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(54) Titre : ANTICORPS ANTI-RECEPTEUR IGF-I
(54) Title: ANTI-IGF-I RECEPTOR ANTIBODY

(57) **Abrégé/Abstract:**

The present invention provides an anti-IGF-I receptor antibody that binds specifically to an IGF-I receptor of a vertebrate and has the proliferation-inducing activity of a vertebrate-derived cell, or a fragment thereof, or derivatives of these.

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ABSTRACT

The present invention provides an anti-IGF-I receptor antibody that binds specifically to an IGF-I receptor of a vertebrate and has the proliferation-inducing activity of a vertebrate-
5 derived cell, or a fragment thereof, or derivatives of these.

DESCRIPTION

TITLE

ANTI-IGF-I RECEPTOR ANTIBODY

5

FIELD

[0001]

The present invention relates to an anti-IGF-I receptor antibody and, more specifically, to
10 an anti-IGF-I receptor antibody which specifically binds to an IGF-I receptor of a vertebrate.

BACKGROUND

1. IGF-I

15

[0002]

IGF-I is an insulin-like growth factor secreted mainly from the liver, and affects an IGF-I
receptor to thereby express a variety of physiological functions in various organs. Because of
this, IGF-I is expected to be used for the treatment of a variety of diseases. Since the amino acid
sequence of IGF-I has a high similarity of about 40% to that of proinsulin, IGF-I can bind to an
20 insulin receptor and thereby express insulin-like effects (Non-Patent Literature 1). In addition,
since the amino acid sequence of the IGF-I receptor has a high similarity of about 60% to that of
an insulin receptor, these receptors can form a heterodimer (Non-Patent Literature 1). Insulin can
act on the insulin receptor to thereby express a strong effect of lowering the level of blood
glucose, and is therefore used as a hypoglycemic drug.

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2. IGF-I receptor**[0003]**

An IGF-I receptor is a transmembrane protein consisting of an alpha chain and a beta
chain, and has six extracellular domains (L1, CR, L2, Fn1, Fn2, and Fn3), a transmembrane
30 domain, and an intracellular domain (Non-Patent Literature 2). The intracellular domain of the
IGF-I receptor incorporates a tyrosine kinase. The extracellular domain is a CR (cysteine-rich)
domain and participates in activation of the intracellular tyrosine kinase associated with
conformational change of the IGF-I receptor, which occurs when IGF-I binds to the IGF-I
receptor. The IGF-I receptor forms a homodimeric complex (homo form). IGF-I binding to the
35 IGF-I receptor (homo form) triggers signaling via activation of the receptor kinase. The IGF-I
receptor also forms a heterodimeric complex (hetero form) with the insulin receptor. Insulin or

IGF-I binding to the IGF-I receptor (hetero form) triggers signaling via activation of the receptor kinase (Non-Patent Literatures 3 and 4).

3. Physiological effects of IGF-I

[0004]

IGF-I has been shown to exhibit growth promoting effects, such as increasing the body length and the body weight, and insulin-like metabolic effects, such as glucose metabolism acceleration and hypoglycemic effects. It has been revealed that mecasermin, a human recombinant IGF-I, improves symptoms related to insulin receptor abnormality, such as hyperglycemia, hyperinsulinemia, acanthosis nigricans and hirsutism. IGF-I has also been shown to improve growth disorder of dwarfism resistant to growth hormone (Non-Patent Literature 5).

As its growth promoting effects, IGF-I is known to promote the DNA synthetic capacity of human cartilage cells. It is also known that administration of IGF-I to a hypophysectomized rat increases its body weight and femur length (Non-Patent Literature 5).

4. Effect of IGF-I on increasing muscle mass

[0005]

Enhancement of cell proliferation activity with IGF-I requires continuous activation of the IGF-I receptor (Non-Patent Literature 6). An animal engineered to overexpress the IGF-I receptor exhibits increased muscle mass (Non-Patent Literature 7). Sustained administration of IGF-I/IGFBP3 to a patient with proximal femur fracture enhances her/his grip strength and improves her/his ability of standing from a seated position without assistance (Non-Patent Literature 8). The muscle IGF-I levels of the elderly humans and mice are known to be lower than those of the young (Non-Patent Literatures 9 and 10). Over expression of IGF-I specifically in muscle tissues of elderly mice improved their muscle masses compared to wild-type mice (Non-Patent Literature 11).

5. Precedent products for increasing muscle mass

[0006]

Anamorelin, a ghrelin receptor agonist, increased lean body mass in a clinical trial for cachexia, which is a disuse muscle atrophy. However, it involves adverse effects such as inducing nausea and hyperglycemia (Non-Patent Literature 12).

Myostatin, a negative control factor of skeletal myogenesis, affects activin receptor II (ActRII) to thereby inhibit Akt/mTOR (Non-Patent Literatures 13 to 15).

LY2495655, an anti-myostatin antibody, increases the muscle masses of patients who received total hip replacement arthroplasty and those of elderly subjects (Non-Patent Literatures

16 and 17).

Bimagrumab, an anti-ActRII antibody, increases the muscle mass of neuromuscular disease patients (Non-Patent Literature 18).

5 However, there is no drug so far which promotes formation of skeletal muscles and can thereby be used for the treatment of a subject in need thereof.

6. Precedent products for promoting growth

[0007]

10 Human recombinant growth hormone (GH) formulation activates a GH receptor and induces IGF-I secretion, thereby exhibiting growth promoting effects. However, since the formulation requires once-daily administration via subcutaneous injection, it often causes poor drug compliance (e.g., unintentional omission of medication) and results in reduction in growth effects (Non-Patent Literature 19). There is an ongoing attempt to develop a long-acting GH formulation with improved kinetics which is to be administered once every one or two weeks.

15 However, there is no drug so far which exhibits growth promoting effects and can thereby be used for the treatment of a subject in need thereof with improved drug compliance. In addition, the GH formulation has been found to exhibit reduced growth effects on patients of GH receptor abnormality with reduced sensitivity to activation of the GH receptor, or patients resistant to GH treatment (Non-Patent Literature 20).

20 IGF-I is the only therapeutic agent which has growth promoting effect on a patient having reduced sensitivity to the GH receptor activation, since it acts on any point downstream of the GH receptor. However, the IGF-I formulation is a parenteral solution to be administered twice daily and therefore likely to cause poor drug compliance. In addition, it has been shown to cause hypoglycemia as an adverse effect (Non-Patent Literature 21). There is no drug so far
25 which has improved drug compliance and reduced occurrence of hypoglycemia than IGF-I and can be used as an alternative therapeutic agent.

7. Hypoglycemic effect of IGF-I

[0008]

30 IGF-I is known to have hypoglycemic effect as an insulin-like effect. IGF-I enhances glucose uptake effect of rat muscle-derived cells (Non-Patent Literature 5). Administration of IGF-I also reduces the blood glucose level of rats (Non-Patent Literature 5).

35 It has been reported that the glucose lowering effect of IGF-I cause hypoglycemia as a clinical adverse effect (Non-Patent Literature 21). Likewise, administration of IGF-I to a human subject causes hypoglycemia. Therefore, at the onset of IGF-I treatment, it is necessary to keep controlling the dosage starting from a low dosage with observing various clinical findings

including the blood glucose level after administration (Non-Patent Literature 5).

IGF-I expresses hypoglycemic effect via promotion of Akt phosphorylation, which is a downstream signal of the IGF-I receptor. An active variant of Akt enhances glucose uptake by 3T3-L1 cells (Non-Patent Literature 22). On the other hand, an Akt2-deficient mouse exhibited elevated blood glucose level (Non-Patent Literature 23). An Akt inhibitor inhibits insulin-induced glucose uptake by rat muscle-derived cells (Non-Patent Literature 24). IGF-I is also known to activate an insulin receptor which plays a role in hypoglycemic effect. These findings suggest that the hypoglycemic effect of IGF-I involves overactivation of Akt and activation of the insulin receptor.

8. Short half-life of IGF-I in blood

[0009]

IGF-I has a short half-life in blood, and therefore requires frequent administrations when used in treatment. In fact, mecasermin, a human recombinant IGF-I, has a blood half-life of about 11 hours to about 16 hours, and therefore needs to be administered once to twice daily in the treatment of dwarfism (Non-Patent Literature 5).

About 70 to 80% of IGF-I is bound to IGFBP3 in blood, while a free form of IGF-I exhibits physiological effect. Binding of IGF-I to IGFBP3 maintains its half-life in blood to a time period of from about 10 hours to about 16 hours (Non-Patent Literature 1).

IPLEX, a combination drug of IGF-I with IGFBP3, exhibited a blood half-life extended from that of IGF-I to a time period of about 21 hours to about 26 hours, and thereby allowed for reduction of administration frequency to once daily (Non-Patent Literature 23). However, IPLEX was already withdrawn from the market.

There has been also an attempt to develop a PEGylated IGF-I with improved IGF-I kinetics, but no drug has successfully been developed so far and is currently available (Patent Literature 1).

9. Therapeutic effects expected to be achieved via IGF-I's effects

[0010]

IGF-I is known to affect various organs and exerts a wide variety of physiological functions (Non-Patent Literature 21).

IGF-I has been reported to have neuroprotective effect on the central nervous system by protecting mitochondria and antioxidant effect via activation of the IGF-I receptor (Non-Patent Literatures 26 and 27). IGF-I promotes regeneration of injured neurites (Non-Patent Literature 28).

IGF-I is a main factor of growth promotion (Non-Patent Literatures 29 and 30). In fact,

mecasermin, a human recombinant IGF-I, is clinically used as a drug for the treatment of dwarfism.

IGF-I is deemed to be effective in the treatment of hepatic cirrhosis, which evolves from liver damage or chronic liver disease and involves hepatic fibrosis. Administration of IGF-I improved hepatic fibrosis in a model animal of hepatic cirrhosis (Non-Patent Literature 31).

IGF-I is also known to play a role in the development and functions of kidney. IGF-I has protective effect against oxidative stress and apoptosis due to glucotoxicity in mesangial cells of kidney (Non-Patent Literature 32). IGF-I is expected as a drug for the treatment of nephropathy.

10 [0011]

Examples of conditions expected to be improved via IGF-I administration include: dwarfism, Laron syndrome, hepatic cirrhosis, hepatic fibrosis, aging, intrauterine growth restriction (IUGR), neurological disease, cerebral stroke, spinal cord injury, cardiovascular protection, diabetes, insulin resistant, metabolic syndrome, nephropathy, osteoporosis, cystic fibrosis, wound healing, myotonic dystrophy, AIDS-associated sarcopenia, HIV-associated fat redistribution syndrome, burn, Crohn's disease, Werner's syndrome, X-linked combined immunodeficiency disease, hearing loss, anorexia nervosa, and retinopathy of prematurity (Non-Patent Literature 21).

Thus, IGF-I is expected as a drug for the treatment of a variety of diseases because of its wide spectrum of physiological effects. However, problems such as its adverse hypoglycemic effect and its short half-life requiring multiple administrations have prevented its clinical applications.

10. IGF-I receptor agonist antibodies

25 [0012]

In general, antibody formulations have long half-life, and prove effective if administered once to twice a month. Although some IGF-I receptor agonist antibodies have been reported to be effective in activating the receptor *in vitro*, no antibodies have been reported to exhibit agonistic activity against the IGF-I receptor *in vivo* (Non-Patent Literatures 33 to 37).

Specifically, antibodies 3B7 and 2D1 enhance cellular DNA synthesis *in vitro* (Non-Patent Literature 34).

Antibodies 11A1, 11A4, 11A11, and 24-57 enhance tyrosine phosphorylation of IGF-I receptor *in vitro* (Non-Patent Literature 35).

Antibodies 16-13, 17-69, 24-57, 24-60, 24-31, and 26-3 are shown to be effective in promoting cellular DNA synthesis and glucose uptake *in vitro*, and have the potential to exhibit hypoglycemic effect (Non-Patent Literatures 36 and 37).

[0013]

However, no IGF-I receptor agonist antibody has been reported to exhibit cell proliferation effects in an *in vitro* experiment using primary cultured cells, inter alia, human myoblasts, let alone muscle-mass increasing effects *in vivo*.

11. IGF-I receptor antagonist antibodies

[0014]

There are attempts to use an antibody which binds to the IGF-I receptor for the treatment of malignancies, based on its antagonist effect of inhibiting binding of IGF-I to the IGF-I receptor. However, existing IGF-I receptor antagonist antibodies have various adverse effects such as hyperglycemia in monotherapy (Non-Patent Literature 38), and exhibit increased incidence of hyperglycemia when used in combination with other anticancer agents (Non-Patent Literature 39). Accordingly, their therapeutic applications are expected to be limited.

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[0016]

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20

SUMMARY

PROBLEM TO BE SOLVED BY THE INVENTION

25 **[0017]**

An objective of the present invention is to provide an anti-IGF-I receptor antibody or its fragment or a derivative thereof which specifically binds to an IGF-I receptor of a vertebrate. Another objective of the present invention is to provide an antibody which increases the muscle mass or the thickness of growth plate cartilage via the IGF-I receptor while not reducing the blood glucose level.

30

MEANS TO SOLVE THE PROBLEM

[0018]

35 The present invention relates to the following:

Aspect [1] An anti-IGF-I receptor antibody or its fragment or a derivative thereof which

specifically binds to an IGF-I receptor of a vertebrate, and exhibits an activity to induce growth of vertebrate-derived cells.

Aspect [2] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to Aspect [1], wherein the activity of the antibody, fragment, or derivative to induce growth of vertebrate-derived cells is equal to or higher than the corresponding activity of a wild-type IGF-I.

Aspect [3] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to Aspect [1] or Aspect [2], wherein the EC₅₀ value of the antibody, fragment, or derivative for inducing growth of vertebrate-derived cells *in vitro* is 1/20 or less of the corresponding value of a wild-type IGF-I.

Aspect [4] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [3], wherein when the antibody, fragment, or derivative is contacted with cultured vertebrate-derived cells, the duration of activity of the antibody, fragment, or derivative to induce growth of the cultured cells relative to the duration of contact is improved than a wild-type IGF-I.

Aspect [5] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [2] to Aspect [4], wherein the wild-type IGF-I is a human IGF-I having an amino acid sequence defined in SEQ ID NO:1.

Aspect [6] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [5], wherein the EC₅₀ value of the antibody, fragment, or derivative for inducing growth of vertebrate-derived cells *in vitro* is 0.1 nmol/L or lower.

Aspect [7] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [6], which exhibits an activity to induce an increase in the muscle mass and/or the body length of a vertebrate when parenterally administered to the vertebrate.

Aspect [8] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [7], which is administered to a vertebrate at a frequency of once a week or less.

Aspect [9] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [8], wherein the vertebrate is a human; a non-human animal including a guinea pig, a monkey, a rabbit, a cow, a pig, a horse, a sheep, a dog, or a fowl; or a non-human animal engineered to express a human IGF-I receptor.

Aspect [10] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [9], which does not induce glucose uptake by differentiated muscle cells when administered at a dosage sufficient to induce growth of vertebrate-derived cells.

Aspect [11] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to

Aspect [10], which does not induce glucose uptake by differentiated muscle cells when administered at a dosage of 100 times or more of the EC₅₀ value for inducing growth of vertebrate-derived cells *in vitro*.

5 Aspect [12] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to Aspect [10] or Aspect [11], wherein the vertebrate-derived cells are myoblasts derived from a human or a non-human mammal.

Aspect [13] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [7] to Aspect [12], which does not lower the blood glucose level of a vertebrate when parenterally administered to the vertebrate at a dosage sufficient to induce an
10 increase in the muscle mass and/or the body length of the vertebrate.

Aspect [14] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to Aspect [13], which does not change the blood glucose level of a vertebrate when parenterally administered to the vertebrate at a dosage of 10 times or more of an effective dosage sufficient to induce an increase in the muscle mass and/or the body length of the vertebrate.

15 Aspect [15] An anti-IGF-I receptor antibody or its fragment or a derivative thereof, according to any one of Aspect [1] to Aspect [14], which binds to a CR domain of an IGF-I receptor.

Aspect [16] An anti-IGF-I receptor antibody or its fragment or a derivative thereof, which binds to a CR domain of an IGF-I receptor, and inhibits binding of IGF-I or IGF-II to an IGF-I receptor.

20 Aspect [17] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to Aspect [16], which binds to an epitope containing ProSerGlyPheIleArgAsnX₁X₂GlnSerMet (where X₁ represents Gly or Ser and X₂ represents Ser or Thr), or a part in the vicinity thereof, in the sequence of the CR domain of the IGF-I receptor.

25 Aspect [18] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to Aspect [17], which binds to an epitope containing ProSerGlyPheIleArgAsnGlySerGlnSerMet, or a part in the vicinity thereof, in the sequence of the CR domain of the IGF-I receptor.

Aspect [19] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [18], which has a cross-reactivity with an IGF-I receptor of a human or a non-human animal including a guinea pig, a monkey, a rabbit, a cow, a pig, a horse,
30 a sheep, a dog, or a fowl.

Aspect [20] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [19], which causes an antigen-antibody reaction with an affinity intensity at a equilibrium dissociation constant (KD) of 1×10^{-8} M or less.

35 Aspect [21] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [16] to Aspect [20], which has at least one of the features of:

- 1) exhibiting an activity to induce growth of vertebrate-derived cells;

- 2) exhibiting an activity to induce an increase in the muscle mass and/or the body length of a vertebrate when parenterally administered to the vertebrate;
- 3) not inducing glucose uptake by differentiated muscle cells when administered at a dosage sufficient to induce growth of vertebrate-derived cells; and
- 5 4) not changing the blood glucose level of a vertebrate when parenterally administered to the vertebrate at a dosage sufficient to induce an increase in the muscle mass and/or the body length of the vertebrate.

Aspect [22] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [16] to Aspect [21], which has at least one of the features of:

- 10 1) inhibiting growth of vertebrate-derived cells induced by IGF-I;
- 2) inhibiting IGF-I-induced cell proliferation in a vertebrate suffering a cell proliferative disease when parenterally administered to the vertebrate;
- 3) not affecting glucose uptake by differentiated muscle cells at a dosage sufficient to inhibit growth of vertebrate-derived cells induced by IGF-I; and
- 15 4) not changing the blood glucose level of a vertebrate suffering a cell proliferative disease when parenterally administered to the vertebrate at a dosage sufficient to inhibit IGF-I-induced cell proliferation in the vertebrate.

Aspect [23] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [22], which is a Fab, scFv, diabody or bispecific antibody, or a
20 derivative thereof.

Aspect [24] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [23], consisting of an amino acid sequence comprising:

as a heavy chain variable region CDR-1 (CDR-H1) sequence, an amino acid sequence defined in SEQ ID NO:3 or an amino acid sequence derived from SEQ ID NO:3 via substitution,
25 deletion or insertion of any one amino acid residue;

as a heavy chain variable region CDR-2 (CDR-H2) sequence, an amino acid sequence defined in SEQ ID NO:4 or an amino acid sequence derived from SEQ ID NO:4 via substitution, deletion or insertion of any one or two amino acid residues;

as a heavy chain variable region CDR-3 (CDR-H3) sequence, an amino acid sequence defined in SEQ ID NO:5 or an amino acid sequence derived from SEQ ID NO:5 via substitution,
30 deletion or insertion of any one or two amino acid residues;

as a light chain variable region CDR-1 (CDR-L1) sequence, an amino acid sequence defined in SEQ ID NO:6 or an amino acid sequence derived from SEQ ID NO:6 via substitution, deletion or insertion of any one or two amino acid residues;

35 as a light chain variable region CDR-2 (CDR-L2) sequence, an amino acid sequence defined in SEQ ID NO:7 or an amino acid sequence derived from SEQ ID NO:7 via substitution,

deletion or insertion of any one amino acid residue; and

as a light chain variable region CDR-3 (CDR-L3) sequence, an amino acid sequence defined in SEQ ID NO:8 or an amino acid sequence derived from SEQ ID NO:8 via substitution, deletion or insertion of any one or two amino acid residues.

5 Aspect [25] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to Aspect [24] further comprises a framework sequence of immunoglobulin.

Aspect [26] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to Aspect [25], wherein the framework sequence of immunoglobulin is a framework sequence of each class of immunoglobulin from a human or a non-human animal including a guinea pig, a
10 monkey, a rabbit, a cow, a pig, a horse, a sheep, a dog, a fowl, a mouse, or a rat.

Aspect [27] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [26], which consists of an amino acid sequence comprising:

as a heavy chain variable region, an amino acid sequence defined in SEQ ID NO:9 or an amino acid sequence having a similarity of 90% or more to SEQ ID NO:9; and

15 as a light chain variable region, an amino acid sequence defined in SEQ ID NO:10 or an amino acid sequence having a similarity of 90% or more to SEQ ID NO:10.

Aspect [28] The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [27] further comprising a constant region of each class of immunoglobulin a human or a non-human animal including a guinea pig, a monkey, a rabbit, a
20 cow, a pig, a horse, a sheep, a dog, a fowl, a mouse, or a rat.

Aspect [29] A nucleic acid molecule consisting of a polynucleotide sequence encoding an anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [28].

Aspect [30] A cloning vector or expression vector comprising at least one nucleic acid molecule
25 according to Aspect [29].

Aspect [31] A recombinant cell derived from a host cell via transfection of a vector according to Aspect [30].

Aspect [32] A process of producing an anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [28], comprising:

30 culturing a recombinant cell according to Aspect [31]; and

purifying the anti-IGF-I receptor antibody, fragment, or derivative produced from the recombinant cell.

Aspect [33] A pharmaceutical composition comprising an anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [28], a nucleic acid
35 molecule according to Aspect [29], a vector according to Aspect [30], or a recombinant cell according to Aspect [31].

Aspect [34] The pharmaceutical composition according to Aspect [33], further comprising an additional active ingredient other than the anti-IGF-I receptor antibody, fragment, or derivative according to any one of Aspect [1] to Aspect [28], the nucleic acid molecule according to Aspect [29], the vector according to Aspect [30], or the recombinant cell according to Aspect [31].

- 5 Aspect [35] The pharmaceutical composition according to Aspect [34], wherein the active ingredient is one or more selected from a growth hormone or an analog thereof, insulin or an analog thereof, IGF-II or an analog thereof, an anti-myostatin antibody, a myostatin antagonist, an anti-activin type IIB receptor antibody, an activin type IIB receptor antagonist, a soluble activin type IIB receptor or an analog thereof, ghrelin or an analog thereof, follistatin or an analog thereof, a beta-2 agonist, and a selective androgen receptor modulator.
- 10 Aspect [36] The pharmaceutical composition according to Aspect [34] or Aspect [35], wherein the active ingredient comprises an ingredient selected from the group consisting of: corticosteroid, antiemetic, ondansetron hydrochloride, granisetron hydrochloride, metoclopramide, domperidone, haloperidol, cyclizine, lorazepam, prochlorperazine, dexamethasone, levomepromazine, tropisetron, cancer vaccine, GM-CSF inhibitor, GM-CSF DNA vaccine, cell-based vaccine, dendritic cell vaccine, recombinant virus vaccine, heat shock protein (HSP) vaccine, homologous tumor vaccine, autologous tumor vaccine, analgesic, ibuprofen, naproxen, choline magnesium trisalicylate, oxycodone hydrochloride, anti-angiogenic, antithrombotic, anti-PD-1 antibody, nivolumab, pembrolizumab, anti-PD-L1 antibody, atezolizumab, anti-CTLA4 antibody, ipilimumab, anti-CD20 antibody, rituximab, anti-HER2 antibody, trastuzumab, anti-CCR4 antibody, mogamulizumab, anti-VEGF antibody, bevacizumab, anti-VEGF receptor antibody, soluble VEGF receptor fragment, anti-TWEAK antibody, anti-TWEAK receptor antibody, soluble TWEAK receptor fragment, AMG 706, AMG 386, antiproliferative, farnesyl protein transferase inhibitor, alpha v beta 3 inhibitor, alpha v beta 5 inhibitor, p53 inhibitor, Kit receptor inhibitor, ret receptor inhibitor, PDGFR inhibitor, growth hormone secretion inhibitor, angiopoietin inhibitor, tumor-infiltrating macrophage inhibitor, c-fms inhibitor, anti-c-fms antibody, CSF-1 inhibitor, anti-CSF-1 antibody, soluble c-fms fragment, pegvisomant, gemcitabine, panitumumab, irinotecan, and SN-38.
- 15 20 25 30 Aspect [37] A medical drug for use in the treatment or prevention of a condition associated with IGF-I or IGF-II, comprising comprising an anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of Aspect [1] to Aspect [28], a nucleic acid molecule according to Aspect [29], a vector according to Aspect [30], or a recombinant cell according to Aspect [31].
- 35 Aspect [38] The medical drug according to Aspect [37], wherein the condition associated with IGF-I is selected from: disuse muscle atrophy, dwarfism, diabetic nephropathy, chronic renal failure, Laron syndrome, hepatic cirrhosis, hepatic fibrosis, aging, intrauterine growth restriction

(IUGR), neurological disease, cerebral stroke, spinal cord injury, cardiovascular protection, diabetes, insulin resistant, metabolic syndrome, osteoporosis, cystic fibrosis, wound healing, myotonic dystrophy, AIDS-associated sarcopenia, HIV-associated fat redistribution syndrome, burn, Crohn's disease, Werner's syndrome, X-linked combined immunodeficiency disease, hearing loss, anorexia nervosa and retinopathy of prematurity, Turner's syndrome, Prader-Willi syndrome, Silver-Russell syndrome, idiopathic short stature, obesity, multiple sclerosis, fibromyalgia, ulcerous colitis, low muscle mass, myocardial ischemia and decreased bone density.

