on day 5. Mice were euthanized and metastases enumerated and sized on day 18 post tumor injection.

[00150] Results are shown in the table below and in Fig. 20. Trap H reduced the number and size of hepatic metastases for H-59 and MC-38 tumors, compared to vehicle alone, in the mouse model.

Experimental group	**Mice with hepatic metastases (incidence)**	**No. of metastases/liver (mean(range))**	**Size of metastases (mm)**
Tumor H-59 Vehicle only	7/7	**46** (12-80)	0.91
Tumor H-59 Trap H- 5mg/kg	6/7	**22*** (0-53)	0.41
Tumor MC-38 Vehicle only	6/6	**34** (7-119)	0.65
Tumor MC-38 Trap H- 5mg/kg	3/5	**10*** (0-31)	0.79

*$p<0.05$ as compared to control (Mann-Whitney test)

Shown in Fig. 20 are representative H&E stained formalin fixed paraffin embedded sections of livers derived from MC-38 colon carcinoma-injected mice 19 days post tumor injection.

Example 7. An IGF-Trap protein inhibit IGF-IR signaling in tumor cells in vivo.

[00151] C57BL6 female mice were injected intrasplenically with 10^5 GFP-tagged H-59 cells followed by injection of 5mg/kg IGF-Trap or vehicle only (untreated) on days 1 and 3 post tumor injection (3 mice per group). The mice

were sacrificed on day 6, livers removed and snap frozen and 10 µM cryostat sections prepared and immunostained with a rabbit polyclonal anti-mouse pIGF1R antibody (ab39398-Abcam, Cambridge, MA) diluted 1:100 followed by a goat anti-rabbit Alexa Fluor 647 (far-red) antibody (Molecular Probes Invitrogen, Eugene, OR) diluted 1:200. Incubations were each for 1 h at room temperature in a humidified chamber in the presence of DAPI (1:2000). The sections were washed and mounted with the GOLD anti-fade reagent (Invitrogen) and analyzed with a Carl Zeiss LSM 510 Meta, confocal microscope (Carl Zeiss Canada Ltd, Toronto, ON, Canada) equipped with a Zen image analysis station. For each treatment group, 12-16 sections were analysed and the percentage of GFP^+ tumor cells that were pIGFIR positive was calculated. Representative merged confocal images are shown in Fig. 21A, and the calculated means of percent of $pIGF\text{-}IR^+$ green fluorescent tumor cells in each group is shown in Fig. 21B. The results show that as a consequence of treatment with the IGF-Trap, activation and signalling of IGF-I receptors on the tumor cells were significantly reduced.

Example 8. An IGF-Trap increases tumor cell apoptosis in vivo.

[00152] Liver cryostat sections were obtained as described above in Example 7. The sections were incubated first with a rabbit polyclonal anti-mouse cleaved caspase 3 antibody (ab4501-Abcam) diluted 1:100 and then with a goat anti-rabbit Alexa Fluor 647 antibody (Molecular Probes) diluted 1:200. Incubation and processing of the sections were as described in Example 7. For each treatment group 11-14 sections were analysed and the percentage of GFP^+ tumor cells that were cleaved caspase 3 positive (an indicator of apoptosis) was calculated. Representative merged confocal images are shown in Fig. 22A, and the calculated means of percent of cleaved-caspase 3^+ green fluorescent tumor cells in each group is shown in Fig. 22B. The results show that treatment with the IGF-Trap caused a significant increase in the proportion of tumor cells undergoing apoptosis.

Example 9. An IGF-Trap inhibits tumor cell proliferation in vivo.

[00153] Liver cryostat sections were obtained as described above in Example 7. Sections were incubated first with a rabbit polyclonal anti-mouse Ki67 antibody (ab15580 - Abcam) diluted 1:100 and then with a goat anti-rabbit Alexa Fluor 647 antibody (Molecular Probes) diluted 1:200. Incubation and processing of the sections were as described in Example 7. For each treatment group 14 sections were analysed and the percentage of GFP^+ tumor cells that were Ki67 positive (a marker of proliferation) was calculated. Representative merged confocal images are shown in Fig. 23A and the calculated means of percent of $Ki67^+$ green fluorescent tumor cells in each group is shown in Fig. 23B. The results show that tumor cell proliferation was significantly reduced in IGF-Trap treated mice.

Example 10. An IGF-Trap blocks angiogenesis in vivo.

[00154] Liver cryostat sections were obtained as described above in Example 7. Sections were incubated first with a rat monoclonal anti-mouse CD31 antibody (Clone MEC 13.3, from BD Biosciences, San Jose, CA) diluted 1:100 and then with a goat anti-rat Alexa Fluor 568 (orange-red) antibody (Molecular Probes, Invitrogen) diluted 1:200. (Fig. 24). The number of $CD31^+$ endothelial cells within tumor micrometastases (Fig. 24A) per field (20X objective) was counted in 16 sections per treatment group and the mean number calculated. Representative merged confocal images are shown in Fig. 24A and the calculated means of $CD31^+$ cells per field in each group is shown in Fig. 24B. The results show that tumor-associated angiogenesis was significantly reduced in IGF-Trap treated mice.

Example 11. Tumor growth arrest in mice injected with murine mammary carcinoma 4T1 cells.

[00155] Balb/c female mice were injected into the mammary fatpad (MFP) with 10^5 mouse mammary carcinoma 4T1 cells (Tabaries, S. et al., Oncogene 30(11):1318-28, 2011) Four hours and 3 days later the treatment group received an i.v. injection of 10mg/kg of the IGF-Trap followed by 2 injections of 5 mg/kg on days 6 and 10 post tumor inoculation (Fig. 25A-arrows).Tumors were measured three times weekly using a caliper and the tumor volumes

calculated using the formula 1/2(length x width^2). In all non-treated mice tumors grew rapidly resulting in death of all mice by day 14 post tumor injection (Fig. 25 A, B) with macroscopic liver metastases. In the treatment group, tumors did not significantly progress while IGF-Trap was administered. Tumor growth was seen only after cessation of treatment (day 14 onward, Fig. 25A). Mice survived up to 35 days post tumor injection (Fig. 25B) ($p < 0.01$ using both Mantel-Cox and Gehan-Breslow-Wilcoxon Tests).

Example 12. Growth arrest and regression in nude mice injected with human breast carcinoma MDA-MB-231 cells.

[00156] One million MD-MBA-231 human breast cancer cells (Mourskaia, A.A. et al., Oncogene, 28(7): 1005-15, 2009) were orthotopically implanted with Matrigel in the mammary fatpads of nu/nu mice. Tumors were measured three times weekly using a caliper and the tumor volumes calculated using the formula 1/2(length x width^2). When tumors were established (50-100mm^3) (Fig. 26A-day 11-arrow), the animals were randomized and treated with 5mg/kg of IGF-Trap or vehicle (i.v.) twice weekly up to day 33. Mice in the control group were all moribund by day 44 (Fig. 26A-dashed line). In the IGF-Trap group, growth of all tumors was arrested during treatment. In some animals, tumors began to progress 20 days after administration of the last treatment (Day 55). All treated mice survived at least until day 70 (study still ongoing). Complete regression (cure) was seen in 1/5 mice and tumor stabilization (growth arrest) was seen in 1/5 mice. In all the mice, tumors were also monitored using longitudinal bioluminescence imaging showing an increase in bioluminescence signal intensity in the control group and a marked reduction in signal in the IGF-Trap treated group over time (Fig. 26B).

[00157] Based upon efficacy in cell-based assays, high-affinity ligand binding to both the IGF-1 and IGF-2 ligands, in vivo stability, and efficacy in mouse tumor models, the Fc-Trap proteins described herein are attractive therapeutic candidates for the treatment and/or prevention of cancer, metastasis and/or angiogenesis-associated disorders.

Example 13. Rational design of sIGF1R-ed-Fc variants for eliminating high-molecular-weight (HMW) species.

[00158] As shown above, fused forms of sIGF1R to Fc IgG1 or IgG2 (sIGF1R-hFc-IgG1 or sIGF1R-hFc-IgG2, respectively) expressed in CHO cells displayed about 50% of disulphide linked high molecular weight species (HMW). Under reducing conditions these HMW could be separated into non-disulphide linked sIGF1R-hFc.

[00159] In order to address the HMW heterogeneity of the original sIGF1R-Fc fusions having an 11 amino acid (aa) linker between the sIGF1R ectodomain (sIGF1R-ed) and the IgG-Fc fragment, we explored several possibilities. Using the crystal structure of the homologous insulin receptor ectodomain (IR-ed), we inferred that the distance between the C-termini of the sIGF1R-ed dimer should be about 120 Å (Fig. 27). Hence, given the geometrical constraints imposed by the sIGF1R-ed dimer, we hypothesized that it is unlikely that in the original 11aa-linked construct the intra-molecular pairing of two Fc moieties can occur. The unpaired Fc chains may become available to open-ended inter-molecular associations, particularly enhanced by the presence of the available cysteine residues in the hinge region of the Fc, thereby explaining the observed HMW ladder.

[00160] To test this idea, and to design modified sIGF1R-hFc-IgG1 variant proteins, we first replaced the cysteines in the hinge region of the Fc with serine residues (variant Mod#1; see Fig. 27). As an alternative, in order to promote intra-molecular Fc dimerization by increasing the length of the linker, we effectively replaced the 11aa linker with a 22aa flexible (GS) linker, as incorporated in the modified variant protein called Mod#2 (Figs. 27, 28). Both of these approaches (mutation of Fc hinge Cys residues, and utilization of a longer flexible linker) were combined into a third modified protein, the Mod#3 variant (Fig. 28). Finally, we attempted to reduce the HMW disulfide-linked species by truncating the Fc hinge region to retain only the lower Cys residue and accordingly further increasing the length of the flexible linker to 27aa (Mod#4; Figs. 27, 28). In addition to the intended reduction of HMW species, the

designed longer linkers (22aa in Mod#2 and Mod#3, and 27aa in Mod#4) are intended to be sufficiently long and flexible to allow not only binding to the FcRn receptor for improved pharmacokinetic properties (half-life), but also to allow simultaneous binding of the Fc portions to the FcRγIII receptor ectodomain that may confer other beneficial properties (e.g., complement function).

[00161] Materials and Methods for this and the following Examples are as follows:

[00162] Generation of pMPG-CR5 vectors expressing four modified sIGF1R-hFc-IgG1 sequences. To generate a pMPG-CR5 vector expressing the four modified sIGF1R-hFc-IgG1 sequences, different subcloning steps were required for each construct. Briefly, the SmaI site of PUC19 was removed by SmaI-NdeI digestion to accept subsequence subcloning. In the next step, the full length of sIGF1R-hFc-IgG1 was cloned into the BamHI site of the modified PUC19. The 542nt SmaI fragment, which contains the junction of sIGF1R and hFc was removed from the sIGF1R-hFc-IgG1 sequence. This modified PUC19-sIGF1R-hFc-IgG1 vector with SmaI deleted fragment was used as backbone for further subcloning. Four modified fragments of sIGF1R-hFc-IgG1 were synthesized by Genescript. These fragments were inserted in the SmaI site of the modified PUC19-sIGF1R-hFc-IgG1 with the SmaI fragment deletion. Finally, the full length of 4 modified sIGF1R-hFc-IgG1 was excised with BamHI digestion and sub-cloned into a pMPG-CR5 expression vector to generate pMPG-CR5- sIGF1R-hFc-IgG1-Mod#1, pMPG-CR5- sIGF1R-hFc-IgG1-Mod#2, pMPG-CR5- sIGF1R-hFc-IgG1-Mod#3, and pMPG-CR5- sIGF1R-hFc-IgG1-Mod#4.

[00163] Transient expression of the 4 modified sIGF1R-hFc-IgG1 proteins in a CHO-BRI-rcTA-55E3 cell line. CHO-BRI-rcTA cells were transfected with each of the plasmids encoding the 4 modified sIGF1R-hFc-IgG1 proteins (sIGF1R-hFc-IgG1-Mod#1, Mod#2, Mod#3 & Mod#4) using PEIpro. Five days after transfection, the expression level of the 4 modified sIGF1R-hFc-IgG1 proteins and formation of high molecular weight species were analyzed by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and

Western Blotting. 200 ml of each supernatant were purified using protein A columns.

[00164] SDS–PAGE and Western blotting. To evaluate the gel migration patterns of the 4 modified sIGF1R-hFc-IgG1 proteins, and the purified proteins (5 and 10 µg of each) were separated by 4-12% SDS-PAGE. To compare the intensity or absence of HMW species, a sample of parent sIGF1R-hFc-IgG1 purified by Hydroxyapatite chromatography followed by gel filtration and a sample of parent sIGF1R-hFc-IgG1 purified by protein A were used as controls (Fig.29). Twenty µl of CHO-BRI-rcTA-sIGF1R-hFc-IgG1-Mod#1, Mod#2, Mod#3 and Mod#4 supernatants were separated by 4-12% SDS-PAGE and transferred onto a membrane. For immunoblotting, the primary antibodies rabbit polyclonal anti-α IGF1R chain (SC-7952 Santa-cruz 1/600) or rabbit polyclonal anti-α IGF1R chain (SC-9038, Santa-cruz, 1/400) were used. Cy5-anti-Rabbit (Jackson, 1/100) was used as a secondary antibody. The IgG1-Fc portion of the sIGF1R-hFc-IgG1 fusion protein was detected with Cy5-goat-anti-Human IgG (H+L, Jackson, 1/400) (Fig. 30).

[00165] After purification of the 4 modified sIGF1R-hFc-IgG1 proteins using protein A, 200ng of each protein was subjected to SDS-PAGE followed by Western Blotting. The sIGF1R-α chain and Fc portions of the fusion proteins were detected as described in the previous paragraph. Purified and non-purified parent constructs were used as controls (Fig. 31). In addition, a set of samples was run under reducing conditions (300mM of DTT).

[00166] Generation of four industry-grade, CHO-Cum2-sIGF1R-hFc-IgG1 cell lines producing the parent protein. Four pools of industry grade stable cell lines expressing IGF1R-hFc-IgG1 were generated by transfection of a CHO-Cum2-L72 cell line (Mullick, A. et al., BMC Biotechnol. 6 :43, 2006) with pMPG-CR5-IGF1R-hFc-IgG1 vector. Cells were kept under hygromycin selection for 3 weeks. The CHO-Cum2 pools of stable cell lines were subcloned to isolate the best producer clone. The subclones with higher expression levels were kept for 2 months in culture for stability testing. The subclone with highest stability and productivity was scaled up, CHO supernatants were concentrated,

and sIGFIR-hFc-IgG1 was purified using a protein A purification method (Fig. 32).

Example 14. Engineering and Testing 4 modified sIGF1R-hFc-IgG1 proteins suggested by sequence modeling.

[00167] Presence of HMW species at > 1% is not recommended for manufacturing of recombinant proteins. As discussed above, unfortunately about half of the original sIGF1R-hFc-IgG1 and sIGF1R-hFc-IgG2 fusion proteins in the parent preparations were present as HMW species. Our success in removing these HMW species by adding a step of elution at pH 4.5 following protein A chromatography was partial and was not scalable because only a small fraction of protein was eluted at this pH.

[00168] To prevent or at least reduce the formation of HMW species, four modified sIGF1R-hFc-IgG1 proteins with different modifications in the junction of the sIGF1R and IgG1 sequences were constructed, as described above. There is one SmaI restriction site at 3′ of sIGF1R sequence and another one at 5′ end of hFc-IgG1 sequence. The presence of these two sites gave us the opportunity to modify this region by swapping any newly synthesized SmaI fragment with the original sequence. As a first step, the sequence of PUC-19 was modified to accommodate sub-cloning subsequences of full length sIGF1R-hFc-IgG1 and swapping the original sequence with the synthesized (modified) SmaI fragment. Finally the full length modified sequences were sub-cloned into a pMPG-CR5 expression vector.

[00169] In the supernatants of CHO-BRI-rCTA-55E3 cells (also referred to herein as CHO-BRI-rCTA cells, for brevity) transiently transfected with the 4 modified sIGF1R-hFc-IgG1 proteins, the proteins could not be detected by SDS-PAGE. To have enough material for SDS-PAGE analysis, 200ml of each supernatant were then purified with protein A. The level of HMW species in parental sIGF1R-hFc-IgG1 purified by Hydroxyapatite chromatography followed by gel filtration and parent sIGF1R-hFc-IgG1 purified by protein A, was compared with the 4 modified sIGF1R-hFc-IgG1 proteins (Fig. 29). Formation of HMW species was completely absent in modified proteins Mod#1 and Mod#3,

in which both cysteines in the core hinge were replaced with serines. HMW species were still present in sIGF1R-hFc-IgG1-Mod#2 and Mod#4 proteins, but their level of production was lower than in the parental form of the proteins (sIGF1R-hFc-IgG1). However, two low molecular weight (LMW) bands with MW about 80-90kDa and 210-220kDa were found in gels of Mod#1 and Mod#3, and to a lesser extent in gels of Mod#4. In addition to these LMW bands, a protein of about 30kD was also detected in the SDS-PAGE profile for Mod#1.

[00170] Western Blots on supernatants containing the 4 modified sIGF1R-hFc-IgG1 constructs were performed using antibodies against the α and β chains of IGF-IR and the Fc portion of the fusion proteins (Fig. 30). No HMW bands were detectable in supernatants containing Mod#1 and Mod#3. The level of HMW bands in Mod#4-containing supernatants was lower than in supernatants containing the parent form of the fusion protein. The anti-β and anti-Fc antibodies also detected some LMW species. On the basis of the Western blot results, the band of approximately 80-90kD appears to be of a single β chain fused to Fc and the band of 210-220 kDa is probably a monomer form of sIGF1R-hFc-IgG1 (Fc+β+α chain). The intensity of these LMW forms in the supernatant of CHO cells was approximately half of the tetramer+Fc protein as assessed by Western Blot.

[00171] To determine the abundance of these bands in the purified protein fractions and compare it to levels obtained with the parental construct, non-purified and purified parental sIGF1R-hFc-IgG1 and the 4 modified sIGF1R-hFc-IgG1 proteins were analyzed by Western Blotting using anti-α subunit and anti-Fc antibodies (Fig. 31). Under non-reducing conditions, the parental sIGF1R-hFc-IgG1, non-purified or purified fractions showed a similar pattern and no LMW species were detected. However, under non-reducing conditions when anti-Fc antibody was used, LMW bands were detectable in purified preparations of the modified sIGF1R-hFc-IgG1 proteins (Mod#1 and Mod#3). The mechanism for formation of these LMW species is not clear. Perhaps replacing both Cys residues in the core hinge with Ser (as in Mod#1 and Mod#3) renders the remaining disulphide bonds of sIGF1R-hFc more sensitive to reduction in the cell culture medium. Interestingly, for Mod#4 where one

cysteine (i.e., one disulphide bond) is retained, the concentration of LMW species was reduced (relative to Mod#1 and Mod#3) but some HMW species appeared in SDS-PAGE gels and Western blots. Notably however, the levels of LMW bands significantly decreased following fractionation of protein A columns, suggesting that they have different binding dynamics (e.g., affinity) to protein A and could likely be eliminated by protein A purification.

[00172] Under fully reducing conditions, when all disulphide bonds are reduced, HMW species should appear as two bands, one at 130-140kDa corresponding to the full length α-chain (not detectable with anti-Fc antibody) and another at 80-90kDa corresponding to the β subunit-Fc fusion protein. However, a band of 210-220 kDa (corresponding to a sIGF1R-hFc-IgG1 monomer) was detectable in the gels. This finding suggested that disulphide bonds formed between the α-chain and the β-Fc fusion protein of each monomer were more resistant to reduction by DDT at 300mM than the disulphide bonds between α-chains of two separate monomers. A low MW band of approximately 30kDa was also detected in the non-purified protein fraction and in Mod#1 and probably represents a truncated form of the Fc-β-fusion protein.

[00173] Although a rational design was employed in constructing all 4 modified proteins, results indicated that only 2 of the new constructs produced proteins that did not form HMW Species. For example, in the case of modified protein Mod#2, in which a longer linker was introduced but the hinge Cys residues were not substituted, HMW species could still be observed, albeit at a lower level than in the parent protein. This finding suggests that, while some intra-molecular Fc dimers may have been established in the Mod#2 variant (as postulated), there was still a significant level of Fc protein available for inter-molecular association. On the other hand, the fact that the Cys-Ser substitutions in the hinge domain of Fc resulted in complete elimination of HMW species in modified proteins Mod#1 and Mod#3, together with the finding of an intermediate level of HMW species in Mod#4 that retained only one of the two hinge Cys (Figs. 29, 30, 31) indicates that hinge Cys residues are indeed involved in promoting inter-molecular oligomerization, as predicted by our

molecular modelling. Interestingly, the 30 kDa protein originating from the Fc fragment was seen only in Mod#1 and not in Mod#3. This may indicate that the intra-molecular dimerization that occurs in the Mod#3 protein due to its longer linker protects the Fc fragment from proteolytic degradation. Proteolytic cleavage appears to have occurred more readily in the Mod#1 protein where the Fc fragment is unpaired both intra-molecularly due to the short linker and inter-molecularly due to the absence of hinge cysteines.

[00174] In summary, these results suggest that the modified protein Mod#3 may be the most suitable candidate for scaled-up production of a protein which is a single band, which is desirable for development as a therapeutic.

Example 15. Generation of industry grade four modified CHO-Cum2-sIGF1R-hFc-IgG1 cell lines.

[00175] Four pools of industry-grade stable cell lines expressing the modified sIGF1R-hFc-IgG1-Mod#1, Mod#2, Mod#3 & Mod#4 proteins were generated in the CHO-BRI-rcTA-55E3 cells. Transfected cells were kept under hygromycin selection for 2-3 weeks. The level of production of each of the modified sIGF1R-hFc-IgG1 proteins was measured in supernatants of cells cultured in presence of 1 µg/ml cumate (cum) (for induction of protein production). After 8 days in culture, protein concentrations in the conditioned media were 21, 17, 20 & 31 µg/ml for modified sIGF1R-hFc-IgG1 Mod#1, Mod#2, Mod#3 & Mod#4, respectively. Subcloning of these producing cell pools and selection of high producer clones is expected to result in increases of 3-5 fold in production levels of the selected proteins.

Example 16. Determination of binding affinity for modified sIGF1R-hFc-IgG1 proteins using surface plasmon resonance.

[00176] As discussed above, modified fusion proteins Mod#1 and Mod#3 produced one major band at the expected MW for the sIGF1R-hFc-IgG1 protein and no detectable production of HMW species. In order to determine whether the binding affinity (and therefore biological activity) of these modified proteins was unchanged as compared to the parent protein, all four modified proteins

(Mod#1, Mod#2, Mod#3 and Mod#4) were amine-coupled to surface plasmon resonance (SPR) sensors and rapid, single-cycle screening was used to compare the profiles of the 4 modified proteins (Fig. 33). These results showed that the 4 modified proteins (Mod #1, Mod#2, Mod#3 and Mod#4) had similar binding affinities to ligands, and that their binding affinities were also highly similar to those of Trap H (the parent trap protein, used as a positive control). Specific, dose-dependent binding responses were strongest with hIGF-1 in all cases (Table II), weaker for other ligands (hIGF-2, mIGF-1, human insulin), and no binding responses were observed with maltose binding protein (MBP; negative control).

Table II. Equilibrium dissociation constants (K_D +/- standard error) for IGF1R ligands binding to immobilized sIGF1R-hFc-IgG1 proteins. Experimental data (5-point single-cycle SPR titrations; n = 2) was fit to the "1:1 Titration" model in the BIAevaluation software.

Purified TRAP protein	**hIGF-1** K_D (nM +/- SE)	**hIGF-2** K_D (nM +/- SE)	**mIGF-1** K_D (nM +/- SE)	**h-insulin** K_D (nM +/- SE)
Mod#1	24 +/- 1	195 +/- 56	252 +/- 21	6375 +/- 176
Mod#2	17 +/- 1	97 +/- 9	172 +/- 11	5362 +/- 222
Mod#3	19 +/- 1	169 +/- 29	894 +/- 89	29902 +/- 1694
Mod#4	18 +/- 1	126 +/- 12	557 +/- 62	21695 +/- 1205
Trap H (parent protein)	11 +/- 1	98 +/- 8	540 +/- 53	15424 +/- 508

[00177] Based upon SDS-PAGE analysis of the four modified proteins and the results of the rapid, single-cycle screening, we selected the Mod#3 and Mod#4 proteins for more extensive multi-cycle testing (Figs. 34, 35; Table III). Consistent with results seen for Trap H, the binding affinity of hIGF-1 to Mod#3 and Mod#4 was highest (~6 nM; Table III); weaker binding was observed with hIGF-2 (~37 nM) and mIGF-1 (~150 nM); while binding affinity to human insulin (~7 uM) was about 100-fold lower than that to hIGF-I.

Table III. Equilibrium dissociation constants (K_D +/- standard error) for IGF1R ligands binding to immobilized sIGF1R-hFc-IgG1 proteins. Experimental data (10-point (hIGF-1 and hIGF-2) or 5-point (mIGF-1 and h-insulin) multi-cycle SPR titrations; n = 2) was fit to the "1:1 Kinetic" model in the BIAevaluation software.

Purified TRAP proteins	**hIGF-1** K_D (nM +/- SE)	**hIGF-2** K_D (nM +/- SE)	**mIGF-1** K_D (nM +/- SE)	**h-insulin** K_D (nM +/- SE)
Mod#3	6.2 +/- 0.1	42 +/- 1	206 +/- 72	7575 +/- 987
Mod#4	6.5 +/- 0.1	37 +/- 1	162 +/- 38	7692 +/- 1201
Trap H (parent protein)	5.7 +/- 0.1	32 +/- 1	74 +/- 15	5050 +/- 676

[00178] All references and documents referred to herein are hereby incorporated by reference in their entirety.

[00179] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A fusion protein comprising an Fc portion of an antibody and a soluble IGF-IR protein, wherein the fusion protein comprises the sequence set forth in SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, or SEQ ID NO: 18.

2. A fusion protein comprising an Fc portion of an antibody and a soluble IGF-IR protein, wherein the fusion protein consists of the sequence set forth in SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, or SEQ ID NO: 18.

3. A biologically active fragment or analog of the fusion protein as defined in claim 1 or 2.

4. The biologically active fragment or analog of claim 3, wherein the biologically active fragment or analog has at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to the fusion protein.

5. The biologically active fragment or analog of claim 3 or 4, wherein the biologically active fragment or analog retains the binding specificity of the fusion protein.

6. A nucleic acid encoding the fusion protein or biologically active fragment or analog thereof as defined in any one of claims 1 to 5.

7. The nucleic acid of claim 6, wherein the nucleic acid has the sequence set forth in SEQ ID NO: 11, 13, 15, or 17, or is a degenerate variant thereof.

8. A nucleic acid comprising the sequence set forth in SEQ ID NO: 11, 13, 15, or 17, or a degenerate variant thereof.

9. A nucleic acid consisting of the sequence set forth in SEQ ID NO: 11, 13, 15, or 17, or a degenerate variant thereof.

10. A nucleic acid having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to the sequence set forth in SEQ ID NO: 11, 13, 15, or 17.

11. The nucleic acid of any one of claims 8 to 10, wherein the nucleic acid encodes a polypeptide having the biological activity or the binding specificity of the fusion protein defined in claim 1 or 2.

12. A pharmaceutical composition comprising the fusion protein or biologically active fragment or analog thereof defined in any one of claims 1 to 5, and a pharmaceutically acceptable carrier.

13. Use of the fusion protein or biologically active fragment or analog thereof of any one of claims 1 to 5 or the pharmaceutical composition of claim 12 for treating an angiogenic associated disorder or a metastatic disease in a subject.

14. The use of claim 13, wherein the fusion protein or biologically active fragment or analog thereof or the composition is used to treat tumor metastasis, colorectal carcinoma, lung carcinoma, breast cancer, multiple myeloma, glioblastoma multiforme, hepatic cancer, liver cancer, bladder cancer, lung cancer, or pancreatic cancer.

15. The use of claim 14, wherein said hepatic cancer is liver metastasis.

16. A method of inhibiting angiogenesis in a subject having an angiogenic associated disorder, comprising administering to said subject a therapeutically effective amount of the fusion protein or biologically active fragment or analog thereof of any one of claims 1 to 5.

17. The method of claim 16, wherein said angiogenic associated disorder is cancer.

18. The method of claim 16, wherein said angiogenic associated disorder is tumor metastasis, colorectal carcinoma, lung carcinoma, breast cancer, multiple myeloma, glioblastoma multiforme, hepatic cancer, liver cancer, bladder cancer, lung cancer, or pancreatic cancer.