Aspect [39] The medical drug according to Aspect [37] or Aspect [38], which is parenterally administered.

Aspect [40] The medical drug according to any one of Aspect [37] to Aspect [39], which is a veterinary drug to be administered to a non-human animal.

Aspect [41] The medical drug according to Aspect [40], wherein the veterinary drug is administered for the purpose of, increasing muscle mass and/or body length, promoting growth, increasing milk production, promoting reproduction, or preventing aging.

Aspect [42] The medical drug according to Aspect [40] or Aspect [41], wherein the non-human animal is a guinea pig, a monkey, a rabbit, a cow, a pig, a horse, a sheep, a dog, or a fowl.

Aspect [43] The medical drug according to any one of Aspect [37] to Aspect [42], for the treatment or prevention of a disease caused by an effect of IGF-I or IGF-II on an IGF-I receptor.

Aspect [44] The medical drug according to Aspect [43], wherein the disease caused by an effect of IGF-I or IGF-II on an IGF-I receptor is selected from the group consisting of: liver cancer, neuroblastoma, striated muscle sarcoma, bone cancer, childhood cancer, acromegalia, ovary cancer, pancreas cancer, benignant prostatic hypertrophy, breast cancer, prostate cancer, bone cancer, lung cancer, colorectal cancer, cervix cancer, synovial sarcoma, urinary bladder cancer, stomach cancer, Wilms' tumor, diarrhea associated with metastatic carcinoid and vasoactive intestinal peptide secreting tumor, vipoma, Verner-Morrison syndrome, Beckwith-Wiedemann syndrome, kidney cancer, renal cell cancer, transitional cell cancer, Ewing's sarcoma, leukemia, acute lymphoblastic leukemia, brain tumor, glioblastoma, non-glioblastomatous brain tumor, meningioma, pituitary adenoma, vestibular schwannoma, primitive neuroectodermal tumor, medulloblastoma, astrocytoma, oligodendroglioma, ependymoma, choroid plexus papilloma, gigantism, psoriasis, atherosclerosis, vascular smooth muscle restenosis, inappropriate microvascular growth, diabetic retinopathy, Graves' disease, multiple sclerosis, systemic lupus erythematosus, chronic thyroiditis, myasthenia gravis, autoimmune thyroiditis and Behcet's disease.

Aspect [45] A method of culturing vertebrate-derived cells *in vitro*, comprising contacting the vertebrate-derived cells with an anti-IGF-I receptor antibody or its fragment or a derivative

thereof according to any one of Aspect [1] to Aspect [28], a nucleic acid molecule according to Aspect [29], a vector according to Aspect [30], and a recombinant cell according to Aspect [31] when culturing the cells.

5 Aspect [46] The method according to Aspect [45], wherein said contacting is carried out for the purpose of promoting growth or inducing differentiation of the vertebrate-derived cells.

Aspect [47] The method according to Aspect [45] or Aspect [46], wherein the anti-IGF-I receptor antibody, fragment, or derivative is adsorbed by, or immobilized to, a solid phase.

10 Aspect [48] A transgenic animal comprising an IGF-I receptor gene which has been mutated in a CR domain thereof via gene engineering such that the CR domain includes an amino acid sequence represented by ProSerGlyPheIleArgAsnGlySerGlnSerMet.

Aspect [49] A transgenic animal into which a heterologous IGF-I receptor gene has been transfected, wherein the amino acid sequence encoded by the heterologous IGF-I receptor gene differs from the amino acid sequence encoded by the animal's inherent IGF-I receptor gene in amino acid residue(s) X₁ and/or X₂ of a sequence represented by
15 ProSerGlyPheIleArgAsnX₁X₂GlnSerMet in a CR domain.

EFFECT OF THE INVENTION

[0019]

20 The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to the present invention has an effect of specifically binding to an IGF-I receptor of a vertebrate.

BRIEF EXPLANATION OF THE DRAWINGS

25 [0020]

Figure 1 illustrates aligned amino acid sequences of CR domains of the mouse, rat, human, guinea pig and rabbit IGF-I receptors, in which the amino acid sequences are indicated using the one letter code;

30 Figure 2 is a graph indicating the results of ELISA using variants of a presumptive epitope of IGF11-16;

Figure 3 is a graph indicating the growth activity of human myoblasts after removal of IGF11-16 and IGF-I;

Figure 4 is a graph indicating the glucose uptake effect by human differentiated muscle cells after addition of IGF-I and IGF11-16;

35 Figure 5 is a graph indicating the weights of extensor digitorum longus muscles of guinea pigs which received sustained administration of IGF-I using an osmotic pump for two weeks or

single-dose subcutaneous or intravenous administration of IGF11-16 two weeks ago;

Figure 6 is a graph indicating the time course of the blood glucose level of guinea pigs under fasting conditions after single-dose subcutaneous administration of IGF-I;

Figure 7 is a graph indicating the time course of the blood glucose level of guinea pigs under fasting conditions after single-dose subcutaneous administration of IGF11-16;

Figure 8 is a graph indicating the time course of the blood glucose level of guinea pigs under fasting conditions after single-dose intravenous administration of IGF11-16;

Figure 9 is a graph indicating the effects of IGF11-16 in increasing the thickness of growth plate cartilage of hypophysectomized guinea pigs (HPX);

Figure 10 is a graph indicating the effects of IGF11-16 in increasing the length of tibia in hypophysectomized guinea pigs (HPX);

Figure 11 is a graph indicating the blood kinetics of IGF-I in guinea pigs under fasting conditions after single-dose subcutaneous administration; and

Figure 12 is a graph indicating the blood kinetics of IGF11-16 in guinea pigs under fasting conditions after single-dose subcutaneous administration.

MODES FOR CARRYING OUT THE INVENTION

[0021]

In the following description, the present invention will be explained with reference to specific embodiments, although the present invention should not be limited to these embodiments in any way. All the documents cited in the present specification, including patent publications, unexamined application publications, and non-patent literatures, are hereby incorporated by reference in their entirety for all purposes.

[IGF]

[0022]

IGF refers to an insulin-like growth factor, which may be either IGF-I or IGF-II. Both IGF-I and IGF-II are biological ligands having agonist activities which bind to an IGF-I receptor (insulin-like growth factor-I receptor) and transduce signals such as cell division and metabolism into the cell. IGF-I and IGF-II are also known to have cross-binding activity to an insulin receptor (INSR), which is structurally similar to the IGF-I receptor. The present specification will mainly discuss IGF-I, since its properties such as physiological functions are known more than those of IGF-II. However, in the context of discussion about various effects and diseases mediated via binding of a ligand to the IGF-I receptor, both IGF-I and IGF-II may collectively be mentioned.

[0023]

IGF-I, also referred to as somatomedin C, is a single polypeptide hormone consisting of 70 amino acids. The sequence of human IGF-I is available, e.g., on the EMBL-EBI with UniProtKB accession number P50919. The amino acid sequence of mature IGF-I is shown in SEQ ID NO:1 of the sequence listing attached hereto. This 70 amino acid sequence is conserved in many species. In the present invention, the term "IGF-I" without any limitation means an IGF-I protein having such hormone activity, unless specified otherwise.

[0024]

IGF-I is produced by a variety of cells in the living body, including liver cells, and exists in blood and other body fluids. Therefore, wild-type IGF-I can be obtained via purification from body fluid of an animal or from a primary cultured cell or a cultured cell line derived from an animal. Since a growth hormone induces IGF-I production by cells, IGF-I can also be purified from body fluid of an animal to which a growth hormone has been administered, or from a primary cultured animal cell or an animal cell line incubated in the presence of a growth hormone. As a different method, IGF-I can also be obtained from a recombinant cell prepared by transfection of an expression vector carrying a nucleic acid molecule encoding an amino acid sequence of IGF-I into a host such as a prokaryotic organism (e.g., *E. coli*) or a eukaryotic cell including a yeast, an insect cell, or a cultured mammal-derived cell, or from a transgenic animal or a transgenic plant into which an IGF-I gene has been transfected. Human IGF-I is also available as a research reagent (Enzo Life Sciences, catalog: ADI-908-059-0100, Abnova, catalog:P3452, etc.) or as a pharmaceutical product (Somazon[®] mecasermin, INCRELEX[®], etc.). The *in vivo* and *in vitro* activities of IGF-I for use can be evaluated as specific activities relative to an IGF-I standard substance under NIBSC code: 91/554, whose activity corresponds to one international unit/microgram. The standard substance is available from World Health Organization's National Institute for Biological Standards and Control (NIBSC). In the context of the present invention, IGF-I is considered as having a specific activity equivalent to the IGF-I of NIBSC code: 91/554.

[IGF-I receptor]**[0025]**

The term "IGF-I receptor" refers to an insulin-like growth factor -I receptor. The term "IGF-I receptor" used herein means an IGF-I receptor protein, unless specified otherwise. The IGF-I receptor is a protein formed with two subunits, each consisting of an alpha chain and a beta chain. The amino acid sequence of a human IGF-I receptor is indicated in SEQ ID NO:2, of

which a subsequence consisting of the 31st to 735th amino acid residues represents the alpha chain, while a subsequence starting from the 740th amino acid residue represents the beta chain. The alpha chain of the IGF-I receptor has a portion to which IGF-I binds, while the beta chain has a transmembrane structure and exhibits a function to transmit signals into the cell. The alpha chain of the IGF-I receptor can be divided into L1, CR, L2, FnIII-1, and FnIII-2a/ID/FnIII-2b domains. According to the amino acid sequence of the human IGF-I receptor defined in SEQ ID NO:2, the 31st to 179th residues correspond to the L1 domain, the 180th to 328th residues correspond to the CR domain, the 329th to 491st residues correspond to the L2 domain, the 492nd to 607th residues correspond to the FnIII-1 domain, and the 608th to 735th residues correspond to the FnIII-2a/ID/FnIII-2b domain. Among them, the CR (cysteine-rich) domain is involved in the activation of an intracellular tyrosine kinase in the beta chain, which is associated with a conformational change of the IGF-I receptor occurring when IGF-I binds to the receptor. The amino acid sequence of human IGF-I receptor is available, e.g., on EMBL-EBI with UniProtKB-accession number P08069, and is also indicated in the sequence listing as SEQ ID NO:2.

[0026]

The IGF-I receptor is known to be expressed in a wide range of tissues and cells in the living body, and receives various stimuli via IGF-I, such as induction of cell proliferation and activation of intracellular signals. In particular, effects of IGF-I on myoblasts via the IGF-I receptor can be evaluated using cell proliferation activities as indicators. For this reason, myoblasts are useful in analyzing the effects of antibodies binding to the IGF-I receptor. Cells expressing an IGF-I receptor derived from human or any other vertebrate can be prepared artificially, by transfection of an expression vector carrying a nucleic acid molecule encoding the amino acid sequence of an IGF-I receptor derived from human or any other vertebrate into a eukaryotic host cell, such as a cultured insect cell or a mammal-derived cell, to prepare a recombinant cell expressing the IGF-I receptor encoded by the transfected nucleic acid on its cell membrane. The resultant cell expressing the IGF-I receptor can be used for analysis of the binding ability and intracellular signal transmissibility of antibodies.

[Anti-IGF-I receptor antibody]

[0027]

An antibody is a glycoprotein containing at least two heavy (H) chains and two light (L) chains coupled together via disulfide bindings. Each heavy chain has a heavy chain variable region (abbreviated as VH) and a heavy chain constant region. The heavy chain constant region contains three domains, i.e., CH1, CH2, and CH3. Each light chain contains a light chain variable region (abbreviated as VL) and a light chain constant region. A light chain constant

region has one domain, i.e., CL. There are two types of light chain constant regions, i.e., λ (lambda) chain and κ (kappa) chain. Heavy chain constant regions are classified into γ (gamma) chain, μ (mu) chain, α (alpha) chain, δ (delta) chain and ϵ (epsilon) chain, and different types of heavy chain constant regions result in different isotypes of antibodies, i.e., IgG, IgM, IgA, IgD, and IgE, respectively. Each of the VH and VL regions is also divided into four relatively conserved regions (FR-1, FR-2, FR-3, and FR-4), collectively referred to as framework regions (FR), and three highly variable regions (CDR-1, CDR-2, and CDR-3), collectively referred to as complementarity determining regions (CDR). The VH region includes the three CDRs and the four FRs arranged in the order of FR-1, CDR-1 (CDR-H1), FR-2, CDR-2 (CDR-H2), FR-3, CDR-3 (CDR-H3), and FR-4 from the amino terminal to the carboxyl terminal. The VL includes the three CDRs and the four FRs arranged in the order of FR-1, CDR-1 (CDR-L1), FR-2, CDR-2 (CDR-L2), FR-3, CDR-3 (CDR-L3), and FR-4 from the amino terminal to the carboxyl terminal. The variable region of each of the heavy chain and the light chain includes a binding domain, which interacts with an antigen.

[0028]

The antibody according to the present invention may be a fragment and/or derivative of an antibody. Examples of antibody fragments include F(ab')₂, Fab, and Fv. Examples of antibody derivatives include: antibodies to which an amino acid mutation has been introduced in its constant region; antibodies in which the domain arrangement of the constant regions has been modified; antibodies having two or more Fc's per molecule; antibodies consisting only of a heavy chain or only of a light chain; antibodies with modified glycosylation; bispecific antibodies; conjugates of antibodies or antibody fragments with compounds or proteins other than antibodies; antibody enzymes; nanobodies; tandem scFv's; bispecific tandem scFv's; diabodies; and VHHs. The term "antibody" used herein encompasses such fragments and/or derivatives of antibodies, unless otherwise specified.

[0029]

The term "monoclonal antibody" conventionally means antibody molecules obtained from a clone derived from a single antibody-producing cell, i.e., a single variety of antibody molecules having a combination of VH and VL with specific amino acid sequences. A monoclonal antibody can also be produced via genetic engineering procedure, by preparing a nucleic acid molecule having a gene sequence encoding the amino acid sequence of the monoclonal antibody protein. A person skilled in the art would also be familiar with techniques for modifying a monoclonal antibody using genetic information about, e.g., H chains, L chains, variable regions thereof, and CDR sequences thereof to thereby improve the binding ability and

specificity of the antibody, and techniques for preparing an antibody suitable for a therapeutic agent by altering an animal antibody such as a mouse antibody into a human-type antibody. A human-type monoclonal antibody can also be prepared by sensitizing a non-human transgenic animal carrying a human antibody gene to an antigen. Another method which does not require sensitization of an animal is a technique involving: preparing a phage library expressing an antigen binding region of a human antibody or a part thereof (human antibody phage display); obtaining a phage clone expressing a peptide which specifically binds to a corresponding antigen or an antibody having a desired amino acid sequence; and producing a desired human antibody based on the information of the selected phage clone. A person skilled in the art can employ such a technique as appropriate (see, e.g., a review by Taketo Tanaka et al., Keio J. Med., Vol.60, pp.37-46). A person skilled in the art can also design an antibody to be administered to a non-human animal in a similar manner to a humanized antibody, by using information about amino acid sequences of CDRs and variable regions as appropriate.

[0030]

The term "antigen-antibody reaction" used herein means that an antibody binds to an IGF-I receptor with an affinity represented by an equilibrium dissociation constant (KD) of 1×10^{-8} M or less. The antibody of the present invention should preferably bind to an IGF-I receptor with a KD of usually 1×10^{-8} M or less, particularly 1×10^{-9} M or less, more particularly 1×10^{-10} M or less.

[0031]

The term "specificity" of an antibody used herein means that an antibody causes a strong binding based on antigen-antibody reaction to a specific antigen. In the context of the present invention, the IGF-I receptor-specific antibody means an antibody which, when used at a concentration sufficient to significantly cause antigen-antibody reaction with cells expressing an IGF-I receptor, causes antigen-antibody reaction with an INSR at a reactivity of 1.5 times or less the reactivity with a Mock cell. An INSR has a high similarity to an IGF-I receptor in primary structure (amino acid sequence) and higher-order structure.

[0032]

A person skilled in the art would be able to carry out measurement of antigen-antibody reaction by selecting an appropriate binding assay in a system of a solid phase or liquid phase. Examples of such assays include, although not limited to: enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), surface plasmon resonance (SPR), fluorescence resonance energy transfer (FRET), and luminescence resonance energy transfer (LRET).

Measurement of antigen-antibody binding affinity can be carried out by, e.g., labelling an antibody and/or an antigen with, e.g., an enzyme, a fluorescent material, a luminescent material, or a radioisotope, and detecting the antigen-antibody reaction using a method suitable for measuring the physical and/or chemical properties characteristic to the label used.

5

[0033]

The anti-IGF-I receptor antibodies according to the present invention encompass both an agonist antibody and an antagonist antibody. When used singly, the IGF-I receptor agonist antibody of the present invention has an effect of enhancing the growth activity of myoblasts. When used in combination with IGF-I, the IGF-I receptor antagonist antibody of the present invention has an effect of inhibiting the IGF-I-induced growth activity of myoblasts.

10

[0034]

The IGF-I receptor agonist antibody according to the present invention which binds strongly to a specific domain of the IGF-I receptor has an effect of enhancing the growth activity of myoblasts *in vitro*.

15

[0035]

The IGF-I receptor agonist antibody of the present invention does not have an effect of enhancing glucose uptake by differentiated muscle cells *in vitro* at an effective concentration sufficient to enhance the growth activity of myoblasts, preferably at a concentration 10 times as high as the effective concentration, more preferably at a concentration 100 times as high as the effective concentration.

20

[0036]

While IGF-I has remarkable hypoglycemic effect at a dosage sufficient to exhibit muscle-mass increasing effect, the IGF-I receptor agonist antibody of the present invention does not have hypoglycemic effect at an effective dosage sufficient to exhibit an effect of increasing muscle mass, preferably at a dosage 10 times as high as the effective dosage.

25

[0037]

In addition, the IGF-I receptor agonist antibody, when administered to a guinea pig at a single dose, exhibit an *in vivo* activity to increase the muscle mass effect which corresponds to the activity achieved by sustained administration of IGF-I. The IGF-I receptor agonist antibody of the present invention also has a long half-life in blood, and exhibits muscle-mass increasing effect via single-dose administration to an animal.

30

35

[0038]

Thus, the IGF-I receptor agonist antibody of the present invention has a potential as a therapeutic or prophylactic agent for a variety of diseases associated with the IGF-I receptor such as disuse muscle atrophy and dwarfism, for which IGF-I has also been expected to be effective. In addition, the IGF-I receptor agonist antibody of the present invention can solve the problems involved in IGF-I by, e.g., overcoming the hypoglycemic effect and extending the blood half-life.

[0039]

The IGF-I receptor antagonist antibody of the present invention inhibits the binding of IGF-I to the IGF-I receptor. According to one embodiment of the IGF-I receptor antagonist antibody of the present invention, the antibody activates the IGF-I receptor while inhibiting the effects of IGF-I on the IGF-I receptor. In this embodiment, the antibody has an effect of negating an additive agonistic activity with IGF-I, e.g., an effect of negating the activity of IGF-I to induce growth of myoblasts. Another embodiment of the IGF-I receptor antagonist antibody of the present invention binds to, but does not activate, the IGF-I receptor. Examples of such antagonist antibodies which does not cause activation of the IGF-I receptor via cross-linking include, although not limited to: antibodies having monovalent antigen-binding ability, such as Fab and scFv; and antibodies having divalent binding sites, such as bispecific antibodies, in which only one of the binding sites binds to a specific domain of the IGF-I receptor, or in which the binding sites are spaced with a controlled interval using a linker. When preparing the IGF-I receptor antagonist antibody according to the present invention, it is possible to confirm whether the antibody binds to the IGF-I receptor but lacks agonistic activity by: determining whether the antibody has binding ability to the IGF-I receptor using a method of measuring antigen-antibody reactivity between the antibody and the IGF-I receptor; or determining whether the antibody lacks an activity to induce cell proliferation using a cell proliferation test with, e.g., myoblasts. On the other hand, the IGF-I receptor antagonist antibody does not affect glucose uptake by differentiated muscle cells *in vitro* or the blood glucose level *in vivo*. Therefore, the IGF-I receptor antagonist antibody of the present invention has a potential as a therapeutic or prophylactic agent without adverse effects such as hyperglycemia, and can be used for treating malignant tumors such as breast cancer, bowel cancer, sarcoma, lung cancer, prostate cancer, thyroid cancer, and myeloma.

[Binding ability of the anti-IGF-I receptor antibody]

[0040]

The anti-IGF-I receptor antibody according to the present invention binds to the CR domain of the IGF-I receptor as an epitope. On the other hand, the IGF-I receptor agonist antibody does not have an ability to bind to INSR, which has a high similarity to the IGF-I receptor in primary structure (amino acid sequence) and higher-order structure.

5 By binding to the CR domain of the IGF-I receptor, the anti-IGF-I receptor antibody according to the present invention is deemed to activate a homo-type receptor, which is a dimer of two copies of the IGF-I receptor, or a hetero-type receptor, which is a dimer between the IGF-I receptor and INSR.

10 **[Sequence of the anti-IGF-I receptor antibody]** **[0041]**

The sequence of the anti-IGF-I receptor antibody according to the present invention is not particularly limited, as long as it specifically binds to an IGF-I receptor of a vertebrate and has an activity to induce cell proliferation.

15 However, the antibody should preferably have specific amino acid sequences as CDR sequences, as will be explained in details below. In the context of the present invention, the term “identity” of amino acid sequences used herein means the ratio of identical amino acid residues between the sequences, while the term “similarity” of amino acid sequences used herein means the ratio of identical or similar amino acid residues between the sequences. The similarity and
20 identity of amino acid sequences can be determined, e.g., using BLAST method (with default conditions of PBLAST provided by NCBI).

[0042]

The term “similar amino acid residues” used herein means a group of amino acid residues
25 having side chains with similar chemical properties (e.g., electric charge or hydrophobicity). Groups of similar amino acid residues include:

- 1) amino acid residues having aliphatic side chains: glycine, alanine, valine, leucine, and isoleucine residues;
 - 2) amino acid residues having aliphatic hydroxyl side chains: serine and threonine residues;
 - 30 3) amino acid residues having amide-containing side chains: asparagine and glutamine residues;
 - 4) amino acid residues having aromatic side chains: phenylalanine, tyrosine, and tryptophan residues;
 - 5) amino acid residues having basic side chains: lysine, arginine, and histidine residues;
 - 35 6) amino acid residues having acidic side chains: aspartic acid and glutamic acid residues;
- and

7) amino acid residues having sulfur-containing side chains: cysteine and methionine residues.

[0043]

5 According to the present invention, the sequence of CDR-1 of the heavy chain variable region (CDR-H1) should preferably be the amino acid sequence defined in SEQ ID NO:3 (SerTyrTrpMetHis) or an amino acid sequence derived from SEQ ID NO:3 via substitution, deletion or insertion of any one amino acid residue. The sequence of CDR-H1 should also preferably have a similarity of 80% or higher to SEQ ID NO:3. In the context of the present
10 invention, when an amino acid residue (hereinafter “the first amino acid residue”) of an amino acid sequence is substituted with another amino acid residue (hereinafter “the second amino acid residue”), the first amino acid residue before the substitution and the second amino acid residue after the substitution should more preferably be similar to each other in structure and/or characteristics.

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[0044]

The sequence of CDR-2 of the heavy chain variable region (CDR-H2) should preferably be the amino acid sequence defined in SEQ ID NO:4 (GluThrAsnProSerAsnSerValThrAsnTyrAsnGluLysPheLysSer) or an amino acid sequence
20 derived from SEQ ID NO:4 via substitution, deletion or insertion of any one or two amino acid residues. The sequence of CDR-H2 should also preferably have a similarity of 82% or higher, particularly 88% or higher, more particularly 94% or higher to SEQ ID NO:4.

[0045]

25 The sequence of CDR-3 of the heavy chain variable region (CDR-H3) should preferably be the amino acid sequence defined in SEQ ID NO:5 (GlyArgGlyArgGlyPheAlaTyr) or an amino acid sequence derived from SEQ ID NO:5 via substitution, deletion or insertion of any one or two amino acid residues. The sequence of CDR-H3 should also preferably have a similarity of 75% or higher, particularly 87% or higher to SEQ ID NO:5.

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[0046]

The sequence of CDR-1 of the light chain variable region (CDR-L1) should preferably be the amino acid sequence defined in SEQ ID NO:6 (ArgAlaSerGlnAsnIleAsnPheTrpLeuSer) or an amino acid sequence derived from SEQ ID NO:6 via substitution, deletion or insertion of any
35 one or two amino acid residues. The sequence of CDR- L1 should also preferably have a similarity of 81% or higher, particularly 90% or higher to SEQ ID NO:6.

[0047]

The sequence of CDR-2 of the light chain variable region (CDR-L2) should preferably be the amino acid sequence defined in SEQ ID NO:7 (LysAlaSerAsnLeuHisThr) or an amino acid sequence derived from SEQ ID NO:7 via substitution, deletion or insertion of any one amino acid residue. The sequence of CDR- L2 should also preferably have a similarity of 85% or higher to SEQ ID NO:7.

[0048]

The sequence of CDR-3 of the light chain variable region (CDR-L3) should preferably be the amino acid sequence defined in SEQ ID NO:8 (LeuGlnGlyGlnSerTyrProTyrThr) or an amino acid sequence derived from SEQ ID NO:8 via substitution, deletion or insertion of any one or two amino acid residues. The sequence of CDR- L3 should also preferably have a similarity of 77% or higher, particularly 88% or higher to SEQ ID NO:8.

[0049]

Still more preferably, the anti-IGF-I receptor antibody according to the present invention should have the combination of CDR sequences of:

as the CDR-H1 sequence, the amino acid sequence defined in SEQ ID NO:3;

as the CDR-H2 sequence, the amino acid sequence defined in SEQ ID NO:4;

as the CDR-H3 sequence, the amino acid sequence defined in SEQ ID NO:5;

as the CDR-L1 sequence, the amino acid sequence defined in SEQ ID NO:6;

as the CDR-L2 sequence, the amino acid sequence defined in SEQ ID NO:7; and

as the CDR-L3 sequence, the amino acid sequence defined in SEQ ID NO:8.

[0050]

Methods for identifying the sequence of each of CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2, and CDR-L3 of an antibody include: Kabat method (Kabat et al., The Journal of Immunology, 1991, Vol.147, No.5, pp.1709-1719) and Chothia method (Al-Lazikani et al., Journal of Molecular Biology, 1997, Vol.273, No.4, pp.927-948). These methods are within the technical common knowledge to persons skilled in the art, the summaries thereof being available, e.g., on the website of Dr. Andrew C.R. Martin's Group (<http://www.bioinf.org.uk/abs/>).

[0051]

The framework sequences of immunoglobulin for the antibody of the present invention

should preferably be the framework sequences of each class of immunoglobulin of a vertebrate, more preferably the framework sequences of each class of immunoglobulin of a human or a non-human animal including guinea pig, monkey, rabbit, cow, pig, horse, sheep, dog, fowl, mouse, or rat.

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[0052]

The anti-IGF-I receptor antibody according to the present invention should preferably have specific amino acid sequences as the heavy chain variable region and the light chain variable region, as will be specified below.

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[0053]

The heavy chain variable region should preferably have the amino acid sequence defined in SEQ ID NO:9, an amino acid sequence derived from SEQ ID NO:9 via substitution, deletion or insertion of any one or two amino acid residues, or an amino acid sequence having a similarity of 90% or higher to SEQ ID NO:9. The light chain variable region should preferably have the amino acid sequence defined in SEQ ID NO:10, an amino acid sequence derived from SEQ ID NO: 10 via substitution, deletion or insertion of any one or two amino acid residues, or an amino acid sequence having a similarity of 90% or higher to SEQ ID NO:10. The anti-IGF-I receptor antibody according to the present invention should more preferably be IGF11-16, that is, should include the combination of SEQ ID NO:9 as the heavy chain variable region and SEQ ID NO:10 as the light chain variable region.

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[0054]

A person skilled in the art will be able to design a humanized anti-IGF-I receptor antibody according to the present invention by selecting amino acid sequences of CDRs and/or variable regions of a heavy chain and a light chain from those mentioned above and combining them with amino acid sequences of framework regions and/or constant regions of a heavy chain and a light chain of a human antibody as appropriate. Amino acid sequences of framework regions and/or constant regions of a heavy chain and a light chain of a humanized antibody can be selected from, e.g., those of human IgG, IgA, IgM, IgE, and IgD classes or variants thereof.

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[0055]

When the anti-IGF-I receptor antibody according to the present invention is an IGF-I receptor agonist antibody, the antibody of the present invention or its antigen binding fragment should preferably be human IgG class or a variant thereof, more preferably human IgG4 subclass, human IgG1 subclass, or a variant thereof. According to one example, a stabilized

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IgG4 constant region has proline at position 241 in the hinge region according to Kabat's numbering system. This position corresponds to position 228 in the hinge region according to EU numbering system (Kabat et al., Sequences of Proteins of Immunological Interest, DIANE Publishing, 1992, Edelman et al., Proc. Natl. Acad. Sci USA, 63, 78-85, 1969). The residue at this position in human IgG4 is usually serine, while substitution of serine with proline can induce stabilization of the resultant antibody. According to another example, introduction of N297A mutation into the constant region of IgG1 serves to minimize the ability to bind to the Fc receptor and/or the ability to fix a complement.

10 **[Competitive binding]**
[0056]

A humanized antibody which causes competitive binding to the IGF-I receptor with the humanized anti-IGF-I receptor antibody according to the present invention is also included in the scope of the present invention. The term "competitive binding" used herein means the phenomenon that when there are two or more monoclonal antibodies together with an antigen, the binding of one of the antibodies to the antigen is inhibited by the binding of the other antibody to the antigen. The competitive binding can usually be measured by, e.g., adding, to a constant amount (concentration) of a monoclonal antibody, another monoclonal antibody with varying the amount (concentration) thereof, and determining the amount (concentration) of the latter monoclonal antibody at which the binding amount of the former monoclonal antibody, existing in the constant amount, is decreased. The degree of inhibition thereof can be expressed in the unit of IC₅₀ or K_i. The monoclonal antibody which causes competitive binding with the humanized anti-IGF-I receptor antibody according to the present invention means an antibody having an IC₅₀ of 1000nM or less, particularly 100nM or less, more particularly 10nM or less when measuring an antigen-antibody binding using the humanized anti-IGF-I receptor antibody according to the present invention, e.g., the IGF11-16 antibody, at 10nM. Measurement of competitive binding can also be made by labelling the antibody for use with, e.g., an enzyme, a fluorescent substance, a luminescent substance, a radioactive isotope, etc., and detecting the label using a measurement method suitable for detection of the physical and/or chemical properties of the label.

[Cross-reaction]
[0057]

The anti-IGF-I receptor antibody according to the present invention should preferably cross-react with the IGF-I receptor of another vertebrate. The term "*cross-reaction*" means that while the antibody causes antigen-antibody reaction with the IGF-I receptor from a target animal

(such as human), the antibody also has an ability to bind to an antigen derived from another animal different from the target animal. The antibody should preferably has a cross-reactivity with the IGF-I receptor of a different animal from the target animal whose IGF-I receptor is the target of the antigen-antibody reaction by the antibody, such as human or a non-human animal including guinea pig, monkey, rabbit, cow, pig, horse, sheep, dog, or fowl. Example 7 demonstrates that an anti-IGF-I receptor antibody, IGF11-16, was shown to bind to the ProSerGlyPheIleArgAsnGlySerGlnSerMet sequence in the CR domain of the human IGF-I receptor. Since this ProSerGlyPheIleArgAsnGlySerGlnSerMet sequence is conserved in the homologous parts of the IGF-I receptors of monkey (cynomolgus monkey), rabbit, guinea pig, cow, sheep, horse, and dog, this antibody has cross-binding ability to the IGF-I receptors from these species. In addition, since the amino acid sequences of the homologous parts of mouse and rat are both ProSerGlyPheIleArgAsnSerThrGlnSerMet, screening for an anti-IGF-I receptor antibody which binds to this part makes it possible to obtain an antibody which binds to the IGF-I receptors of, e.g., mouse and rat, and also has similar characteristics and functions as those of IGF11-16.

[0058]

Alternatively, a cell or an animal of a species which does not cross-react with the anti-IGF-I receptor antibody according to the present invention can be altered via genetic engineering into a cell or an animal expressing an IGF-I receptor with which the anti-IGF-I receptor antibody according to the present invention cross-reacts.

[Activity to induce growth of vertebrate-derived cells and activity to induce an increase in the muscle mass and/or the body length]

[0059]

An anti-IGF-I receptor antibody according to an embodiment of the present invention has an activity to induce growth of vertebrate-derived cells. Although IGF-I receptor agonist antibodies were already known, no antibody has been reported to show an activity to induce growth primary cultured cells, particularly myoblasts. Also, there has been no known antibody reported so far as having cell growth-inducing activity at a dosage lower than the EC₅₀ value of IGF-I *in vitro*. The vertebrate-derived cells in the context of the present invention should preferably be cells derived from mammals, birds, reptiles, amphibia, or fish, more preferably cells derived from mammals or birds, still more preferably cells derived from human, monkey, rabbit, guinea pig, cow, pig, sheep, horse or dog. Cells derived from these species which express an IGF-I receptor with which the anti-IGF-I receptor antibody according to the present invention cross-reacts can be induced to proliferate by the anti-IGF-I receptor antibody according to the

present invention. The vertebrate-derived cells according to the present invention also encompass: cells and animals engineered to express an IGF-I receptor of a species with which the anti-IGF-I receptor antibody according to the present invention cross-reacts; and modified animal cells derived from such engineered cells and animals.

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[0060]

An antibody's activity to induce growth of vertebrate-derived cells can be analyzed *in vitro* using primary cultured cells, established cell lines, or transformants derived from such cells. The term "primary cultured cells" means cells which were isolated from an organ or a
 10 tissue of a living organism, and can typically be subcultured for some passages. Primary cultured cells derived from a vertebrate can be obtained from an organ or a tissue of the vertebrate via enzyme treatment, dispersion with physical means, or explant method. An organ or a tissue or a fragment thereof obtained from the vertebrate can also be used for analyzing the antibody's activity above. Preferable examples of organs and tissues from which primary cells are prepared
 15 include: endocrine tissues such as thyroid, parathyroid, and adrenal gland; immune tissues such as appendix, tonsil, lymph nodes, and spleen; respiratory organs such as trachea and lung; digestive organs such as stomach, duodenum, small intestine, and large intestine; urinary organs such as kidney and urinary bladder; male genital organs such as vas deferens, testicle, and prostate; female genital organs such as breast and fallopian tube; and muscle tissues such as heart
 20 muscle and skeletal muscles. More preferable examples include liver, kidney, or digestive organs or muscle tissues, among which muscle tissues are still more preferable. Primary cultured cells which can be used for analyzing the growth-inducing activity of an anti-IGF-I receptor antibody in the context of the present invention are cells which express an IGF-I receptor and can be induced to proliferate by IGF-I binding to the IGF-I receptor. Typical examples thereof are
 25 skeletal muscle myoblasts, which are primary cultured cells isolated from muscle tissue. Human- or animal-derived primary cultured cells available by assignment or commercially on the market can also be obtained and used. Human primary cultured cells are available from various institutions and companies, e.g., ATCC[®], ECACC, Lonza, Gibco[®], Cell Applications, ScienCell research laboratories, and PromoCell.

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[0061]

The term "cell line" means a line of cultured cells which were derived from a living organism and then immortalized such that they can semipermanently proliferate with maintaining their specific properties. Cell lines are divided into non-tumor-derived cell lines and
 35 tumor-derived cell lines. Vertebrate-derived cell lines which can be used for analyzing the growth-inducing activity of the anti-IGF-I receptor antibody according to the present invention

are cells which express an IGF-I receptor and can be induced to proliferate by IGF-I binding to the IGF-I receptor. Examples of cell lines which express an IGF-I receptor and can be induced to proliferate by IGF-I include, although not limited to: human neuroblastoma SH-SY5Y, human epidermal keratinocyte line HaCaT, human alveolar basal epithelial adenocarcinoma cell line A549, human colon-adenocarcinoma cell line Caco-2, human hepatocellular cancer cell line HepG2, human cervical cancer cell line Hela, human cervical cancer cell line SiHa, human breast cancer cell line MCF7, human pluripotent human embryonal carcinoma line NTERA-2, and human bone cancer cell line U-2-OS.

10 **[0062]**

Other cells which can be used for analyzing the growth-inducing activity of the anti-IGF-I receptor antibody according to the present invention are transformants derived from primary cultured cells and cell lines. Examples of such transformants include: iPS cells produced from primary cultured cells; and cells and tissues differentiated from such iPS cells. Other transformants include primary cultured cells and cell lines engineered to incorporate a gene so as to transiently or permanently express the gene. Examples of genes to be introduced into and expressed by such cells include IGF-I receptor genes of human and other species.

15 **[0063]**

Methods for determining the ability of the anti-IGF-I receptor antibody according to the present invention to induce vertebrate-derived cells to proliferate include: cell counting, measurement of DNA synthesis, and measurement of change in the metabolic enzyme activity. Methods for cell counting include methods using blood cell counting plates or cell counting devices such as Coulter counters. Methods for measuring DNA synthesis include methods based on uptake of [3H]-thymidine or 5-bromo-2'-deoxyulysine (BrdU). Method for measuring the change in metabolic enzyme activity include MTT method, XTT method, and WST method. A person skilled in the art could also employ other methods as appropriate. An activity to induce cell proliferation is detected if the growth of the cultured cells reacted with the anti-IGF-I receptor antibody according to the present invention is higher than the growth of the cells in the absence of the antibody. In this case, it is convenient to also measure the induction activity under the same conditions using IGF-I, which is an original legand for the IGF-I receptor, as a control.

20 **[0064]**

The cultured cells to be tested are reacted with either the anti-IGF-I receptor antibody according to the present invention or IGF-I with varying its concentration, and the concentration at which 50% of the maximum growth activity is exhibited is determined as an EC₅₀ value.

When human skeletal muscle myoblasts are used for evaluating the growth activity, the EC₅₀ value of the anti-IGF-I receptor antibody according to the present invention for inducing cell proliferation should preferably be comparable to that of IGF-I, more preferably 1/10 or less of that of IGF-I, still more preferably 1/20 or less, still further more preferably 1/50 or less of that of IGF-I. When human skeletal muscle myoblasts are used for evaluating the growth activity, the EC₅₀ value of the anti-IGF-I receptor antibody according to the present invention should preferably be 0.5nmol/L or less, more preferably 0.3nmol/L or less, still more preferably 0.1nmol/L or less.

10 [0065]

Methods for measuring the activity to induce growth of vertebrate-derived cells *in vivo* include: a method involving parenterally administering the anti-IGF-I receptor antibody according to the present invention to a vertebrate and measuring changes in the weight, size, cell count, etc., for the entire body of the individual which received the administration or for an organ or a tissue isolated from the individual; and a method involving using an animal with a graft of vertebrate cells and measuring changes in the weight, size, cell count, etc., of the graft including vertebrate cells. Measurements for the entire body of an individual include: measurements of the body weight, the body length, and the circumferences of four limbs; measurement of the body composition, using impedance method; and measurement of the creatinine height coefficient. Measurements for an organ, a tissue, or a graft from an individual include: in the case of a non-human animal, a method involving directly recovering the target organ, tissue or graft and measuring its weight, size, or the number of cells included therein. Non-invasive measurements for an organ, a tissue, or a graft from an individual include: image analysis using X-ray photography, CT, and MRI; and contrast methods using tracers with isotopes or fluorescent substances. If the target tissue is skeletal muscle, then a change in the muscle force can also be used as an indicator. A person skilled in the art could also employ any other methods as appropriate for analyzing the activity of the anti-IGF-I receptor antibody according to the present invention to induce growth of vertebrate-derived cells *in vivo*. Methods for measuring the activity of the anti-IGF-I receptor antibody according to the present invention to induce growth of vertebrate-derived cells *in vivo* include: carrying out measurements using, e.g., the methods mentioned above for individuals who received administration of the anti-IGF-I receptor antibody according to the present invention and individuals who received administration of a different antibody other than the anti-IGF-I receptor antibody according to the present invention or any other control substance, and comparing the resultant measurements between these individuals.

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[0066]

The anti-IGF-I receptor antibody according to the present invention is characterized by having a longer duration of cell-growth inducing effect relative to the time of contact with the cells compared to the duration of the wild-type IGF-I, and thereby exhibits improved sustainability. According to Example 12, which demonstrates cell proliferation induction activities *in vitro*, when cells were contacted with the wild-type IGF-I and then washed with culture medium without IGF-I, the cell proliferation induction activity of the wild-type IGF-I disappeared after the washing. On the other hand, when cells were contacted with IGF11-16 (anti-IGF-I receptor antibody according to the present invention) and then washed with culture medium without IGF11-16, the cell growth-inducing activity continued even after the washing. According to Example 16, which compares the kinetics of IGF-I and IGF11-16 (anti-IGF-I receptor antibody according to the present invention) in blood, about 50% or higher of the wild-type IGF-I administered to an animal disappeared from the blood within 24 hours after the administration, while 60% or higher of the IGF11-16 antibody administered to an animal remained in the blood even 48 hours after the administration. Thus, the IGF11-16 antibody was shown to remain in the blood for a long time. These results indicate that the anti-IGF-I receptor antibody according to the present invention exhibits a long-term effect of inducing cell proliferation both *in vitro* and *in vivo*.

[0067]

The anti-IGF-I receptor antibody according to the present invention is also expected to exhibit an *in vivo* effect of increasing the muscle mass and/or the body length. Specifically, IGF-I has an effect of inducing the growth and differentiation of myoblasts in skeletal muscles as mentioned above, as well as an effect of broadening muscle fibers. It is expected that these effects collectively lead to the effect of increasing the muscle mass. Like IGF-I, when the anti-IGF-I receptor antibody according to the present invention is administered to an animal, it also exhibits an effect of increasing the muscle mass of the animal. The anti-IGF-I receptor antibody according to the present invention is the first IGF-I receptor agonist antibody which has been shown to exhibit an *in vivo* effect of increasing the muscle mass.

[0068]

Methods for measuring the activity of the anti-IGF-I receptor antibody according to the present invention to increase the muscle mass include: for the entire body of the individual which received the administration, measurement of the body weight, the body length, and the circumferences of four limbs; measurement of the body composition, using impedance method; and measurement of the creatinine, and height coefficient. Other methods include: image analysis using X-ray photography, CT, and MRI; contrast methods using tracers with isotopes or

fluorescent substances; and measurement of a change in the muscle force. In the case of a non-human animal, a method involving directly recovering the target organ, tissue or graft and measuring its weight and/or size can also be used. The effect of increasing the muscle mass can be evaluated by: comparing the muscle mass increases between an individual to which the anti-IGF-I receptor antibody according to the present invention was administered and an individual to which the antibody was not administered; or comparing the muscle masses of an individual before and after administration of the anti-IGF-I receptor antibody according to the present invention. The effect of increasing the muscle mass can be determined if there is any increase in the muscle mass of an individual before and after the administration of the anti-IGF-I receptor antibody according to the present invention. Preferably, the effect achieved by administration of the anti-IGF-I receptor antibody according to the present invention can be determined when there is a difference of preferably 103% or higher, more preferably 104% or higher of the muscle mass between an individual to which the anti-IGF-I receptor antibody according to the present invention was administered and an individual to which the antibody was not administered, or of the same individual between before and after administration of the anti-IGF-I receptor antibody according to the present invention. IGF-I also plays a role in the bone growth, and has an effect of increasing the body length (the body height in the case of the human). Therefore, the anti-IGF-I receptor antibody according to the present invention also exhibits an effect of increasing the body length when administered to an animal. The effect of the anti-IGF-I receptor antibody according to the present invention in increasing the body length of an individual can be determined by measuring the body weight, the body length, and the circumferences of four limbs of the individual.

[Effects on the glucose uptake by vertebrate-derived cells and/or the blood glucose level of an animal]
[0069]

An anti-IGF-I receptor antibody according to an embodiment of the present invention is characterized by not affecting the intracellular glucose uptake by differentiated muscle cells derived from a vertebrate and/or the blood glucose level of a vertebrate. Specifically, IGF-I is known to has an effect of increasing the intracellular glucose uptake and an effect of lowering the blood glucose level as part of its agonist effects to the IGF-I receptor. On the other hand, although the anti-IGF-I receptor antibody of the present invention functions as an agonist of an IGF-I receptor antibody, it unexpectedly does not induce the glucose uptake by differentiated muscle cells even at a dosage of 100 times or more as high as the *in vitro* EC₅₀ value for cell growth-inducing activity. More particularly, when parenterally administered to an animal at a dosage of 10 times or more as high as the effective dosage sufficient to induce a muscle mass

increase, the antibody unexpectedly does not alter the blood glucose level. In addition, as properties as an IGF-I receptor antagonist antibody, its characteristics of not affecting the intracellular glucose uptake by differentiated muscle cells derived from a vertebrate and/or the blood glucose level of a vertebrate are advantageous effects which serve to avoid the problem of causing hyperglycemia, which problem was desired to be solved for the antibody to be used for human therapy, but was not solved by conventional IGF-I receptor antagonist antibodies. The vertebrate-derived cells according to the present invention should preferably be cells derived from mammals, birds, reptiles, amphibia or fish, more preferably cells derived from mammals or birds, still more preferably cells derived from human, monkey, rabbit, guinea pig, cow, pig, sheep, horse or dog. The vertebrate-derived cells according to the present invention also encompasses: cells and animals engineered to express an IGF-I receptor derived from a vertebrate species having a cross-reactivity with the anti-IGF-I receptor antibody according to the present invention; cells and animals engineered to express an IGF-I receptor mutated so as to has a binding ability; and cells derived from such engineered animals.

[0070]

In order to analyze the effect of the anti-IGF-I receptor antibody of the present invention in not affecting the intracellular glucose uptake by vertebrate-derived cells *in vitro*, it is possible to use primary cultured cells, cell lines, and transformants derived from such cells. The term “primary cultured cells” means cells which were isolated from an organ or a tissue of a living organism, and can typically be subcultured for some passages. Primary cultured cells derived from a vertebrate can be obtained from an organ or a tissue of the vertebrate via enzyme treatment, dispersion with physical means, or explant method. Preferable examples of organs and tissues from which primary cells are prepared include: endocrine tissues such as thyroid, parathyroid, and adrenal gland; immune tissues such as appendix, tonsil, lymph nodes, and spleen; respiratory organs such as trachea and lung; digestive organs such as stomach, duodenum, small intestine, and large intestine; urinary organs such as kidney and urinary bladder; male genital organs such as vas deferens, testicle, and prostate; female genital organs such as breast and fallopian tube; and muscle tissues such as heart muscle and skeletal muscles. More preferable examples include liver, kidney, or digestive organs or muscle tissues, among which muscle tissues are still more preferable.

[0071]

Primary cultured cells which can be used for analyzing the feature of an anti-IGF-I receptor antibody for not affecting the intracellular glucose uptake in the context of the present invention are cells which express an IGF-I receptor and can be induced to cause intracellular

glucose uptake by IGF-I binding to the IGF-I receptor. Typical examples thereof are muscle cells differentiated from skeletal muscle myoblasts, which are primary cultured cells isolated from muscle tissue.

5 [0072]

The “differentiated muscle cells” of the present invention are muscle cells which have caused differentiation, and include those which have not been completely differentiated. For the sake of convenience, the term “differentiated muscle cells” in the context of the present invention refers to cells which have experienced differentiation for at least about six days from
10 the start of differentiation. Human- or animal-derived primary cultured cells available by assignment or commercially on the market can also be obtained and used. Human primary cultured cells are available from various institutions and companies, e.g., ATCC[®], ECACC, Lonza, Gibco[®], Cell Applications, ScienCell research laboratories, and PromoCell.

15 [0073]

The term “cell line” means a line of cultured cells which were derived from a living organism and then immortalized such that they can semipermanently proliferate while maintaining their specific properties. Cell lines are divided into non-tumor-derived cell lines and tumor-derived cell lines. Vertebrate-derived cell lines which can be used for analyzing the effect
20 of the anti-IGF-I receptor antibody according to the present invention on the intracellular glucose uptake are cells which express an IGF-I receptor and can be induced to cause intracellular glucose uptake by IGF-I binding to the IGF-I receptor. Examples of cell lines which express an IGF-I receptor and can be induced to cause intracellular glucose uptake by IGF-I include, although not limited to: skeletal muscle cells, fat cells, and epidermal keratinocytes.