19. The method of claim 18, wherein said hepatic cancer is liver metastasis.

20. The method of any one of claims 16 to 19, further comprising administering the fusion protein or biologically active fragment or analog thereof in combination with another angiogenesis inhibitor.

21. A method of inhibiting angiogenesis in a subject having an angiogenic associated disorder comprising administering to said subject an autologous cell genetically modified to express the fusion protein or biologically active fragment or analog thereof of any one of claims 1 to 5.

22. The method of claim 21, wherein said autologous cell is a bone marrow derived mesenchymal stromal cell.

23. The method of claim 21 or 22, wherein said angiogenic associated disorder is cancer.

24. The method of claim 23, wherein the cancer is tumor metastasis, colorectal carcinoma, lung carcinoma, breast cancer, multiple myeloma, glioblastoma multiforme, hepatic cancer, liver cancer, bladder cancer, lung cancer, or pancreatic cancer.

25. The method of claim 24, wherein the hepatic cancer is liver metastasis.

26. The method of any one of claims 21 to 25, further comprising administering the fusion protein or biologically active fragment or analog thereof in combination with another angiogenesis inhibitor.

27. A method of treating cancer in a subject in need thereof, comprising administering to said subject the fusion protein or biologically active fragment or analog thereof of any one of claims 1 to 5.

28. The method of claim 27, wherein said cancer is metastatic disease.

29. The method of claim 27, wherein said cancer is tumor metastasis, colorectal carcinoma, lung carcinoma, breast cancer, liver cancer, bladder cancer, lung cancer, pancreatic cancer, multiple myeloma, glioblastoma multiforme, or liver metastasis.

30. The method of any one of claims 27 to 29, further comprising administering the fusion protein or biologically active fragment or analog thereof in combination with an angiogenesis inhibitor, wherein the angiogenesis inhibitor and the fusion protein or biologically active fragment or analog thereof are administered concomitantly or sequentially.

31. The method of claim 20 or 26, wherein the fusion protein or biologically active fragment or analog thereof and the other angiogenesis inhibitor are administered concomitantly or sequentially.

32. A method of preventing or treating an angiogenic associated disorder in a subject, the method comprising administering the fusion protein or biologically active fragment or analog thereof of any one of claims 1 to 5 to the subject, wherein angiogenesis is inhibited in the subject, such that the angiogenic associated disorder is prevented or treated.

33. A method of preventing or treating tumor metastasis, colorectal carcinoma, lung carcinoma, breast cancer, multiple myeloma,

glioblastoma multiforme, hepatic cancer, liver cancer, bladder cancer, lung cancer, or pancreatic cancer in a subject, the method comprising administering the fusion protein or biologically active fragment or analog thereof of any one of claims 1 to 5 to the subject, such that tumor metastasis, colorectal carcinoma, lung carcinoma, breast cancer, multiple myeloma, glioblastoma multiforme, hepatic cancer, liver cancer, bladder cancer, lung cancer, or pancreatic cancer is prevented or treated.

34. The method of any one of claims 16 to 33, wherein the fusion protein or biologically active fragment or analog thereof is administered via injection.

35. The method of claim 34, wherein the injection is intravenous or intraperitoneal.

36. The method of any one of claims 16 to 33, wherein the fusion protein or biologically active fragment or analog thereof is administered orally.

37. The method of any one of claims 16 to 36, further comprising administering the fusion protein or biologically active fragment or analog thereof in combination with one or more other anti-cancer agents or therapies.

38. The method of claim 37, wherein the fusion protein or biologically active fragment or analog thereof and the one or more other anti-cancer agents or therapies are administered concomitantly or sequentially.

39. The method of claim 37 or 38, wherein the anti-cancer agent or therapy is surgery, radiology, chemotherapy, or a targeted cancer treatment.

40. The method of claim 39, wherein the targeted cancer treatment is a small molecule, a monoclonal antibody, a cancer vaccine, an antisense nucleotide, an siRNA, an aptamer, or a gene therapy.

41. A pharmaceutical composition for treating metastasis in a subject, comprising the fusion protein or biologically active fragment or analog thereof of any one of claims 1 to 5; and a pharmaceutically acceptable carrier.

42. A pharmaceutical composition for treating cancer in a subject, comprising the fusion protein or biologically active fragment or analog thereof of any one of claims 1 to 5; and a pharmaceutically acceptable carrier.

43. The pharmaceutical composition of claim 42, wherein the cancer is tumor metastasis, colorectal carcinoma, lung carcinoma, breast cancer, multiple myeloma, glioblastoma multiforme, hepatic cancer, liver cancer, bladder cancer, lung cancer, or pancreatic cancer

44. The pharmaceutical composition of claim 43, wherein said hepatic cancer is liver metastasis.

45. A vector comprising the sequence set forth in SEQ ID NO: 11, 13, 15, or 17, or a degenerate variant thereof.

46. A fusion protein comprising an Fc portion of an antibody and a soluble IGF-IR protein.

47. The fusion protein of claim 46, wherein the antibody is a human IgG antibody.

48. The fusion protein of claim 47, wherein the antibody is an IgG1 or an IgG2 antibody.

49. The fusion protein of any one of claims 46 to 48, wherein the fusion protein binds specifically to IGF-1 and IGF-2.

50. The fusion protein of any one of claims 46 to 49, wherein the fusion protein binds to IGF-1 and IGF-2 with at least about the same affinity, or wherein the affinity of the fusion protein for IGF-2 is higher than the affinity of the fusion protein for IGF-1.

51. The fusion protein of any one of claims 46 to 50, wherein the affinity of the fusion protein for insulin is at least about 1000-fold lower than the fusion protein's affinity for IGF-1 or IGF-2.

52. The fusion protein of any one of claims 46 to 51, wherein the fusion protein does not bind detectably to insulin.

53. The fusion protein of any one of claims 46 to 52, wherein the soluble IGF-IR protein comprises the extracellular domain of IGF-IR having the amino acid sequence of SEQ ID NO: 1 or 6, or a biologically active fragment or analog thereof, and/or wherein said soluble IGF-IR protein forms the tetrameric structure of SEQ ID NO: 1 or 6.

54. The fusion protein of any one of claims 46 to 53, wherein said soluble IGF-IR protein consists of SEQ ID NO: 1 or 6 or a biologically active fragment or analog thereof.

55. The fusion protein of any one of claims 46 to 54, wherein the soluble IGF-IR protein comprises the extracellular domain of IGF-IR having the amino acid sequence of SEQ ID NO: 4, or a biologically active fragment or analog thereof.

56. The fusion protein of any one of claims 46 to 55, wherein said soluble IGF-IR protein forms the tetrameric structure of SEQ ID NO: 1 or 6.

57. The fusion protein of any one of claims 46 to 52 and 56, wherein said soluble IGF-IR protein consists of SEQ ID NO: 1 or 6 or a biologically active fragment or analog thereof.

58. The fusion protein of claim 46, wherein the fusion protein comprises the sequence set forth in SEQ ID NO: 8 or SEQ ID NO: 10.

59. The fusion protein of claim 1, wherein the fusion protein consists of the sequence set forth in SEQ ID NO: 8 or SEQ ID NO: 10.

60. A biologically active fragment or analog of the fusion protein as defined in any one of claims 46 to 59.

61. The biologically active fragment or analog of claim 60, wherein the biologically active fragment or analog has at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% sequence identity to the fusion protein.

62. The biologically active fragment or analog of claim 60 or 61, wherein the biologically active fragment or analog retains the binding specificity of the fusion protein.

63. A nucleic acid encoding the fusion protein or biologically active fragment or analog thereof as defined in any one of claims 46 to 62.

64. The fusion protein or biologically active fragment or analog thereof defined in any one of claims 46 to 63, wherein said fusion protein or biologically active fragment or analog thereof is encoded by a nucleic acid having the sequence set forth in SEQ ID NO: 5, 7, or 9, or a degenerate variant thereof.

65. A nucleic acid having the nucleotide sequence set forth in SEQ ID NO: 5, 7, or 9, or a degenerate variant thereof.

66. A pharmaceutical composition comprising the fusion protein or biologically active fragment or analog thereof defined in any one of claims 46 to 62 and 64, and a pharmaceutically acceptable carrier.

67. A pharmaceutical composition for treating cancer in a subject, comprising the fusion protein or biologically active fragment or analog thereof defined in any one of claims 46 to 62 and 64, and a pharmaceutically acceptable carrier.

68. A method of inhibiting angiogenesis in a subject having an angiogenic associated disorder, comprising administering to said subject a therapeutically effective amount of the fusion protein or biologically active fragment or analog thereof as defined in any one of claims 46 to 62 and 64.

69. A method of treating cancer in a subject in need thereof, comprising administering to said subject the fusion protein or biologically active fragment or analog thereof as defined in any one of claims 46 to 62 and 64.

70. The method of claim 68, wherein said angiogenic associated disorder is cancer.

71. The method of claim 70, wherein said cancer is tumor metastasis, colorectal carcinoma, lung carcinoma, breast cancer, liver cancer, bladder cancer, lung cancer, pancreatic cancer, multiple myeloma, glioblastoma multiforme, or liver metastasis.

72. The method of any one of claims 68 to 71, wherein the fusion protein or biologically active fragment or analog thereof is administered via injection, intravenously, intraperitoneally, or orally.

73. The method of any one of claims 68 to 72, wherein the fusion protein or biologically active fragment or analog thereof is administered in combination with one or more other anti-cancer agents or therapies.

FIGURE 1

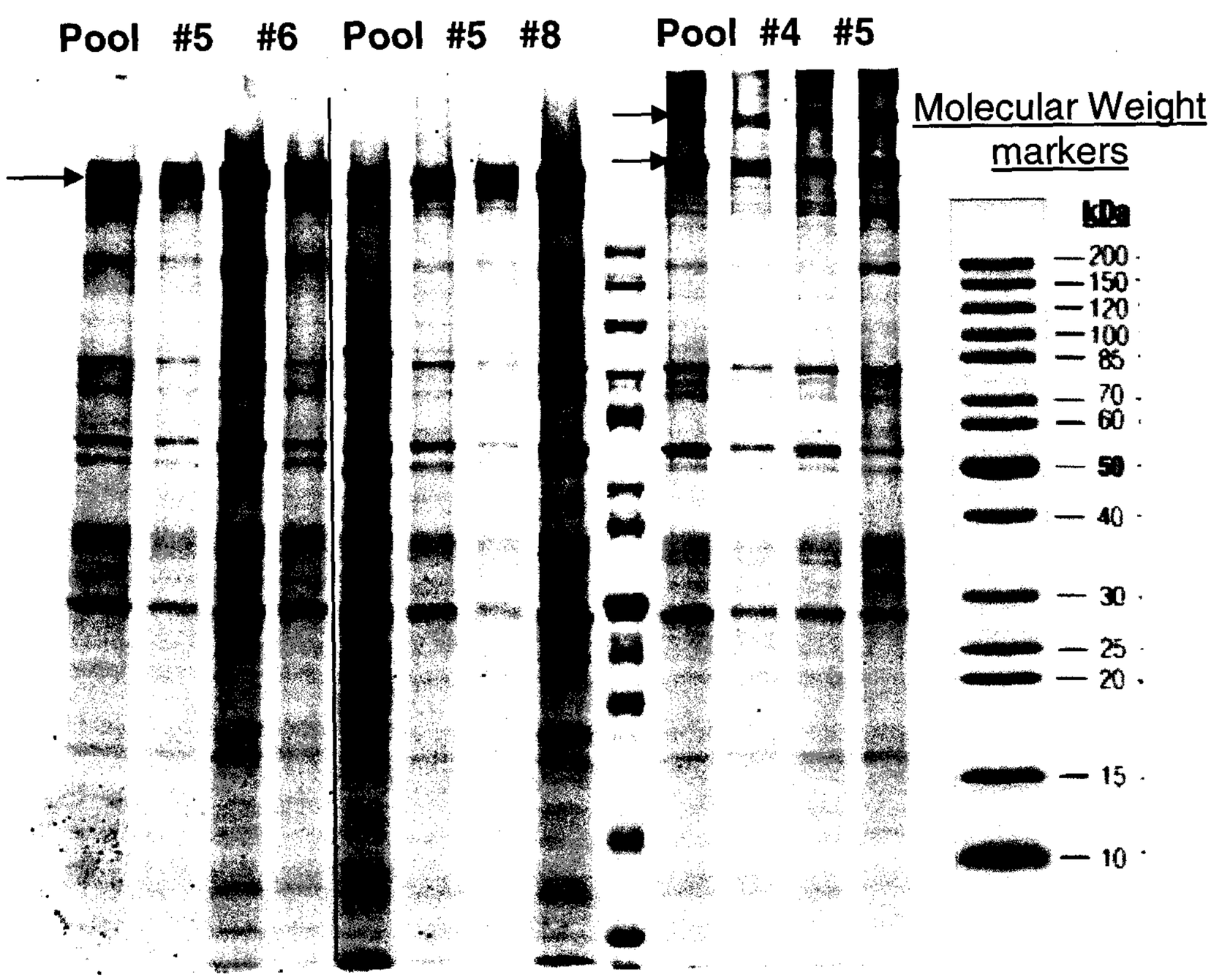
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FIGURE 2

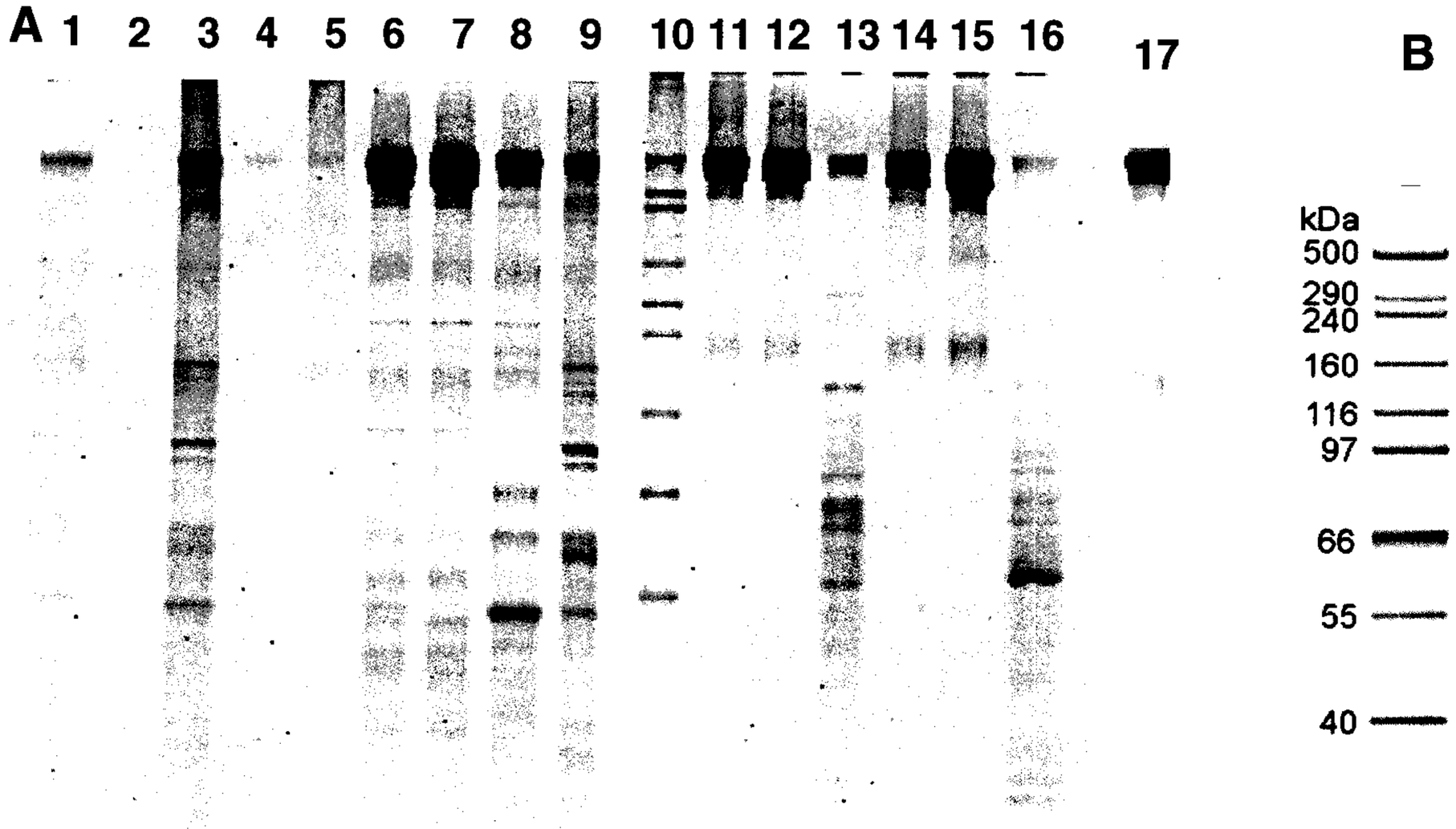
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FIGURE 3

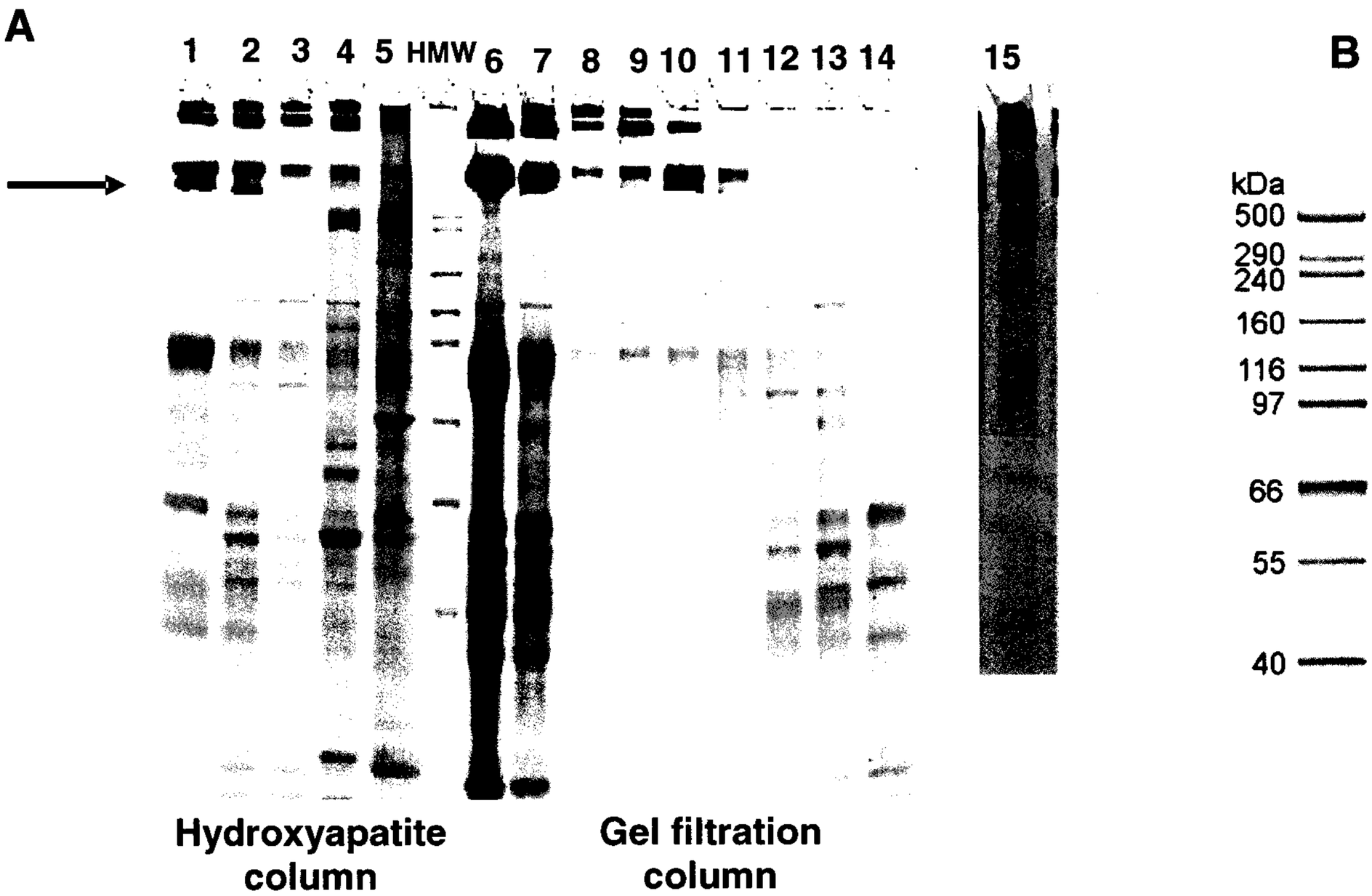
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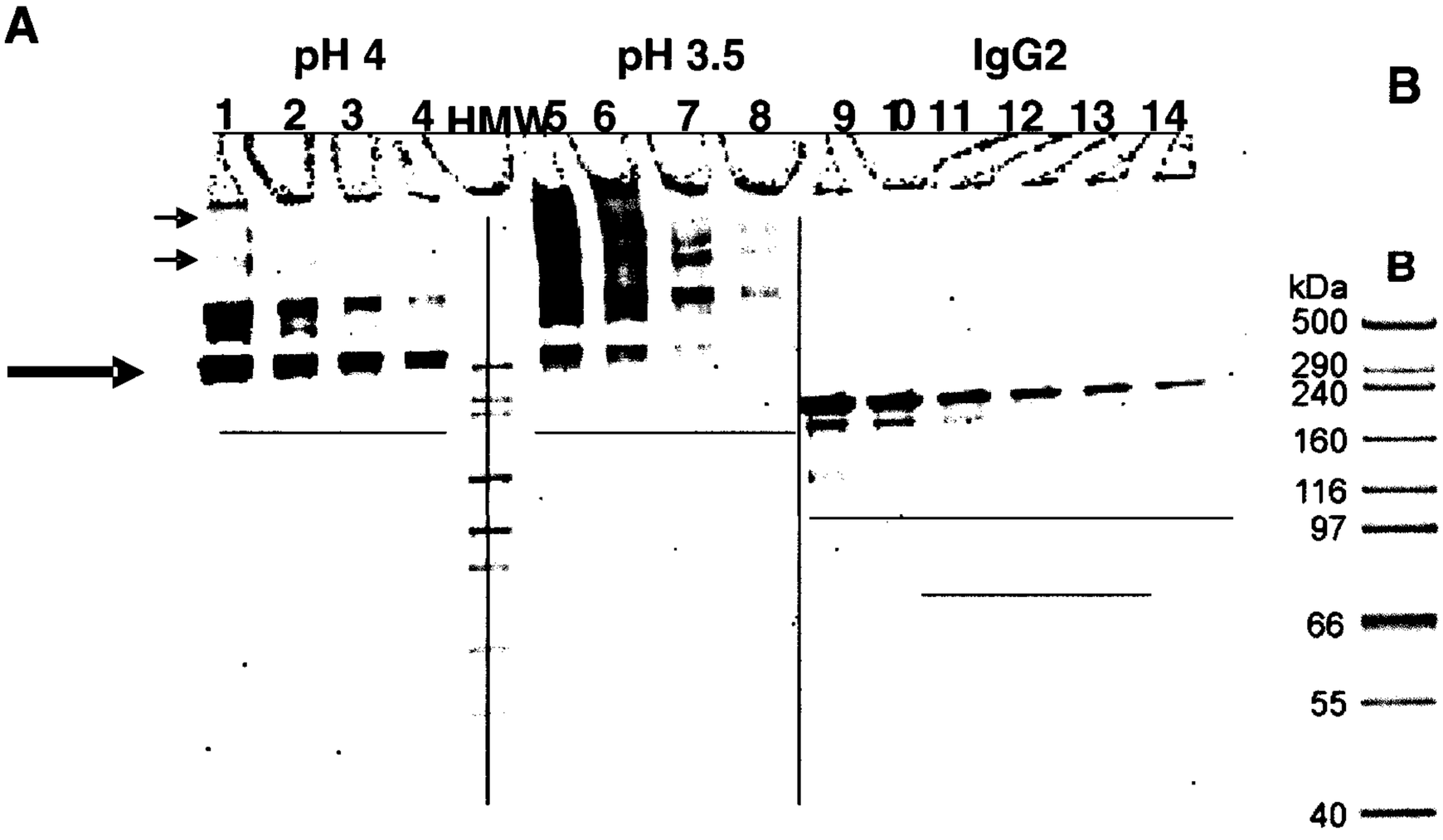
FIGURE 4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116
97
66
55
40

FIGURE 5

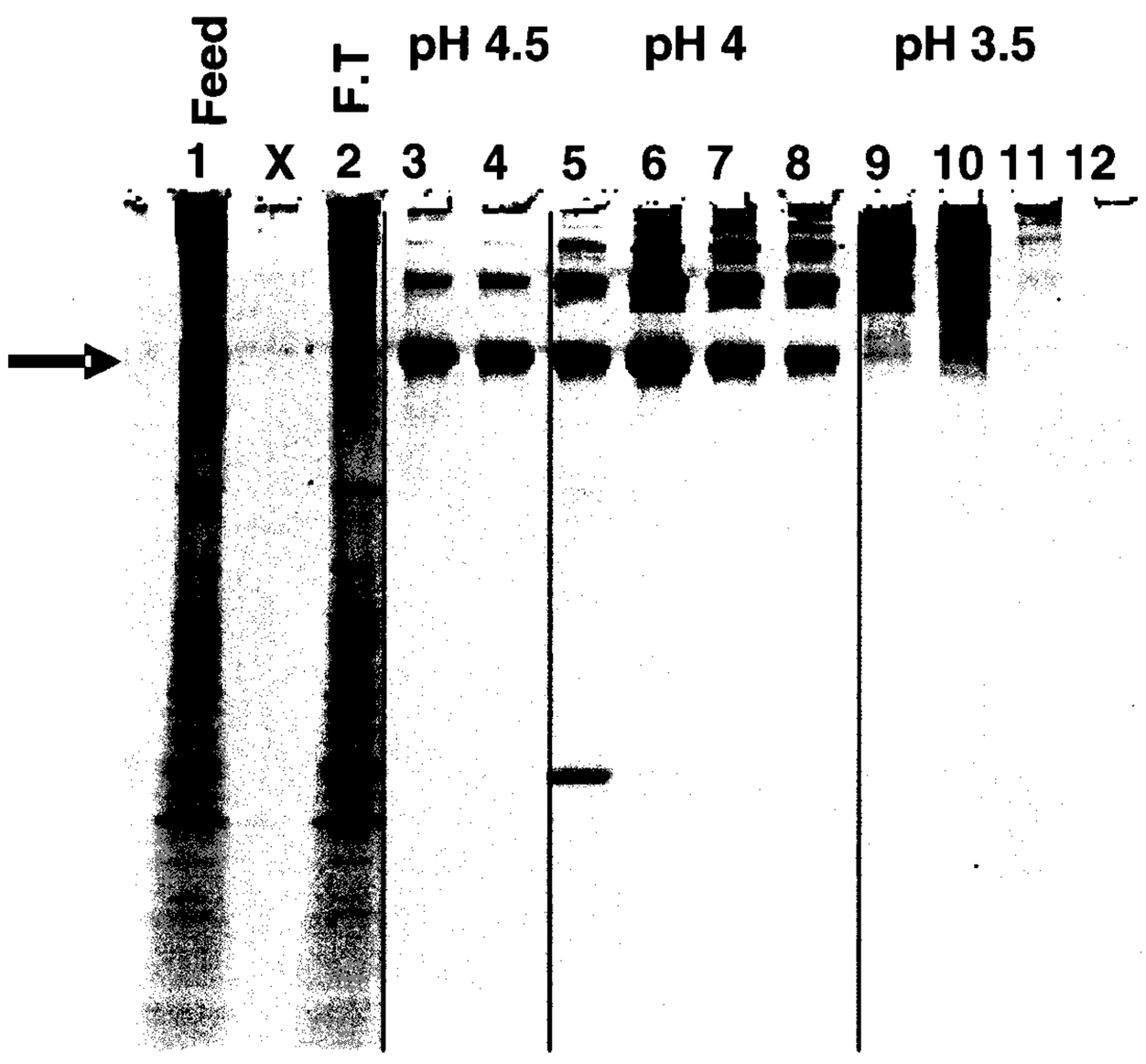
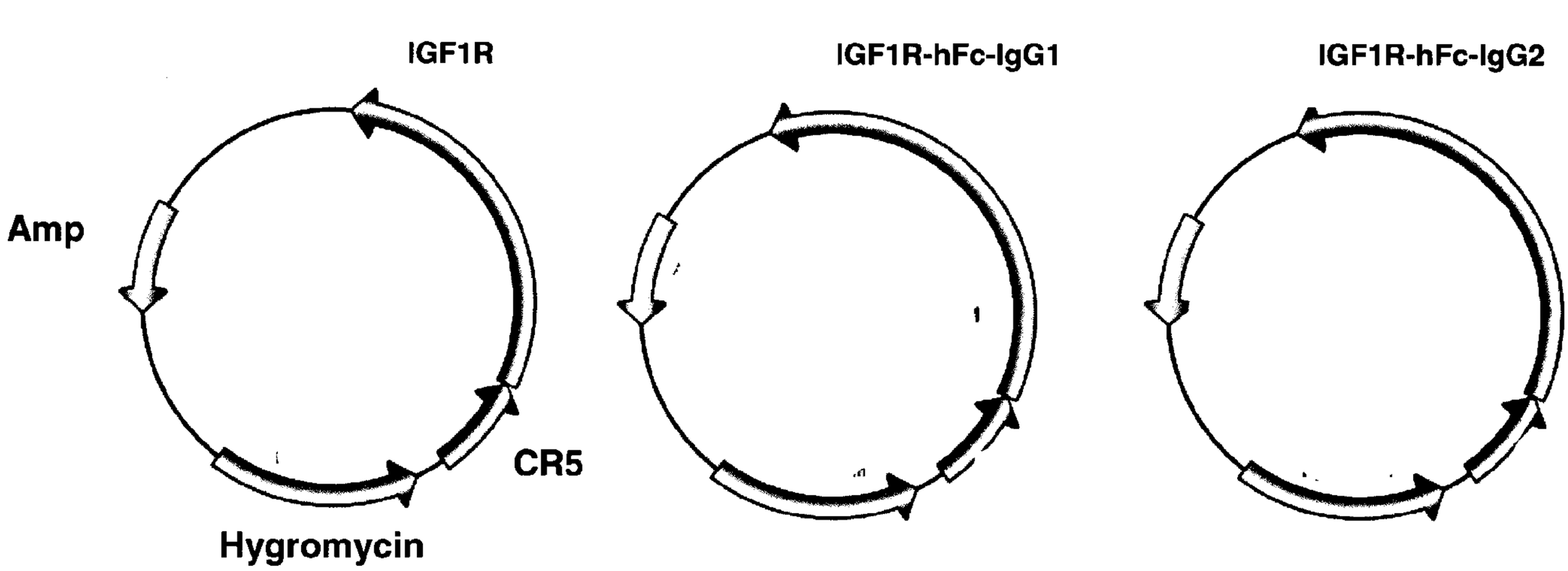