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[0074]

Other cells which can be used for analyzing the effect of the anti-IGF-I receptor antibody according to the present invention on the intracellular glucose uptake are transformants derived from primary cultured cells and cell lines. Examples of such transformants include: iPS cells
30 produced from primary cultured cells; and cells and tissues induced to differentiate from such iPS cells. Other transformants include primary cultured cells and cell lines engineered to incorporate a gene so as to transiently or permanently express the gene. Examples of genes to be introduced into and expressed by such cells include IGF-I receptor genes of human and other species.

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[0075]

Methods for determining the effect of the anti-IGF-I receptor antibody according to the present invention on the glucose uptake by vertebrate-derived cells include: measurement of the intracellular glucose concentration; measurement of the intracellular uptake of a glucose analog tracer substance; and measurement of a change in the amount of a glucose transporter. Methods
 5 for measuring the glucose concentration include absorbance measurement methods such as enzyme method. Methods for measuring the intracellular uptake amount of a glucose analog tracer substance include measurement of the uptake amount of [3H]-2'-deoxyglucose. Methods for measuring a change in the amount of a glucose transporter include immunocytostaining and western blotting. A person skilled in the art could also employ other methods as appropriate. The
 10 fact that there is no effect on the intracellular glucose uptake can be confirmed if the intracellular glucose uptake of the cultured cells reacted with the anti-IGF-I receptor antibody according to the present invention is almost the same of the intracellular glucose uptake of the cultured cells in the absence of the antibody. In this case, it is convenient to also carry out the measurement under the same conditions using IGF-I, which is an original legend for the IGF-I receptor, as a
 15 control.

[0076]

The cultured cells to be tested are treated with either the anti-IGF-I receptor antibody according to the present invention or IGF-I with varying its concentration, and the glucose
 20 uptake of the treatment group is indicated as a percentage when the intracellular glucose uptake of the non-treatment group is determined as 100%. When human differentiated muscle cells are used for evaluating the glucose uptake, the glucose uptake achieved by the anti-IGF-I receptor antibody according to the present invention should preferably be equal to or less than the glucose uptake achieved by IGF-I at the same concentration. More preferably, the glucose uptake
 25 achieved by the anti-IGF-I receptor antibody according to the present invention should be 110% or less, still more preferably 100%, of the glucose uptake amount of the non-treatment group. When human differentiated muscle cells are used for evaluating the glucose uptake, the glucose uptake achieved by the anti-IGF-I receptor antibody according to the present invention added at an amount of 100nmol/L should preferably be 110% or less, more preferably 105% or less, still
 30 more preferably from 95% to 100%.

[0077]

Methods for determining the glucose uptake by vertebrate-derived cells *in vivo* include: methods involving parenterally administering the anti-IGF-I receptor antibody according to the
 35 present invention to a vertebrate and determining a change in the glucose content of an organ or a tissue of the individual. Methods of measurement for the entire body of the individual which

received the administration include: measurement of the blood glucose level; and hemoglobin A1C using glycosylated proteins as indicators. Methods of measuring the glucose uptake for an organ or a tissue of an individual include: in the case of a non-human animal, directly recovering the target organ or tissue, and calculating the concentration of glucose or a tracer. Non-invasive methods for measuring the glucose uptake individual for an organ or a tissue of an individual include: image analysis using X-ray photography, CT, and MRI; and contrast methods using tracers with isotopes or fluorescent substances. If the target tissue is a skeletal muscle, then the glucose clamp can also be used as an indicator. A person skilled in the art could also employ any other methods as appropriate for analyzing the effect of the anti-IGF-I receptor antibody according to the present invention on the glucose uptake by vertebrate-derived cells *in vivo*.

[0078]

The anti-IGF-I receptor antibody according to the present invention is also characterized in that when parenterally administered to a vertebrate even at an effective dosage sufficient to increase the muscle mass of the vertebrate, preferably at a dosage of 10 times or more the effective dosage, it does not change the blood glucose level of the vertebrate. When evaluating the effect of the anti-IGF-I receptor antibody of the present invention in changing the blood glucose level of a vertebrate, it is preferred to use an animal belonging to mammals, birds, reptiles, amphibia or fish, more preferably an animal belonging to mammals or birds, still more preferably human, monkey, rabbit, guinea pig, cow, pig, sheep, horse or dog. An animal engineered to express an IGF-I receptor of a species which has cross-reactivity with the anti-IGF-I receptor antibody according to the present invention can also be used as an animal for evaluating the effect of the anti-IGF-I receptor antibody of the present invention in changing the blood glucose level. Invasive methods for measuring the blood glucose level include colorimetric method and electrode method. Examples of enzyme methods used for detection include glucose oxidase method (GOD method) and glucose dehydrogenase method (GDH method). Non-invasive methods include optical measurement methods. A person skilled in the art can also select any other method as appropriate. In the case of human, the normal range of fasting blood glucose level is from 100mg/dL to 109mg/dL. With regard to adverse events in the blood glucose level resulting from a drug administration (Common Terminology Criteria for Adverse Events v4.0), the blood glucose level of lower than the range of from 77mg/dL to 55mg/dL is defined as an indicative of low blood glucose, while a blood glucose level of higher than the range of from 109mg/dL to 160mg/dL is defined as an indicative of high blood glucose. A drug administration is considered as not affecting the blood glucose level when the blood glucose level after the drug administration is higher than 55mg/dL and lower than 160mg/dL, more preferably higher than 77mg/dL and lower than 109mg/dL. However, the normal value of blood

glucose level and its range of fluctuation vary depending on the animal to which a drug is administered, and even a human subject may not always have a blood glucose level within a normal range at the time of the drug administration. Accordingly, in the context of the present invention, the anti-IGF-I receptor antibody according to the present invention should preferably
 5 be considered as not changing the blood glucose level of a vertebrate to which the antibody is administered when the change in the blood glucose level of the vertebrate is preferably 30% or less, more preferably 20% or less, still more preferably 10% or less.

[Process for producing the anti-IGF-I receptor antibody]

10 **[0079]**

The antibody according to the present invention can be produced using various techniques well-known to a person skilled in the art. Specifically, the antibody according to the present invention may be a polyclonal antibody or a monoclonal antibody (Milstein et al., Nature (England), October 6, 1983, Vol.305, No.5934, pp.537-540). A polyclonal antibody according to
 15 the present invention can be obtained, for example, by sensitizing a mammal with a peptide of the IGF-I receptor defined in SEQ ID NO:2 as an antigen, and recovering the resultant antibody from, e.g., the animal's serum. When the peptide is used as an antigen, the peptide may be bound to a carrier protein such as BSA or KLH or coupled with polylysine. Specific examples of peptides which can be used as an antigen include, although not limited thereto,
 20 ProSerGlyPheIleArgAsnGlySerGlnSerMet, a partial sequence of SEQ ID NO:2. A monoclonal antibody according to the present invention can be obtained, for example, by sensitizing a mammal with such an antigen, recovering an immune cell from the mammal, and fusing the immune cell with a myeloma cell to produce a hybridoma, cloning and culturing the hybridoma, and recovering the resultant antibody from the cultured hybridoma. An example of such a
 25 method for obtaining a monoclonal antibody is described in Example 1, and examples of monoclonal antibodies obtained thereby include, although not limited thereto, a monoclonal antibody having the VH amino acid sequence defined in SEQ ID NO:9 and the VL amino acid sequence defined in SEQ ID NO:10 (IGF11-16).

30 **[0080]**

Once such a monoclonal antibody is obtained, then a nucleic acid molecule having a gene sequence encoding the amino acid sequence of the antibody protein, and such a nucleic acid molecule can also be used for producing the antibody via genetic engineering technique. A person skilled in the art would appreciate various techniques for utilizing gene information about
 35 the antibody, such as information of the H chain and the L chain, the variable regions thereof, and the CDR sequences, for modifying the antibody in order to improve its binding ability or

specificity, or altering an animal antibody such as a mouse antibody into a human-type antibody, to thereby prepare an antibody having a structure suitable as a therapeutic agent for human. A human-type monoclonal antibody can also be prepared by using, as an animal to be sensitized with an antigen, a non-human transgenic animal into which a human antibody gene has been introduced. Another method which does not require sensitization of an animal is a technique involving using a phage library expressing an antigen binding region of a human antibody or a part thereof (human antibody phage display) and obtaining a phage clone expressing a peptide which specifically binds to a corresponding antigen or an antibody having a desired amino acid sequence, and producing a desired human antibody based on the information of the selected phage clone. A person skilled in the art can employ such a technique as appropriate (see, e.g., a review by Taketo Tanaka et al., Keio J. Med., Vol.60, pp.37-46).

[0081]

A method for producing a monoclonal antibody as mentioned above includes culturing a hybridoma which produces the desired antibody and purifying the resultant antibody from the culture supernatant via conventional means. Another method for producing a monoclonal antibody as mentioned above includes providing a hybridoma which produces the desired antibody or a phage clone obtained from a human antibody phage display, obtaining a gene encoding such an antibody, more specifically, a gene encoding a heavy chain and/or a light chain of immunoglobulin, preparing a vector expressing the gene, and introducing the vector into a host cell (mammal cell, insect cell, microorganism, etc.) for production of the antibody. A person skilled in the art could also modify this method by genetically engineering the gene encoding a heavy chain and/or a light chain of immunoglobulin for introducing a desired trait, and producing a humanized antibody, an antibody chimeric protein, a low-molecular antibody, or a scaffold antibody using structure information about variable regions or CDR regions of a heavy chain and/or a light chain of immunoglobulin, by using known techniques. In order to improve the performance of the antibody or avoid adverse effects, a person skilled in the art could also introduce an alteration into the structures of constant regions or sugar chains of the antibody, by using techniques well-known to a person skilled in the art.

[0082]

The anti-IGF-I receptor antibody according to the present invention can be obtained using a method well-known to persons skilled in the art. Specifically, while the humanized anti-IGF-I receptor antibody according to the present invention is typically a monoclonal antibody (Milstein et al., Nature, 1983, Vol.305, No.5934, pp.537-540), such a monoclonal antibody can be prepared by, e.g., the following method.

[0083]

This method starts with, for example, preparation of a nucleic acid molecule encoding the amino acid sequence(s) of a heavy chain and/or a light chain constituting an immunoglobulin of the anti-IGF-I receptor antibody according to the present invention. The nucleic acid molecule may then be cloned into various vector or plasmids to produce a vector or plasmid containing the nucleic acid molecule. Next, the nucleic acid molecule, vector, or plasmid is used to transform a host cell, which may be selected from, e.g., eukaryotic cells such as mammal cells, insect cells, yeast cells, and plant cells, and bacterium cells. The transformed host cell is then cultured under appropriate conditions which can allow production of the anti-IGF-I receptor antibody according to the present invention. If necessary, the resultant anti-IGF-I receptor antibody according to the present invention may be isolated from the host cell. Various methods that can be used for this procedure are well-known to persons skilled in the art.

[0084]

A method based on immunization of an animal includes preparing a non-human transgenic animal into which a human antibody gene has been introduced as the subject animal to be immunized, immunizing the animal using the IGF-I receptor and/or its partial peptide as an antigen, recovering an immune cell from the animal and fusing it with a myeloma cell to form a hybridoma, which is then cloned to produce an antibody, which is then recovered from the culture supernatant using a routine purification procedure. Example of such a method of obtaining a monoclonal antibody is described in, e.g., WO2013/180238A.

[0085]

Another available method includes using a phage library expressing a variable region of a desired humanized antibody or a part thereof (human antibody phage display) to thereby obtain an antibody which specifically binds to a corresponding antigen or a phage clone having a specific amino acid sequence, whose information is then used for producing the desired humanized antibody (see, e.g., the review by Taketo Tanaka et al., The Keio Journal of Medicine, Vol.60, pp.37-46)

[0086]

In this connection, a person skilled in the art can produce various antibodies such as antibody chimeric proteins, low molecule antibodies, and scaffold antibodies using known techniques, e.g., by making a genetic modification to a gene encoding a heavy chain and/or a light chain of an immunoglobulin for introducing a desired trait, or by using structure

information of variable regions or CDR regions of a heavy chain and/or a light chain of an immunoglobulin. In addition, in order to improve the performance of the antibody or avoiding side effects, it is possible to introduce a modification into the structure of a constant region of an antibody or to introduce glycosylation sites of an antibody, using techniques well-known to persons skilled in the art as appropriate.

[Drug containing the anti-IGF-I receptor antibody]

[0087]

The anti-IGF-I receptor antibody according to the present invention can be used as a therapeutic or prophylactic agent for a condition associated with IGF-I or a disease caused by any effect on an IGF-I receptor. Specifically, conditions associated with IGF-I or diseases that can be the target of therapy or prevention using the IGF-I receptor agonist antibody include: disuse muscle atrophy, dwarfism, hepatic cirrhosis, hepatic fibrosis, diabetic nephropathy, chronic renal failure, Laron syndrome, aging, intrauterine growth restriction (IUGR), cardiovascular protection, diabetes, insulin resistant, metabolic syndrome, osteoporosis, cystic fibrosis, myotonic dystrophy, AIDS-associated sarcopenia, HIV-associated fat redistribution syndrome, Crohn's disease, Werner's syndrome, X-linked combined immunodeficiency disease, hearing loss, anorexia nervosa and retinopathy of prematurity, Turner's syndrome, Prader-Willi syndrome, Silver-Russell syndrome, idiopathic short stature, obesity, multiple sclerosis, ulcerous colitis, low muscle mass, myocardial ischemia, and decreased bone density. Diseases that can be the target of therapy or prevention using the IGF-I receptor antagonist antibody include: neuroblastoma, striated muscle sarcoma, bone cancer, childhood cancer, acromegalia, ovary cancer, pancreas cancer, benignant prostatic hypertrophy, breast cancer, prostate cancer, bone cancer, lung cancer, colorectal cancer, cervix cancer, synovial sarcoma, urinary bladder cancer, stomach cancer, Wilms' tumor, diarrhea associated with metastatic carcinoid and vasoactive intestinal peptide secreting tumor, vipoma, Verner-Morrison syndrome, Beckwith-Wiedemann syndrome, kidney cancer, renal cell cancer, transitional cell cancer, Ewing's sarcoma, leukemia, acute lymphoblastic leukemia, brain tumor, glioblastoma, non-glioblastomatic brain tumor, meningioma, pituitary adenoma, vestibular schwannoma, primitive neuroectodermal tumor, medulloblastoma, astrocytoma, oligodendroglioma, ependymoma, choroid plexus papilloma, gigantism, psoriasis, atherosclerosis, vascular smooth muscle restenosis, inappropriate microvascular growth, diabetic retinopathy, Graves' disease, systemic lupus erythematosus, chronic thyroiditis, myasthenia gravis, autoimmune thyroiditis, and Behcet's disease. Particularly preferred uses of the anti-IGF-I receptor antibody according to the present invention include use as a therapeutic or prophylactic agent of disuse muscle atrophy and/or dwarfism. The anti-IGF-I receptor antibody according to the present invention is advantageous in that it does not change

the blood glucose level upon administration.

[0088]

A drug containing the anti-IGF-I receptor antibody according to the present invention may be formulated in the form of a pharmaceutical composition which contains, in addition to the anti-IGF-I receptor antibody according to the present invention, a pharmaceutically acceptable carrier and/or any other excipient. Drug formulation using a pharmaceutically acceptable carrier and/or any other excipient can be carried out in accordance with, e.g., a method described in the University of the Sciences in Philadelphia, "Remington: The Science and Practice of Pharmacy, 20th EDITION", Lippincott Williams & Wilkins, 2000. Such a therapeutic or prophylactic agent may be provided as a liquid formulation prepared by dissolving, suspending, or emulsifying the ingredients into sterile aqueous medium or oily medium, or as a lyophilized formulation thereof. A medium or solvent as a diluent for preparing such a formulation may be an aqueous medium, examples of which include distilled water for injection and physiological saline solution, which may optionally be used with addition of an osmoregulating agent (e.g., D-glucose, D-sorbitol, D-mannitol, and sodium chloride), and/or in combination with a suitable dissolving aid such as an alcohol (e.g., ethanol), a polyalcohol (e.g., propylene glycol or polyethylene glycol), or a nonionic surfactant (e.g., polysorbate 80 or polyoxyethylene hydrogenated castor oil 50). Such a formulation can also be prepared with an oily medium or solvent, examples of which include sesame oil and soybean oil, which can optionally be used in combination with a dissolving aid such as benzyl benzoate and benzyl alcohol. Such liquid drugs may often be prepared using appropriate additives such as buffering agents (e.g., phosphate buffering agents and acetate buffering agents), soothing agents (e.g., benzalkonium chloride and procaine hydrochloride), stabilizers (e.g., human serum albumin and polyethylene glycol), preservatives (e.g., ascorbic acid, erythorbic acid, and their salts), coloring agents (e.g., copper chlorophyll β -carotene, Red #2 and Blue #1), antiseptic agents (e.g., paraoxybenzoic acid esters, phenol, benzethonium chloride and benzalkonium chloride), thickeners (e.g., hydroxypropyl cellulose, carboxymethyl cellulose, and their salts), stabilizers (e.g., human serum albumin mannitol and sorbitol), and odor correctives (e.g., menthol and citrus aromas). Other alternative forms include therapeutic agents or prophylactic agent for application onto mucous membranes, such formulations often containing additives such as pressure-sensitive adhesives, pressure-sensitive enhancers, viscosity regulators, thickening agents and the like (e.g., mucin, agar, gelatin, pectin, carrageenan, sodium alginate, locust bean gum, xanthan gum, tragacanth gum, gum arabic, chitosan, pullulan, waxy starch, sucralfate, cellulose and its derivatives (such as hydroxypropyl methyl cellulose), polyglycerol fatty acid esters, acrylic acid-alkyl (meth)acrylate copolymers, or their salts and polyglycerol fatty acid

esters), primarily for the purpose of imparting mucosal adsorption or retention properties. However, the form, solvent and additives for the therapeutic agent or prophylactic agent to be administered to the body are not limited to these, and appropriately selection may be made by a person skilled in the art.

5

[0089]

A drug containing the anti-IGF-I receptor antibody according to the present invention may further contain, in addition to the anti-IGF-I receptor antibody according to the present invention, another known agent (active ingredient). A drug containing the anti-IGF-I receptor antibody according to the present invention may be combined with another known agent in the form of a kit. Examples of active ingredients to be combined with the IGF-I receptor agonist antibody include: growth hormone or an analog thereof, insulin or an analog thereof, IGF-II or an analog thereof, an anti-myostatin antibody, myostatin antagonist, anti-activin type IIB receptor antibody, activin type IIB receptor antagonist, soluble activin type IIB receptor or an analog thereof, ghrelin or an analog thereof, follistatin or an analog thereof, a beta-2 agonist, and a selective androgen receptor modulator. Examples of active ingredients to be combined with the IGF-I receptor antagonist antibody include: corticosteroid, antiemetic, ondansetron hydrochloride, granisetron hydrochloride, metoclopramide, domperidone, haloperidol, cyclizine, lorazepam, prochlorperazine, dexamethasone, levomepromazine, tropisetron, cancer vaccine, GM-CSF inhibitor, GM-CSF DNA vaccine, cell-based vaccine, dendritic cell vaccine, recombinant virus vaccine, heat shock protein (HSP) vaccine, homologous tumor vaccine, autologous tumor vaccine, analgesic, ibuprofen, naproxen, choline magnesium trisalicylate, oxycodone hydrochloride, anti-angiogenic, antithrombotic, anti-PD-1 antibody, nivolumab, pembrolizumab, anti-PD-L1 antibody, atezolizumab, anti-CTLA4 antibody, ipilimumab, anti-CD20 antibody, rituximab, anti-HER2 antibody, trastuzumab, anti-CCR4 antibody, mogamulizumab, anti-VEGF antibody, bevacizumab, anti-VEGF receptor antibody, soluble VEGF receptor fragment, anti-TWEAK antibody, anti-TWEAK receptor antibody, soluble TWEAK receptor fragment, AMG 706, AMG 386, antiproliferative, farnesyl protein transferase inhibitor, alpha v beta 3 inhibitor, alpha v beta 5 inhibitor, p53 inhibitor, Kit receptor inhibitor, ret receptor inhibitor, PDGFR inhibitor, growth hormone secretion inhibitor, angiopoietin inhibitor, tumor-infiltrating macrophage inhibitor, c-fms inhibitor, anti-c-fms antibody, CSF-1 inhibitor, anti-CSF-1 antibody, soluble c-fms fragment, pegvisomant, gemcitabine, panitumumab, irinotecan, and SN-38. The dosage of the other agent used in combination with the anti-IGF-I receptor antibody may be within a dosage used for normal therapy, but can be increased or decreased depending on the situation.

35

[0090]

The therapeutic or prophylactic agent according to the present invention can be parenterally administered for the purpose of improving symptoms. For parenteral administration, a transnasal agent may be prepared, and a liquid drug, suspension or solid formulation may be selected. An injection may be prepared as a different form of parenteral administration, the injection being selected as subcutaneous injection, intravenous injection, infusion, intramuscular injection, intracerebroventricular injection or intraperitoneal injection. Other formulations used for parenteral administration include suppositories, sublingual agents, percutaneous agents and transmucosal administration agents other than transnasal agents. In addition, intravascular local administration is possible by a mode of addition or coating onto a stent or intravascular obturator.

[0091]

The dose for an agent for treatment or prevention according to the invention will differ depending on the patient age, gender, body weight and symptoms, the therapeutic effect, the method of administration, the treatment time, or the types of active ingredients in the medical composition, but normally it may be administered in the range of 0.1 mg to 1 g and preferably in the range of 0.5 mg to 300 mg of active compound per administration for adults, once every one to four weeks, or once every one to two months. Thus, the administration should preferably be carried out less than once weekly. However, since the administration dose and frequency will vary depending on a variety of conditions, lower administration dose and fewer administration frequency than those mentioned above may be sufficient, or administration dose and frequency exceeding these ranges may be necessary.

[Uses for non-human animals]**[0092]**

An anti-IGF-I receptor antibody according to an embodiment of the present invention can be used for livestock or veterinary applications on non-human animals. Animals being the target of the anti-IGF-I receptor antibody according to the present invention for livestock or veterinary applications should preferably be non-human animals belonging to mammals, birds, reptiles, amphibia or fish, more preferably non-human animals belonging to mammals or birds, still more preferably an animal selected from monkey, rabbit, guinea pig, cow, pig, sheep, horse or dog. Although cow growth hormones and pig growth hormones are currently used for increasing milk production of cows and for promoting growth of piglets, respectively, these effects are considered to be achieved by IGF-I, whose expression is induced by a growth hormone (see H. Jiang and X. Ge, Journal of Animal Science, Vol.92, pp21-29, 2014). Therefore, the agonist

effects of the anti-IGF-I receptor antibody according to the present invention can be utilized for the purposes of enhancing milk production of an animal and promoting growth of a fetus or a new-born baby animal. Examples of other applications for which the anti-IGF-I receptor antibody according to the present invention can be used include, although not limited to:

5 increasing the muscle mass of an animal, increasing the weight ratio of muscles to fat of an animal, increasing the transformation efficiency of fed diet into tissues of the body, increasing the reproductive efficiency, enhancing the reproduction ability of an species for preservation thereof, and treating trauma and exhaustive symptoms involved in debilitating diseases. The antagonist effect achieved by another embodiment of the anti-IGF-I receptor antibody according

10 to the present invention can be utilized for treating malignant tumor of an animal, controlling the reproduction frequency of an animal, controlling the growth of an individual, and other uses. A person skilled in the art could also modify the structure of the anti-IGF-I receptor antibody according to the present invention as appropriate to alter the amino acid sequences of the frameworks or constant regions of the antibody and to thereby decrease its immunogenicity,

15 depending on the animal species to which the antibody is administered.

[Method for culturing cells using the anti-IGF-I receptor antibody]

[0093]

IGF-I and its derivatives are widely used in cell culture techniques for maintaining,

20 growing, and/or differentiating vertebrate-derived cells *in vitro*, and commercially marketed as cell culture reagents. However, since IGF-I can lose its effects during long-term culturing due to, e.g., its lack of sufficient stability, it is necessary to, e.g., keep adjusting the concentration thereof in order to carry out cell culturing stably. In addition, since IGF-I induces glucose uptake by cells, there is a possibility that the metabolism and characteristics of the cells may be changed

25 due to an increase in the intracellular glucose concentration, and that the culture conditions may change due to a decrease in the glucose concentration of the culture medium. Compared to IGF-I, the anti-IGF-I receptor antibody according to the present invention is characterized in that it is more stable, can maintain its cell proliferation effect even after contact with cells, can exhibit an activity to induce cell proliferation even at a lower concentration, and does not induce

30 intracellular glucose uptake. The anti-IGF-I receptor antibody according to the present invention can be used for cell culturing, by adding an appropriate amount of the antibody to culture medium or by adsorbing or immobilizing an appropriate amount of the antibody to a solid phase of a culture vessel. Thus, the anti-IGF-I receptor antibody according to the present invention makes it possible to reduce the amount to be used, and effectively induce proliferation of cells

35 adhering to the solid phase. The vertebrate-derived cells according to the present invention should preferably be cells derived from mammals, birds, reptiles, amphibia or fish, more

preferably cells derived from mammals or birds, still more preferably cells derived from human, monkey, rabbit, guinea pig, cow, pig, sheep, horse or dog. The cells used may be primary cultured cells, cell lines, transformants derived from such cells, or cells derived from a transgenic animal. More specifically, examples of subjects that can be cultured using the anti-IGF-I receptor antibody according to the present invention also include an organ or a tissue of a vertebrate or a transgenic animal derived from such a vertebrate. The anti-IGF-I receptor antibody according to the present invention can be used for culturing cells for the purposes of cellular production of a substance or cell therapy and regeneration medicine using such cells.

10

EXAMPLES

Example 1: Production of mouse monoclonal antibody

[0094]

A mouse monoclonal antibody can be produced by a hybridoma technique developed by Kohler, et al. (Nature 256: 495-497, 1975). An IGF-I receptor agonist antibody was produced by immunizing mice with cells expressing a human IGF-I receptor according to standard hybridoma technology. All animal experiments were conducted in accordance with the regulations of the institution. A standard method involving fusion of mouse spleen-derived cells with a mouse myeloma cell line (P3U1) was conducted. Hybridomas were selected using a medium containing hypoxanthine, aminopterin, and thymidine. The hybridoma broth was used for evaluation of the affinity by Cell ELISA using cells expressing an IGF-I receptor and evaluation of the activation of intracellular tyrosine kinase of the IGF-I receptor by PathHunter[®] to select a positive hybridoma-containing well. The hybridomas contained in this well were single-cloned by a limiting dilution technique. This single-cloned positive hybridoma was serum-free cultured, and the monoclonal antibody was purified from the broth through a protein A column (Ab-Capcher, ProteNova). An IGF-I receptor agonist antibody, named IGF11-16, was found by evaluation of human myoblast proliferation activity using the monoclonal antibody.

Example 2: Determination of antibody isotype

30

[0095]

In order to determine the antibody isotype of the IGF-I receptor agonist antibody, ELISA was implemented using antibodies specific to respective antibody isotypes. An anti-mouse-IgG antibody (TAGO, 6150) diluted 2000-fold with PBS was added to a 96-well plate (Nunc, MaxiSorp) in an amount of 50 μ L/well and was left to stand at 4°C overnight. The solution in the 96-well plate was replaced with 3% BSA/PBS, and the plate was used in ELISA. The IGF-I receptor agonist antibody was added to the anti-mouse-IgG antibody-immobilized 96-well plate

in an amount of 30 μ L/well, followed by reaction at room temperature for 1.5 hours. Each well was washed with a washing liquid, and antibodies specifically reacting with respective isotypes of mouse IgG: anti-mouse-IgG1 antibody-ALP conjugate (SBA, 1070-04), anti-mouse-IgG2a antibody-ALP conjugate (SBA, 1080-04), anti-mouse-IgG2b antibody-ALP conjugate (SBA, 1090-04), and anti-mouse-IgG3 antibody-ALP conjugate (SBA, 1100-04), were then added in an amount of 30 μ L/well, followed by reaction at room temperature for 1 hour. A substrate (PNPP) was added in an amount of 100 μ L/well, followed by reaction at room temperature for 45 minutes. The difference between absorbance values at 405 and 550 nm was calculated and was evaluated as avidity.

Since IGF11-16 showed reactivity with the anti-mouse-IgG1 antibody, the isotype of the antibody was IgG1.

Example 3: Determination of sequence of antibody

[0096]

In order to determine the gene sequences of the light chain and heavy chain of the IGF-I receptor agonist antibody, a SMARTer[®] RACE method was implemented. Gene fragments encoding the heavy chain and the light chain of the antibody and containing initiation and termination codons were produced from RNA derived from the hybridoma producing the antibody by the SMARTer[®] RACE method, and the nucleotide sequences thereof were determined. A first strand cDNA was synthesized using the total RNA derived from the hybridoma as a template with SMARTer[®] RACE 5'/3' Kit (634859, Clontech) and was then amplified by PCR reaction. Using the cDNA as a template, PCR reaction was performed with the primer to universal sequence primers attached to the kit and specific to the heavy chain and the light chain of the antibody, respectively. The primers for the light chain (kappa) of the mouse antibody and the IgG1 of the mouse antibody were designed with reference to Accession Nos. BC080787 and LT160966, respectively. The designed nucleotide sequence of the primer for the light chain of the mouse antibody was ggtgaagttgatgtcttgtgagtgg, and the designed nucleotide sequence of the primer for the heavy chain of the mouse antibody was gctcttctcagtatgggtggtgtgc. These primers were used in experiments. The resulting PCR products were used as 5' RACE PCR products in TA cloning.

[0097]

In the TA cloning, the 5' RACE PCR products were subjected to electrophoresis, and cDNA having the target sequence was purified with QIAEX II Gel Extraction Kit (20021, Qiagen). The purified cDNA was subjected to reaction using TaKaRa-Taq (R001A, Takara) at 72°C for 5 minutes to attach adenine to the 5' and 3' ends. The cDNA was cloned into

Topoisomerase I-activated pCR[®] II-TOPO[®] vector (hereinafter, referred to as TOPO vector) using TOPO[®] TA Cloning[®] Kit (450641, Thermofisher) according to the protocol attached to the kit. The TOPO vector cloned with the target cDNA was transformed into E. coli TOP10, followed by culturing in an agar medium containing 50 µg/mL of kanamycin. The insertion of the target cDNA into the TOPO vector was verified by colony PCR. The nucleotide sequence of the cloned cDNA was identified. Similarly, the nucleotide sequence of the 3' RACE PCR product was identified to determine the full-length sequence of the antibody gene. The full-length nucleotide and amino acid sequences of the light chain of IGF11-16 are shown in SEQ ID NO:27 and SEQ ID NO:28, respectively, and the full-length nucleotide and amino acid sequences of the heavy chain of IGF11-16 are shown in SEQ ID NO:29 and SEQ ID NO:30, respectively. The amino acid sequences of CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2, CDR-L3, the heavy chain variable region, and the light chain variable region of IGF11-16 are shown in SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10, respectively.

Example 4: Avidity to IGF-I receptor (ELISA)

[0098]

In order to investigate the avidity of the IGF-I receptor agonist antibody to the IGF-I receptor of human (SEQ ID NO: 2, NP_000866), guinea pig (SEQ ID NO: 11, XP_003475316), cynomolgus monkey (SEQ ID NO: 12, NP_001248281), rabbit (SEQ ID NO: 13, XP_017193273), rat (SEQ ID NO: 14, NP_494694), and mouse (SEQ ID NO: 15, NP_034643), Cell ELISA was implemented using cells expressing the respective IGF-I receptors.

[0099]

The pEF1 expression vector (Thermofisher) containing the IGF-I receptor gene of human (SEQ ID NO: 16), guinea pig (SEQ ID NO: 17), cynomolgus monkey (SEQ ID NO: 18), rabbit (SEQ ID NO: 19), rat (SEQ ID NO: 20), or mouse (SEQ ID NO: 21) was transfected into P3U1 cells by lipofection. After the lipofection, the P3U1 cells were cultured at least overnight and were added to a 96-well plate (coated with poly-D-lysine) at a concentration of 0.8×10^5 cells/well and immobilized with 10% buffered formalin (Mildform[®] 10NM, Wako), followed by blocking with phosphate buffer containing 3% BSA. The resulting plate was used for ELISA.

[0100]

In ELISA, 30 µL of an IGF11-16 antibody solution adjusted to 10 nM with 0.1% skimmed milk/3% BSA/PBS was added to each well and was subjected to reaction at room temperature for about 1.5 hours. After washing with a washing liquid twice, anti-mouse-IgG

antibody-HRP conjugate solutions (30 μ L) adjusted to predetermined concentrations with 0.1% skimmed milk/3% BSA/PBS were added to respective wells and were subjected to reaction at room temperature for about 1 hour. After washing with a washing liquid twice, 50 μ L of a substrate (TMB) was added to each well to start the reaction. After about 20 minutes, 50 μ L of 0.5 M sulfuric acid was added to each well. The absorbances at 450 nm and 550 nm were measured, and the difference between absorbance values at 450 nm and 550 nm was calculated. The avidity was calculated based on the difference between the absorbance values at 450 nm and 550 nm for cells (Mock cells, SEQ ID NO: 22) transfected with a vector not containing the IGF-I receptor gene as a standard value 1 (Table 1).

[0101]

[Table 1]

Type	Mouse	Rat	Guinea pig	Rabbit	Cynomolgus monkey	Human
Avidity	0.9	1.0	5.3	5.4	5.4	5.4

[0102]

IGF11-16 increased the avidity to the cells expressing the IGF-I receptors of human, guinea pig, cynomolgus monkey, and rabbit by more than 5-fold compared with Mock cells. In contrast, the avidity of IGF11-16 to the cells expressing the IGF-I receptors of rat and mouse was almost equivalent to that of Mock cells and was not increased. These results demonstrated that IGF11-16 binds to the human, guinea pig, cynomolgus monkey, and rabbit IGF-I receptors but does not bind to the rat and mouse IGF-I receptors.

Example 5: Avidity to insulin receptor (ELISA)

[0103]

In order to investigate the avidity of the IGF-I receptor agonist antibody to an insulin receptor, Cell ELISA was implemented using cells expressing human insulin receptor.

[0104]

The pEF1 expression vector (Thermofisher) containing the human insulin receptor gene was transfected into HEK 293T cells by lipofection. The HEK 293T cells after the lipofection were added to a 96-well plate (coated with poly-D-lysine) at a concentration of 0.8×10^5 cells/well (about 180 μ L/well) and were immobilized with 10% buffered formalin (Mildform[®] 10NM, Wako), followed by blocking with phosphate buffer containing 3% BSA. The plate was used in ELISA.

[0105]

In ELISA, antibody solutions (30 μ L) adjusted to predetermined concentrations with 0.1% skimmed milk/3% BSA/PBS were added to respective wells and were subjected to reaction at room temperature for about 1 hour. After washing with a washing liquid (tris buffer containing Tween) twice, anti-mouse-IgG antibody-ALP conjugate solutions (30 μ L) adjusted to predetermined concentrations with 0.1% skimmed milk/3% BSA/PBS were added to respective wells and were subjected to reaction at room temperature for about 1 hour. After washing with a washing liquid twice, 100 μ L of a substrate (PNPP) was added to each well to start the reaction. After about 30 minutes, the absorbances at 405 nm and 550 nm were measured, and the difference between absorbance values at 405 and 550 nm was calculated. The avidity was calculated based on the value of the difference between absorbance values at 405 and 550 nm for cells (Mock cells, SEQ ID NO: 22) transfected with a vector not containing the IGF-I receptor gene and the insulin receptor gene as a standard value 1 (Table 2).

[0106]

[Table 2]

IGF11-16	human IGF-I receptor	human insulin receptor
0.5 nM	2.9	1.1
5 nM	3.7	1.4

[0107]

In ELISA using immobilized cells expressing the human IGF-I receptor, the difference between absorbance values at 405 and 550 nm in 0.5 nM and 5 nM of IGF11-16 was increased to about 3-fold or more compared with Mock cells. In contrast, in ELISA using immobilized cells expressing human insulin receptor, the difference between absorbance values at 405 and 550 nm in 0.5 nM and 5 nM of IGF11-16 was not increased to 1.5-fold or more. These results demonstrated that IGF11-16 more strongly binds to the IGF-I receptor compared with the insulin receptor.

Example 6: Analysis of binding site of IGF-I receptor (ELISA)**[0108]**

In order to identify the epitope of the IGF-I receptor agonist antibody against the IGF-I receptor, the avidity of the IGF-I receptor agonist antibody to variants prepared by replacing each domain of the IGF-I receptor with a domain of the insulin receptor having a structure

similar to that of the IGF-I receptor was measured.

[0109]

An extracellular domain of the human IGF-I receptor (NP_000866) was replaced with an
 5 extracellular domain of insulin receptor, or an extracellular domain of human insulin receptor
 (NP_000199) was replaced with an extracellular domain of the IGF-I receptor. The following
 four substitutions were thereby produced.

Substitution 1: hIGFIR[L1-L2]/hINSR, a substitution in which L1 domain to L2 domain of
 human insulin receptor were replaced with L1 domain to L2 domain of the human IGF-I
 10 receptor;

Substitution 2: hINSR[L1-L2]/hIGFIR, a substitution in which L1 domain to L2 domain of the
 human IGF-I receptor were replaced with L1 domain to L2 domain of the human insulin
 receptor;

Substitution 3: hINSR[L1]/hIGFIR, a substitution in which L1 domain of the IGF-I receptor was
 15 replaced with L1 domain of the human insulin receptor; and

Substitution 4: hINSR[L2]/hIGFIR, a substitution in which L2 domain of the human IGF-I
 receptor was replaced with L2 domain of the human insulin receptor.

[0110]

The pEF1 expression vectors (Thermofisher) containing the respective genes of the
 20 above-mentioned four substitutions of the human IGF-I receptor were transfected into P3U1
 cells by lipofection. The gene of hIGFIR[L1-L2]/hINSR as Substitution 1 is shown in SEQ ID
 NO: 23; the gene of hINSR[L1-L2]/hIGFIR as Substitution 2 is shown in SEQ ID NO: 24; the
 gene of hINSR[L1]/hIGFIR as Substitution 3 is shown in SEQ ID NO: 25; and the gene of
 25 hINSR[L2]/hIGFIR as Substitution 4 is shown in SEQ ID NO: 26. After the lipofection, the
 P3U1 cells were cultured at least overnight and were added to a 96-well plate (coated with poly-
 D-lysine) at a concentration of 0.8×10^5 cells/well and immobilized with 10% buffered formalin
 (Mildform[®] 10NM, Wako), followed by blocking with phosphate buffer containing 3% BSA.
 The plate was used in ELISA.

[0111]

In ELISA, 30 μ L of an antibody solution adjusted to 10 nM with 0.1% skimmed milk/3%
 BSA/PBS was added to each well and was subjected to reaction at room temperature for about
 1.5 hours. After washing with a washing liquid twice, 30 μ L of an anti-mouse-IgG antibody-
 35 HRP conjugate solution adjusted to 5 nM with 0.1% skimmed milk/3% BSA/PBS was added to
 each well and was subjected to reaction at room temperature for about 1 hour. After washing

with a washing liquid twice, 50 μ L of a substrate (TMB) was added to each well to start the reaction. After about 20 minutes, 50 μ L of 0.5 M sulfuric acid was added to each well to stop the reaction. The absorbances at 450 nm and 550 nm were measured, and the difference between absorbance values at 450 nm and 550 nm was calculated. The avidity was calculated based on the difference between absorbance values at 450 nm and 550 nm for cells (Mock cells, SEQ ID NO: 22) transfected with a vector not containing the gene of each substitution as a standard value 1 (Table 3).

[0112]

10 [Table 3]

Substitution	IGF11-16
hIGFIR[L1-L2]/hINSR	5.5
hINSR[L1-L2]/hIGFIR	1.5
hINSR[L1]/hIGFIR	5.7
hINSR[L2]/hIGFIR	5.6

[0113]

In ELISA using immobilized cells expressing hIGFIR[L1-L2]/hINSR, hINSR[L1]/hIGFIR, or hINSR[L2]/hIGFIR, the absorbance at 450 to 550 nm in IGF11-16 was increased to 5-fold or more compared with Mock cells. In contrast, the avidity of IGF11-16 to the cells expressing hINSR[L1-L2]/hIGFIR was weak. These results demonstrated that IGF11-16 binds to a CR domain of the IGF-I receptor.

Example 7: Determination of epitope of IGF11-16

20 [0114]

In order to identify in more detail the epitope from the CR domain as an epitope of IGF11-16, the binding sequence was estimated from the species difference in avidity to the IGF-I receptor of IGF11-16. Fig. 1 shows the amino acid sequences of the CR domain of the IGF-I receptor of the respective species.

25

[0115]

IGF11-16 binds to the human, guinea pig, and rabbit IGF-I receptors, but does not bind to the mouse and rat IGF-I receptors. Based on the results, an amino acid sequence common to human, guinea pig, and rabbit but not common to mouse and rat was estimated as the epitope of IGF11-16 from the amino acid sequences of the CR domain of the IGF-I receptor.

30

[0116]

In order to determine the amino acid site of the CR domain of the IGF-I receptor to which IGF11-16 binds, the avidity of the CR domain to each amino acid substitution was measured by ELISA.

5

[0117]

Cell ELISA was implemented using cells expressing an IGF-I receptor in which the amino acid sequence presumed to bind to IGF11-16 was modified in the CR domain.

10 **[0118]**

As the amino acid substitutions of CR domain, the three substitutions shown below were used. In addition, a wild-type human IGF-I receptor and a wild-type rat IGF-I receptor each incorporated into a pEF1 expression vector (Thermofisher) were used as a positive control and a negative control, respectively. The expression level of each IGF-I receptor was determined using the reactivity of an FLAG M2 antibody to the FLAG tag (AspTyrLysAspAspAspLys) attached to the intracellular domain of the IGF-I receptor as an index.

Substitution 1 of the CR domain: in the amino acid sequence of the human IGF-I receptor (NP_000866, SEQ ID NO: 2), aspartic acid at position 245 and alanine at position 247 were replaced with asparagine and threonine, respectively.

20 Substitution 2 of the CR domain: in the amino acid sequence of the human IGF-I receptor (NP_000866, SEQ ID NO: 2), glutamic acid at position 294 was replaced with aspartic acid.

Substitution 3 of the CR domain: in the amino acid sequence of the human IGF-I receptor (NP_000866, SEQ ID NO: 2), glycine at position 315 and serine at position 316 were replaced with serine and threonine, respectively.

25

[0119]

HEK 293T cells were seeded in a 10-cm dish coated with poly-D-lysine at 9×10^6 cells/well. On the next day, each plasmid DNA was transfected into the cells by lipofection. On the following day, the HEK 293T cells were detached with 0.25% trypsin/EDTA and were suspended in a broth. The HEK 293T cells were added to a 96-well plate (coated with poly-D-lysine) at a concentration of 0.8×10^5 cells/well and were incubated at 37°C under conditions of 5% CO₂ overnight. The medium was removed from the 96-well plate, and the cells were immobilized with a 10% buffered formalin (Mildform[®] 10NM, Wako). The 10% buffered formalin was replaced with a blocking buffer (3% BSA/PBS/sodium azide), and the plate was used in ELISA.

35

[0120]

In ELISA, 50 μ L of a solution of the IGF11-16 antibody or the FLAG M2 antibody adjusted to 1 nM with 0.1% skimmed milk/3% BSA/PBS was added to each well and was subjected to reaction at room temperature for about 1 hour. After washing with a washing liquid
 5 twice, anti-mouse-IgG antibody-HRP conjugate solutions (50 μ L) adjusted to predetermined concentrations with 0.1% skimmed milk/3% BSA/PBS were added to respective wells and were subjected to reaction at room temperature for about 1 hour. After washing with a washing liquid twice, 100 μ L of a substrate (TMB) was added to each well to start the reaction. After about 30 minutes, 100 μ L of 0.5 M sulfuric acid was added to each well to stop the reaction, and the
 10 absorbances at 450 nm was measured. The value of the absorbance at 450 nm was evaluated as the avidity.

[0121]

The results are shown Fig. 2. It was confirmed that the reactivities of the FLAG M2
 15 antibody with the cells expressing the respective substitutions of the CR domain are substantially equivalent to one another and that the expression levels of the individual substitutions of the CR domain are substantially the same. IGF11-16 increased the value of absorbance at 450 nm to 2 or more in the wild-type human IGF-I receptor in which no modification was introduced into the CR domain and showed enhancement in the avidity. IGF11-16 increased the value of absorbance
 20 at 450 nm to 2 or more in Substitutions 1 and 2 of the CR domain and showed enhancement in the avidity. In contrast, the value of absorbance at 450 nm of Substitution 3 of the CR domain was about 1 and was the same level as the absorbance of the rat IGF-I receptor as a negative control, and no avidity was recognized. These results demonstrated that the amino acids at
 25 positions 315 and 316 of the IGF-I receptor are important for the avidity of IGF11-16 to the CR domain of the IGF-I receptor.

[0122]

The results suggest that the binding site of IGF11-16 to the human IGF-I receptor is near glycine (Gly) at position 315 and serine (Ser) at position 316. In general, the recognition
 30 sequence of an antibody is composed of eight amino acid residues (average of six to ten residues) and IGF11-16 has cross-reactivity showing no avidity to the rat IGF-I receptor and showing avidity to the rabbit and human IGF-I receptors; hence, the sequence of the binding site of IGF11-16 to the human IGF-I receptor was estimated to be
 ProSerGlyPheIleArgAsnGly*Ser*GlnSerMet (Gly*Ser* indicates the amino acid sequence at
 35 positions 315 and 316).

Example 8: Avidity to IGF-I receptor determined by surface plasmon resonance**[0123]**

The avidity (binding rate and dissociation rate) of an agent to an IGF-I receptor was measured by surface plasmon resonance (SPR).

5

[0124]

An anti-His monoclonal antibody was immobilized to a sensor chip CM3 (GE) with an Amine Coupling Kit (BR-1000-50, GE) and a His Capture Kit (28-9950-56, GE). The immobilization conditions were NHS/EDC: 7 minutes, 50 µg/mL anti-His monoclonal antibody: 3 minutes, ethanolamine: 7 minutes, and target: ≥ 3000 RU. As analytes, the agent was used at predetermined concentrations. As a ligand, a recombinant human IGF-I receptor His tag (305-GR-050, R&D SYSTEMS, hereinafter, referred to as IGF-IR-His) was used. As a negative control, Purified Mouse IgG2a, κ, Isotype Ctrl, Clone: MG2a-53 (401502, BioLegend, hereinafter, referred to as ctrl IgG2a) was used.

15

[0125]

The sensor chip CM3 immobilized with the anti-His monoclonal antibody was set to Biacore T200, the reaction temperature was set to 36°C, and a running buffer (HBS-EP+, BR-1006-69, GE) was fed at a flow rate 30 µL/min. The amount of the binding ligand was set to about 100 RU, and IGF-IR-His (0.5 to 2×10^{-8} mol/L) was added to the sensor chip to be captured by the anti-His monoclonal antibody. Ctrl IgG2a (10 nmol/L) was allowed to react for 1 minute, and HBS-EP+ was fed at a flow rate of 30 µL/min for at least 10 minutes. The analyte and HBS-EP+ were added to flow cells (1 and 2) and flow cells (3 and 4), respectively.

20

[0126]

The reaction conditions were set to a binding time of 600 seconds and a dissociation time of 600 seconds. After completion of the reaction, washing was performed with regeneration buffer 1 (0.2% SDS), regeneration buffer 2 (100 mmol/L Tris-HCl (pH 8.5), 1 mol/L NaCl, 15 mmol/L MgCl₂), and regeneration buffer 3 (10 mmol/L glycine-HCl (pH 1.5)) for 1 minute each at a flow rate of 30 µL/min. The dissociation rate constant (k_a , 1/Ms), binding rate constant (k_d , 1/s), and dissociation constant (K_D , M) were calculated by analysis with a model of 1:1 binding using Biacore T200 Evaluation software (ver 2.0). The results are shown in Table 4.

30

[0127]

[Table 4]

Ligand	Analyte	k_a (1/Ms)	k_d (1/s)	K_D (M)
IGF-I receptor	IGF-I	5.099×10^6	0.009083	1.781×10^{-9}
IGF-I receptor	IGF11-16	1.051×10^6	$< 1 \times 10^{-5*}$	$< 1 \times 10^{-11}$

*: a value lower than the lower limit of the measurement of apparatus.

[0128]

5 The k_a value of IGF11-16 against the human IGF-I receptor was about one-fifth of that of IGF-I, indicating a low binding rate. In contrast, the k_d value of IGF11-16 against the human IGF-I receptor was lower than the lower limit of the measurement of the apparatus and was lower than 1/1000 of that of IGF-I, indicating a significantly low dissociation rate and bare dissociation of IGF11-16 bound to the IGF-I receptor. The K_D value of IGF11-16 against the
10 human IGF-I receptor was lower than 1/50 of that of IGF-I, indicating a high binding strength. It is demonstrated that the avidity of IGF11-16 to an IGF-I receptor is high compared with that of IGF-I.