FIGURE 6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CR5
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B.
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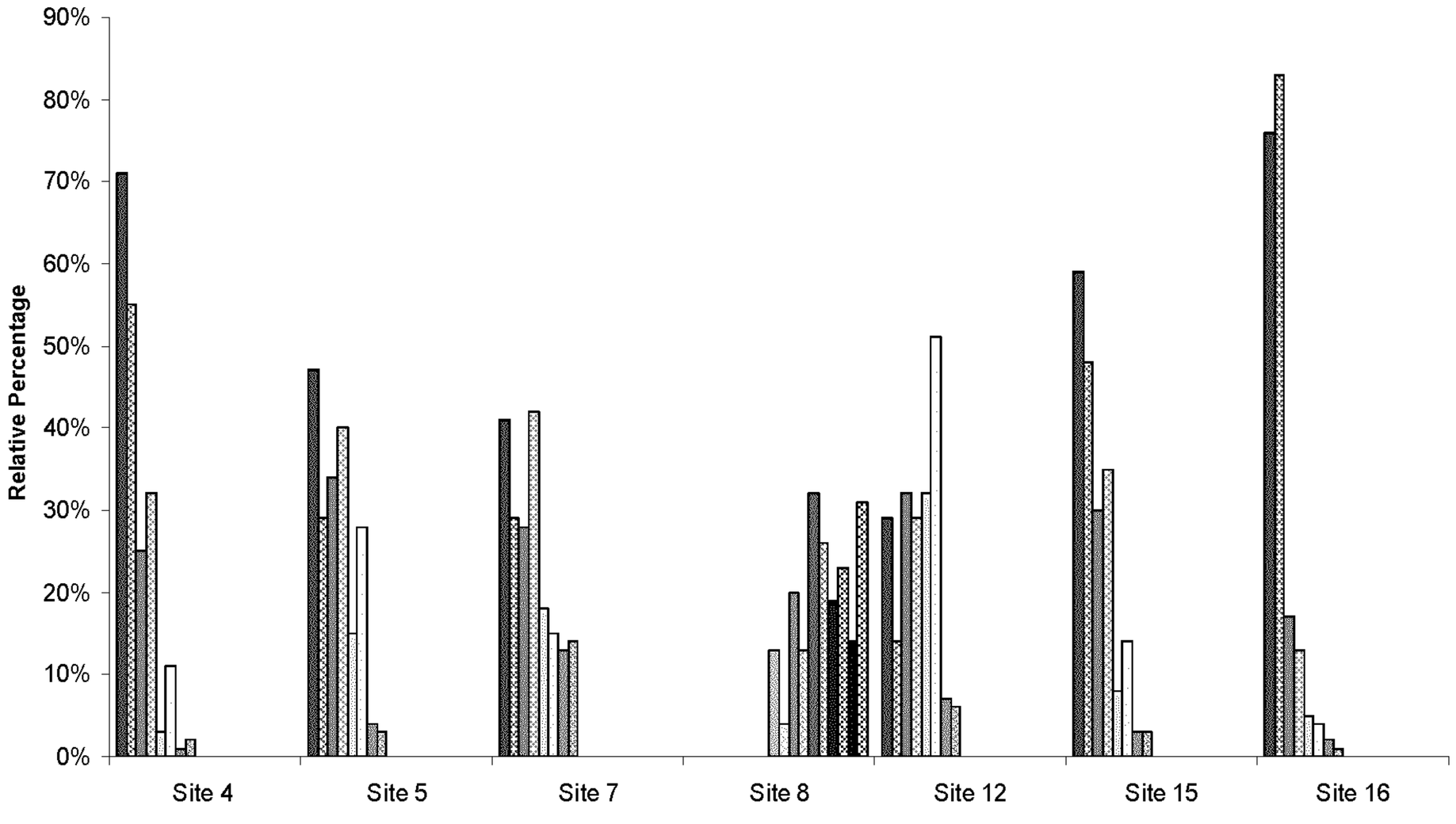
10 kb

FIGURE 7

A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Site 12
Site 15
Site 16

B

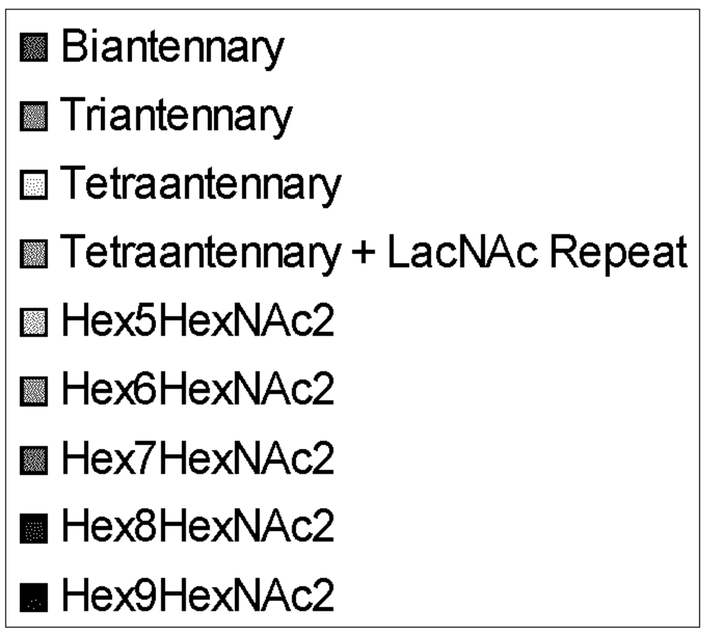
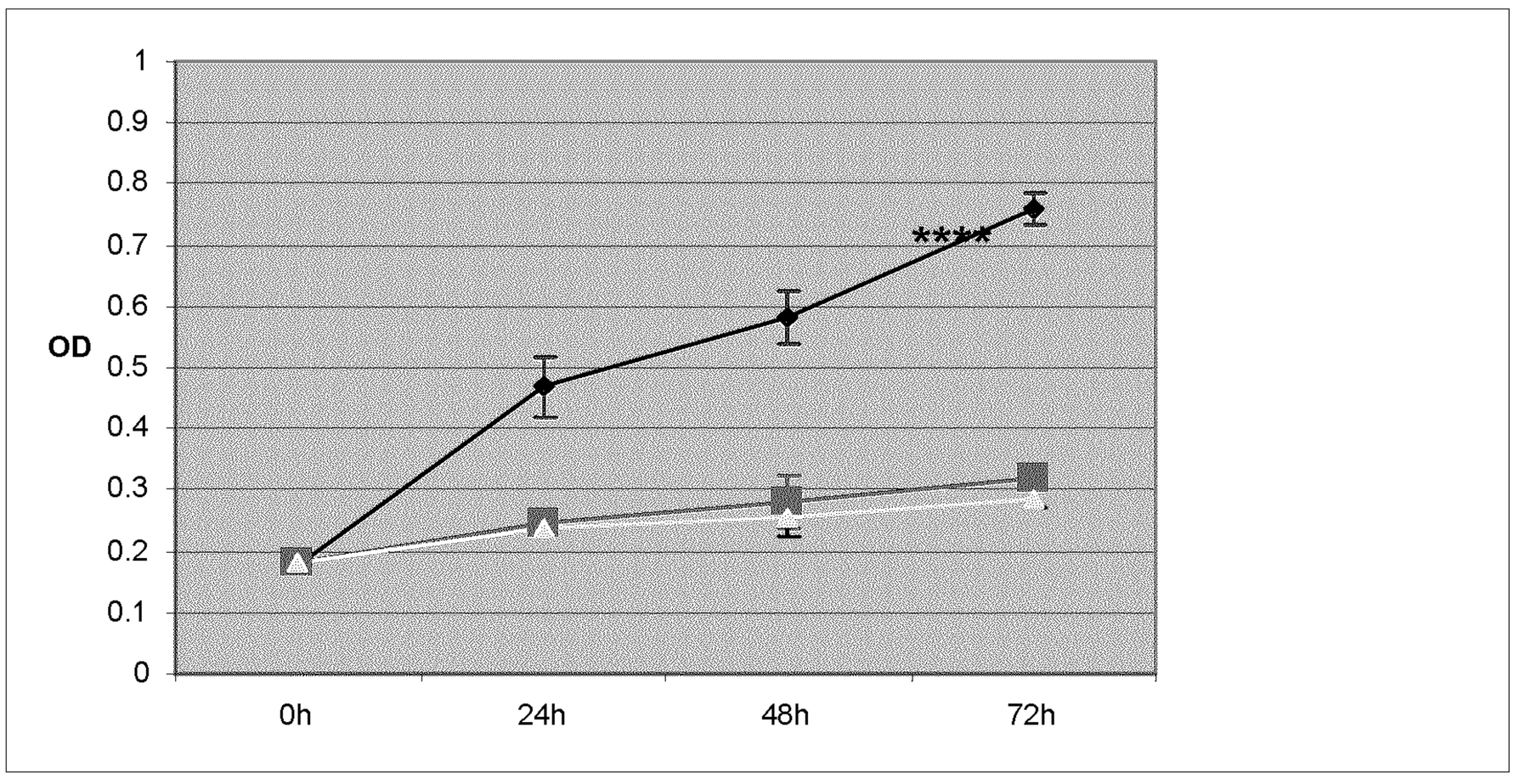

Biantennary
Triantennary
Tetraantennary
Tetraantennary + LacNAc Repeat
Hex5HexNAc2
Hex6HexNAc2
Hex7HexNAc2
Hex8HexNAc2
Hex9HexNAc2

FIGURE 8

A


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD
****
0h
24h
48h
72h

B

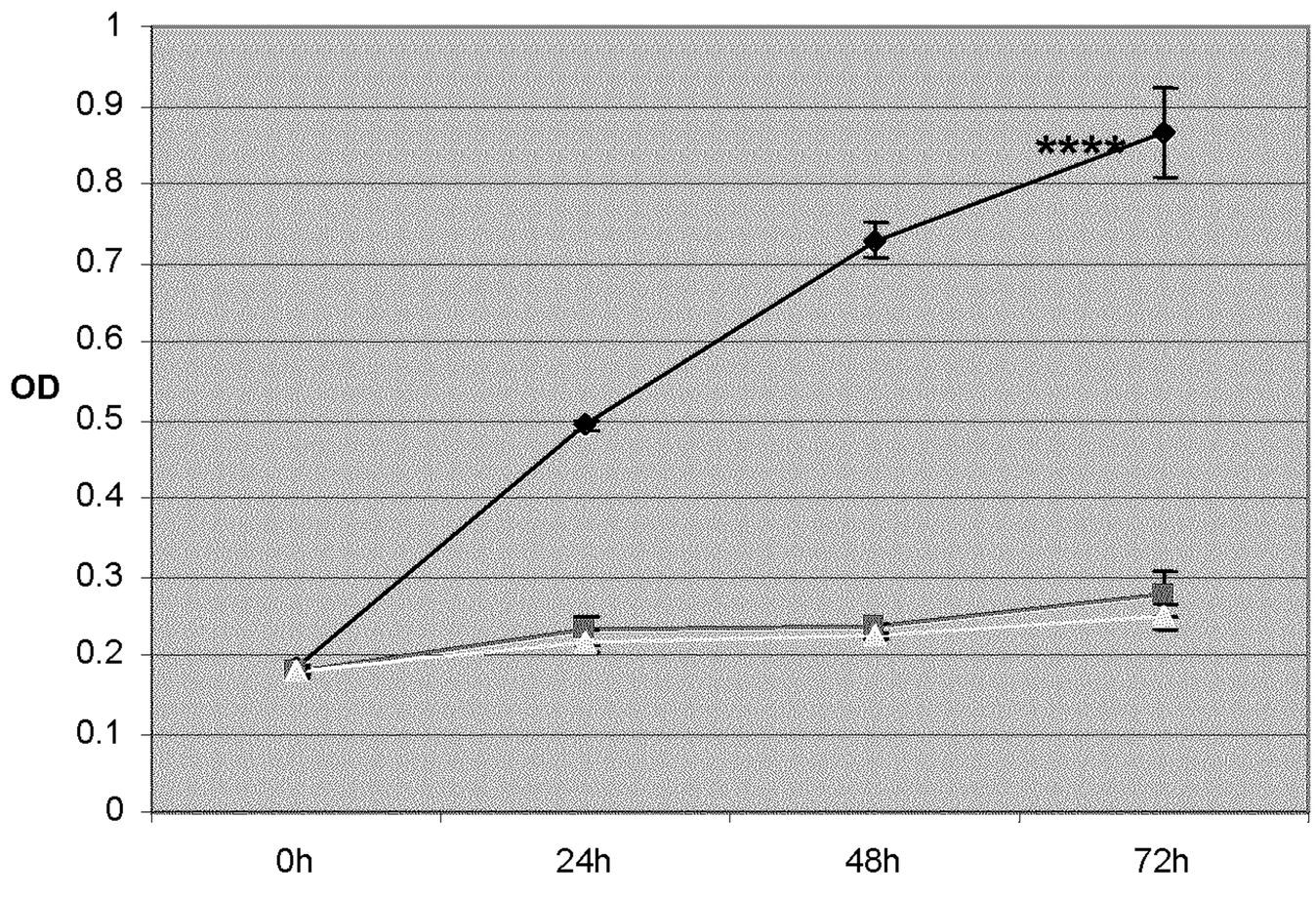

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD
****
0h
24h
48h
72h

FIGURE 9

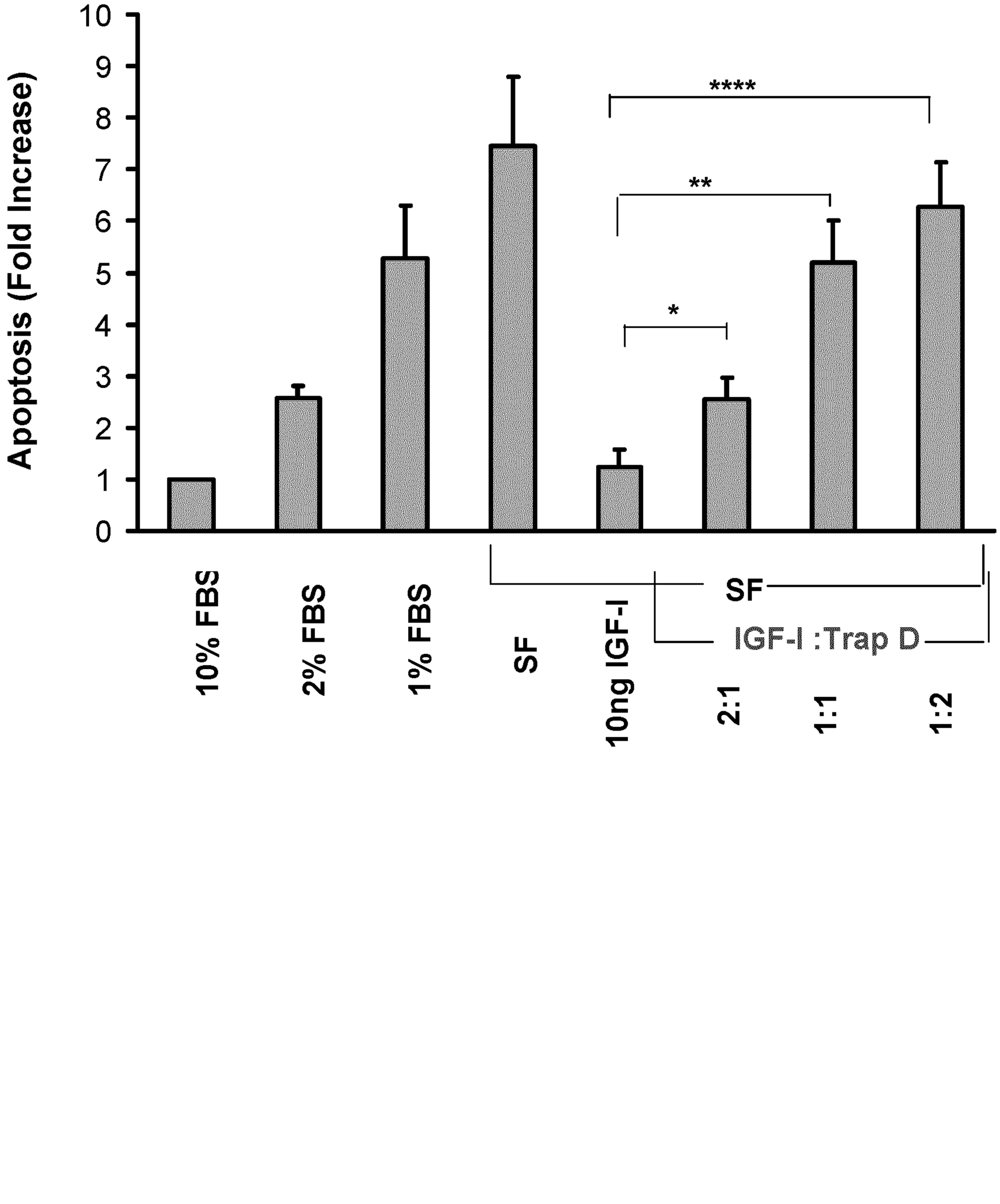

Apoptosis (Fold Increase)
0
1
2
3
4
5
6
7
8
9
10
****
**
*
10% FBS
2% FBS
1% FBS
SF
0ng IGF-I
2:1
1:1
1:2
SF
IGF-I :Trap D

FIGURE 10

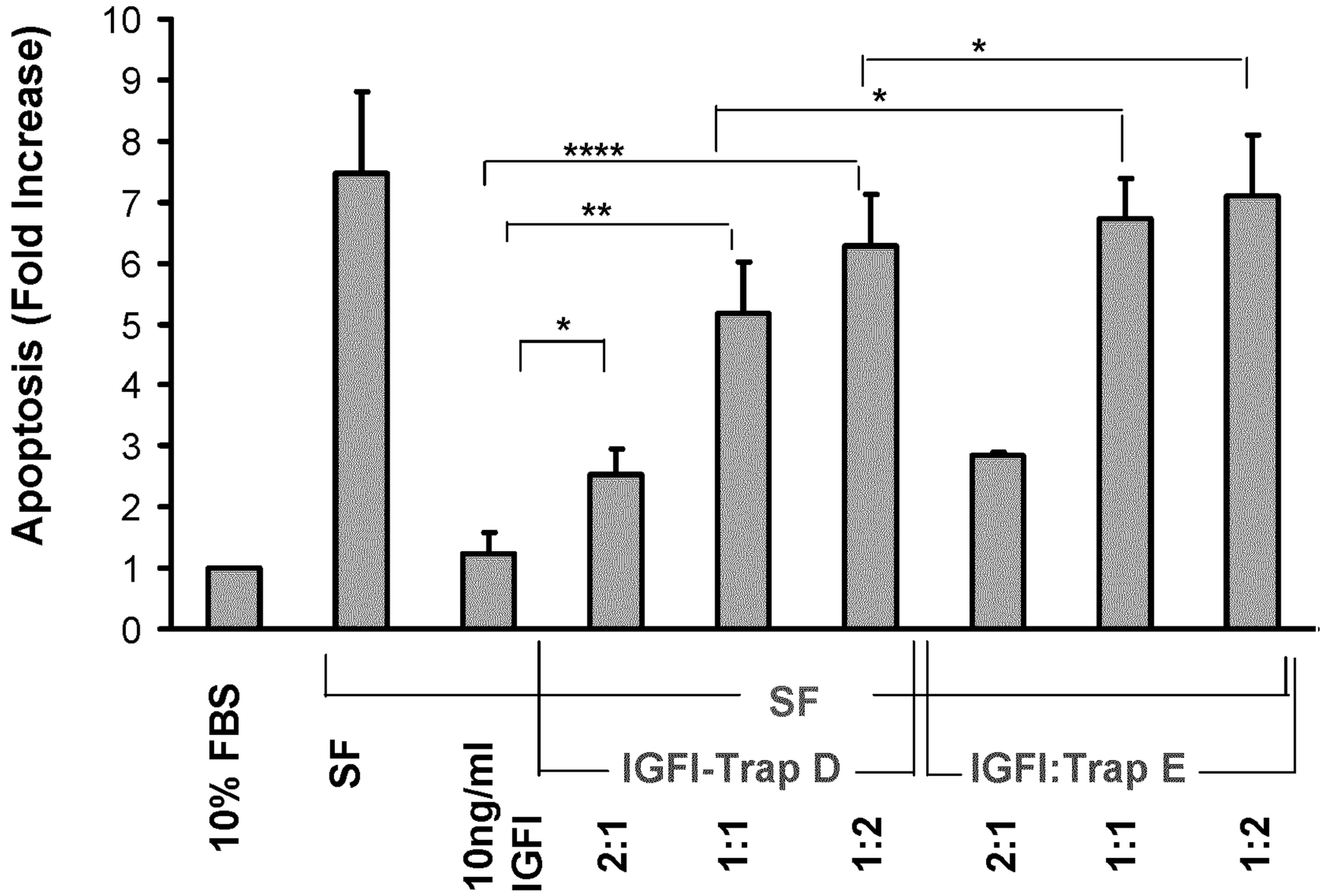

Apoptosis (Fold Increase)
0
1
2
3
4
5
6
7
8
9
10
****
**
*
*
*
*
10% FBS
SF
10ng/ml IGFI
2:1
1:1
1:2
2:1
1:1
1:2
SF
IGFI-Trap D
IGFI:Trap E

FIGURE 11

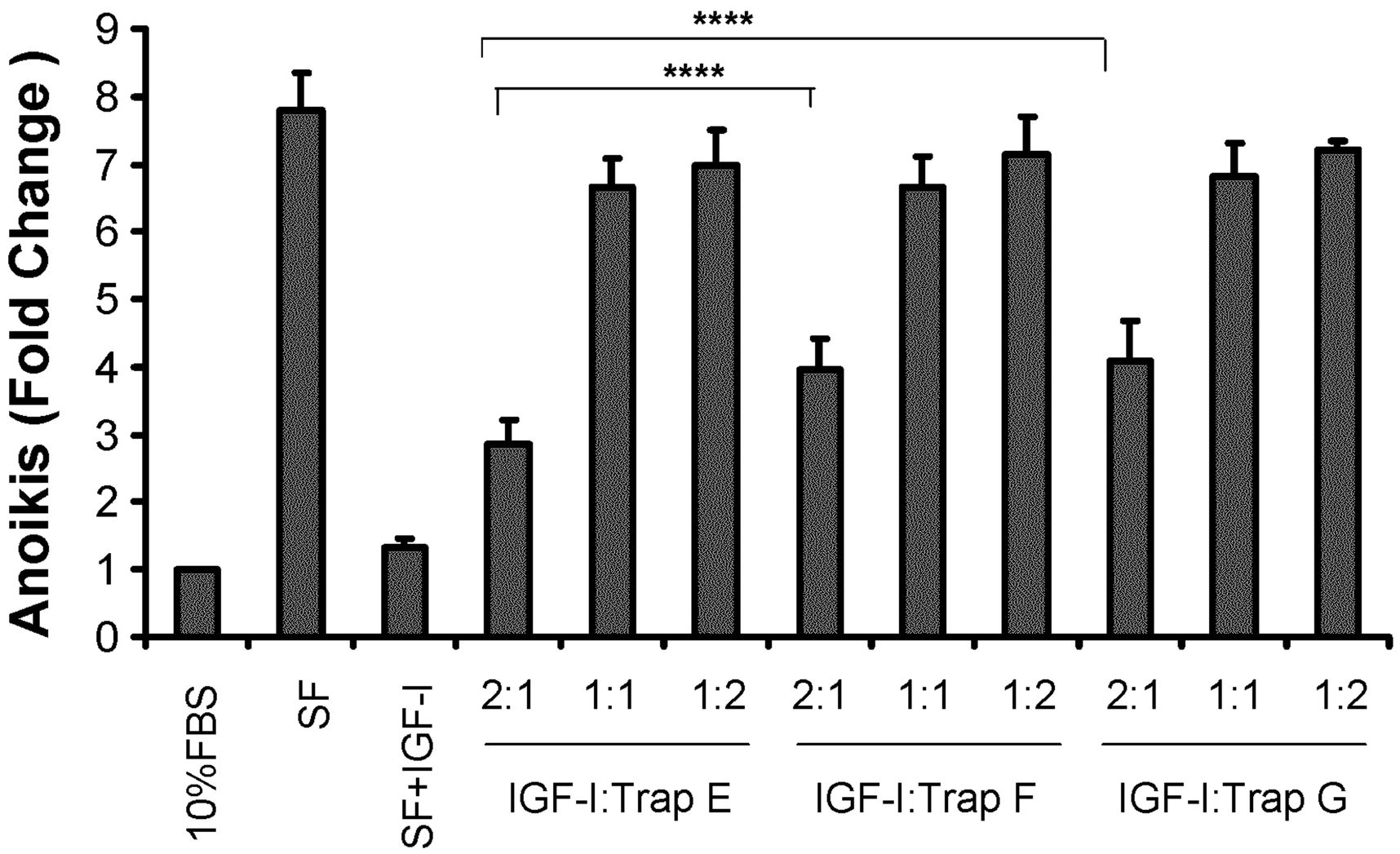

Anoikis (Fold Change)
0
1
2
3
4
5
6
7
8
9
****
****
10%FBS
SF
SF+IGF-I
2:1
1:1
1:2
2:1
1:1
1:2
2:1
1:1
1:2
IGF-I:Trap E
IGF-I:Trap F
IGF-I:Trap G