Example 9: Activation effect on IGF-I receptor or insulin receptor determined by

15 **PathHunter[®]**

[0129]

In order to detect the activation effect of an IGF-I receptor agonist antibody on the IGF-I receptor, the activation of a downstream signal of the IGF-I receptor was measured with PathHunter[®] IGF1R Functional Assay (DiscoverX).

20

[0130]

A cell line was used in which an adapter protein SHC1-Enzyme Acceptor (EA) fusion protein including an IGF-I receptor and an SH2 domain binding to an intracellular tyrosine kinase of the IGF-I receptor was forcibly expressed intracellularly. In order to detect the
25 activation effect of the IGF-I receptor agonist antibody on an insulin receptor, the activation of a downstream signal of the insulin receptor was measured with PathHunter[®] INSR Functional Assay (DiscoverX). Another cell line was used in which an adapter protein PLCG1-EA fusion protein including an insulin receptor and an SH2 domain that binds to an intracellular tyrosine kinase of the insulin receptor was forcibly expressed intracellularly. In each cell line, a ligand
30 binds to the IGF-I receptor or the insulin receptor, which causes dimerization of the receptor; phosphorylation of the receptor to recruit the adapter protein having the SH2 domain; formation of a receptor signal transduction complex; acceleration of the binding between the spatially

adjacent tyrosine kinase and EA; and reconstitution of the activated β -galactosidase. The effect of an agent on a receptor tyrosine kinase can be identified by measuring the level of the chemiluminescent signal of a substrate hydrolyzed by the reconstituted β -galactosidase activity.

5 **[0131]**

Cells expressing the IGF-I receptor or the insulin receptor were seeded in a 96-well plate (Black/clear or White/clear) coated with poly-D-lysine or collagen-I at 90 μ L/well (2×10^4 cells/well or 5×10^3 cells/well) and were incubated at 37°C under conditions of 5% CO₂. On the next day, agents in predetermined concentrations were added to the plate at 10 μ L/well, followed
10 by incubation at 37°C under conditions of 5% CO₂. On the following day, 30 μ L of the culture supernatant was added to 15 μ L of a substrate solution, followed by reaction for 60 minutes, and the luminescent signal was measured with a luminometer (Tristar, Berthold Japan K.K.). The activation of the IGF-I receptor was calculated with the activity of a group in which only a solvent was defined as 100%. The results are shown in Table 5.

15

[0132]

[Table 5]

Agent	Concentration (nM)		
	0.5	5	50
Control antibody (FLAG M2)	105	109	109
Insulin	184	1244	4619
IGF-I	208	3537	5248
IGF11-16	234	2900	2786

[0133]

20 The activation of the insulin receptor was calculated with the activity of a group in which only a solvent was defined as 100%. The results are shown in Table 6.

[0134]

[Table 6]

Agent	Concentration (nM)		
	0.5	5	50
Control antibody (FLAG M2)	105	104	111
Insulin	1432	1655	1405
IGF-I	126	158	240
IGF11-16	95	96	93

[0135]

The activation of the IGF-I receptor by an agent was measured using a cell line expressing the IGF-I receptor. In the cell line expressing the IGF-I receptor, IGF-I and IGF11-16 showed the activation effect on the IGF-I receptor compared with a control.

[0136]

The activation of the insulin receptor by an agent was measured using a cell line expressing the insulin receptor. In the cell line expressing the insulin receptor, the activation effect on the insulin receptor by insulin was observed. IGF-I concentration-dependently activated the insulin receptor, and a significant activation effect was observed at 50 nM. In contrast, IGF11-16 did not activate the insulin receptor.

[0137]

It is known that IGF-I shows reactivity with an insulin receptor. It is also known that the activation of an insulin receptor causes a hypoglycemic effect. It was demonstrated that IGF11-16 specifically acts on the IGF-I receptor and does not have the hypoglycemic effect via the insulin receptor.

20

Example 10: Cell proliferation activity on human myoblast**[0138]**

In order to investigate the proliferation activity of the IGF-I receptor agonist antibody on human myoblasts, an agent was added to human myoblasts, and the amount of ATP in the cells after 4 days was measured.

25

[0139]

Normal human skeletal muscle myoblasts (HSMM, Lonza) were seeded in a 96-well

plate (coated with collagen type I) at 0.1 mL/well (2×10^3 cells/well) using an SkBM-2 (Lonza, CC-3246) medium containing 1% BSA and were incubated at 37°C under conditions of 5% CO₂. On the next day of the cell seeding, each agent was added to the plate at 25 µL/well and was incubated at 37°C under conditions of 5% CO₂ for 4 days. The amount of intracellular ATP was measured as an index of cell proliferation with CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega). The supernatant was removed from the 96-well plate subjected to the incubation for 4 days so that the broth in each well was 50 µL, and the plate was then left to stand at room temperature for at least 30 minutes. CellTiter-Glo[®] reagent was added to the plate at 50 µL/well, followed by reaction for at least 10 minutes. The luminescent signal was then measured with a luminometer (Tristar, Berthold Japan K.K.). The activity was calculated with the activity of a group containing only a solvent defined as 100%. The results are shown in Table 7.

[0140]

[Table 7]

Agent	Concentration (nM)	Experiment 1	Experiment 2
		Cell proliferation inducing activity (%)	Cell proliferation inducing activity (%)
Control antibody (FLAG M2 Ab)	0.005	99	-
IGF-I	0.005	102	103
IGF11-16	0.005	141	130
16-13	0.005	-	102
26-3	0.005	-	108
Solvent control 1* (containing NaN ₃)	-	-	104
Control antibody (FLAG M2 Ab)	0.5	98	-
IGF-I	0.5	133	137
IGF11-16	0.5	146	143
16-13	0.5	-	109
26-3	0.5	-	119
Solvent control 2* (containing NaN ₃)	-	-	112

*: Solvent controls 1 and 2 contain sodium azide in an amount of 0.005 nM and 0.5 nM, respectively, which are the same amounts as those in antibodies 16-13 and 26-3.

5 [0141]

IGF-I and IGF11-16 enhanced the cell proliferation activity, compared with the control antibody (FLAG M2, Sigma-Aldrich).

[0142]

10 The proliferation activity of human myoblasts was concentration-dependently enhanced in 0.00005, 0.0005, 0.005, 0.05, 0.5, 5, 50, and 500 nM IGF11-16. The EC₅₀ values of the myoblast proliferative activity of IGF11-16 and IGF-I were 0.004 nM and 0.61 nM, respectively. The results indicate that the activity of IGF11-16 was above 100 times that of IGF-I.

15 [0143]

The antibodies 16-13 and 26-3 described in Non-Patent Literature 35 did not show

noticeable cell proliferation activity compared with the solvent control (containing sodium azide), and the activity was weak compared with that of IGF11-16.

Example 11: Cell proliferation activity in guinea pig myoblast

5 [0144]

Guinea pig myoblasts (Cell Applications) were seeded in a 96-well plate (coated with collagen type I) at 0.1 mL/well (4×10^3 cells/well) using an SkBM-2 (Lonza, CC-3246) medium containing 1% BSA and were incubated at 37°C under conditions of 5% CO₂. On the next day of the cell seeding, each agent was added to the plate at 25 µL/well and was incubated at 37°C
10 under conditions of 5% CO₂ for 4 days. The amount of intracellular ATP was measured as an index of cell proliferation by CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega). The supernatant was removed from the 96-well plate subjected to the incubation for 4 days so that the broth in each well was 50 µL, and the plate was then left to stand at room temperature for at least 30 minutes. CellTiter-Glo[®] reagent was added to the plate at 50 µL/well, followed by reaction
15 for at least 10 minutes. The luminescent signal was then measured with a luminometer (Tristar, Berthold Japan K.K.).

[0145]

The proliferation activity of guinea pig myoblasts was concentration-dependently
20 enhanced in 0.00005, 0.0005, 0.005, 0.05, 0.5, 5, 50, and 500 nM IGF11-16. The EC₅₀ values of the myoblast proliferative activity of IGF11-16 and IGF-I were 0.004 nM and 0.76 nM, respectively. The results indicate that the activity of IGF11-16 was above 100 times that of IGF-I.

Example 12: In vitro comparison with persistence of effect of IGF-I

[0146]

In order to compare the persistence of the effects of IGF11-16 and IGF-I, the medium was replaced after 18 hours from the addition of IGF11-16 or IGF-I, and the proliferation activity of human myoblasts was measured under the conditions that IGF11-16 and IGF-I were
30 removed.

[0147]

Normal human skeletal muscle myoblasts (Human Skeletal Muscle Myoblast Cells, HSMM, Lonza) were seeded in a 96-well plate (coated with collagen type I) at 0.1 mL/well
35 (2×10^3 cells/well) using an SkBM-2 (Lonza, CC-3246) medium containing 1% BSA and were incubated at 37°C under conditions of 5% CO₂. On the next day of the cell seeding, IGF11-16 or

IGF-I was added to the plate at 25 μ L/well. After 18 hours from the addition, the medium was replaced with a medium not containing IGF11-16 or IGF-I or a medium containing them, followed by incubation at 37°C under conditions of 5% CO₂ for 4 days. The amount of intracellular ATP was measured as an index of cell proliferation by CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega). The supernatant was removed from the 96-well plate subjected to the incubation for 4 days so that the broth in each well was 50 μ L, and the plate was then left to stand at room temperature for at least 30 minutes. CellTiter-Glo[®] reagent was added to the plate at 50 μ L/well, followed by reaction for at least 10 minutes. The luminescent signal was then measured with a luminometer (Tristar, Berthold Japan K.K.). The proportion (control group: 0%) relative to a control group containing only a solvent was calculated as the cell proliferation activity. The results are shown in Fig. 3.

[0148]

The cell proliferation activity increased to 39% and 75%, respectively, in the groups in which 1 nM and 5 nM IGF-I were respectively added for 4 days. The cell proliferation activity increased to 8% and 10%, respectively, in the groups in which 1 nM and 5 nM IGF-I were respectively added for 18 hours and were then washed out, and the activity was lower than 1/5 of those of the groups in which IGF-I was added for 4 days, indicating a noticeable reduction in the effect.

[0149]

In the group in which 0.5 nM IGF11-16 was added for 4 days, the cell proliferation activity increased to 49%. The cell proliferation activity of the group in which 0.5 nM IGF11-16 was added for 18 hours and was then washed out increased to 30%, which corresponded to 60% or more of the activity of the group in which IGF11-16 was added for 4 days.

[0150]

The cell proliferation activities of the group treated with 0.5 nM IGF11-16 in which washing out was performed after addition of an agent were compared with the cell proliferation activities of the groups treated with 1 nM and 5 nM IGF-I. The activity of IGF11-16 was high in statistical significance. These results demonstrated that IGF11-16 maintains the proliferation activity of human myoblasts even after washing out of the agent and has a strong effect compared with IGF-I. IGF11-16 maintained the cell proliferation activity even after washing out. The results indicate that unlike the effect of IGF-I, IGF11-16 strongly binds to the IGF-I receptor and has a persistent activation effect on the IGF-I receptor.

Example 13: Glucose uptake in human differentiated muscle cell**[0151]**

In order to investigate the effect of IGF11-16 on the glucose uptake, the uptake amount of radiolabeled ^3H -2-deoxy glucose was measured using human differentiated muscle cells and was compared with the effect of IGF-I.

[0152]

Normal human skeletal muscle myoblasts (Human Skeletal Muscle Myoblast Cells, HSMM, Lonza) were seeded in a 24-well plate (Costar, 3526) at 0.5 mL/well (2×10^4 cells/well) and were incubated at 37°C under conditions of 5% CO₂. The medium (an SkBM-2 (Lonza, CC-3246) supplemented with FBS (Lonza, CC-4423W), L-Glutamine (Lonza, CC-4422W), Dexamethasone (Lonza, CC-4421W), rhEGF (Lonza, CC-4420W), and GA-1000 (Lonza, CC-4419W)) was replaced with fresh one until the cells were confluent. The medium was replaced with 0.5 mL/well of a medium for differentiation (DMEM/F12 (1:1) (Gibco, 11320) containing 2% horse serum (Sigma, H1270), 50 U/mL penicillin, 50 µg/mL streptomycin (Gibco, 15070-063)), and the confluent HSMM cells were incubated at 37°C under conditions of 5% CO₂ to start differentiation into muscle cells. The cells after about 6 days from the start of differentiation were used as human differentiated muscle cells in a glucose uptake experiment.

[0153]

The medium was replaced with 0.5 mL/well of a starvation medium (1 g/L glucose-containing DMEM (Gibco, 11885) supplemented with 0.1% BSA Fatty Acid free (Seikagaku Corporation, 82-002-5), 50 U/mL penicillin, and 50 µg/mL streptomycin (Gibco, 15070-063)), and the human differentiated muscle cells were incubated at 37°C under conditions of 5% CO₂ overnight. On the next day, the medium was replaced with 0.5 mL/well of a medium for starvation, and the cells were incubated at 37°C under conditions of 5% CO₂ for 2 hours. The wells were washed with 1 mL/well of PBS, and 0.5 mL/well of a treatment medium containing the respective agents was added to the wells, followed by incubation at 37°C under conditions of 5% CO₂ for 2 hours. The treatment medium was prepared to give final concentrations of 0.1 mmol/L glucose, 0.1% BSA, ^3H -2-deoxy glucose (1 µCi/mL), and each concentration of a human recombinant IGF-I or IGF-I receptor agonist antibody using a glucose uptake buffer (containing 20 mmol/L HEPES (DOJINDO, 342-01375), 150 mmol/L NaCl (SIGMA, S5150), 5 mmol/L KCl (Wako, 163-03545), 5 mmol/L MgSO₄ (Wako, 131-00405), 1.2 mmol/L KH₂PO₄ (Wako, 169-04245), 25 mmol/L CaCl₂ (Fluka, 21114), and 2 mmol/L pyruvate (Wako, 190-14881) dissolved in water for injection and having a pH 7.4 adjusted with NaOH). The wells were washed by adding 1 mL/well of cooled PBS three time to stop the glucose uptake. The cells

were lysed by adding 0.25 mL/well of 1 N NaOH. The whole amount of the cell lysate was added to a vial containing 3 mL of liquid scintillator ULTIMA GOLD (PerkinElmer Japan) and was stirred. The radioactivity (DPM) of ^3H was measured for 3 minutes with a liquid scintillation counter. The glucose uptake ratio of a treated group was calculated with the averaged glucose uptake amount (DPM) of an untreated group (control group) defined as 100%. The results are shown in Fig. 4.

[0154]

In 0.8, 4, 20, and 100 nM IGF-I, the glucose uptake was concentration-dependently and significantly enhanced. In contrast, IGF11-16 did not show significant effect until 100 nM. These results suggest that the glucose uptake effect of IGF11-16 in human differentiated muscle cells is extremely weak.

Example 14: In vivo efficacy (effect of increasing muscle mass in guinea pig)

[0155]

In order to verify the in vivo efficacy of the IGF-I receptor agonist antibody, IGF11-16 was administered to guinea pigs in a single administration, and the muscle mass after 2 weeks was measured for comparison with the effect when IGF-I was continuously administered. The effect of increasing muscle mass is defined as an effect of increasing the weight of muscle of a guinea pig by 5% or more compared with that of the control group.

[0156]

IGF11-16 (0.03, 0.1, or 0.3 mg/kg) was subcutaneously or intravenously administered to normal guinea pigs in a single administration. Human recombinant IGF-I (Mecasermin) as a positive control was subcutaneously embedded with an osmotic pump (Alzet) and was continuously administered at 1 mg/kg/day. After two weeks from the administration of the agent, the guinea pigs were euthanized by exsanguination under anesthesia, and the weight of the extensor digitorum longus muscle was measured. The results are shown in Fig. 5.

[0157]

In the group (iv) of intravenous administration of IGF11-16 in an amount of 0.03, 0.1, or 0.3 mg/kg, the muscle mass was dose-dependently and significantly increased, compared with the control group treated with only a solvent. Even in the group (sc) of subcutaneous administration of IGF11-16 at 0.3 mg/kg, the muscle mass was significantly increased compared with the control group.

[0158]

The increased amounts of the muscle in the groups of administration of 0.03 to 0.3 mg/kg of IGF11-16 in a single administration were equivalent to that in the group (infusion) of continuous administration of 1 mg/kg/day of human recombinant IGF-I. The results indicate that IGF11-16 shows efficacy even in in vivo by intravenous or subcutaneous administration in a single administration.

[0159]

It was demonstrated that IGF11-16 shows efficacy equivalent to that by continuous administration of IGF-I in a single administration. In clinical use, IGF-I (Mecasermin) is administered once or twice a day. In contrast, IGF11-16 administered one every other week shows in vivo effectiveness equivalent to that in continuous administration of IGF-I, indicating that IGF11-16 has excellent persistence compared with IGF-I.

Example 15: In vivo hypoglycemic effect (hypoglycemic effect in guinea pig)

[0160]

In order to verify whether the IGF-I receptor agonist antibody has in vivo hypoglycemic effect or not, IGF11-16 was administered to guinea pigs in a single administration, and the blood glucose levels were measured over time and compared with the hypoglycemic effect of IGF-I in a single administration. The hypoglycemic effect is defined as an effect of lowering the blood glucose level to 50 mg/dL or less or causing hypoglycemia.

[0161]

IGF-I was subcutaneously administered to guinea pigs a single time, and the hypoglycemic effect was investigated. The guinea pigs were fasted for 12 hours, and human recombinant IGF-I (Mecasermin) was subcutaneously administered to the guinea pigs at 0.3, 1, 3, and 10 mg/kg a single time. The guinea pigs were fasted for 24 hours after the administration. Blood was collected from the awake guinea pigs at before the administration (0 hour) and at 1, 2, 4, 8, 10, and 24 hours after the administration and was subjected to measurement of the blood glucose level with a Glutest Sensor (Sanwa Kagaku Kenkyusyo). The results are shown in Fig. 6.

[0162]

IGF-I showed a significant glucose lowering effect at 0.3 mg/kg or more. Hypoglycemia was observed at 1 mg/kg or more. Death was caused at 3 mg/kg or more.

[0163]

IGF11-16 was subcutaneously administered to guinea pigs a single time, and the hypoglycemic effect was investigated. The guinea pigs were fasted for 12 hours, and IGF11-16 was subcutaneously administered to the guinea pigs at 10, 30, and 100 mg/kg a single time. The guinea pigs were fasted for 24 hours after the administration. Blood was collected from the awake guinea pigs at before the administration (0 hour) and at 2, 4, 8, 10, and 24 hours after the administration and was subjected to measurement of the blood glucose level with a Glutest Sensor (Sanwa Kagaku Kenkyusyo). The results are shown in Fig. 7.

[0164]

IGF11-16 did not show any significant difference in the blood glucose level, even in the group of 100 mg/kg administration, compared with a control group in which only the solvent was administered. The results indicate that subcutaneous administration of IGF11-16 does not have a hypoglycemic effect and does not affect the blood glucose level.

[0165]

IGF11-16 was intravenously administered to guinea pigs a single time, and the hypoglycemic effect was investigated. The guinea pigs were fasted for 12 hours, and IGF11-16 was intravenously administered to the guinea pigs at 0.1, 1.5, 6, and 20 mg/kg. The guinea pigs were fasted for 24 hours after the administration. Blood was collected from the awake guinea pigs at before the administration (0 hour) and at 0.5, 1, 2, 4, 8, and 24 hours after the administration and was subjected to measurement of the blood glucose level with a Glutest Sensor (Sanwa Kagaku Kenkyusyo). The results are shown in Fig. 8.

[0166]

IGF11-16 did not show any significant difference in the blood glucose level, even in the group of 20 mg/kg administration, compared with a control group in which only the solvent was administered. The results indicate that intravenous administration of IGF11-16 also does not have a hypoglycemic effect and does not affect the blood glucose level.

[0167]

IGF11-16 does not have a noticeable hypoglycemic effect in both subcutaneous and intravenous administrations, unlike IGF-I, and does not affect the blood glucose level, indicating that IGF11-16 has a possibility as an agent that overcomes a side effect of IGF-I, hypoglycemia.

Example 16: In vivo efficacy (growth promoting effect in guinea pig)

[0168]

In order to verify the in vivo efficacy of the IGF-I receptor agonist antibody on bone, the effect was compared with IGF-I continuously administered and growth hormone (GH) repeatedly administered once a day. IGF11-16 was administered to guinea pigs with removed pituitary gland a single time. After two weeks, the length of the tibia and the thickness of the growth plate cartilage were measured as indices of the growth promoting effect. IGF11-16 (0.3 mg/kg and 1 mg/kg) was subcutaneously administered to the guinea pigs with removed pituitary gland a single time. As a control, human recombinant IGF-I (Mecasermin) was subcutaneously embedded with an osmotic pump (Alzet) and was continuously administered at 1 mg/kg/day. Another control, human recombinant GH (Genotropin[®]), was subcutaneously administered at a dose of 1 mg/kg repeatedly once a day. After two weeks from the agent administration, the guinea pigs were euthanized by exsanguination under anesthesia, and the thickness of the growth plate cartilage of the tibia proximal and the length of the tibia were measured. The results are shown in Figs. 9 and 10.

[0169]

In the group (IGF11-16) in which IGF11-16 was subcutaneously administered at 0.3 mg/kg and 1 mg/kg, the thickness of the growth plate cartilage and the length of the tibia were dose-dependently and significantly extended to show a growth promoting effect, compared with those in a control group (vehicle) in which the guinea pigs with removed pituitary gland were treated with only the solvent.

The growth promoting effect of the group of a single administration of IGF11-16 at 0.3 mg/kg was equivalent to that of the group (IGF-I) of continuous administration of human recombinant IGF-I at 1 mg/kg/day. The growth promoting effect of the group of single administration of IGF11-16 at 1 mg/kg was equivalent to that of the group (GH) of repeated administration of human recombinant GH at 1 mg/kg/day. The results indicate that a single administration of IGF11-16 shows efficacy equivalent to that of a continuous administration of IGF-I and that of repeated administration of GH once a day. In clinical use, human recombinant IGF-I (Mecasermin) and human recombinant GH (Genotropin[®]) are administered by subcutaneous injection once or twice a day and six or seven times a week, respectively. In contrast, IGF11-16 administered once every other week shows in vivo effectiveness equivalent to that in continuous administration of IGF-I and that in repeated administration of GH once a day, indicating that IGF11-16 has excellent persistence compared with IGF-I and GH.

Example 17: Kinetics of IGF-I and IGF11-16 in blood

[0170]

Kinetics of IGF-I in blood

Guinea pigs were fasted for 12 hours, and human recombinant IGF-I was subcutaneously administered to the guinea pigs at 0.3, 1, 3, and 10 mg/kg. The guinea pigs were fasted for 24 hours after the administration. Blood was collected from the awake guinea pigs at before the administration (0 hour) and at 1, 2, 4, 8, 10, and 24 hours after the administration. The human IGF-I concentration in plasma was measured by ELISA (DG100, R&D). The results are shown in Fig. 11.

[0171]

The plasma IGF-I concentration increased administration-dose dependently and, after 24 hours from the administration, decreased to about 50% of the maximum plasma IGF-I concentration. In the group of 0.3 mg/kg administration, the IGF-I concentration at 24 hours after the administration was lower than the lower limit of the measurement. In the group of 10 mg/kg administration, the guinea pigs died due to hypoglycemia after 4 hours from the administration, and the plasma could not be collected.

[0172]

Kinetics of IGF11-16 in blood

Guinea pigs were fasted for 12 hours, and the IGF-I receptor agonist antibody was subcutaneously administered to the guinea pigs at 0.3, 1, 3, 10, 30, and 100 mg/kg. The guinea pigs were fasted for 24 hours after the administration and were then refed. Blood was collected from the awake guinea pigs at before the administration (0 hour) and at 2, 4, 8, 10, 24, 48, and 72 hours after the administration. The IGF11-16 concentration in plasma was measured by ELISA. The results are shown in Fig. 12.

The plasma IGF11-16 concentration increased administration-dose dependently, and the plasma IGF11-16 concentration after 48 hours from the administration was retained to be at about 50% or more of that at 24 hours after the administration, indicating that the kinetics of IGF11-16 in blood is excellent in the persistence compared with that of IGF-I.

INDUSTRIAL APPLICABILITY

[0173]

The present invention can provide an antibody which specifically binds to an IGF-I receptor of a vertebrate, and thereby increase the muscle mass or the thickness of growth plate cartilage via the IGF-I receptor, but does not reduce the blood glucose level. Therefore, the present invention can be used for the treatment, prevention, or diagnosis of diseases associated with an anti-IGF-I receptor antibody.