FIGURE 12

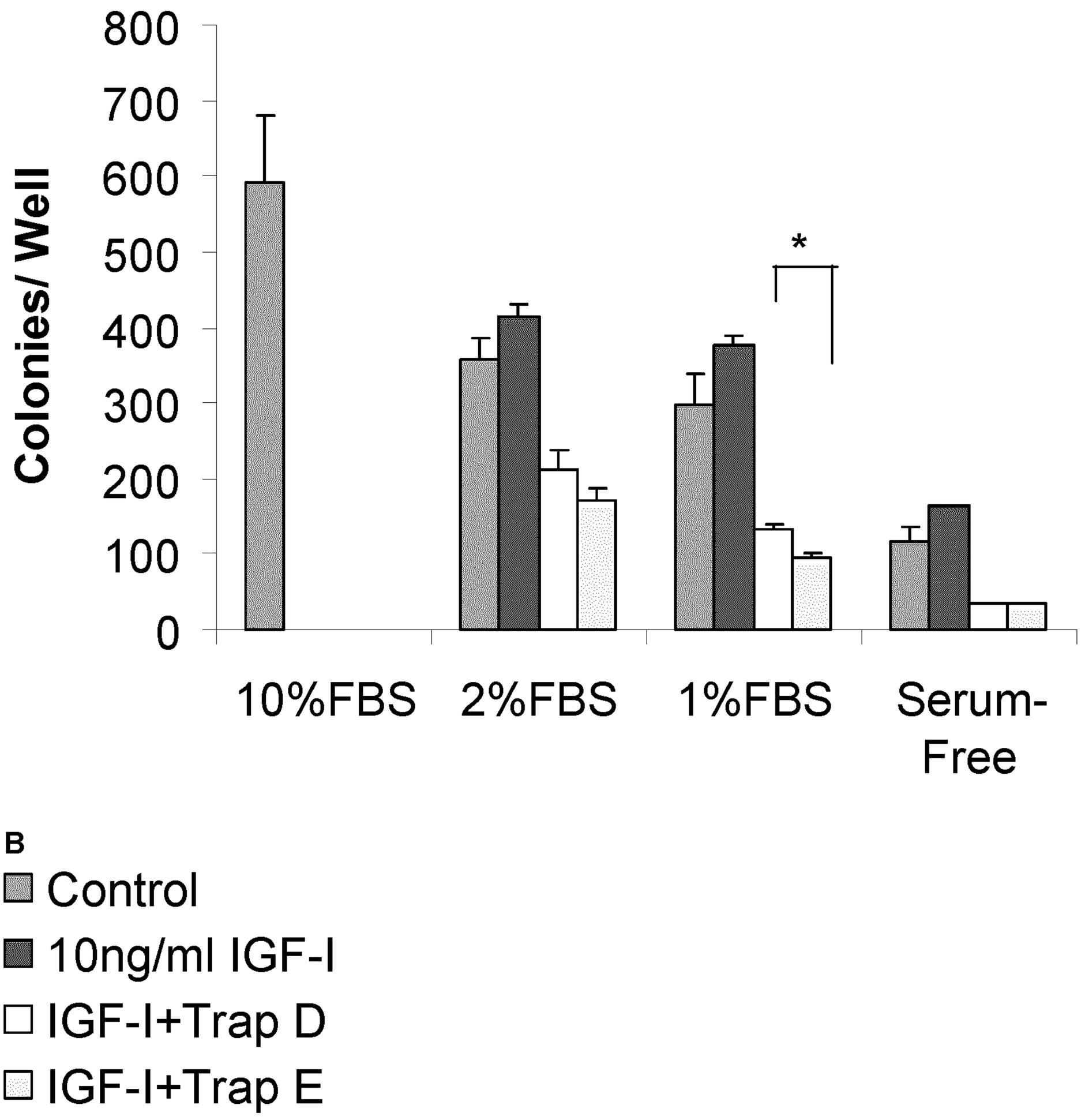

A
800
700
600
500
400
300
200
100
0
Colonies/ Well
*
10%FBS
2%FBS
1%FBS
Serum-Free
B
Control
10ng/ml IGF-I
IGF-I+Trap D
IGF-I+Trap E

FIGURE 13

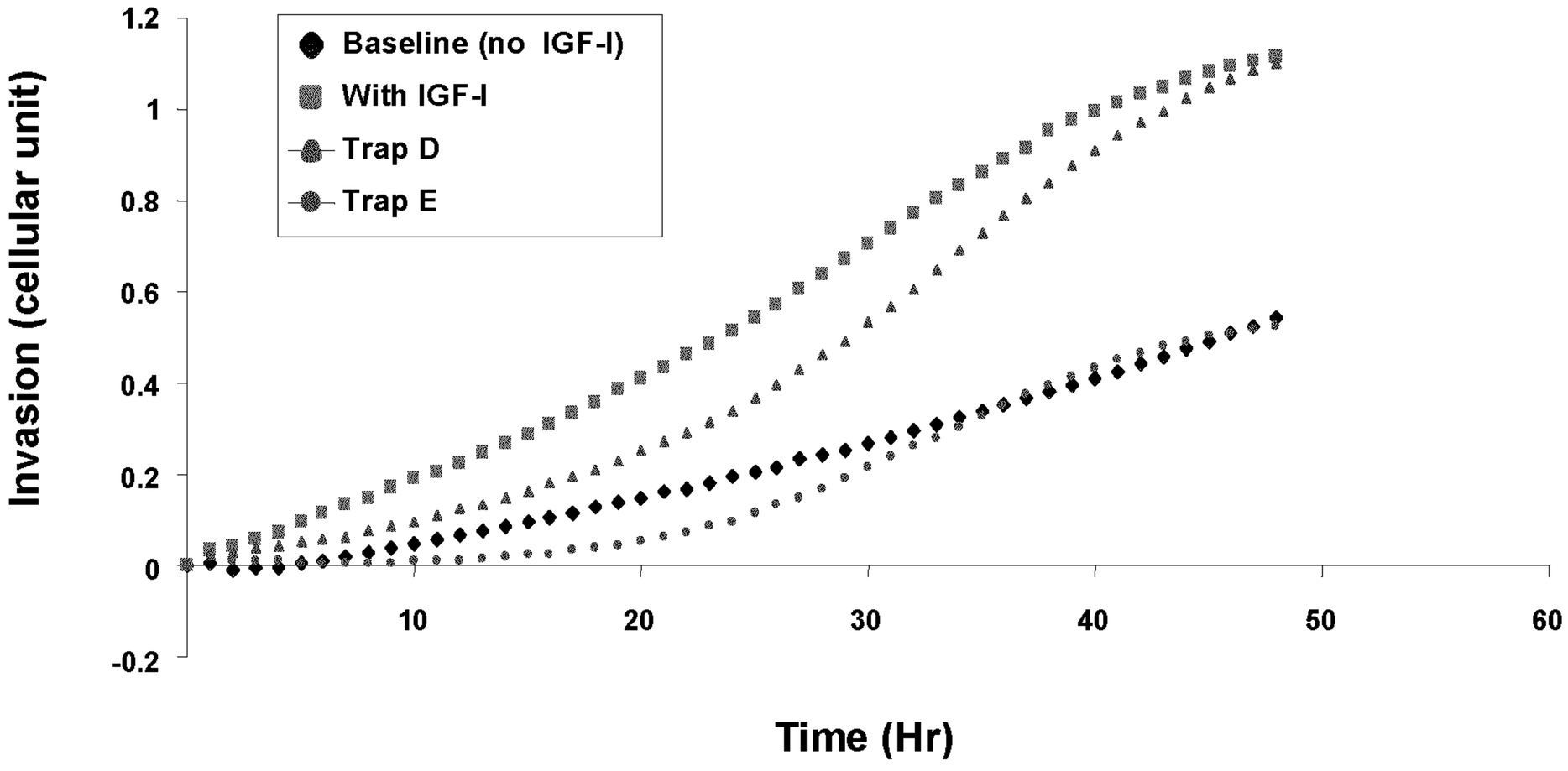
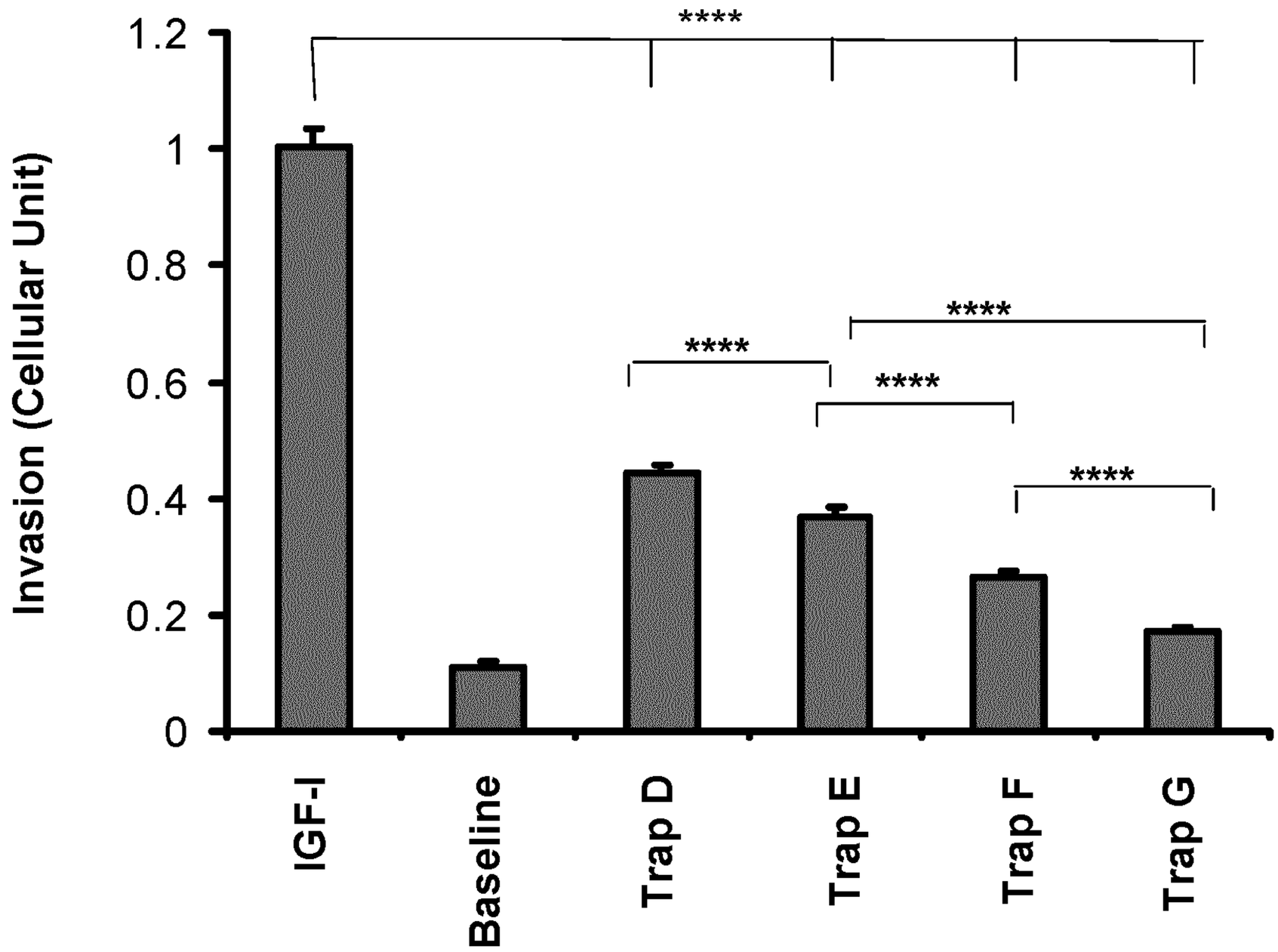

Baseline (no IGF-I)
With IGF-I
Trap D
Trap E
Invasion (cellular unit)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
10
20
30
40
50
60
Time (Hr)

FIGURE 14

A


****
1.2
1
0.8
0.6
0.4
0.2
0
Invasion (Cellular Unit)
****
****
****
****
IGF-I
Baseline
Trap D
Trap E
Trap F
Trap G

B

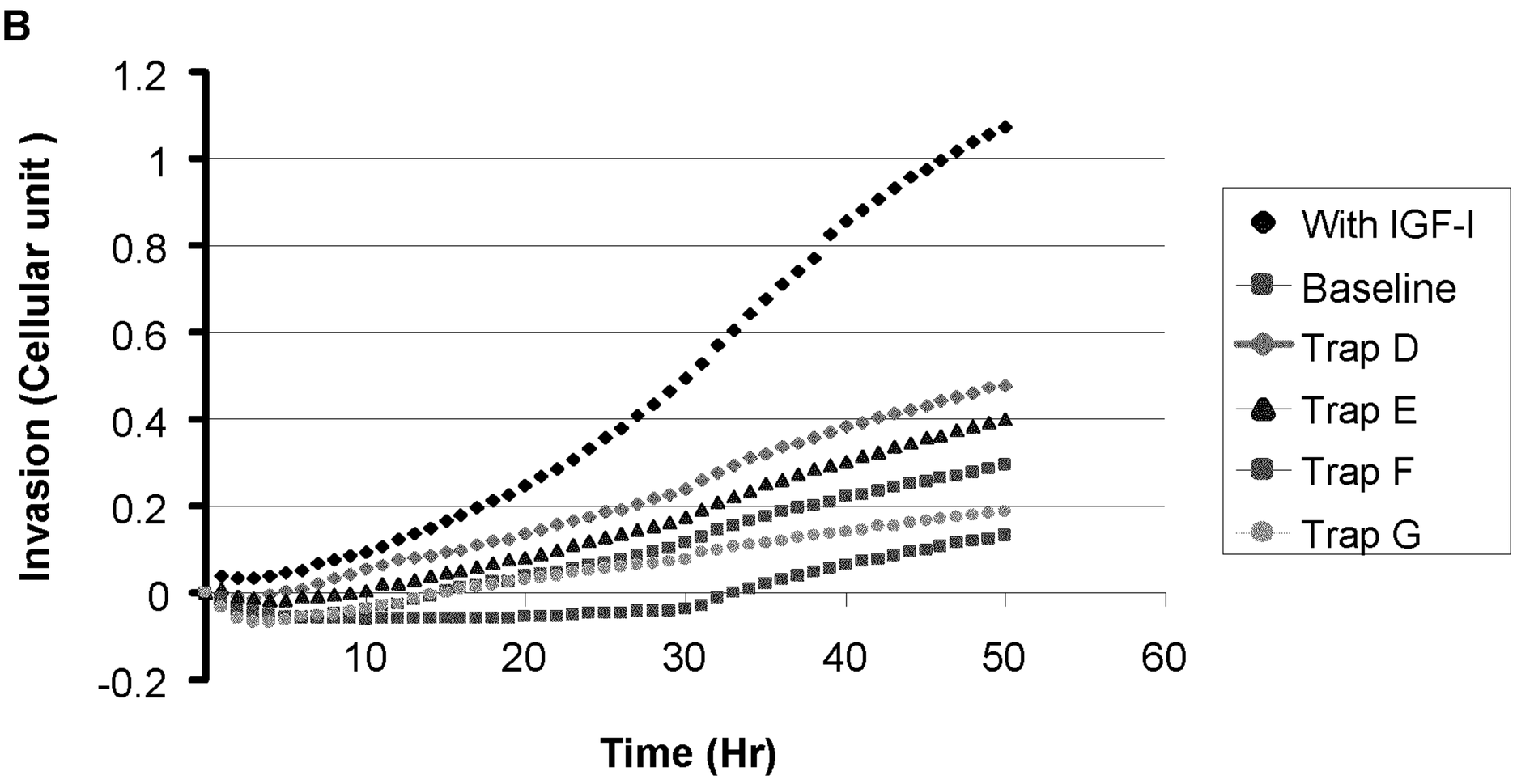
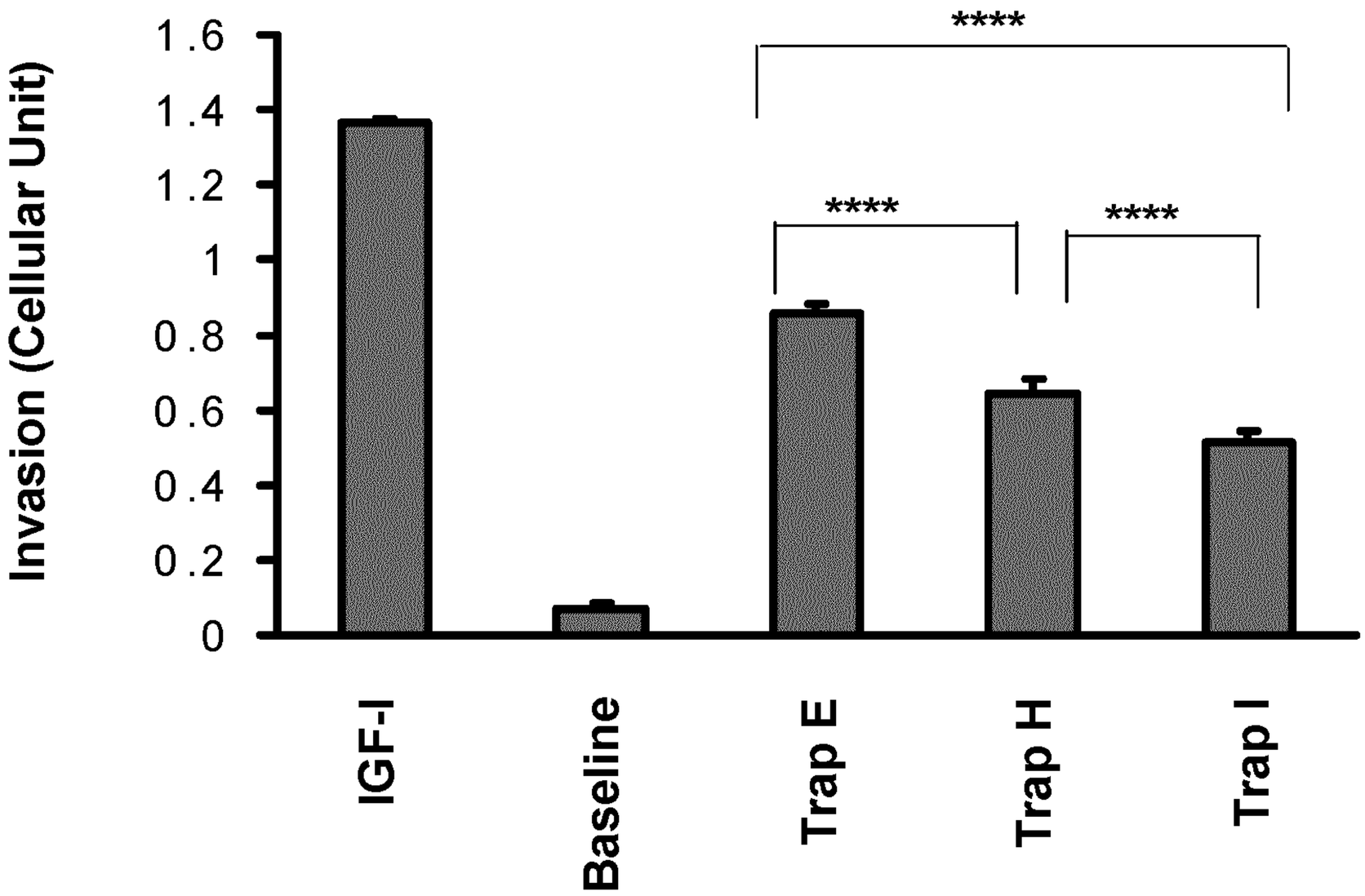

1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Invasion (Cellular unit)
10
20
30
40
50
60
With IGF-I
Baseline
Trap D
Trap E
Trap F
Trap G
Time (Hr)

FIGURE 15

A


Invasion (Cellular Unit)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
****
****
****
IGF-I
Baseline
Trap E
Trap H
Trap I

B

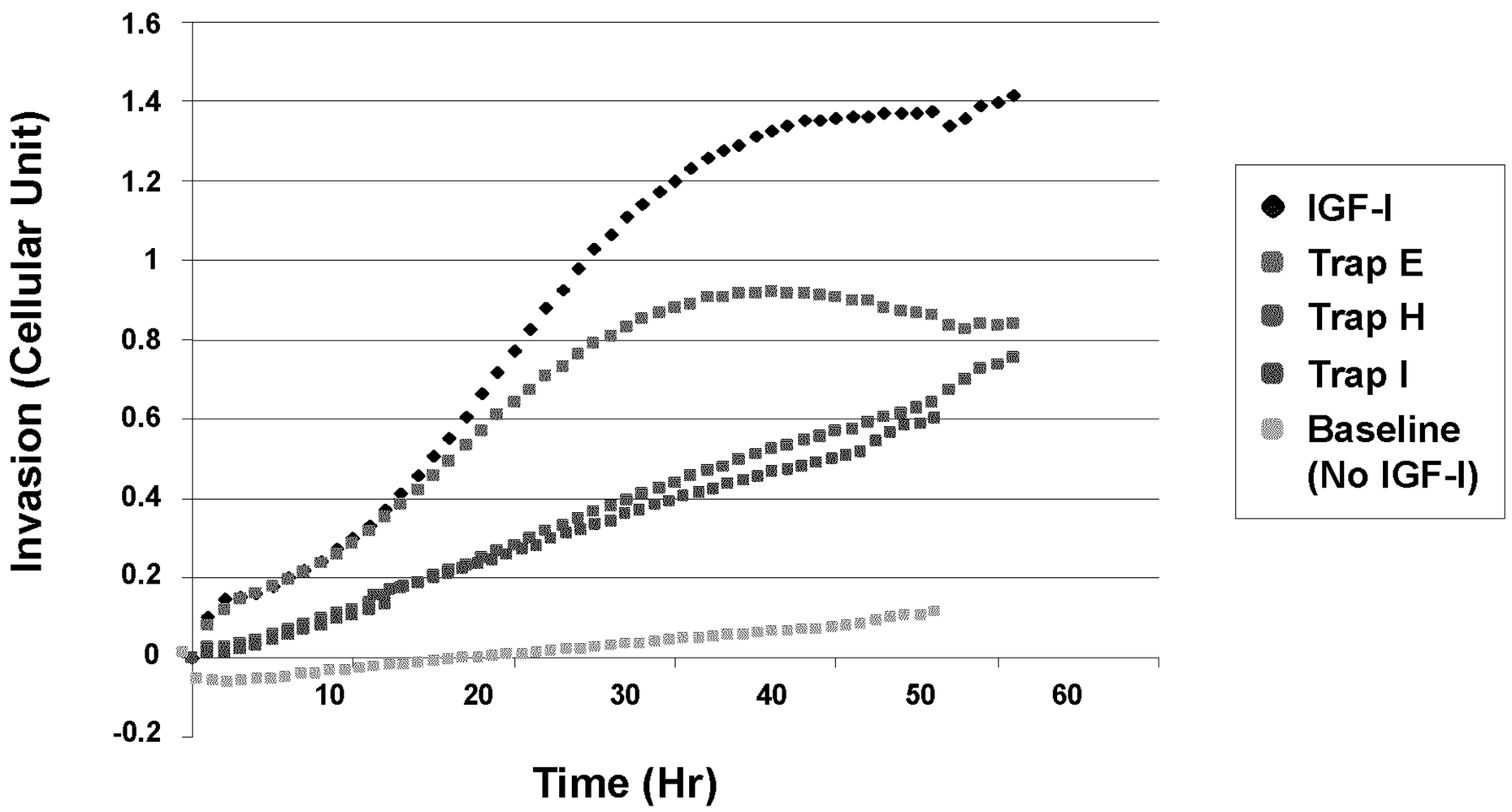

Invasion (Cellular Unit)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
10
20
30
40
50
60
Time (Hr)
IGF-I
Trap E
Trap H
Trap I
Baseline
(No IGF-I)