SEQUENCE LISTING

CLAIMS

[Claim 1]

An anti-IGF-I receptor antibody or its fragment or a derivative thereof which specifically binds to an IGF-I receptor of a vertebrate, and exhibits an activity to induce growth of vertebrate-derived cells.

[Claim 2]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to claim 1, wherein the activity of the antibody, fragment, or derivative to induce growth of vertebrate-derived cells is equal to or higher than the corresponding activity of a wild-type IGF-I.

[Claim 3]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to claim 1 or 2, wherein the EC₅₀ value of the antibody, fragment, or derivative for inducing growth of vertebrate-derived cells *in vitro* is 1/20 or less of the corresponding value of a wild-type IGF-I.

[Claim 4]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 3, wherein when the antibody, fragment, or derivative is contacted with cultured vertebrate-derived cells, the duration of activity of the antibody, fragment, or derivative to induce growth of the cultured cells relative to the duration of contact is improved than a wild-type IGF-I.

[Claim 5]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 2 to 4, wherein the wild-type IGF-I is a human IGF-I having an amino acid sequence defined in SEQ ID NO:1.

[Claim 6]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 5, wherein the EC₅₀ value of the antibody, fragment, or derivative for inducing growth of vertebrate-derived cells *in vitro* is 0.1 nmol/L or lower.

[Claim 7]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any

one of claims 1 to 6, which exhibits an activity to induce an increase in the muscle mass and/or the body length of a vertebrate when parenterally administered to the vertebrate.

[Claim 8]

5 The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 7, which is administered to a vertebrate at a frequency of once a week or less.

[Claim 9]

10 The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 8, wherein the vertebrate is a human; a non-human animal including a guinea pig, a monkey, a rabbit, a cow, a pig, a horse, a sheep, a dog, or a fowl; or a non-human animal engineered to express a human IGF-I receptor.

[Claim 10]

15 The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 9, which does not induce glucose uptake by differentiated muscle cells when administered at a dosage sufficient to induce growth of vertebrate-derived cells.

[Claim 11]

20 The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to claim 10, which does not induce glucose uptake by differentiated muscle cells when administered at a dosage of 100 times or more of the EC₅₀ value for inducing growth of vertebrate-derived cells *in vitro*.

25 [Claim 12]

 The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to claim 10 or 11, wherein the vertebrate-derived cells are myoblasts derived from a human or a non-human mammal.

30 [Claim 13]

 The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 7 to 12, which does not lower the blood glucose level of a vertebrate when parenterally administered to the vertebrate at a dosage sufficient to induce an increase in the muscle mass and/or the body length of the vertebrate.

35

[Claim 14]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to claim 13, which does not change the blood glucose level of a vertebrate when parenterally administered to the vertebrate at a dosage of 10 times or more of an effective dosage sufficient to induce an increase in the muscle mass and/or the body length of the vertebrate.

5

[Claim 15]

An anti-IGF-I receptor antibody or its fragment or a derivative thereof, according to any one of claims 1 to 14, which binds to a CR domain of an IGF-I receptor.

10

[Claim 16]

An anti-IGF-I receptor antibody or its fragment or a derivative thereof, which binds to a CR domain of an IGF-I receptor, and inhibits binding of IGF-I or IGF-II to an IGF-I receptor.

[Claim 17]

15

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to claim 16, which binds to an epitope containing ProSerGlyPheIleArgAsnX₁X₂GlnSerMet (where X₁ represents Gly or Ser and X₂ represents Ser or Thr), or a part in the vicinity thereof, in the sequence of the CR domain of the IGF-I receptor.

20

[Claim 18]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to claim 17, which binds to an epitope containing ProSerGlyPheIleArgAsnGlySerGlnSerMet, or a part in the vicinity thereof, in the sequence of the CR domain of the IGF-I receptor.

25

[Claim 19]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 18, which has a cross-reactivity with an IGF-I receptor of a human or a non-human animal including a guinea pig, a monkey, a rabbit, a cow, a pig, a horse, a sheep, a dog, or a fowl.

30

[Claim 20]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 19, which causes an antigen-antibody reaction with an affinity intensity at a equilibrium dissociation constant (KD) of 1×10^{-8} M or less.

35

[Claim 21]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 16 to 20, which has at least one of the features of:

- 1) exhibiting an activity to induce growth of vertebrate-derived cells;
- 2) exhibiting an activity to induce an increase in the muscle mass and/or the body length of a vertebrate when parenterally administered to the vertebrate;
- 3) not inducing glucose uptake by differentiated muscle cells when administered at a dosage sufficient to induce growth of vertebrate-derived cells; and
- 4) not changing the blood glucose level of a vertebrate when parenterally administered to the vertebrate at a dosage sufficient to induce an increase in the muscle mass and/or the body length of the vertebrate.

[Claim 22]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 16 to 21, which has at least one of the features of:

- 1) inhibiting growth of vertebrate-derived cells induced by IGF-I;
- 2) inhibiting IGF-I-induced cell proliferation in a vertebrate suffering a cell proliferative disease when parenterally administered to the vertebrate;
- 3) not affecting glucose uptake by differentiated muscle cells at a dosage sufficient to inhibit growth of vertebrate-derived cells induced by IGF-I; and
- 4) not changing the blood glucose level of a vertebrate suffering a cell proliferative disease when parenterally administered to the vertebrate at a dosage sufficient to inhibit IGF-I-induced cell proliferation in the vertebrate.

[Claim 23]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 22, which is a Fab, scFv, diabody or bispecific antibody, or a derivative thereof.

[Claim 24]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 23, consisting of an amino acid sequence comprising:

as a heavy chain variable region CDR-1 (CDR-H1) sequence, an amino acid sequence defined in SEQ ID NO:3 or an amino acid sequence derived from SEQ ID NO:3 via substitution, deletion or insertion of any one amino acid residue;

as a heavy chain variable region CDR-2 (CDR-H2) sequence, an amino acid sequence defined in SEQ ID NO:4 or an amino acid sequence derived from SEQ ID NO:4 via

substitution, deletion or insertion of any one or two amino acid residues;

as a heavy chain variable region CDR-3 (CDR-H3) sequence, an amino acid sequence defined in SEQ ID NO:5 or an amino acid sequence derived from SEQ ID NO:5 via substitution, deletion or insertion of any one or two amino acid residues;

5 as a light chain variable region CDR-1 (CDR-L1) sequence, an amino acid sequence defined in SEQ ID NO:6 or an amino acid sequence derived from SEQ ID NO:6 via substitution, deletion or insertion of any one or two amino acid residues;

as a light chain variable region CDR-2 (CDR-L2) sequence, an amino acid sequence defined in SEQ ID NO:7 or an amino acid sequence derived from SEQ ID NO:7 via substitution, deletion or insertion of any one amino acid residue; and

10 as a light chain variable region CDR-3 (CDR-L3) sequence, an amino acid sequence defined in SEQ ID NO:8 or an amino acid sequence derived from SEQ ID NO:8 via substitution, deletion or insertion of any one or two amino acid residues.

15 [Claim 25]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to claim 24 further comprises a framework sequence of immunoglobulin.

[Claim 26]

20 The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to claim 25, wherein the framework sequence of immunoglobulin is a framework sequence of each class of immunoglobulin from a human or a non-human animal including a guinea pig, a monkey, a rabbit, a cow, a pig, a horse, a sheep, a dog, a fowl, a mouse, or a rat.

25 [Claim 27]

The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 26, which consists of an amino acid sequence comprising:

as a heavy chain variable region, an amino acid sequence defined in SEQ ID NO:9 or an amino acid sequence having a similarity of 90% or more to SEQ ID NO:9; and

30 as a light chain variable region, an amino acid sequence defined in SEQ ID NO:10 or an amino acid sequence having a similarity of 90% or more to SEQ ID NO:10.

[Claim 28]

35 The anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 27 further comprising a constant region of each class of immunoglobulin a human or a non-human animal including a guinea pig, a monkey, a rabbit, a cow, a pig, a horse,

a sheep, a dog, a fowl, a mouse, or a rat.

[Claim 29]

5 A nucleic acid molecule consisting of a polynucleotide sequence encoding an anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 28.

[Claim 30]

10 A cloning vector or expression vector comprising at least one nucleic acid molecule according to claim 29.

[Claim 31]

A recombinant cell derived from a host cell via transfection of a vector according to claim 30.

15 [Claim 32]

A process of producing an anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 28, comprising:

culturing a recombinant cell according to claim 31; and

20 purifying the anti-IGF-I receptor antibody, fragment, or derivative produced from the recombinant cell.

[Claim 33]

25 A pharmaceutical composition comprising an anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 28, a nucleic acid molecule according to claim 29, a vector according to claim 30, or a recombinant cell according to claim 31.

[Claim 34]

30 The pharmaceutical composition according to claim 33, further comprising an additional active ingredient other than the anti-IGF-I receptor antibody, fragment, or derivative according to any one of claims 1 to 28, the nucleic acid molecule according to claim 29, the vector according to claim 30, or the recombinant cell according to claim 31.

[Claim 35]

35 The pharmaceutical composition according to claim 34, wherein the active ingredient is one or more selected from a growth hormone or an analog thereof, insulin or an analog thereof,

IGF-II or an analog thereof, an anti-myostatin antibody, a myostatin antagonist, an anti-activin type IIB receptor antibody, an activin type IIB receptor antagonist, a soluble activin type IIB receptor or an analog thereof, ghrelin or an analog thereof, follistatin or an analog thereof, a beta-2 agonist, and a selective androgen receptor modulator.

5

[Claim 36]

The pharmaceutical composition according to claim 34 or 35, wherein the active ingredient comprises an ingredient selected from the group consisting of: corticosteroid, antiemetic, ondansetron hydrochloride, granisetron hydrochloride, metoclopramide, domperidone, haloperidol, cyclizine, lorazepam, prochlorperazine, dexamethasone, levomepromazine, tropisetron, cancer vaccine, GM-CSF inhibitor, GM-CSF DNA vaccine, cell-based vaccine, dendritic cell vaccine, recombinant virus vaccine, heat shock protein (HSP) vaccine, homologous tumor vaccine, autologous tumor vaccine, analgesic, ibuprofen, naproxen, choline magnesium trisalicylate, oxycodone hydrochloride, anti-angiogenic, antithrombotic, anti-PD-1 antibody, nivolumab, pembrolizumab, anti-PD-L1 antibody, atezolizumab, anti-CTLA4 antibody, ipilimumab, anti-CD20 antibody, rituximab, anti-HER2 antibody, trastuzumab, anti-CCR4 antibody, mogamulizumab, anti-VEGF antibody, bevacizumab, anti-VEGF receptor antibody, soluble VEGF receptor fragment, anti-TWEAK antibody, anti-TWEAK receptor antibody, soluble TWEAK receptor fragment, AMG 706, AMG 386, antiproliferative, farnesyl protein transferase inhibitor, alpha v beta 3 inhibitor, alpha v beta 5 inhibitor, p53 inhibitor, Kit receptor inhibitor, ret receptor inhibitor, PDGFR inhibitor, growth hormone secretion inhibitor, angiopoietin inhibitor, tumor-infiltrating macrophage inhibitor, c-fms inhibitor, anti-c-fms antibody, CSF-1 inhibitor, anti-CSF-1 antibody, soluble c-fms fragment, pegvisomant, gemcitabine, panitumumab, irinotecan, and SN-38.

25

[Claim 37]

A medical drug for use in the treatment or prevention of a condition associated with IGF-I or IGF-II, comprising comprising an anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 28, a nucleic acid molecule according to claim 29, a vector according to claim 30, or a recombinant cell according to claim 31.

30

[Claim 38]

The medical drug according to claim 37, wherein the condition associated with IGF-I is selected from: disuse muscle atrophy, dwarfism, diabetic nephropathy, chronic renal failure, Laron syndrome, hepatic cirrhosis, hepatic fibrosis, aging, intrauterine growth restriction (IUGR), neurological disease, cerebral stroke, spinal cord injury, cardiovascular protection,

35

diabetes, insulin resistant, metabolic syndrome, osteoporosis, cystic fibrosis, wound healing, myotonic dystrophy, AIDS-associated sarcopenia, HIV-associated fat redistribution syndrome, burn, Crohn's disease, Werner's syndrome, X-linked combined immunodeficiency disease, hearing loss, anorexia nervosa and retinopathy of prematurity, Turner's syndrome, Prader-Willi syndrome, Silver-Russell syndrome, idiopathic short stature, obesity, multiple sclerosis, fibromyalgia, ulcerous colitis, low muscle mass, myocardial ischemia and decreased bone density.

[Claim 39]

The medical drug according to claim 37 or 38, which is parenterally administered.

[Claim 40]

The medical drug according to any one of claims 37 to 39, which is a veterinary drug to be administered to a non-human animal.

[Claim 41]

The medical drug according to claim 40, wherein the veterinary drug is administered for the purpose of, increasing muscle mass and/or body length, promoting growth, increasing milk production, promoting reproduction, or preventing aging.

[Claim 42]

The medical drug according to claim 40 or 41, wherein the non-human animal is a guinea pig, a monkey, a rabbit, a cow, a pig, a horse, a sheep, a dog, or a fowl.

[Claim 43]

The medical drug according to any one of claims 37 to 42, for the treatment or prevention of a disease caused by an effect of IGF-I or IGF-II on an IGF-I receptor.

[Claim 44]

The medical drug according to claim 43, wherein the disease caused by an effect of IGF-I or IGF-II on an IGF-I receptor is selected from the group consisting of: liver cancer, neuroblastoma, striated muscle sarcoma, bone cancer, childhood cancer, acromegalia, ovary cancer, pancreas cancer, benign prostatic hypertrophy, breast cancer, prostate cancer, bone cancer, lung cancer, colorectal cancer, cervix cancer, synovial sarcoma, urinary bladder cancer, stomach cancer, Wilms' tumor, diarrhea associated with metastatic carcinoid and vasoactive intestinal peptide secreting tumor, vipoma, Verner-Morrison syndrome, Beckwith-Wiedemann

syndrome, kidney cancer, renal cell cancer, transitional cell cancer, Ewing's sarcoma, leukemia, acute ymphoblastic leukemia, brain tumor, glioblastoma, non-glioblastomatic brain tumor, meningioma, pituitary adenoma, vestibular schwannoma, primitive neuroectodermal tumor, medulloblastoma, astrocytoma, oligodendroglioma, ependymoma, choroid plexus papilloma, gigantism, psoriasis, atherosclerosis, vascular smooth muscle restenosis, inappropriate microvascular growth, diabetic retinopathy, Graves' disease, multiple sclerosis, systemic lupus erythematosus, chronic thyroiditis, myasthenia gravis, autoimmune thyroiditis and Behcet's disease.

10 [Claim 45]

A method of culturing vertebrate-derived cells *in vitro*, comprising contacting the vertebrate-derived cells with an anti-IGF-I receptor antibody or its fragment or a derivative thereof according to any one of claims 1 to 28, a nucleic acid molecule according to claim 29, a vector according to claim 30, and a recombinant cell according to claim 31 when culturing the cells.

[Claim 46]

The method according to claim 45, wherein said contacting is carried out for the purpose of promoting growth or inducing differentiation of the vertebrate-derived cells.

[Claim 47]

The method according to claim 45 or 46, wherein the anti-IGF-I receptor antibody, fragment, or derivative is adsorbed by, or immobilized to, a solid phase.

25 [Claim 48]

A transgenic animal comprising an IGF-I receptor gene which has been mutated in a CR domain thereof via gene engineering such that the CR domain includes an amino acid sequence represented by ProSerGlyPheIleArgAsnGlySerGlnSerMet.

30 [Claim 49]

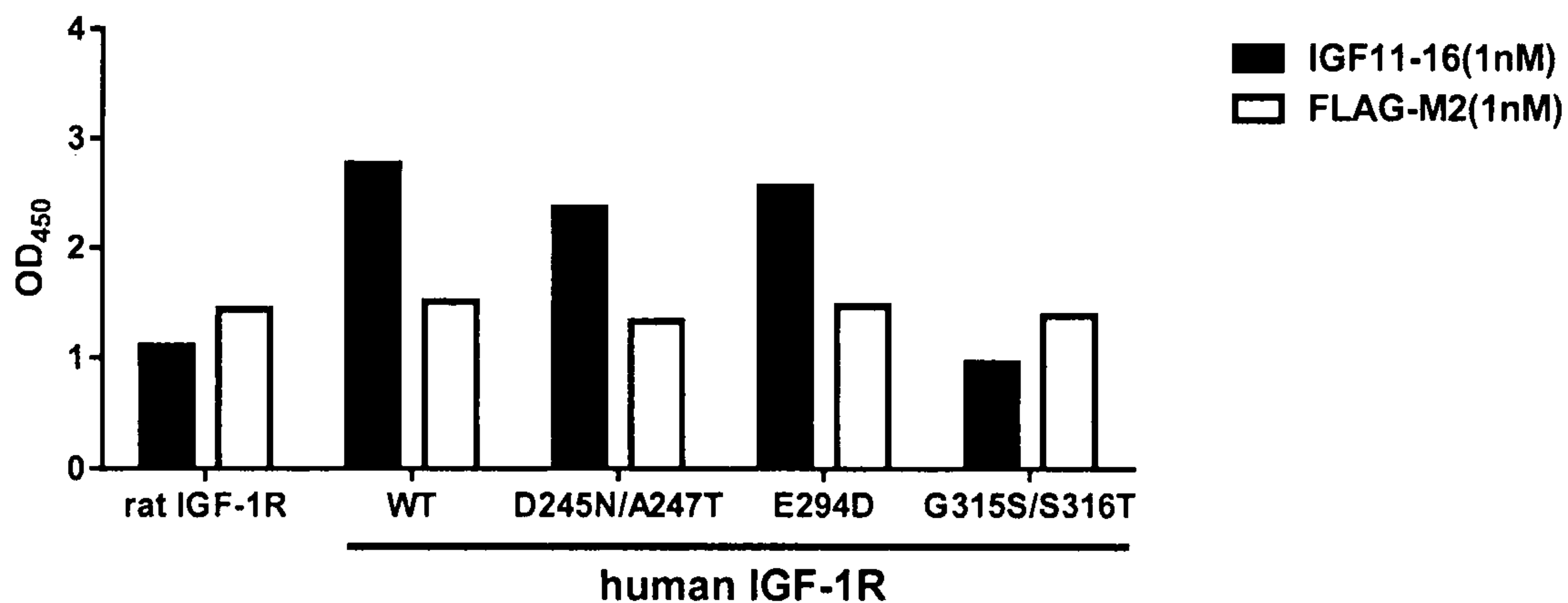
A transgenic animal into which a heterologous IGF-I receptor gene has been transfected, wherein the amino acid sequence encoded by the heterologous IGF-I receptor gene differs from the amino acid sequence encoded by the animal's inherent IGF-I receptor gene in amino acid residue(s) X₁ and/or X₂ of a sequence represented by ProSerGlyPheIleArgAsnX₁X₂GlnSerMet in a CR domain.

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FIG. 1

IGF1R_MOUSE	EMTNLKDIGLYNLRNITRGAIRIEKNADLCYLSTIDWSLILDAVSNNYIVGNKPPKECGD	180
IGF1R_RAT	EMTNLKDIGLYNLRNITRGAIRIEKNADLCYLSTIDWSLILDAVSNNYIVGNKPPKECGD	180
IGF1R_HUMAN	EMTNLKDIGLYNLRNITRGAIRIEKNADLCYLSTVDWSLILDAVSNNYIVGNKPPKECGD	180
IGF1R_CAVY	EMTNLKDIGLYNLRNITRGAIRIEKNADLCYLSTVDWSLILDAVSNNYIVGNKSPKECGD	180
IGF1R_RABIT	EMTNLKDIGLYNLRNITRGAIRIEKNADLCYLSTVDWSLILDAVSNNYIVGNKSPKECGD	180
*****;*****;*****		
IGF1R_MOUSE	LCPGTLEEKPMCEKTTINNEYNYRCWTTNRCQKMCPSVCGKRACTENNECCHPECLGSCH	240
IGF1R_RAT	LCPGTLEEKPMCEKTTINNEYNYRCWTTNRCQKMCPSVCGKRACTENNECCHPECLGSCH	240
IGF1R_HUMAN	LCPGTMEEEKPMCEKTTINNEYNYRCWTTNRCQKMCPSVCGKRACTENNECCHPECLGSCH	240
IGF1R_CAVY	LCPGTMEEEKPLCEKTTINNEYNYRCWTTNRCQKMCPSACGKRACTEYQECCHPECLGSCH	240
IGF1R_RABIT	MCPGTLEEKPLCEKTAINNEYNYRCWTTNRCQKMCPSACGKRACTENNECCHPECLGSCH	240
:****:****:****:*****;*****;*****		
IGF1R_MOUSE	TPDDNTTCVACRHHYYKGVCPACPPGTYRFEGWRCVDRDFCANIPNAESSDSGDFVIHD	300
IGF1R_RAT	TPDDNTTCVACRHHYYKGVCPACPPGTYRFEGWRCVDRDFCANIPNAESSDSGDFVIHD	300
IGF1R_HUMAN	APDNDTACVACRHHYYAGVCPACPPNTYRFEGWRCVDRDFCANILSAESSDSEGDFVIHD	300
IGF1R_CAVY	APDDDTACVACRHHYYAGICVPACPPGTYRFEGWRCVDRDFCANIPNAESSDSEGDFVIHD	300
IGF1R_RABIT	APDDDTACVACRHHYYSGVCPACPPNTYRFEGWRCVDRDFCANIPNADGGDSEGDFVIHD	300
:*:~*:*****:~*:~*:*****;*****;*****~*:~*:*****		
IGF1R_MOUSE	DECMQECPSGFIRNSTQSMYCIPCEGPCPKVCGDEEKTKTIDSVTSAQMLQGCTILKGN	360
IGF1R_RAT	GECMQECPSGFIRNSTQSMYCIPCEGPCPKVCGDEEKTKTIDSVTSAQMLQGCTILKGN	360
IGF1R_HUMAN	GECMQECPSGFIRNGSQSMYCIPCEGPCPKVCE-EEKTKTIDSVTSAQMLQGCTIFKGN	359
IGF1R_CAVY	GECMQECPSGFIRNGSQSMYCIPCEGPCPKVCE-EEKTKTIDSVTSAQMLQGCTIFKGN	359
IGF1R_RABIT	GECMQECPSGFIRNGSQSMFCIPCEGPCPKVCE-EDKTKTIDSVNSAQMLQGCTIFKGN	359
.*****;***:*****~*:*****;*****;***		