FIGURE 16

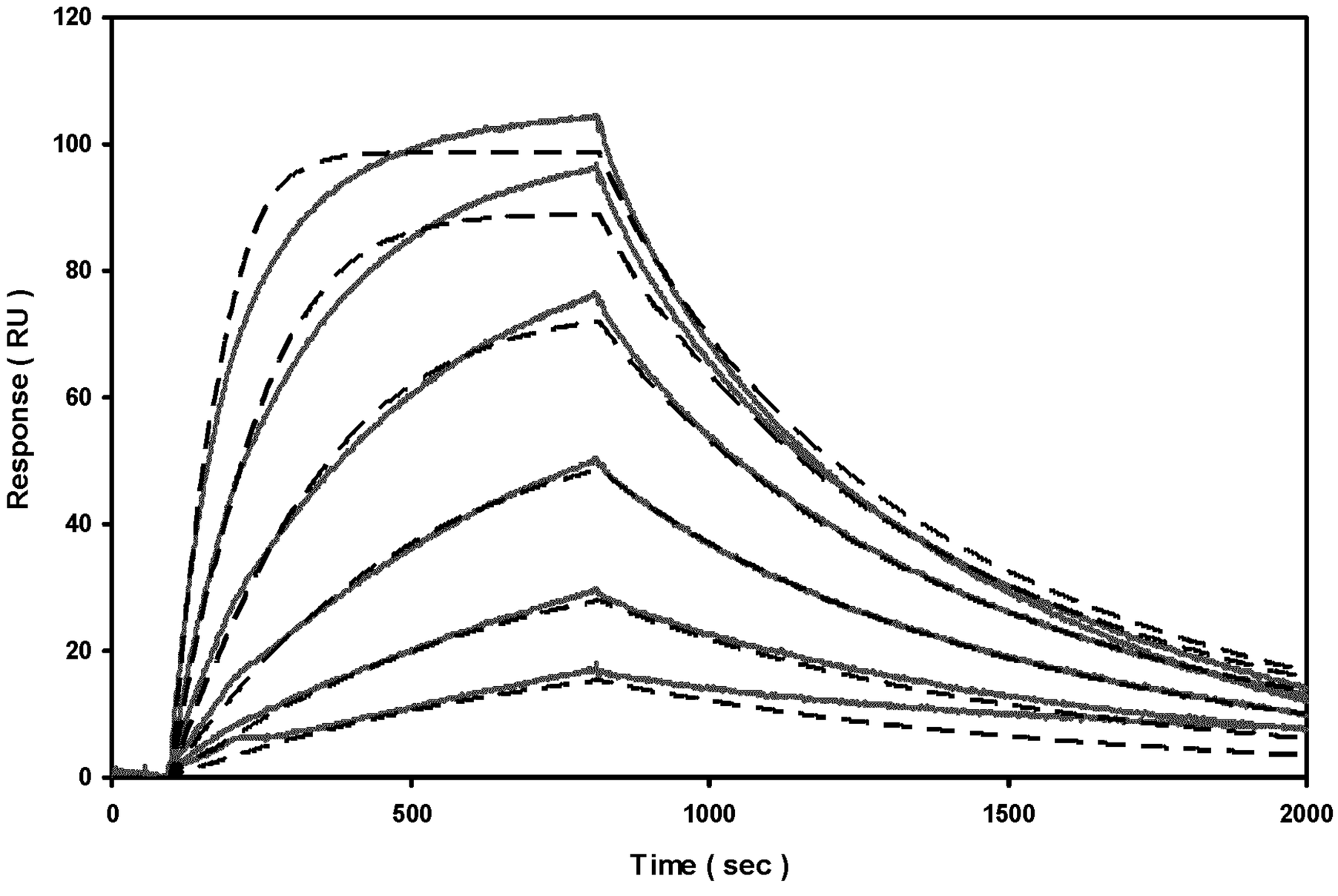


FIGURE 17

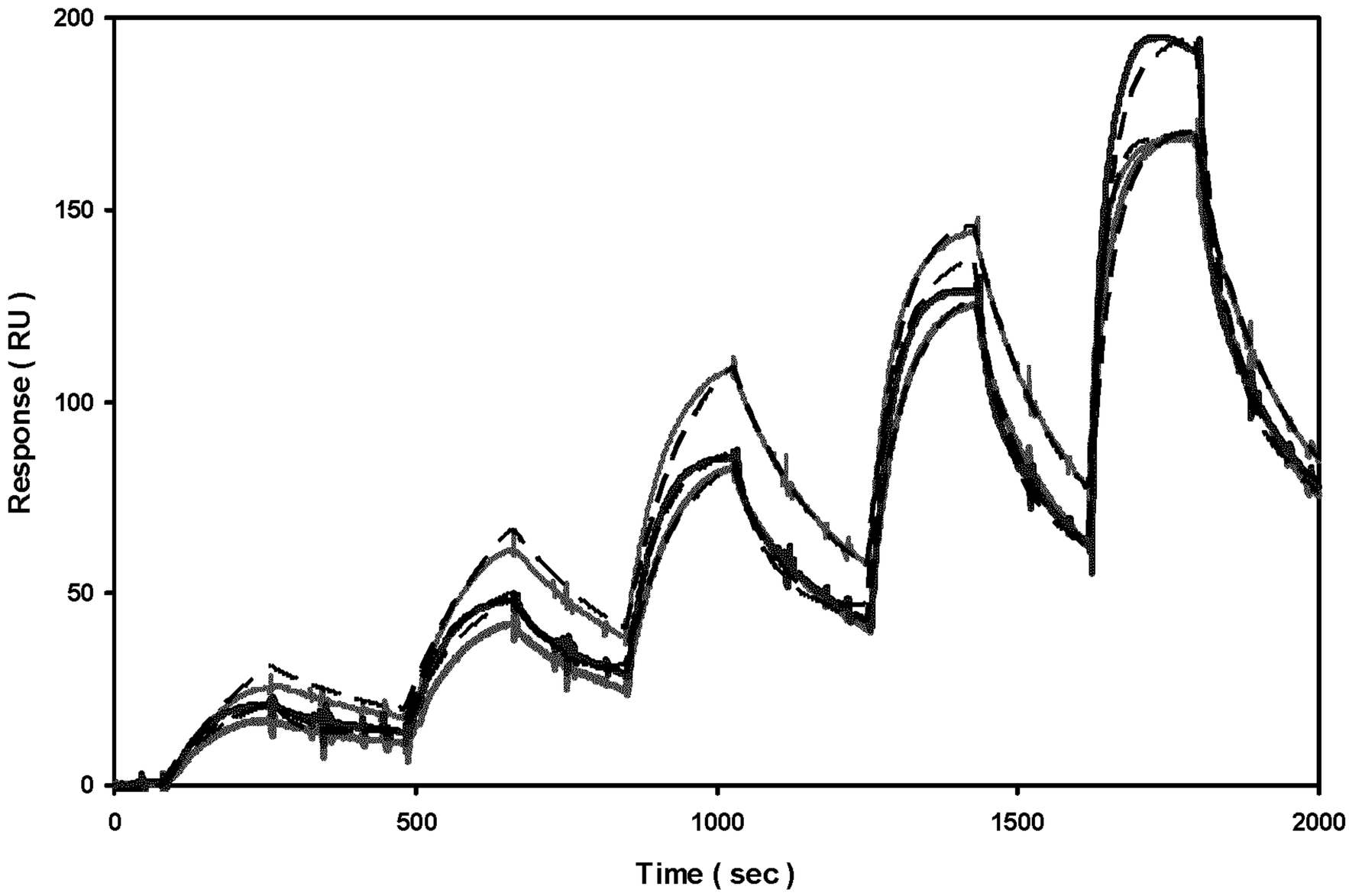
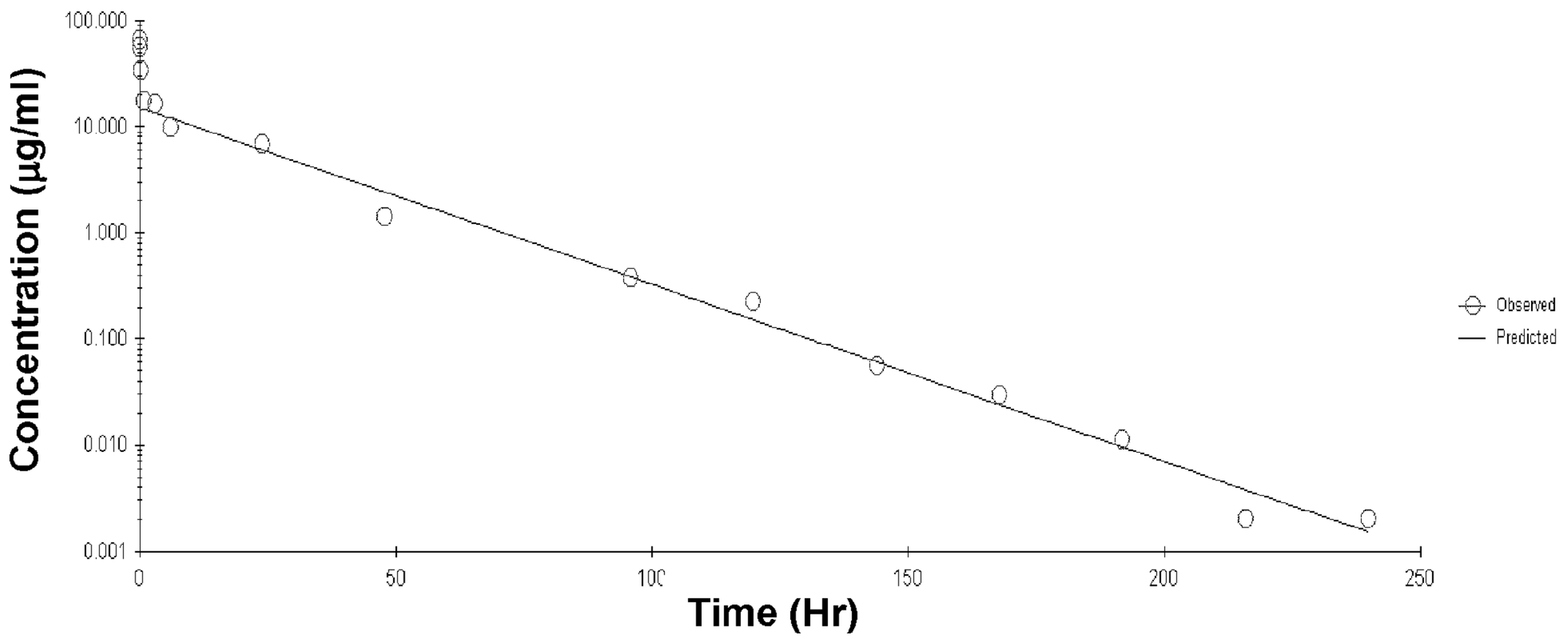


FIGURE 18

A


Concentration (µg/ml)
100.000
10.000
1.000
0.100
0.010
0.001
0
50
100
150
200
250
Time (Hr)
Observed
Predicted

B

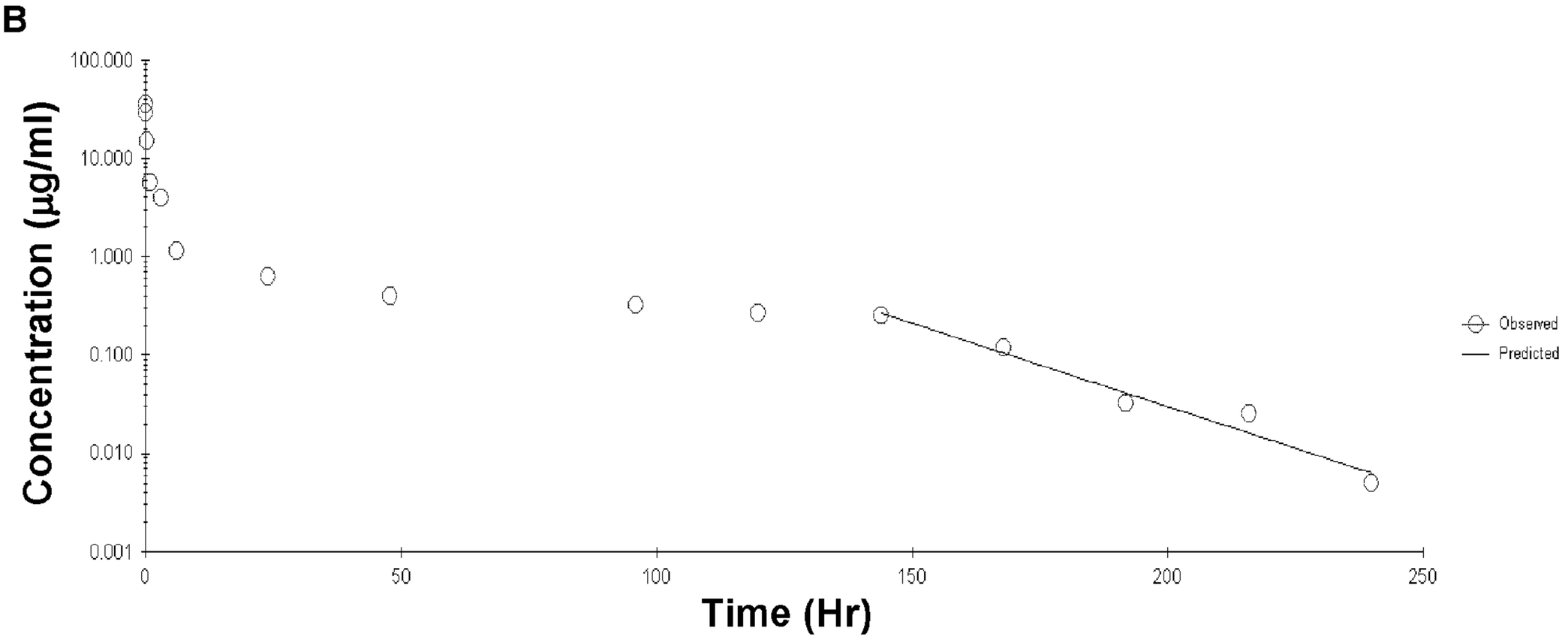
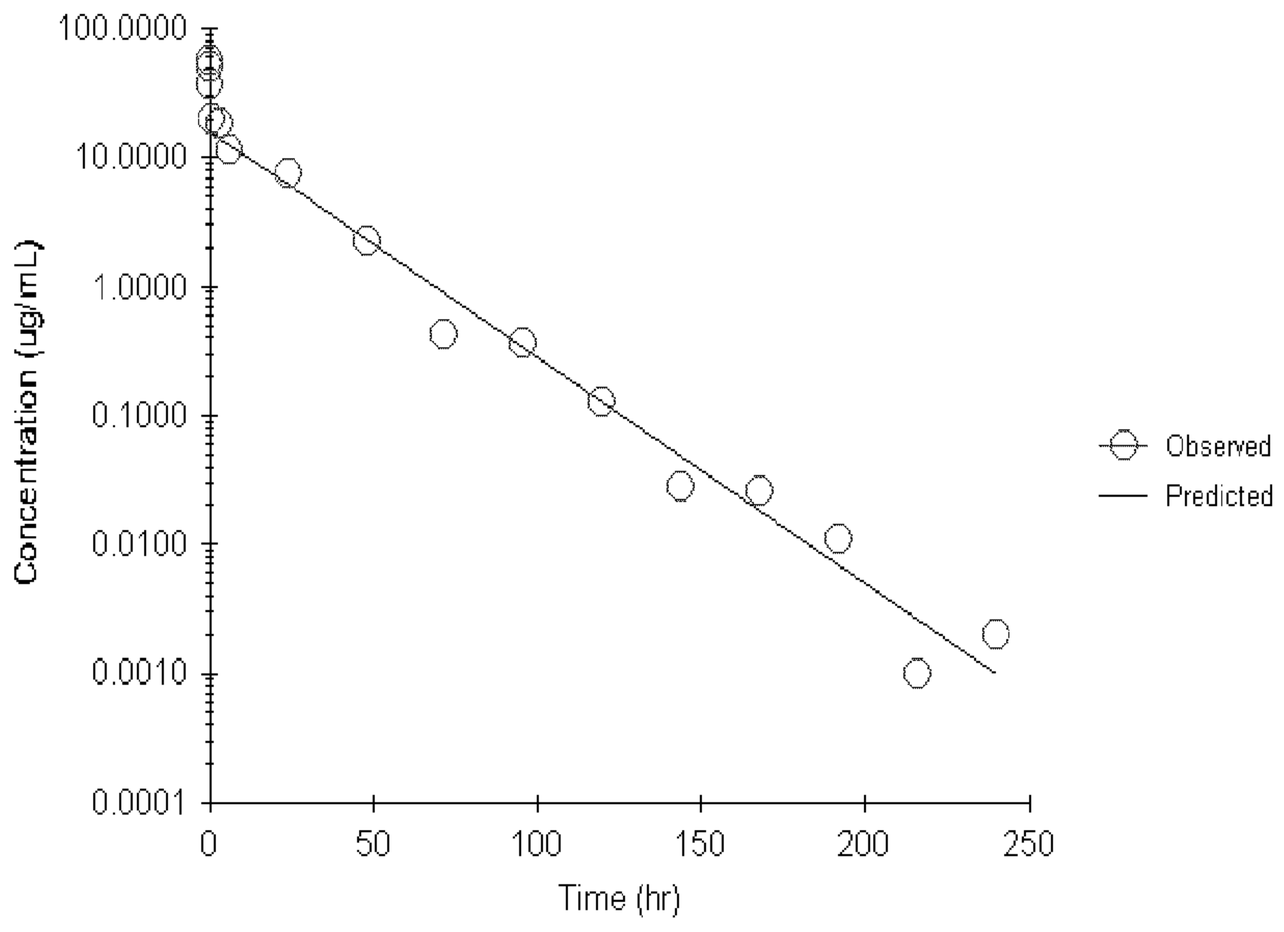

Concentration (µg/ml)
100.000
10.000
1.000
0.100
0.010
0.001
0
50
100
150
200
250
Time (Hr)
Observed
Predicted

FIGURE 19

A


100.0000
10.0000
1.0000
0.1000
0.0100
0.0010
0.0001
Concentration (ug/mL)
0
50
100
150
200
250
Time (hr)
Observed
Predicted

B

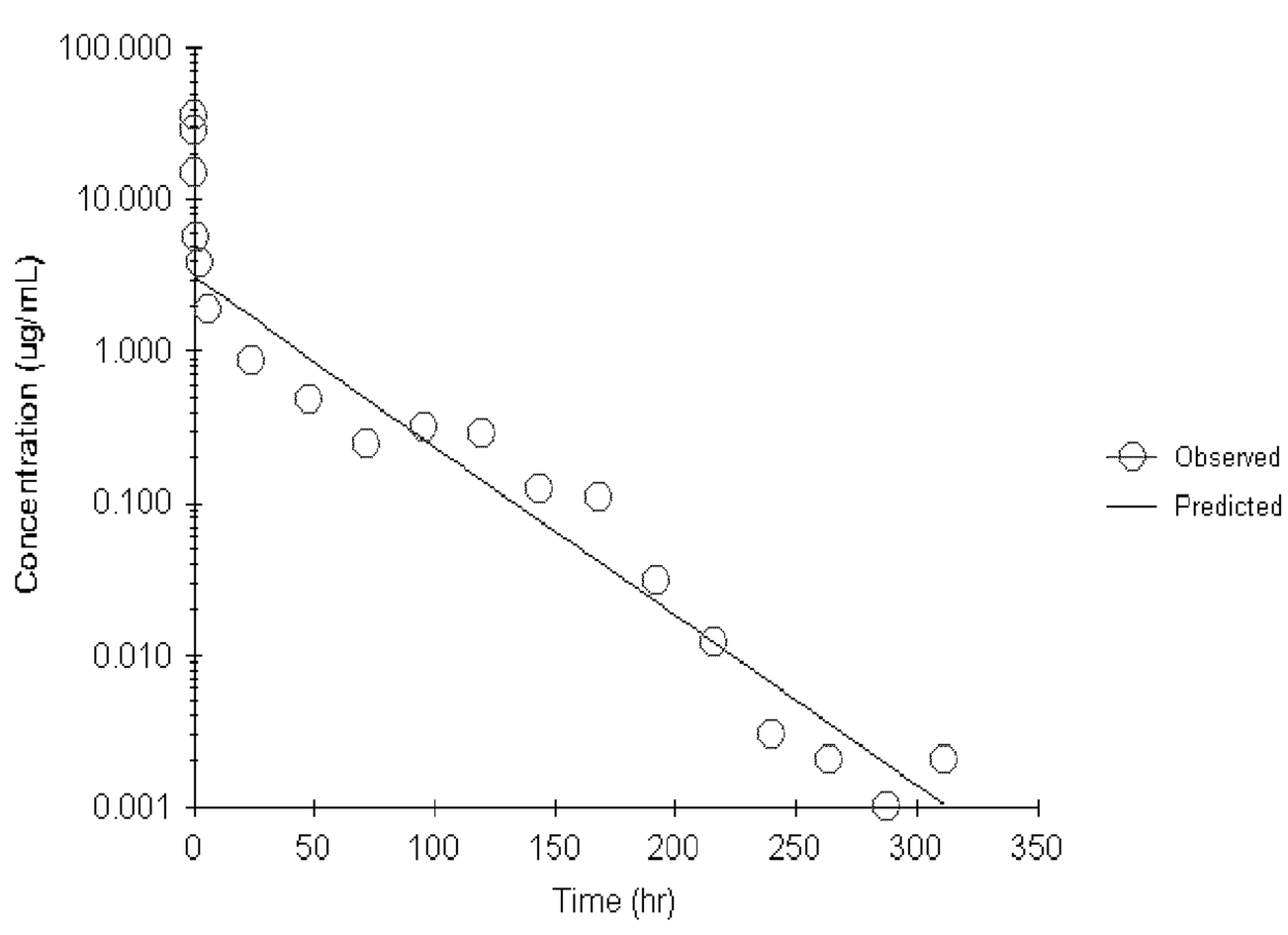
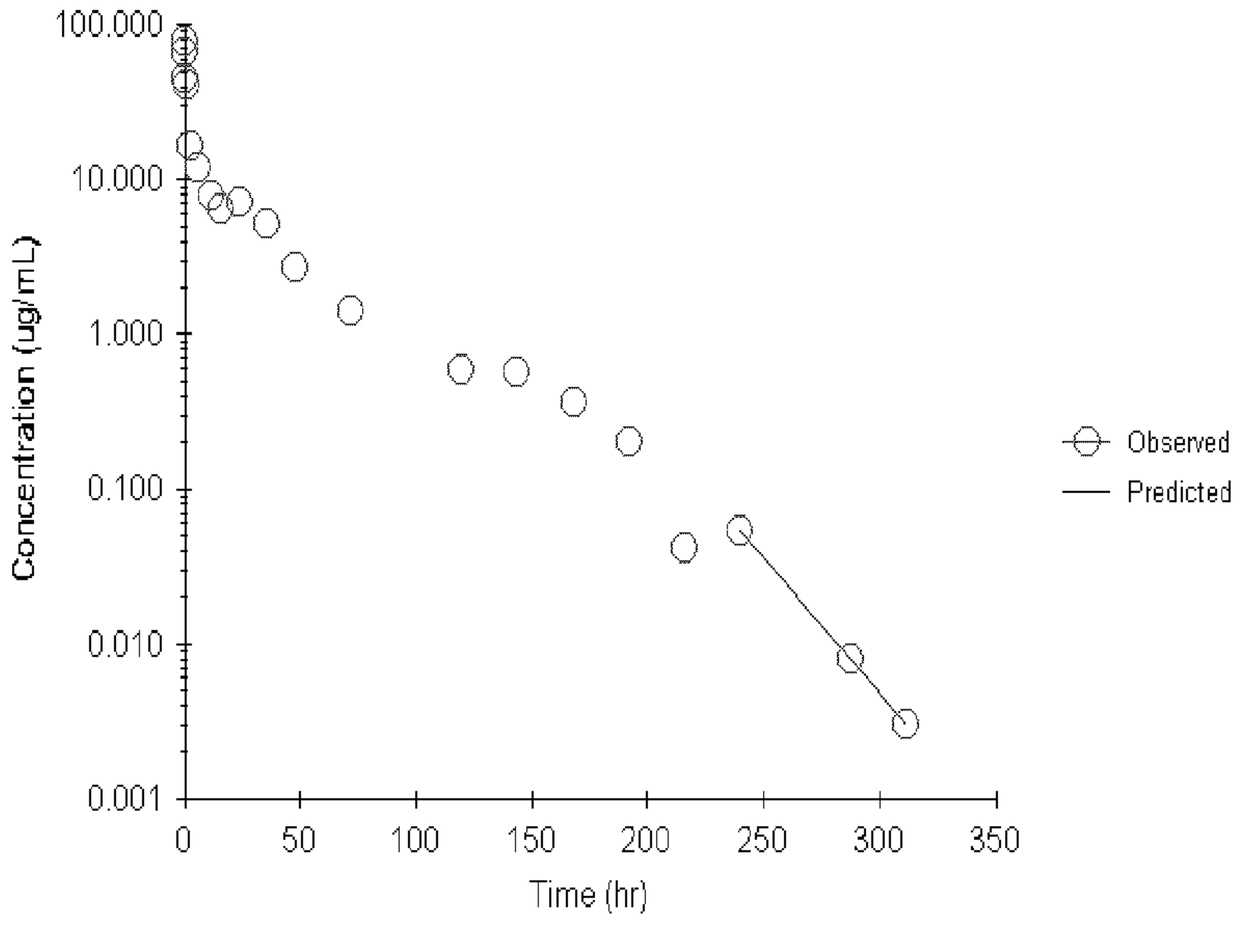

100.000
10.000
1.000
0.100
0.010
0.001
Concentration (ug/mL)
0
50
100
150
200
250
300
350
Time (hr)
Observed
Predicted

FIGURE 19 CONTINUED

C



D

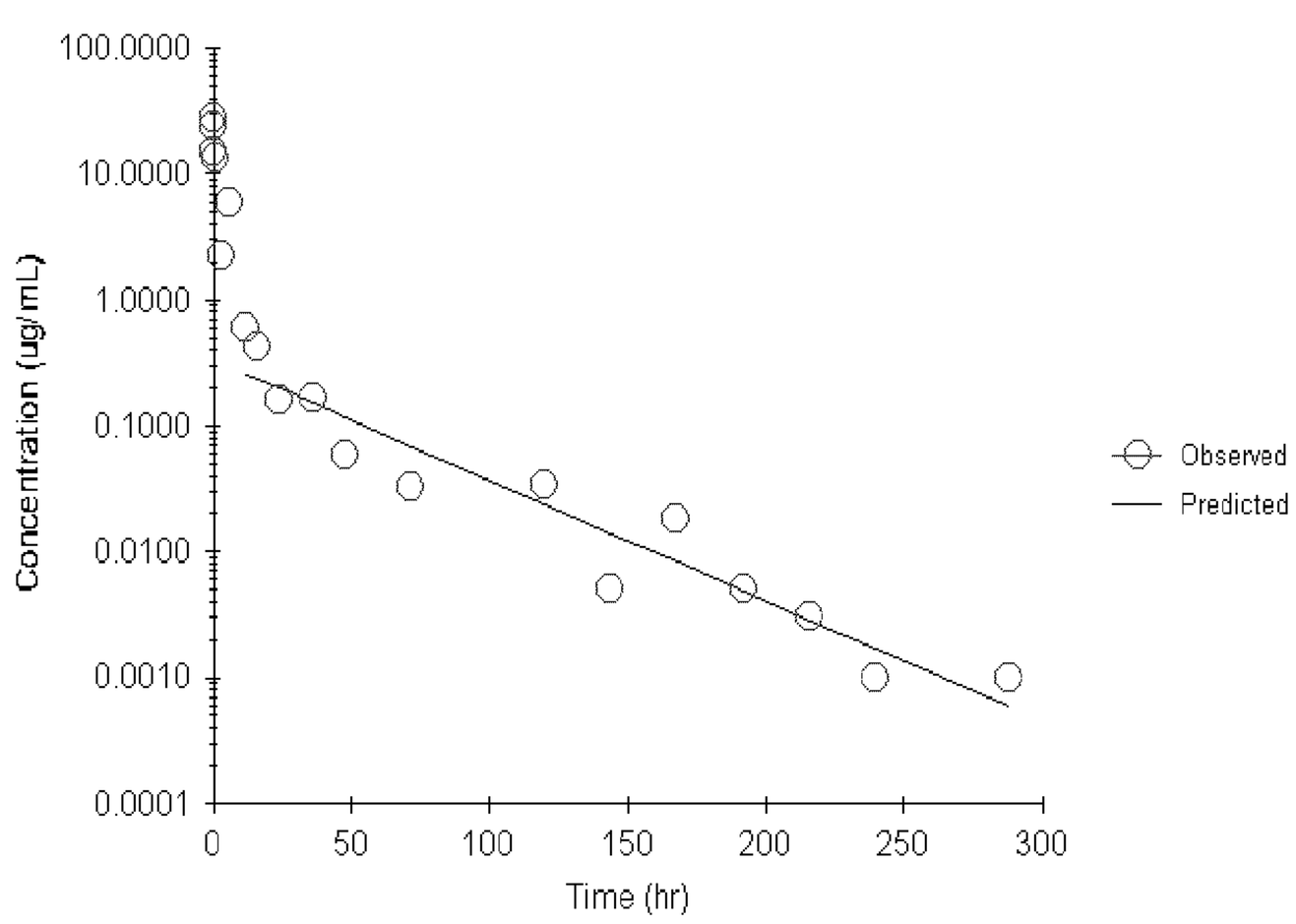


FIGURE 20

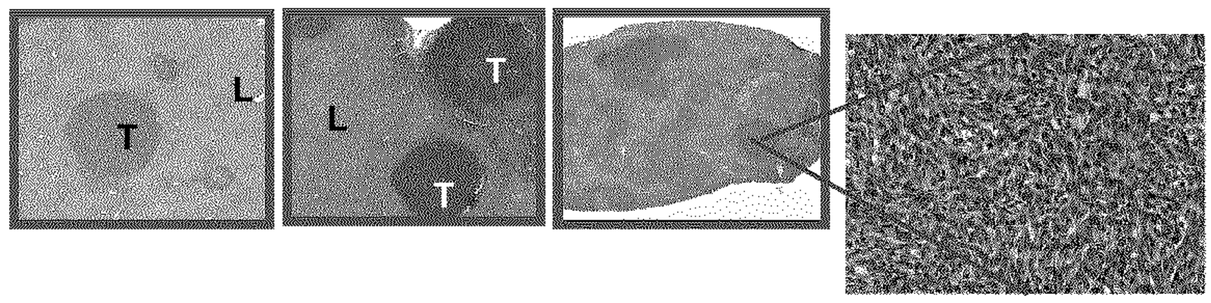
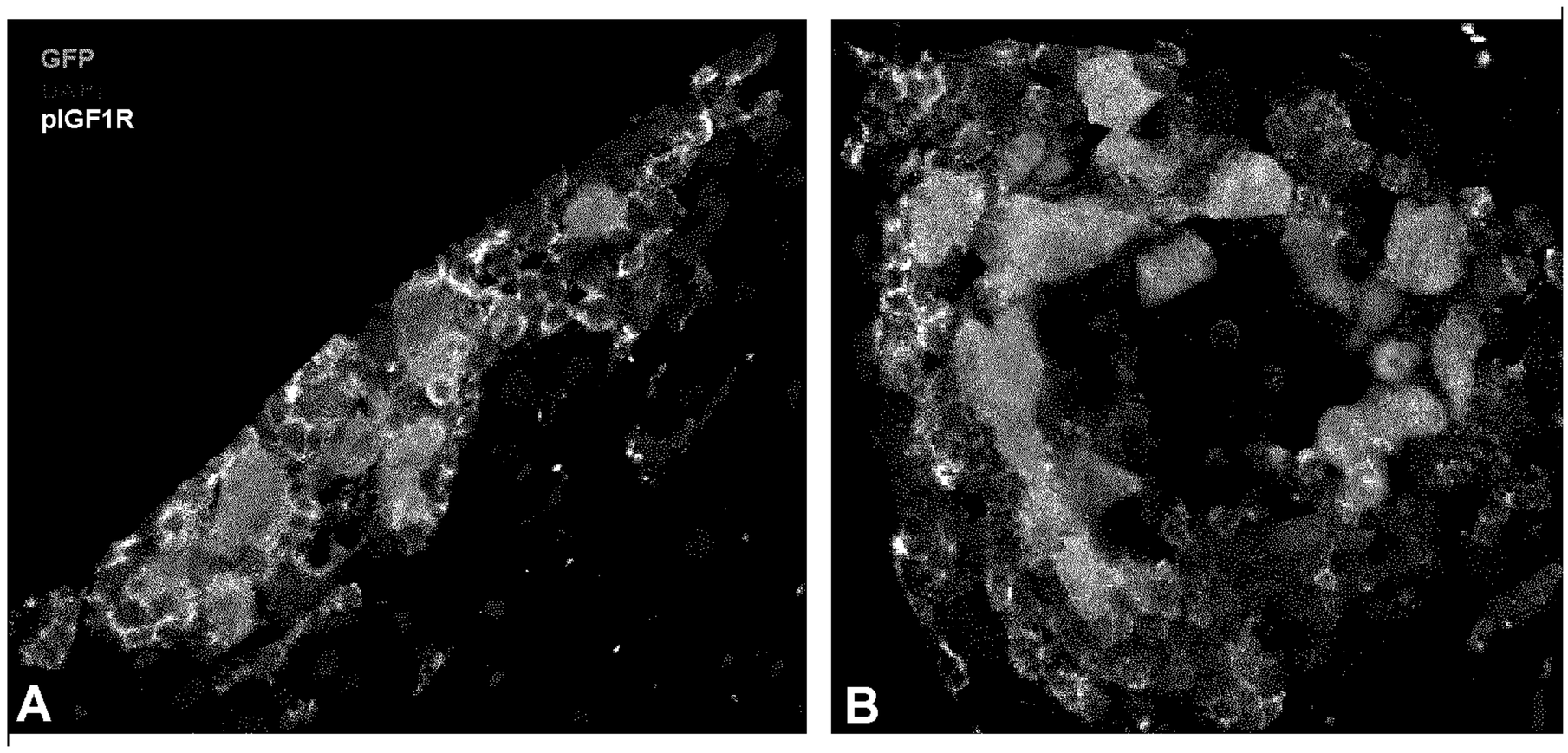


FIGURE 21

A



Non-treated Trap- treated

B

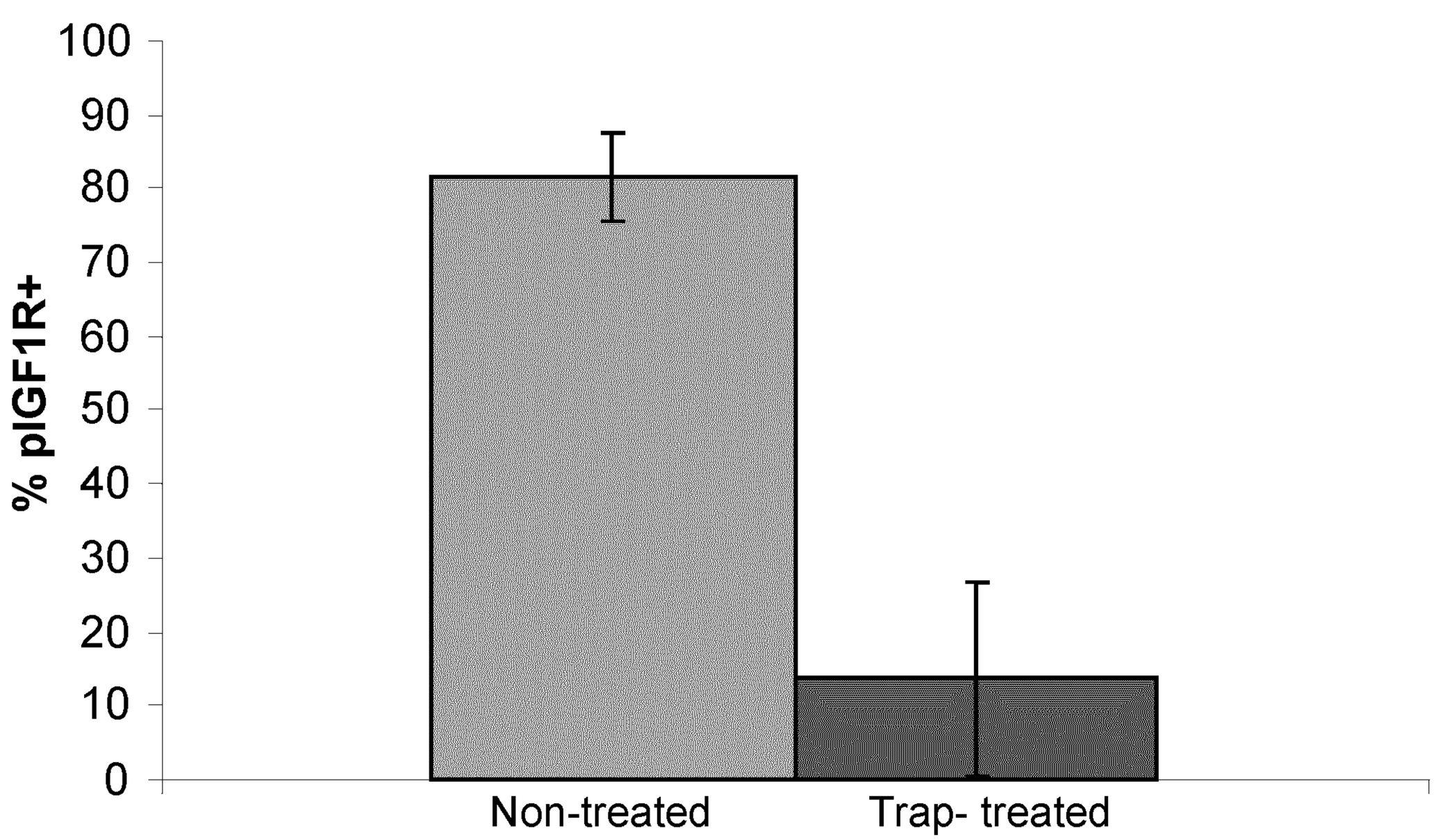
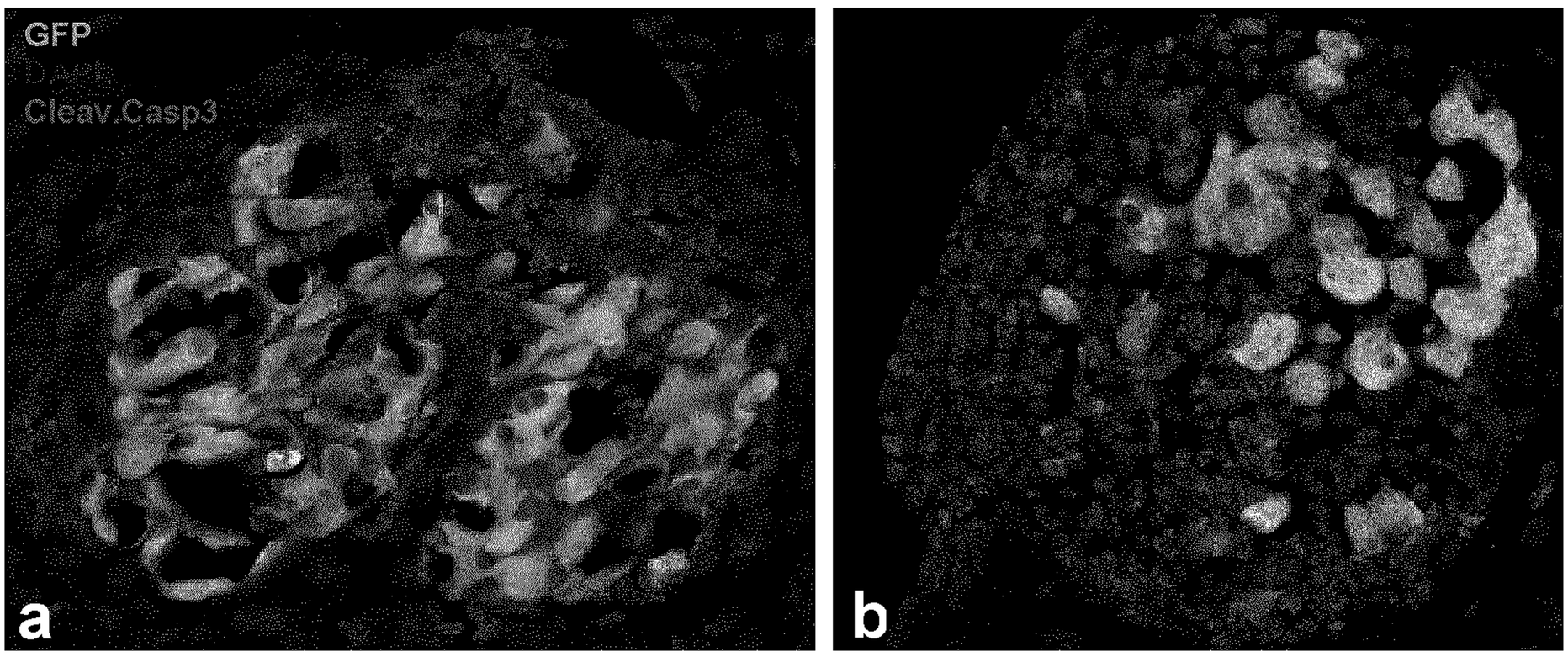


FIGURE 22

A



Non-treated IGF-Trap -treated

B

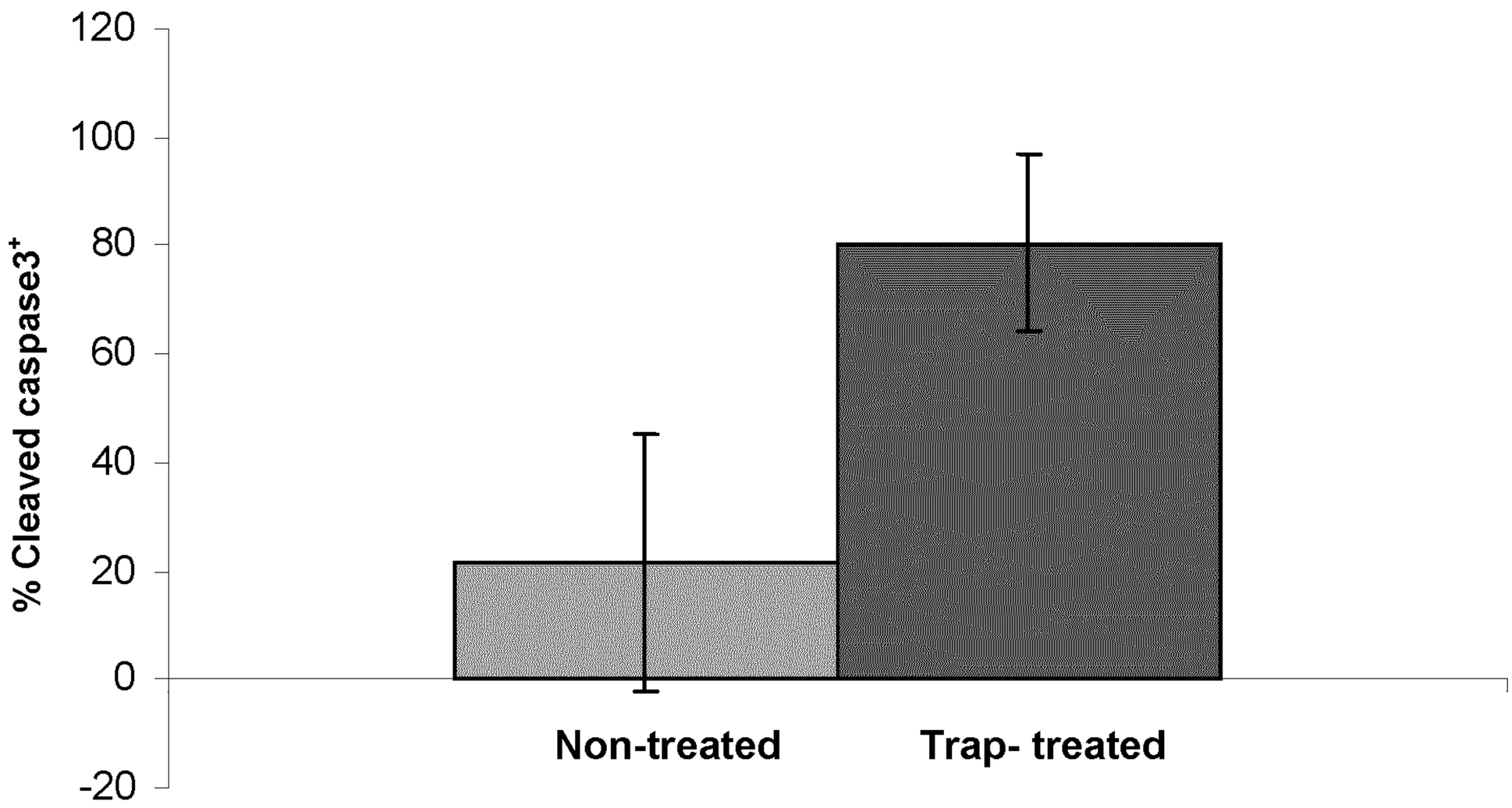
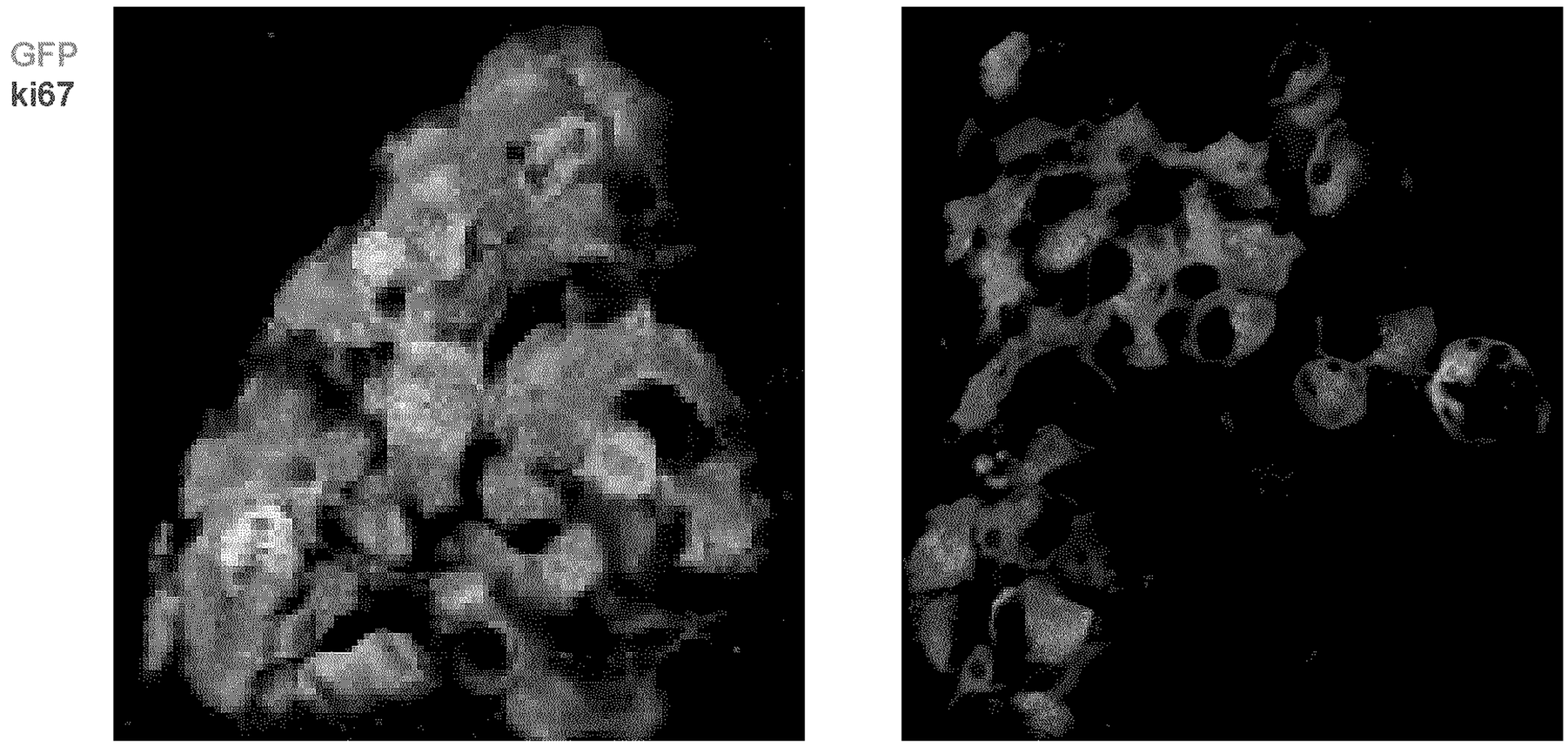


FIGURE 23

A


GFP
ki67
Non- treated
IGF- Trap- treated

B

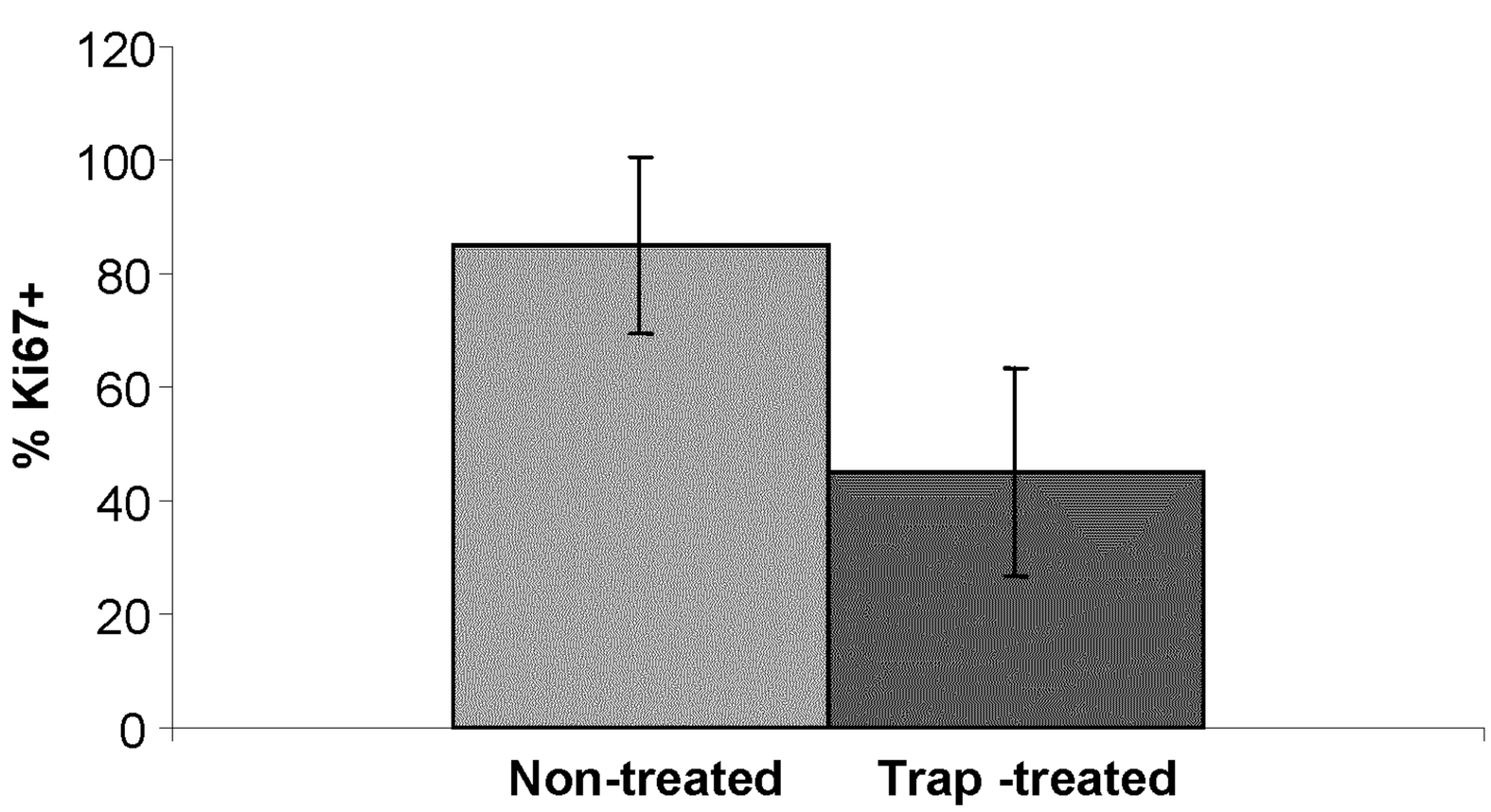
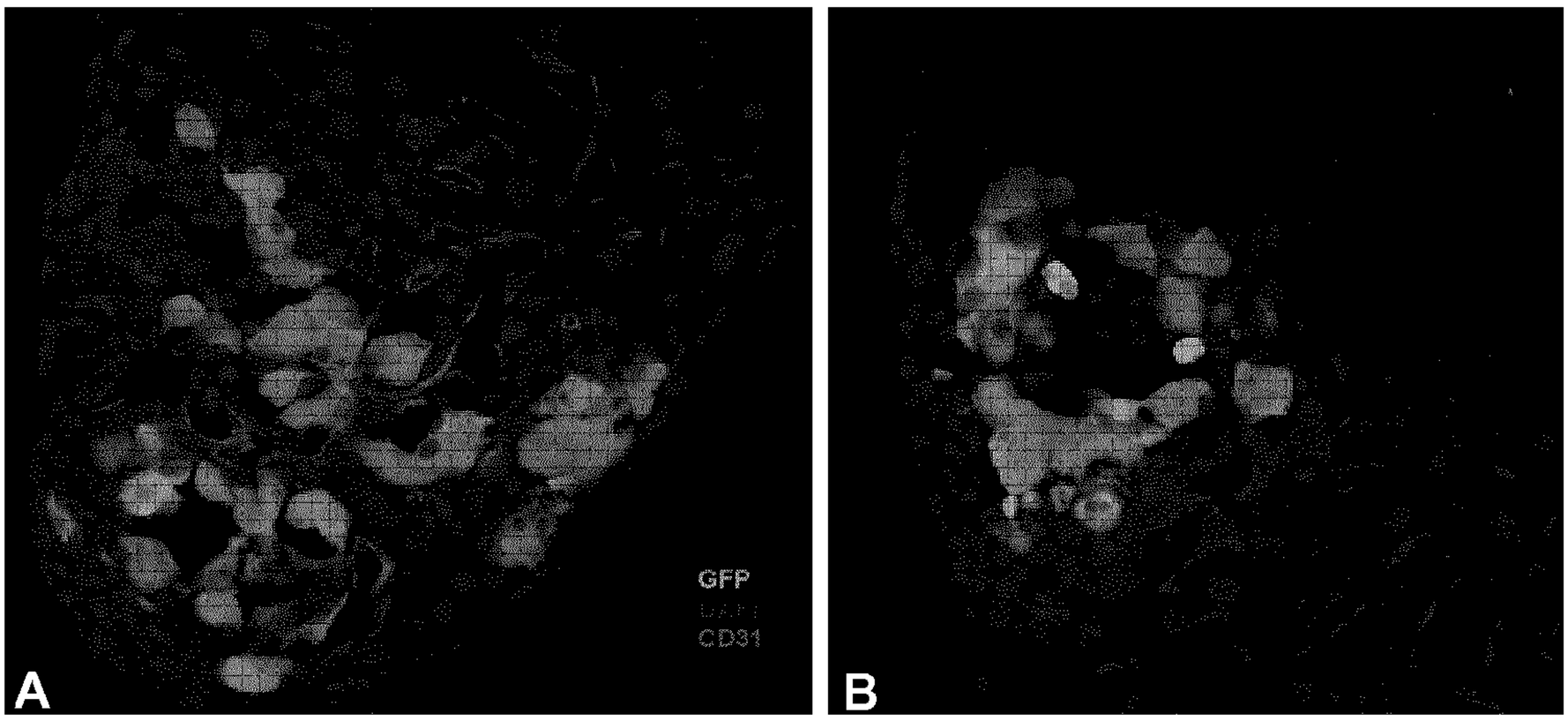

120
100
80
60
40
20
0
% Ki67+
Non-treated
Trap -treated

FIGURE 24

A



Non- treated IGF-Trap- Treated

B

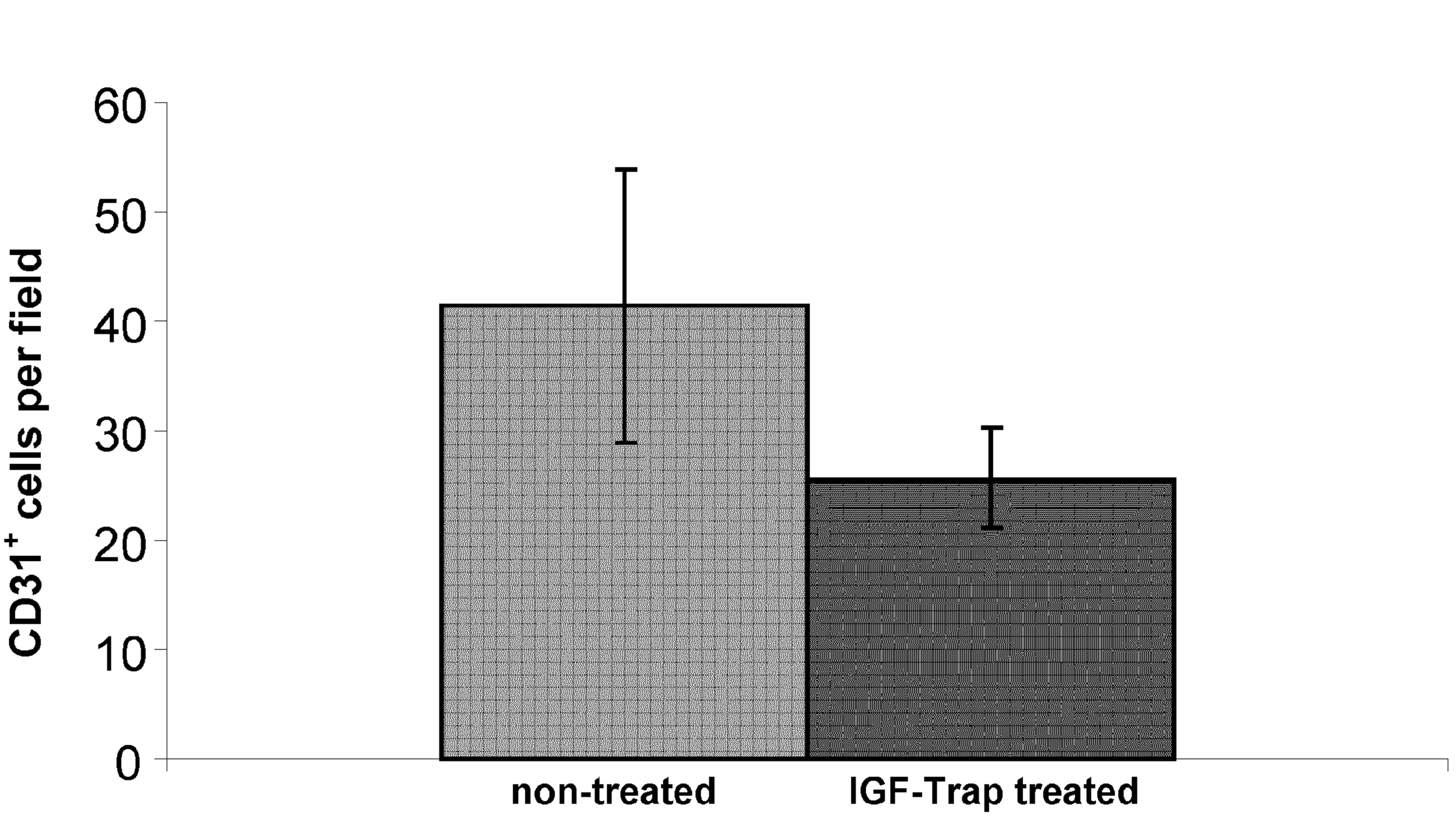
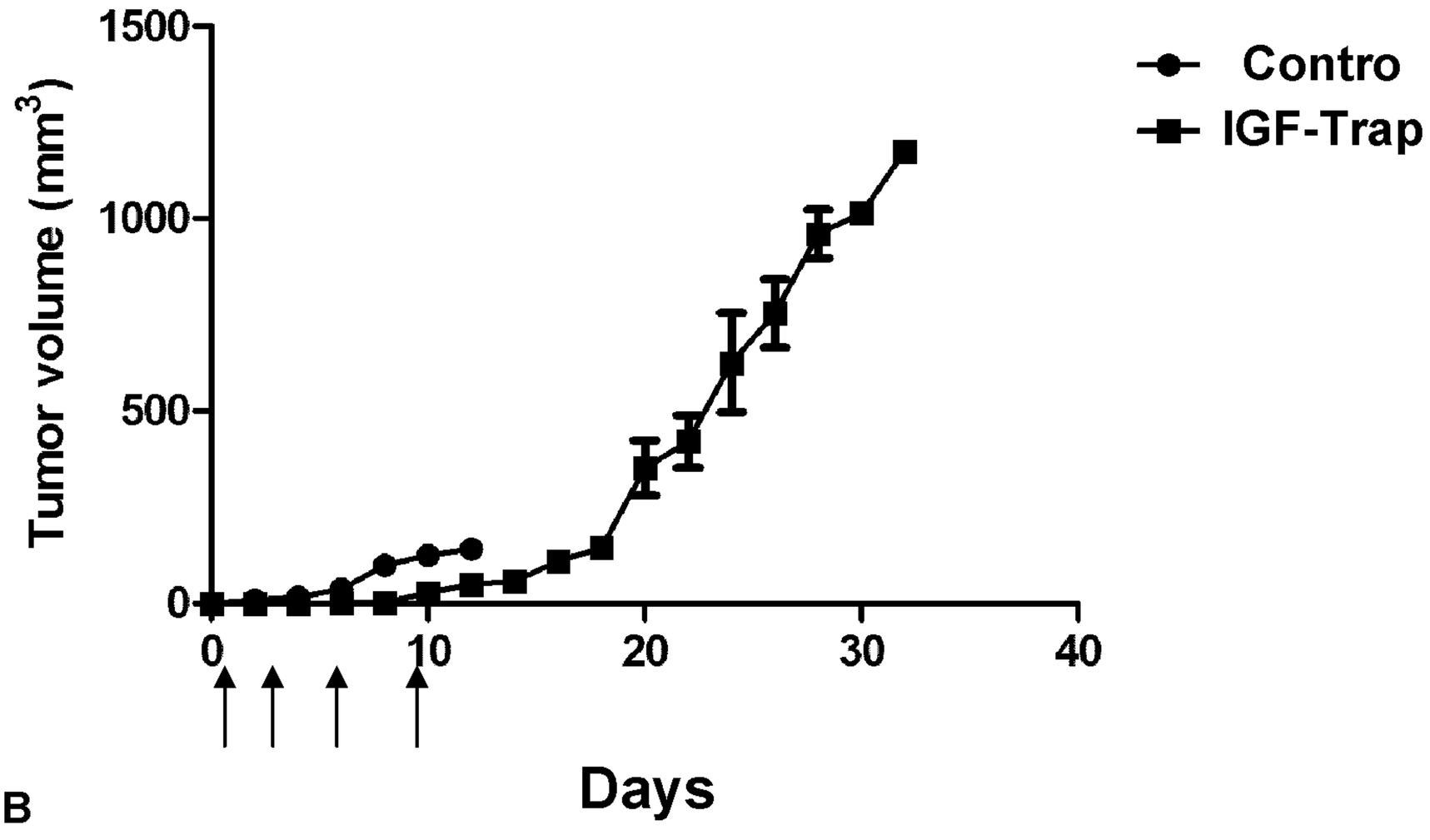


FIGURE 25

A


1500
1000
500
0
Tumor volume (mm3)
0
10
20
30
40
Days
Contro
IGF-Trap

B

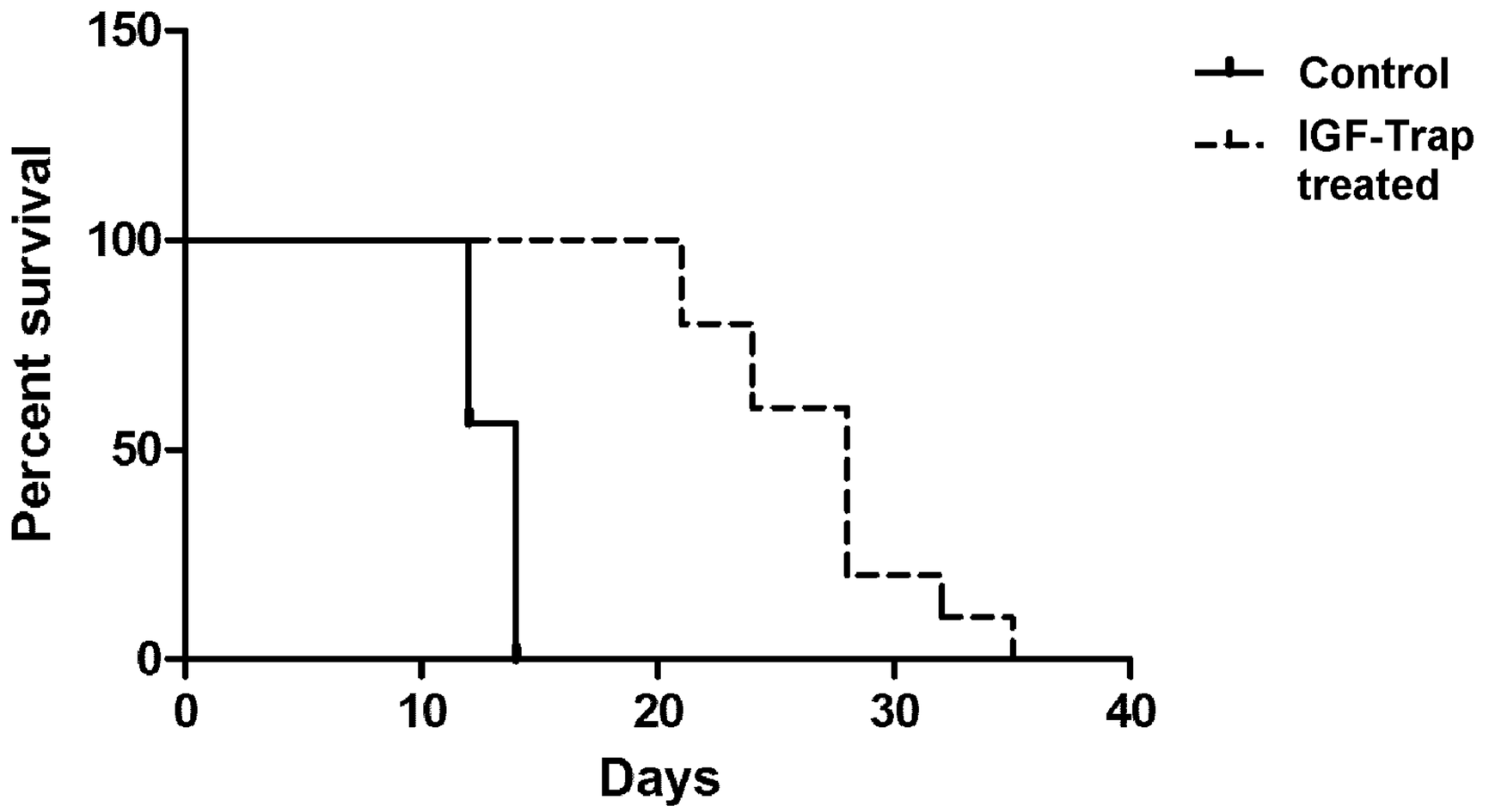
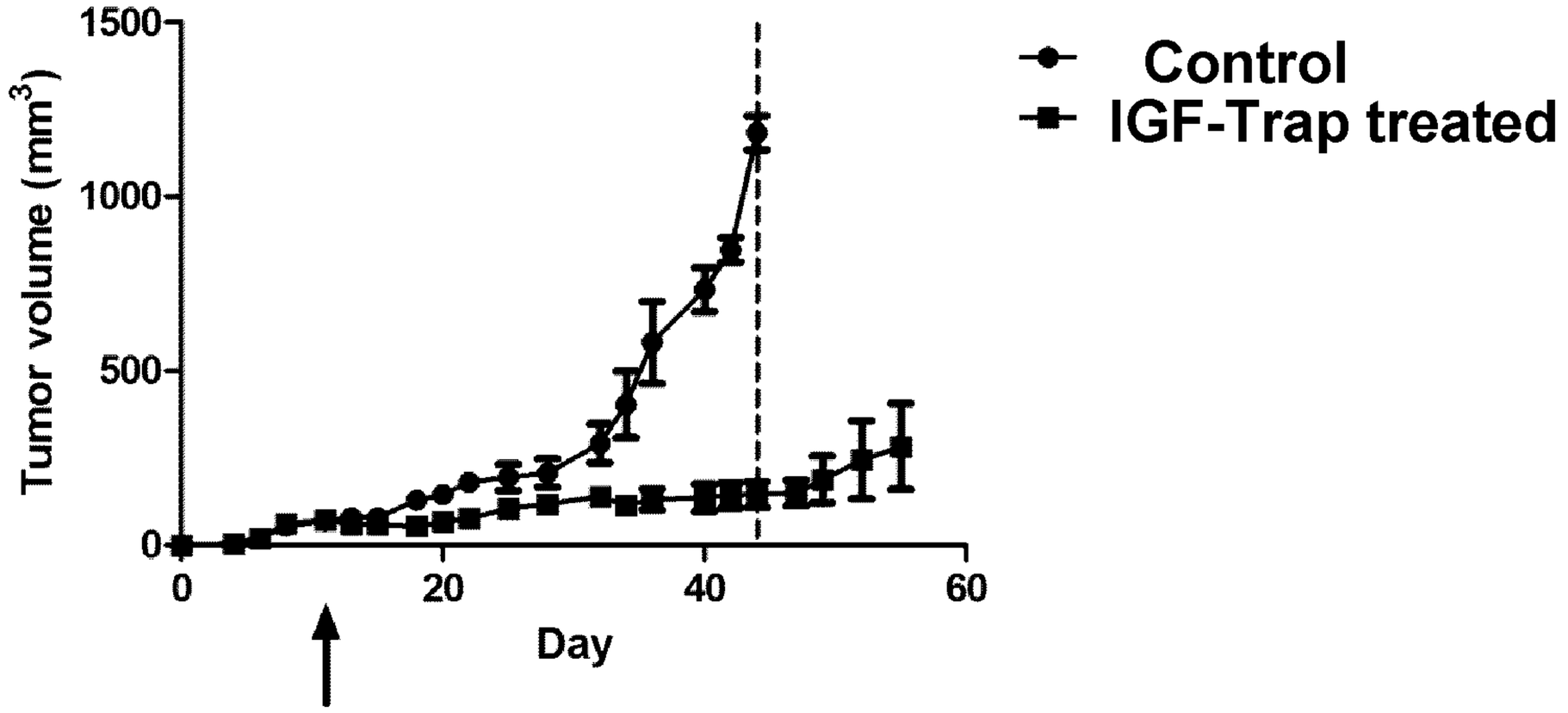

150
100
50
0
Percent survival
0
10
20
30
40
Days
Control
IGF-Trap treated

FIGURE 26
A


1500
1000
500
0
Tumor volume (mm3)
0
20
40
60
Day
Control
IGF-Trap treated

B

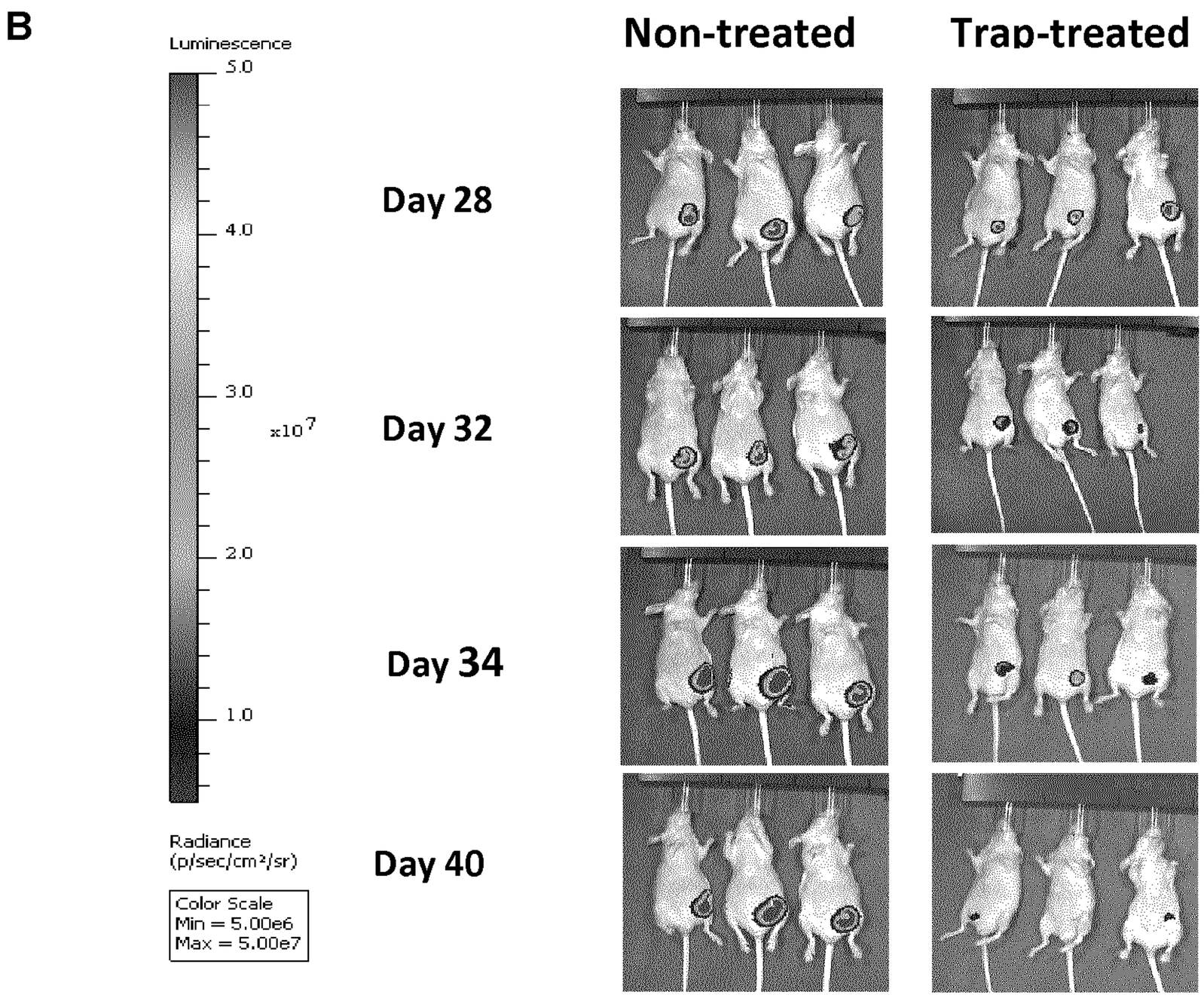

Luminescence
5.0
4.0
3.0
x10 7
2.0
1.0
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 5.00e6
Max = 5.00e7
Non-treated
Trap-treated
Day 28
Day 32
Day 34
Day 40

FIGURE 26 CONTINUED

C

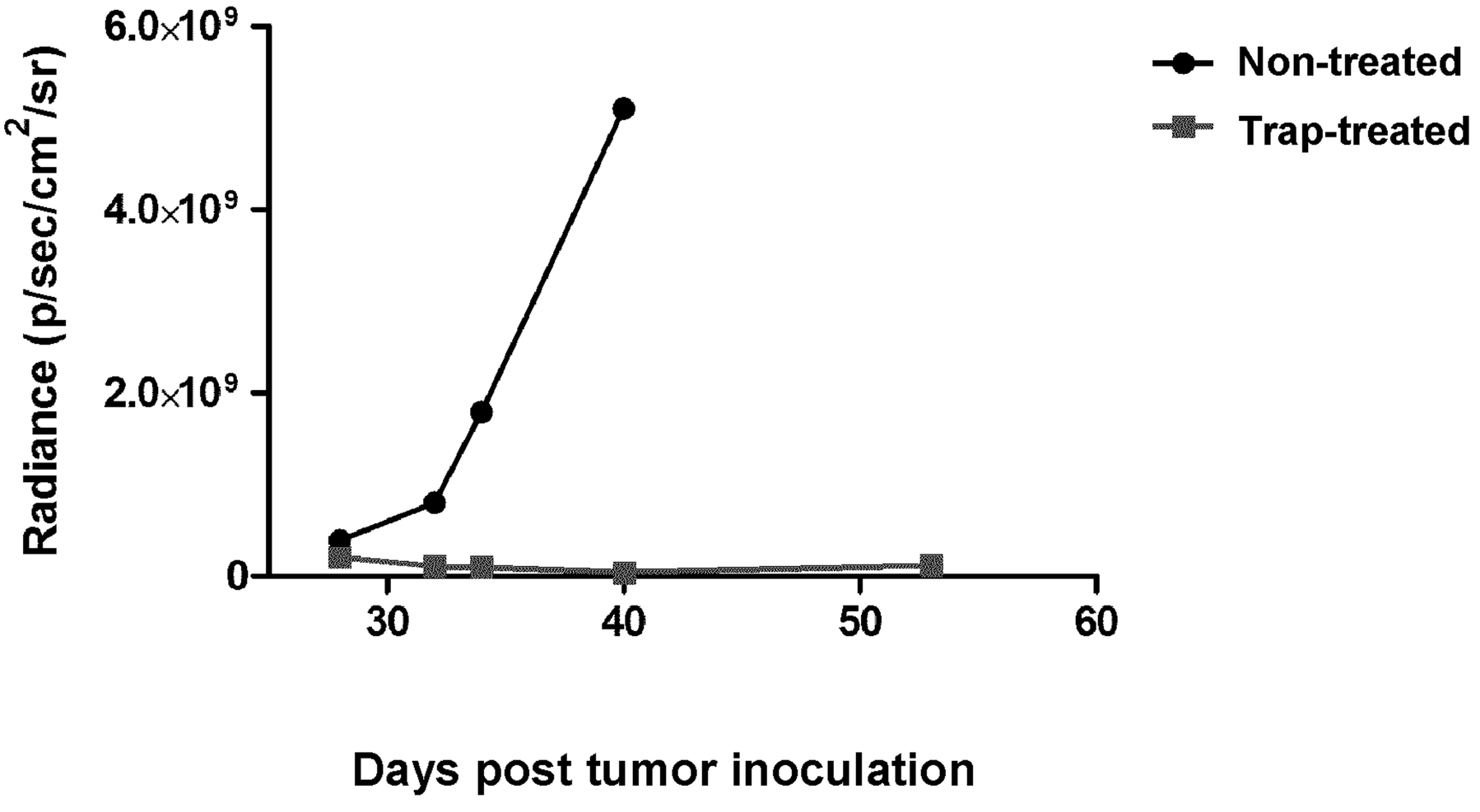


FIGURE 27

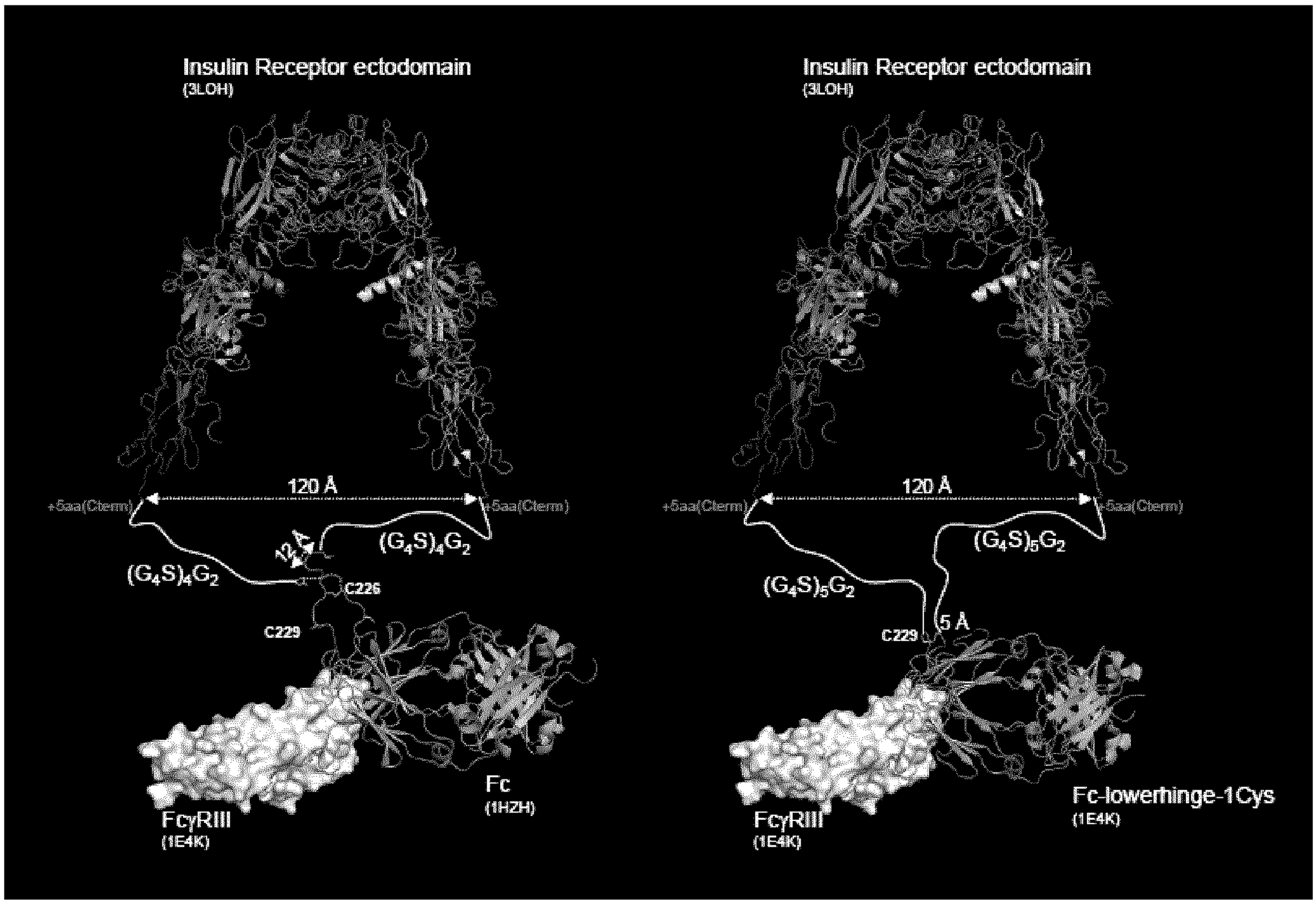

Insulin Receptor ectodomain
(3LOH)
120 Å
+5aa(Cterm)
+5aa(Cterm)
12 Å
(G4S)4G2
(G4S)4G2
C226
C229
Fc
(1HZH)
FcγRIII
(1E4K)
Insulin Receptor ectodomain
(3LOH)
120 Å
+5aa(Cterm)
+5aa(Cterm)
(G4S)5G2
(G4S)5G2
C229
5 Å
Fc-lowerhinge-1Cys
(1E4K)
FcγRIII
(1E4K)

FIGURE 28

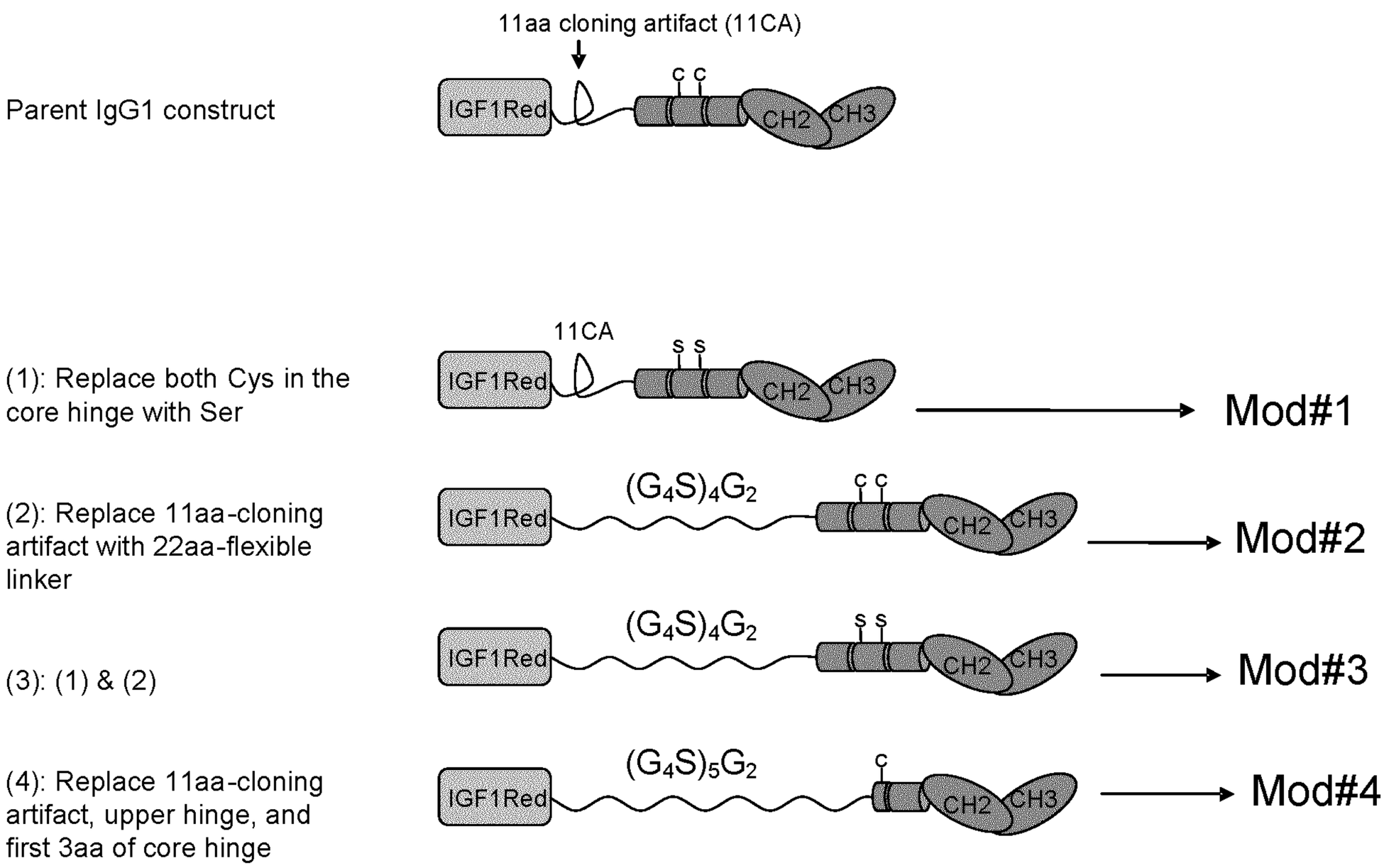

Parent IgG1 construct
11aa cloning artifact (11CA)
IGF1Red
CH2
CH3
(1): Replace both Cys in the core hinge with Ser
11CA
IGF1Red
CH2
CH3
Mod#1
(2): Replace 11aa-cloning artifact with 22aa-flexible linker
(G4S)4G2
IGF1Red
CH2
CH3
Mod#2
(3): (1) & (2)
(G4S)4G2
IGF1Red
CH2
CH3
Mod#3
(4): Replace 11aa-cloning artifact, upper hinge, and first 3aa of core hinge (including first Cys) with 27aa-flexible linker
(G4S)5G2
IGF1Red
CH2
CH3
Mod#4

FIGURE 29

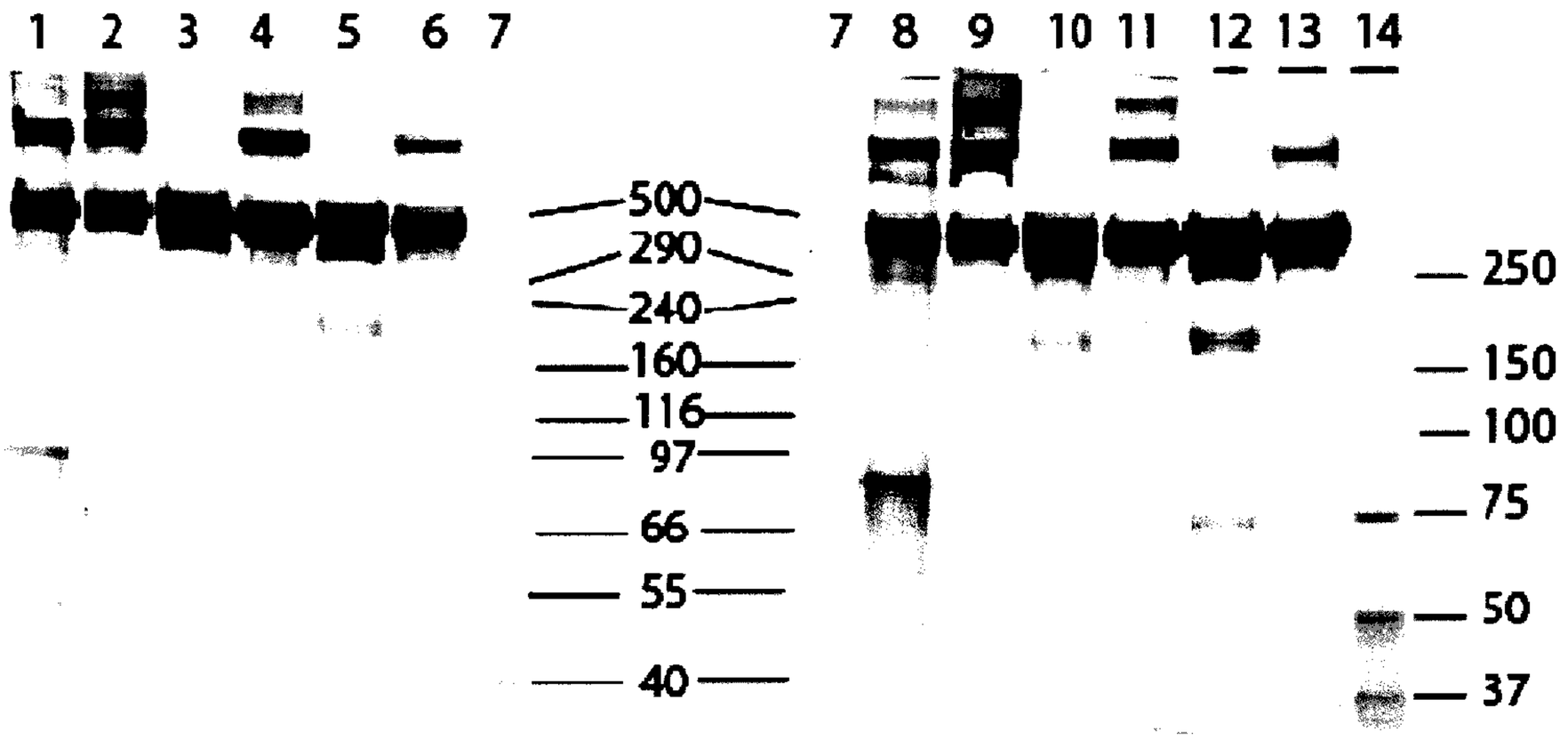

1 2 3 4 5 6 7
7 8 9 10 11 12 13 14
500
290
240
160
116
97
66
55
40
250
150
100
75
50
37
5ug of purified protein/lane
10ug of purified protein/lane

FIGURE 30

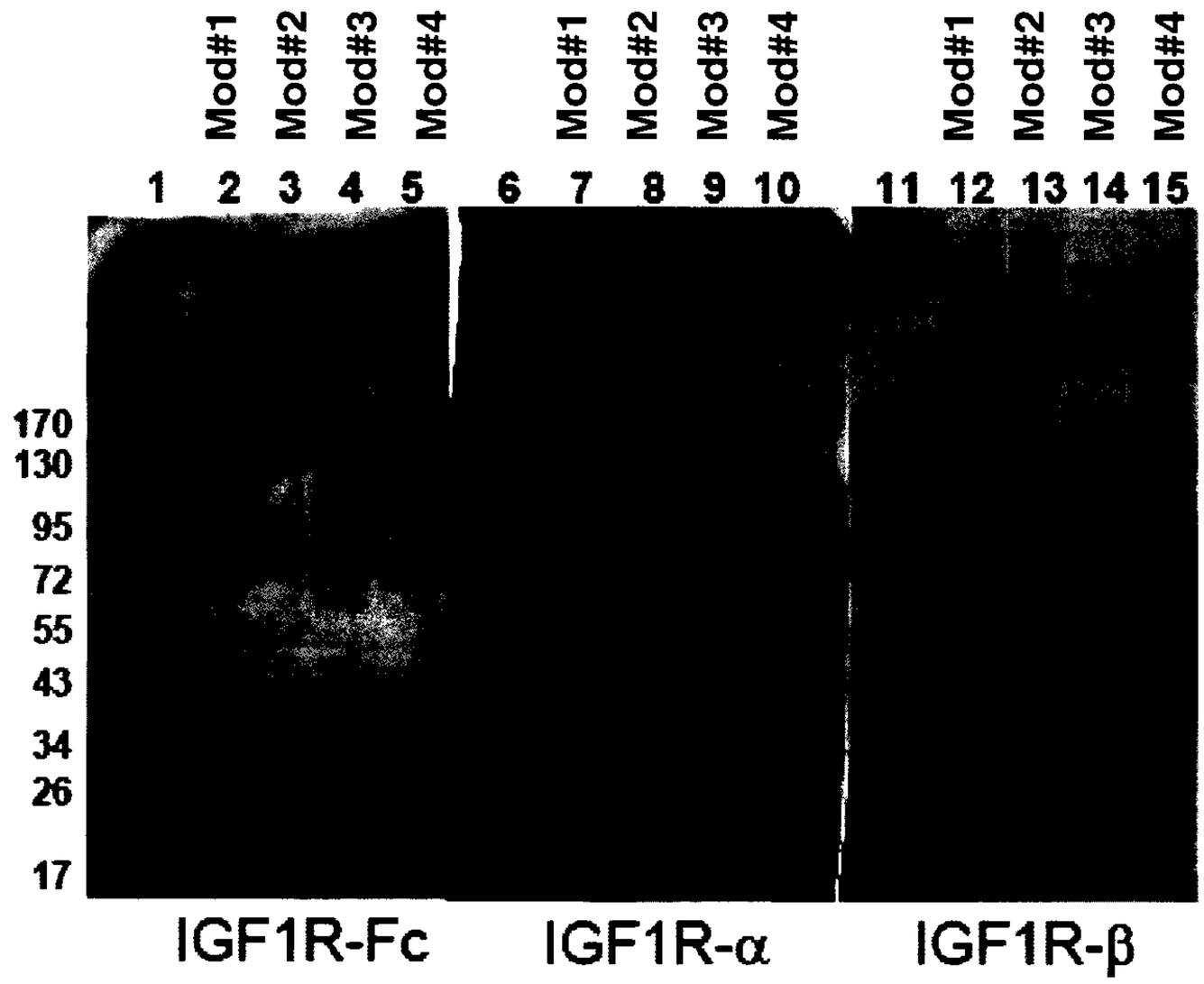
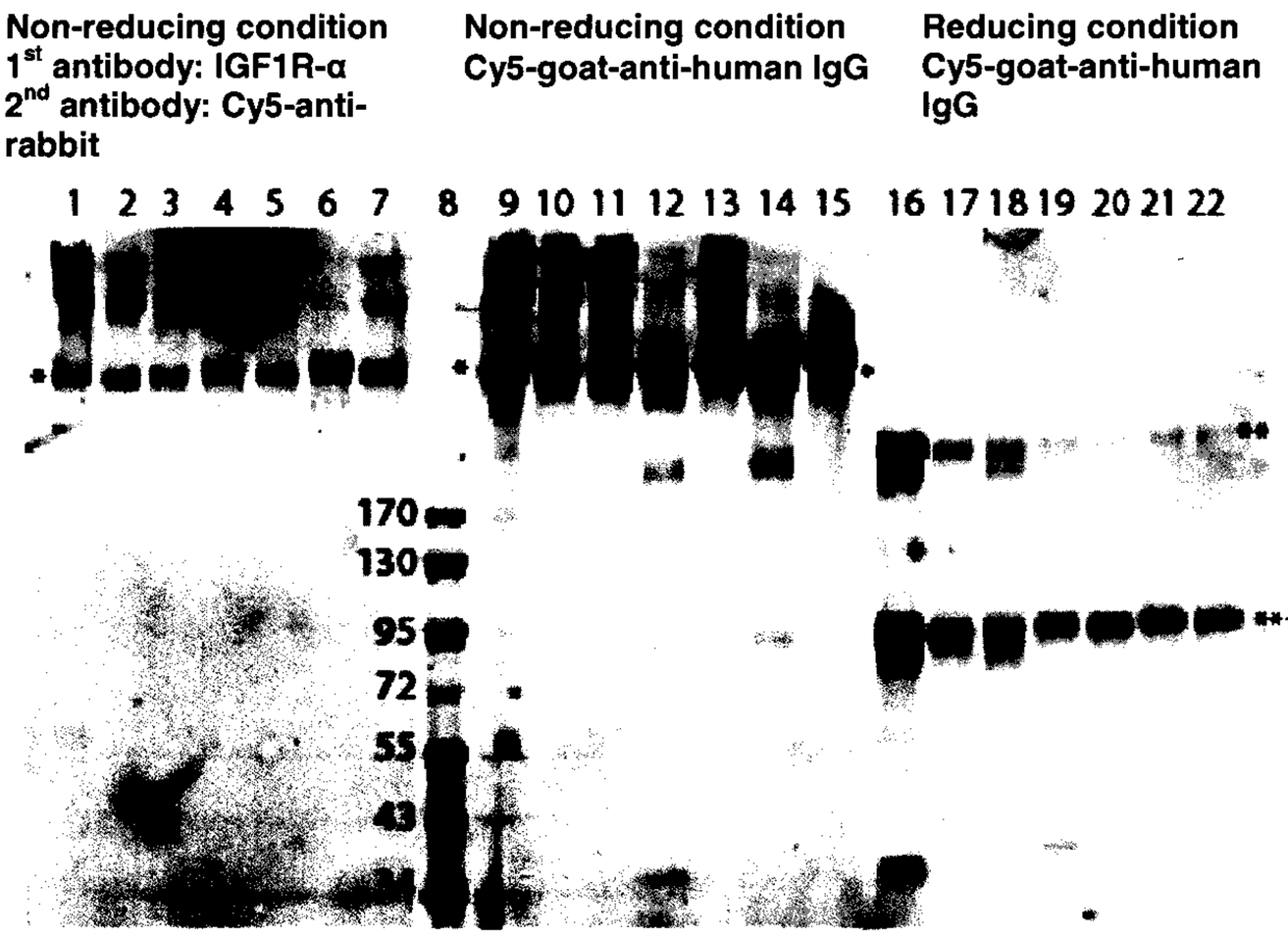
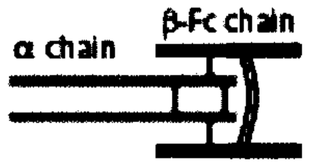
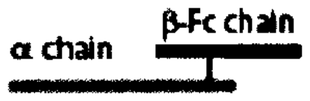

Mod#1
Mod#2
Mod#3
Mod#4
Mod#1
Mod#2
Mod#3
Mod#4
Mod#1
Mod#2
Mod#3
Mod#4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
170
130
95
72
55
43
34
26
17
IGF1R-Fc
IGF1R-α
IGF1R-β

FIGURE 31





* IGF1R-hFc-IgG1 homodimer (Fc-β–α–α–β-Fc)
in non-reducing form (420-440kDa)



** IGF1R-hFc-IgG1 monomer (Fc-β–α)
in partial-reducing form (210-220kDa)

*** IGF1R-hFc-IgG1 monomer (Fc-β & α)
in completely reducing form generate
a Fc-β chain (80-90kDa) and α–chain (130kDa
non detectable with Cy5-goat-anti-human IgG)

FIGURE 32

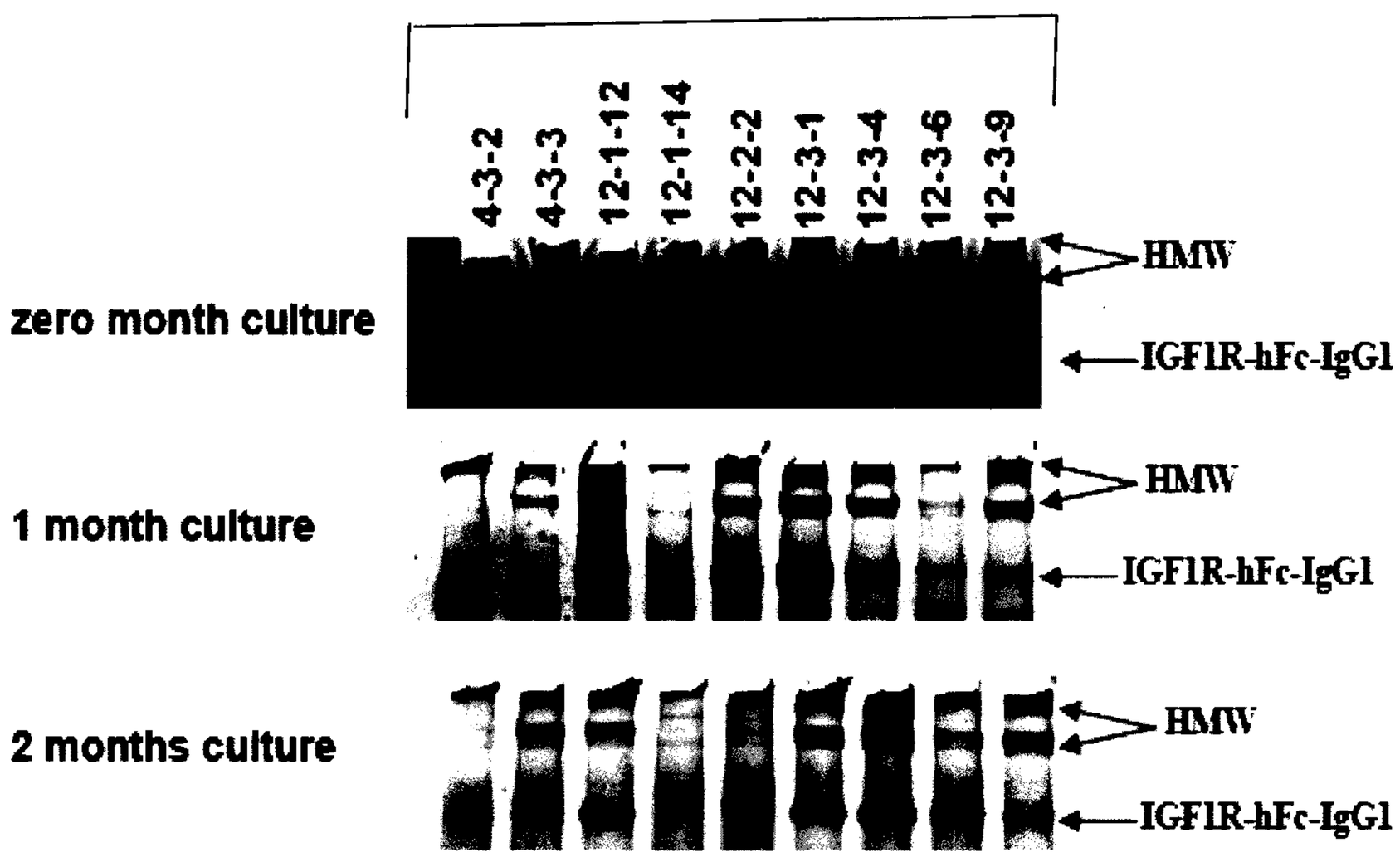

Subclones
4-3-2
4-3-3
12-1-12
12-1-14
12-2-2
12-3-1
12-3-4
12-3-6
12-3-9
zero month culture
1 month culture
2 months culture
HMW
IGF1R-hFc-IgG1
HMW
IGF1R-hFc-IgG1
HMW
IGF1R-hFc-IgG1

FIGURE 33

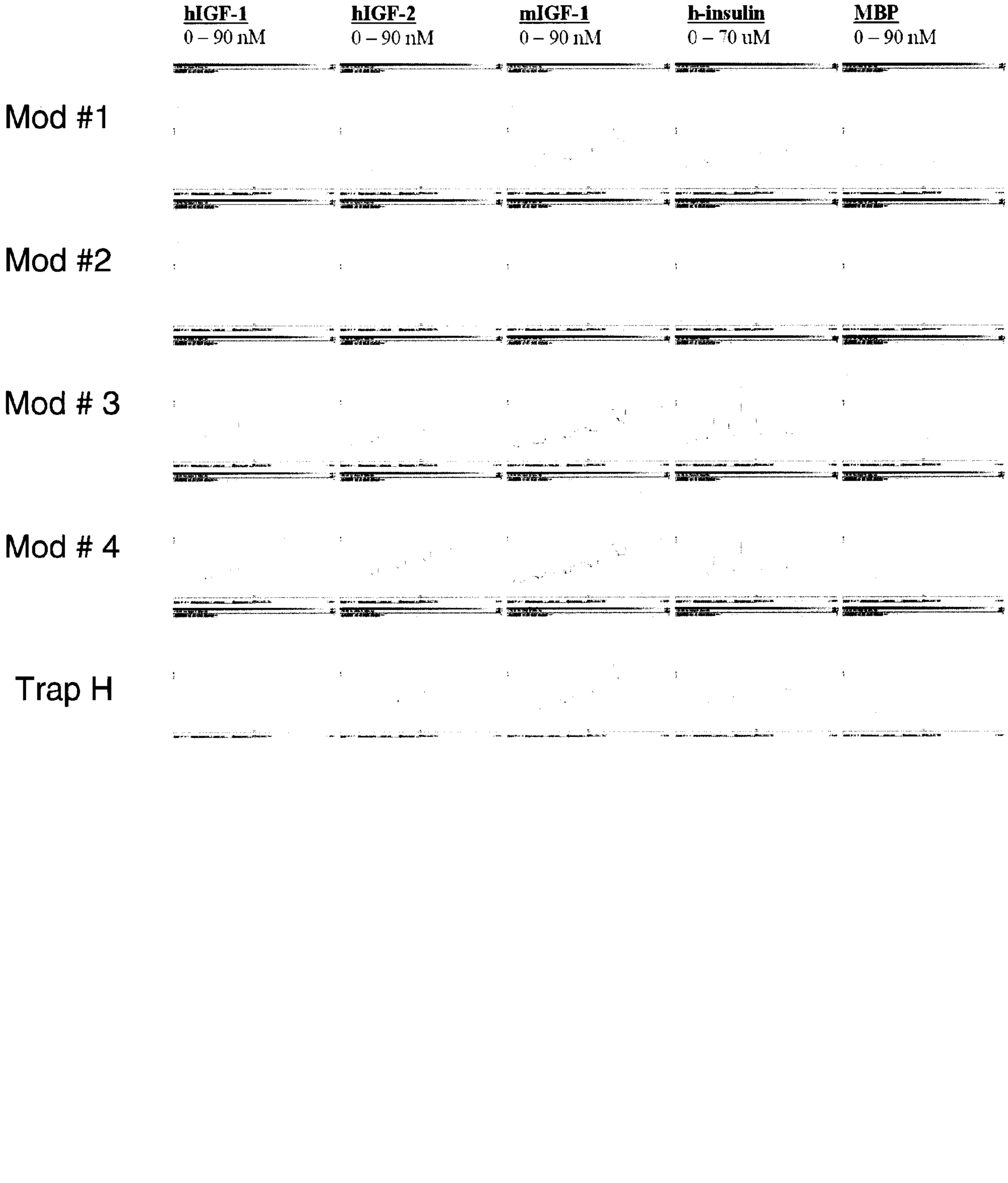

hIGF-1
0 – 90 nM
hIGF-2
0 – 90 nM
mIGF-1
0 – 90 nM
h-insulin
0 – 70 uM
MBP
0 – 90 nM
Mod #1
Mod #2
Mod # 3
Mod # 4
Trap H

FIGURE 34

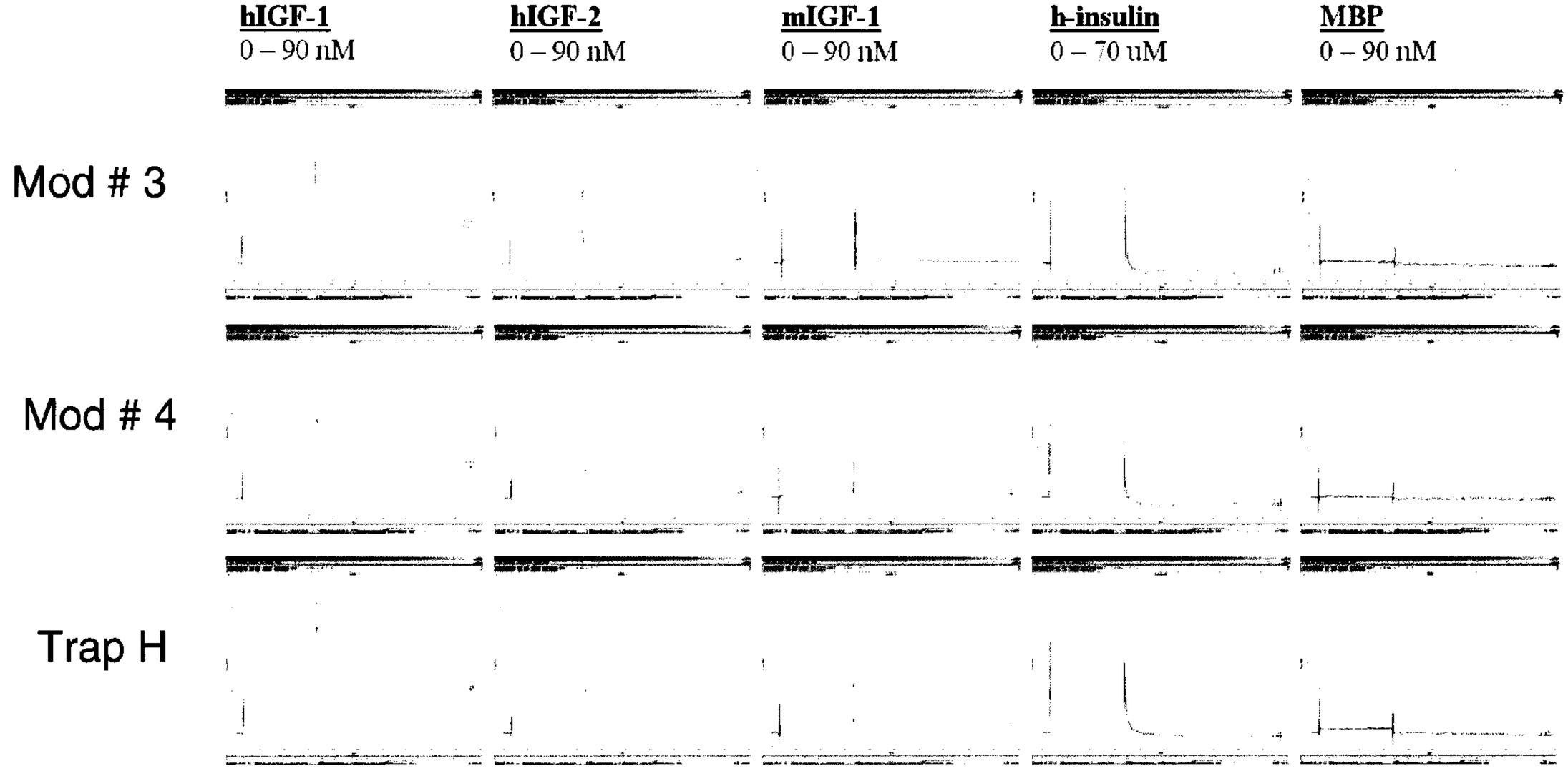

hIGF-1
0 – 90 nM
hIGF-2
0 – 90 nM
mIGF-1
0 – 90 nM
h-insulin
0 – 70 uM
MBP
0 – 90 nM
Mod # 3
Mod # 4
Trap H

FIGURE 35

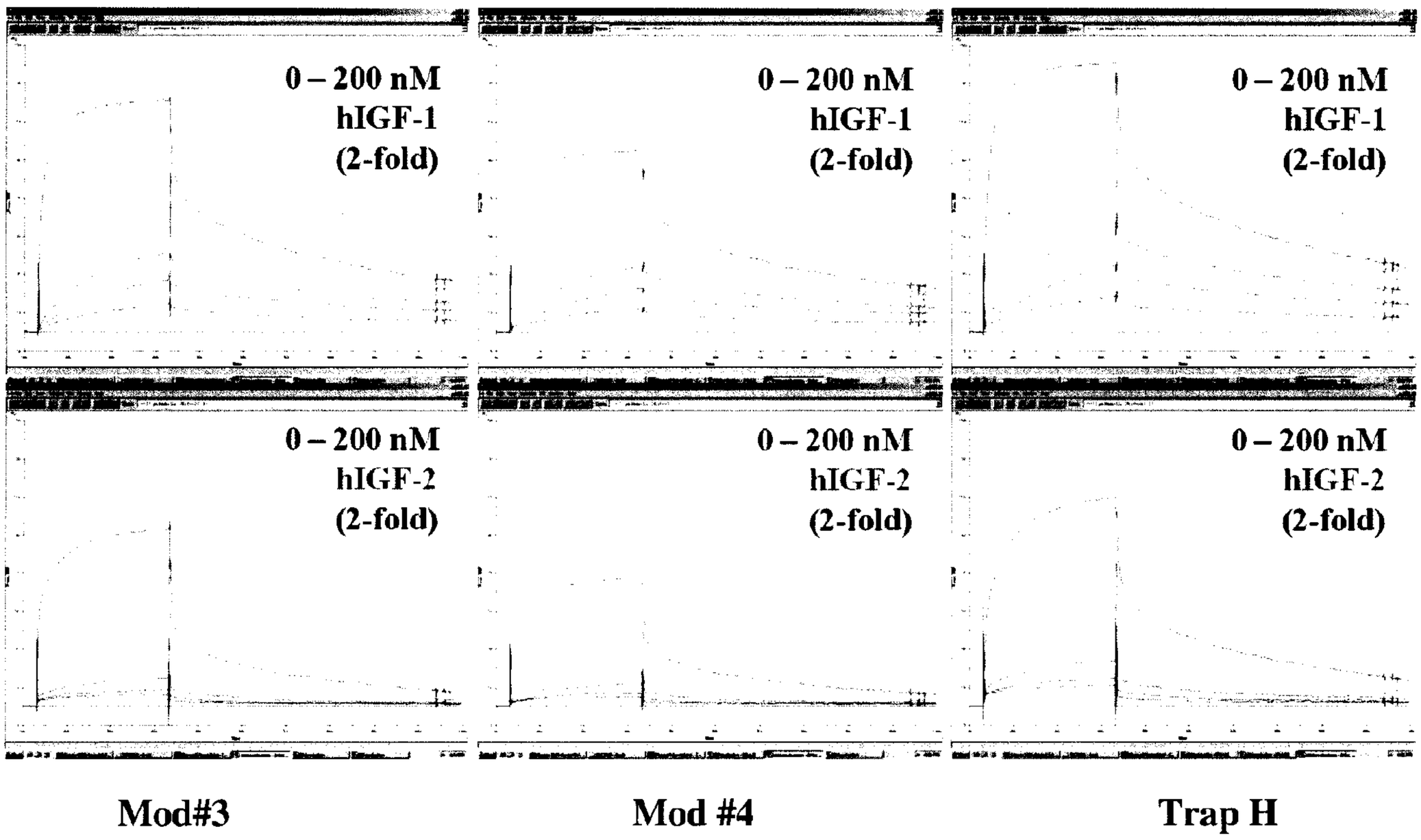
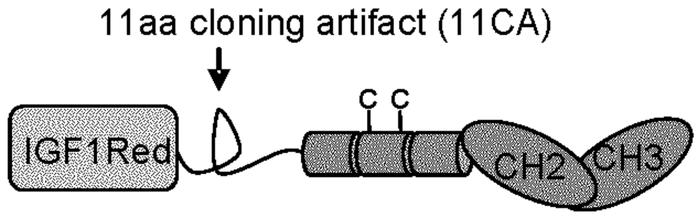
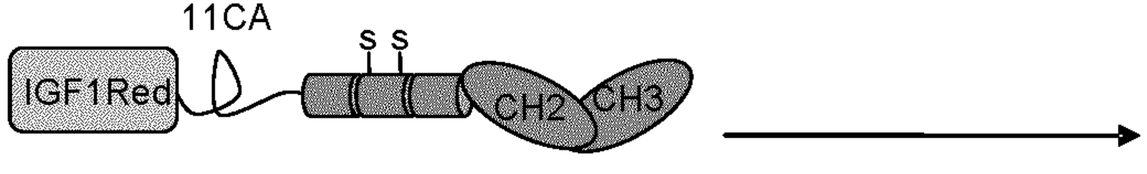

0 – 200 nM
hIGF-1
(2-fold)
0 – 200 nM
hIGF-1
(2-fold)
0 – 200 nM
hIGF-1
(2-fold)
0 – 200 nM
hIGF-2
(2-fold)
0 – 200 nM
hIGF-2
(2-fold)
0 – 200 nM
hIGF-2
(2-fold)
Mod#3
Mod #4
Trap H

FIGURE 28

Parent IgG1 construct

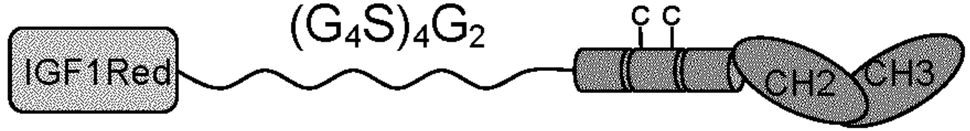


(1): Replace both Cys in the core hinge with Ser



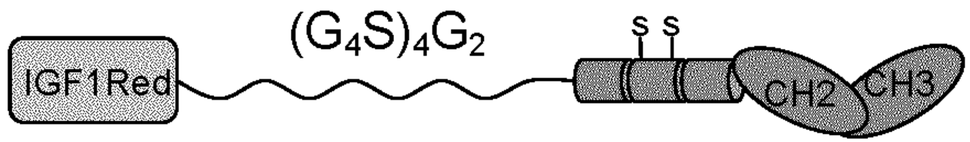
Mod#1

(2): Replace 11aa-cloning artifact with 22aa-flexible linker



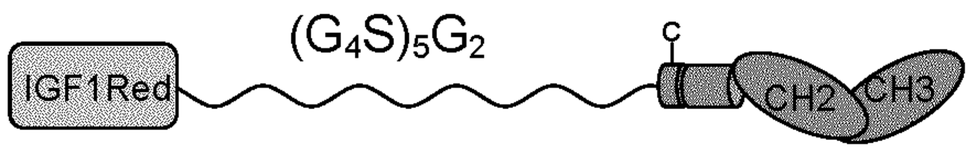
Mod#2

(3): (1) & (2)



Mod#3

(4): Replace 11aa-cloning artifact, upper hinge, and first 3aa of core hinge (including first Cys) with 27aa-flexible linker



Mod#4