FIG. 2



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FIG. 3

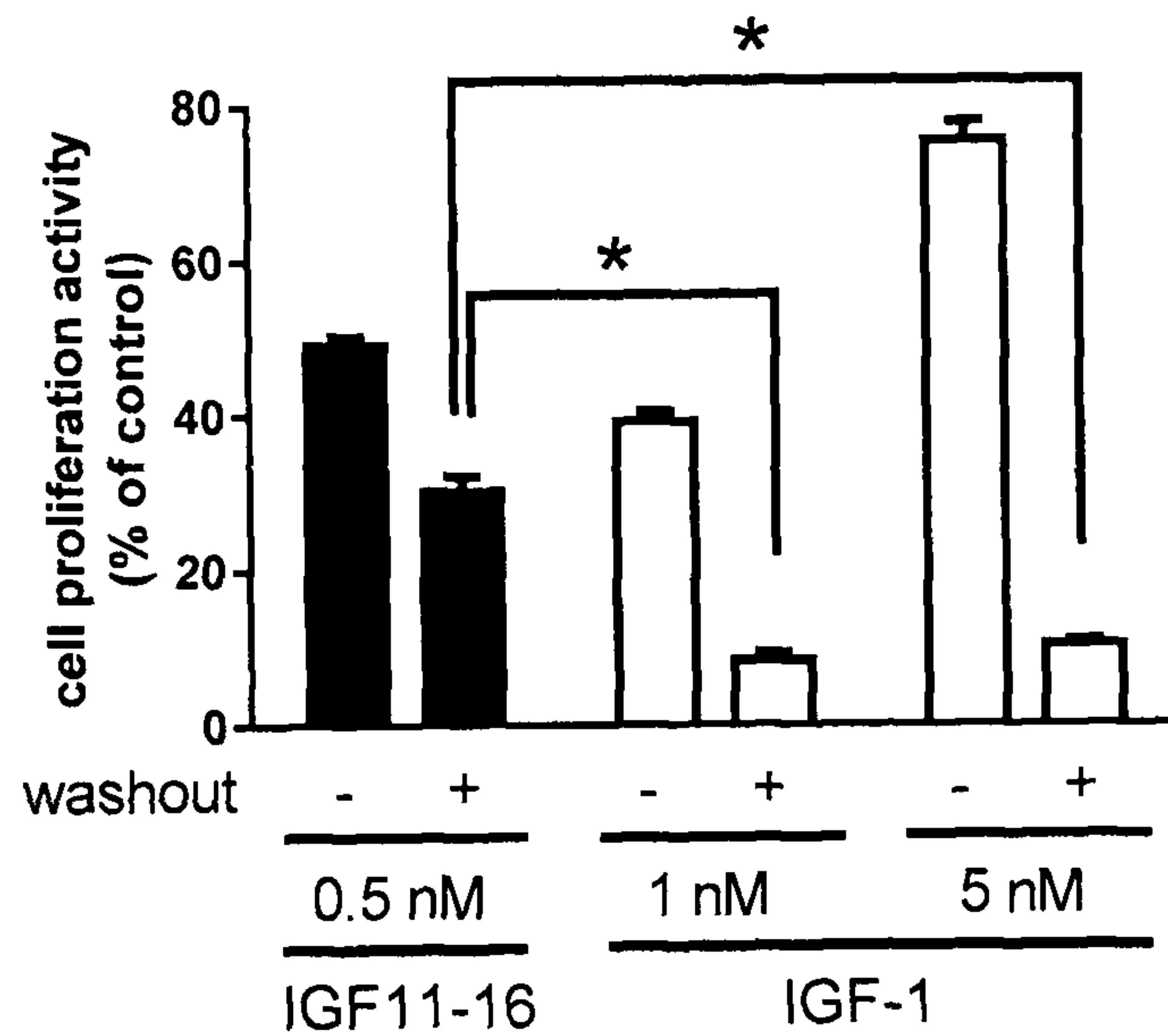
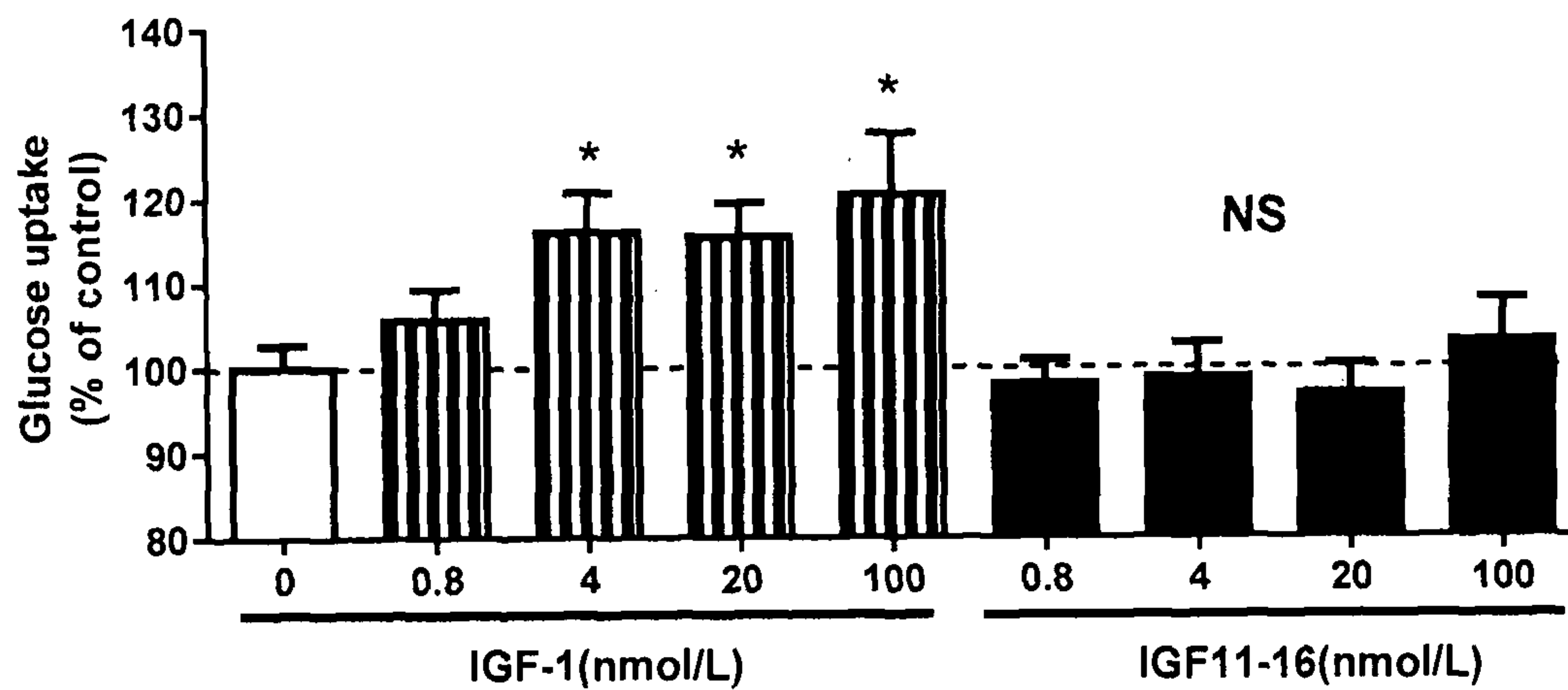


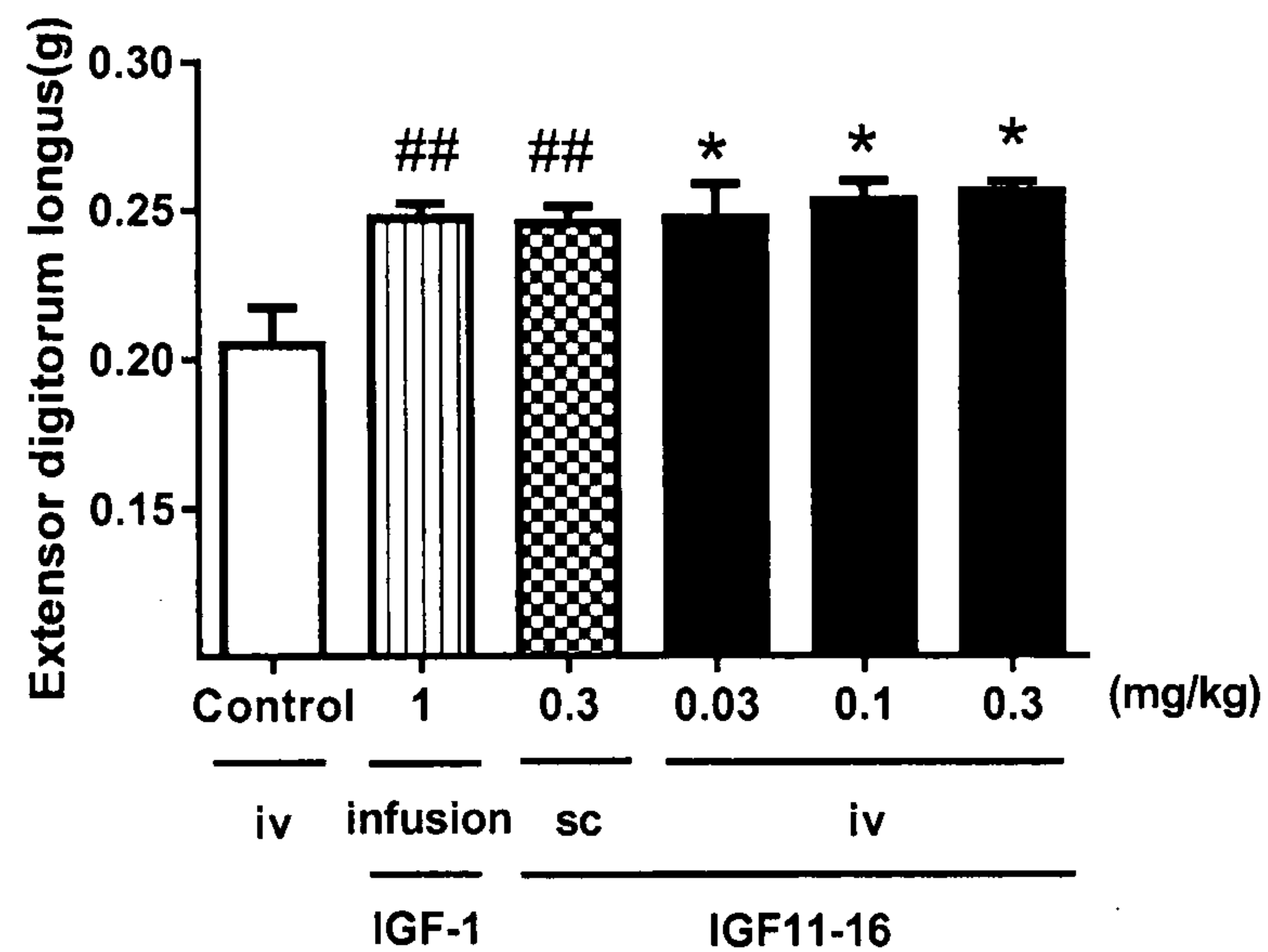
FIG. 4



Results are means and SEM from three independent experiments
 (n=10-16, n=2-8 in each experiment)
 *: p<0.05, Williams test, v.s. control
 NS: p>0.05, Steel test, v.s. control

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FIG. 5

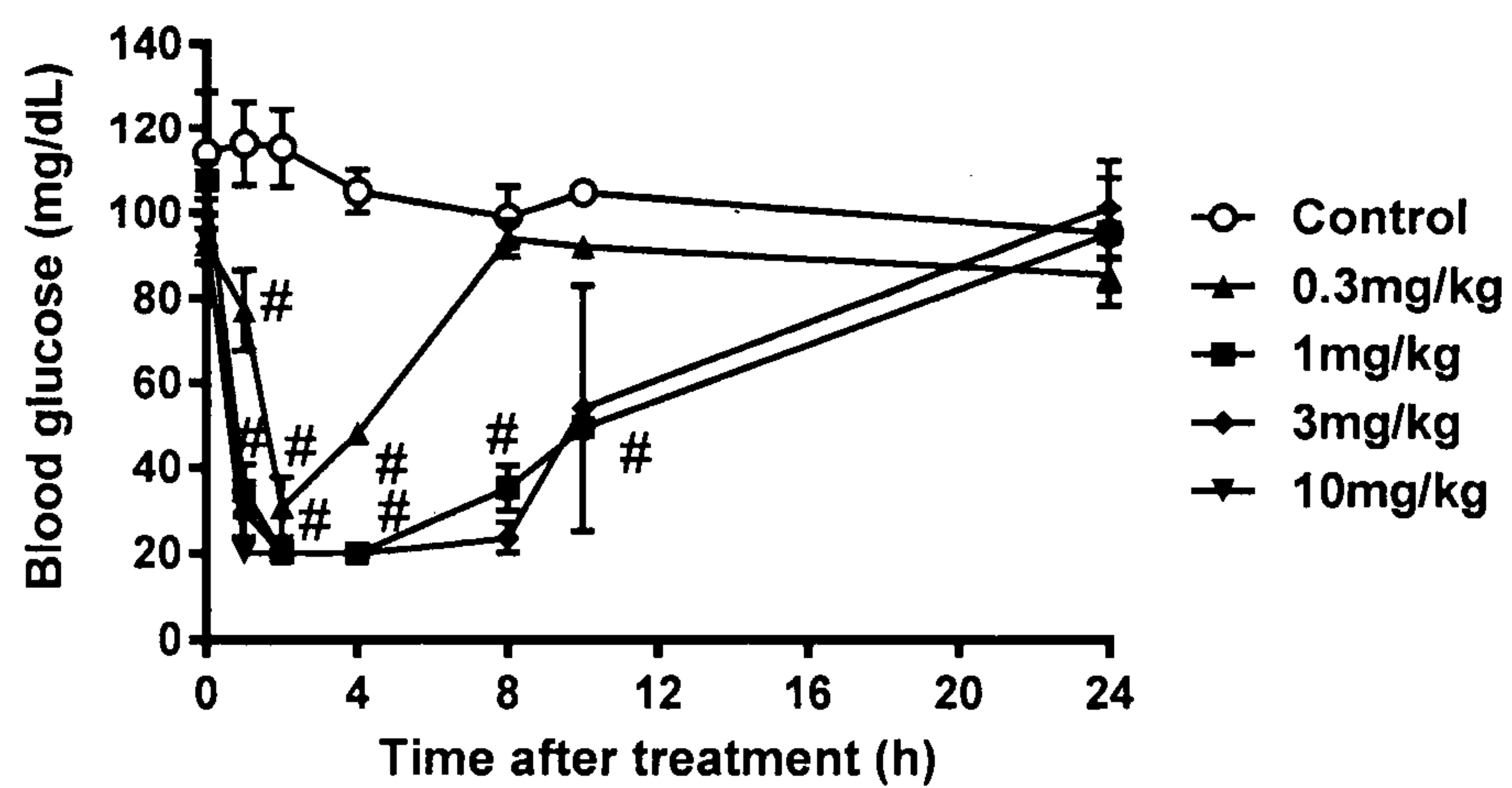


Mean, SEM, n=6-8

##: p<0.01, Dunnett's test, v.s. control

*: p<0.05, Shirley-Williams test, v.s. control

FIG. 6

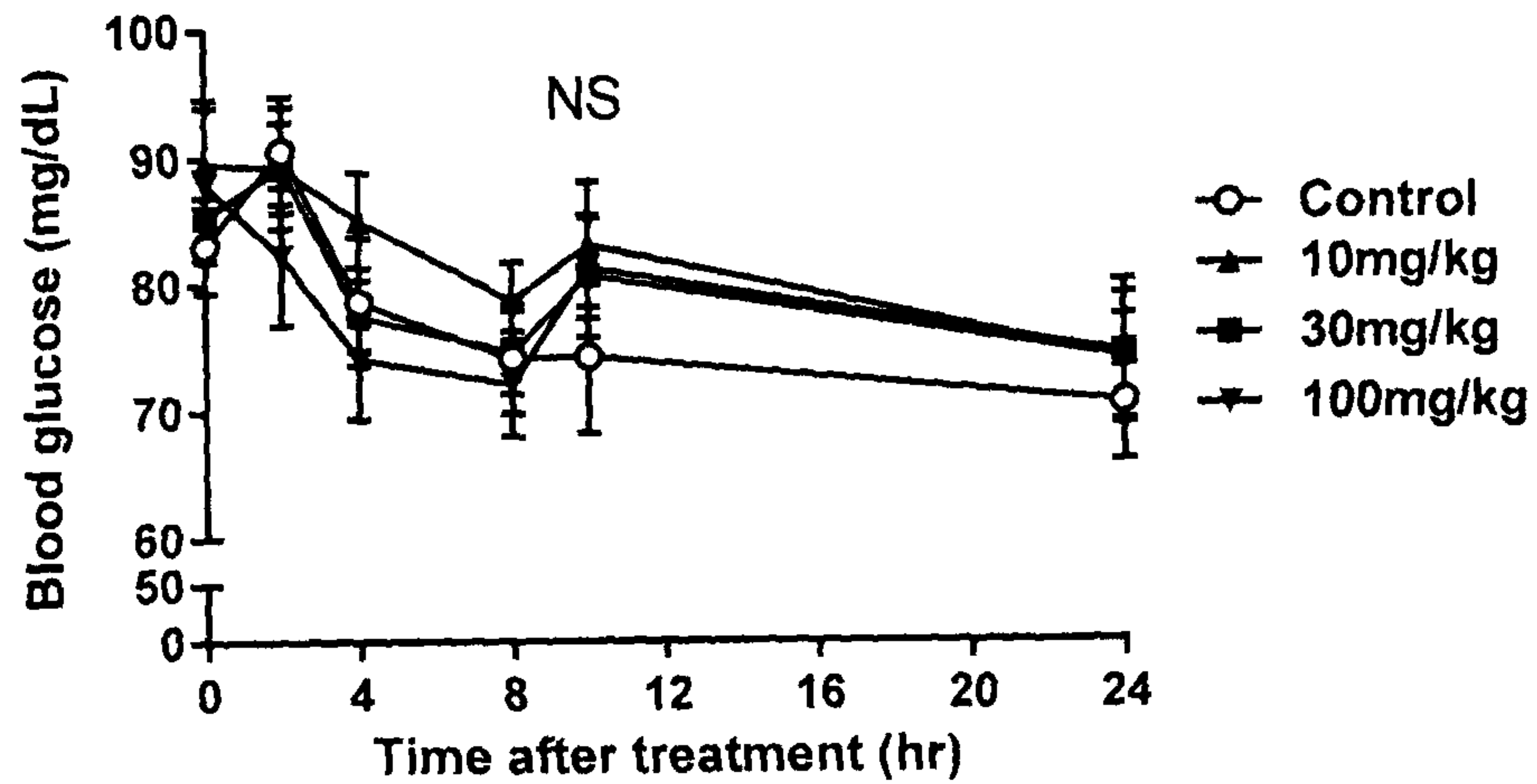


Mean, SEM, n=2-3

#: p<0.05, Two-Way ANOVA, v.s. control

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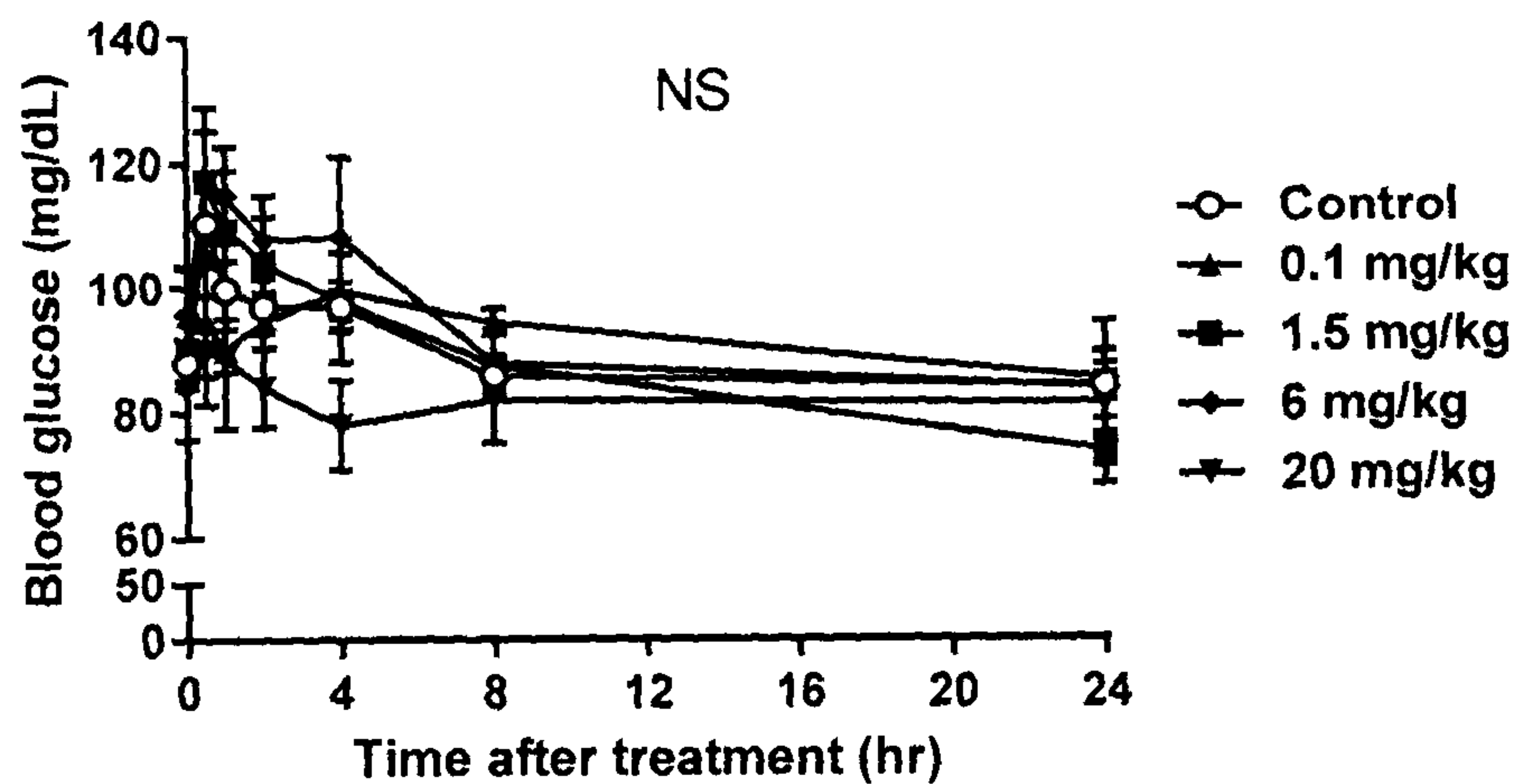
FIG. 7



N=6, Mean, SEM

NS: $p > 0.05$, Two way ANOVA, v.s. control

FIG. 8



Mean, SEM, n=3-4

NS: $p > 0.05$, Two way ANOVA, v.s. control

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FIG. 9

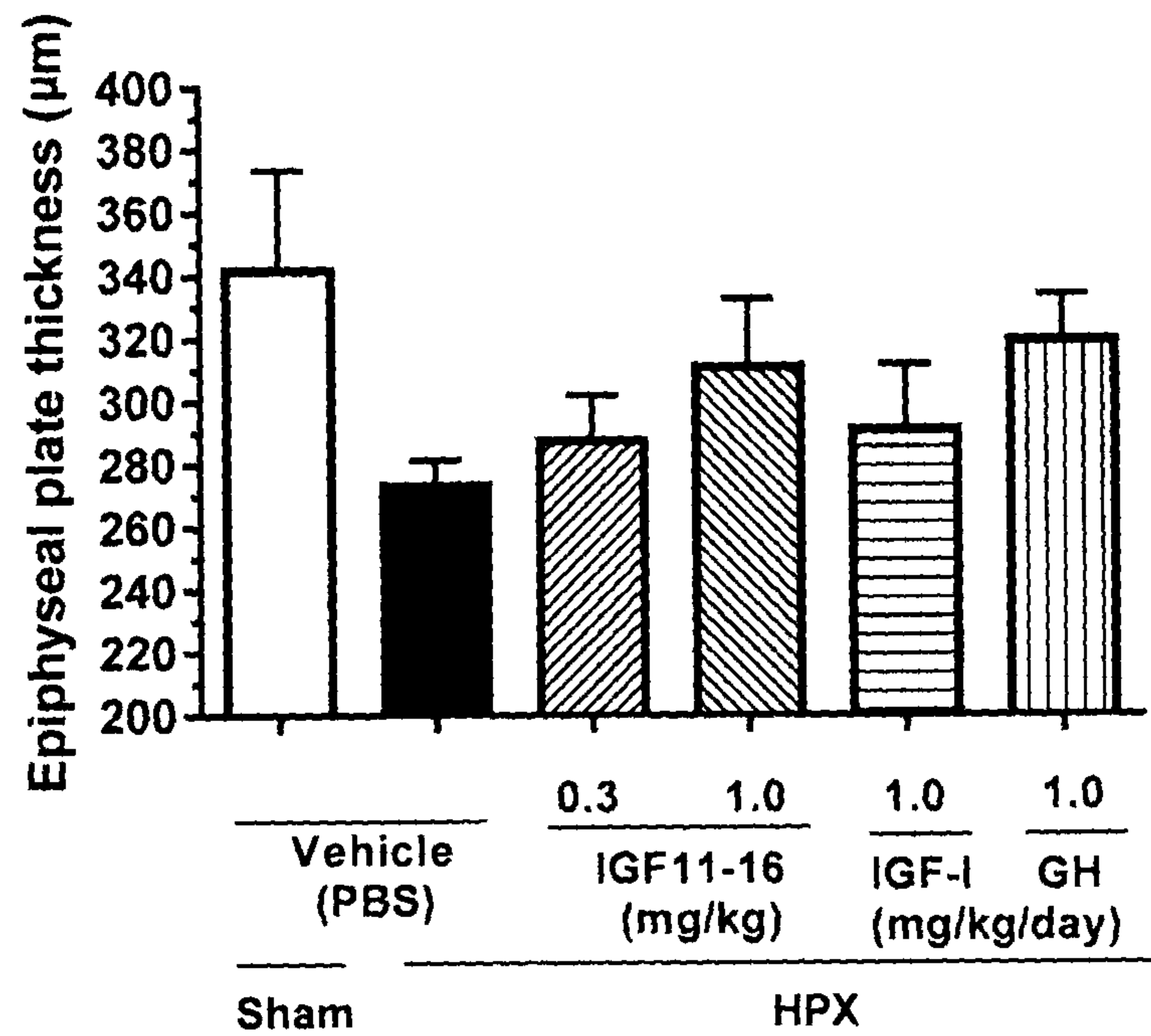
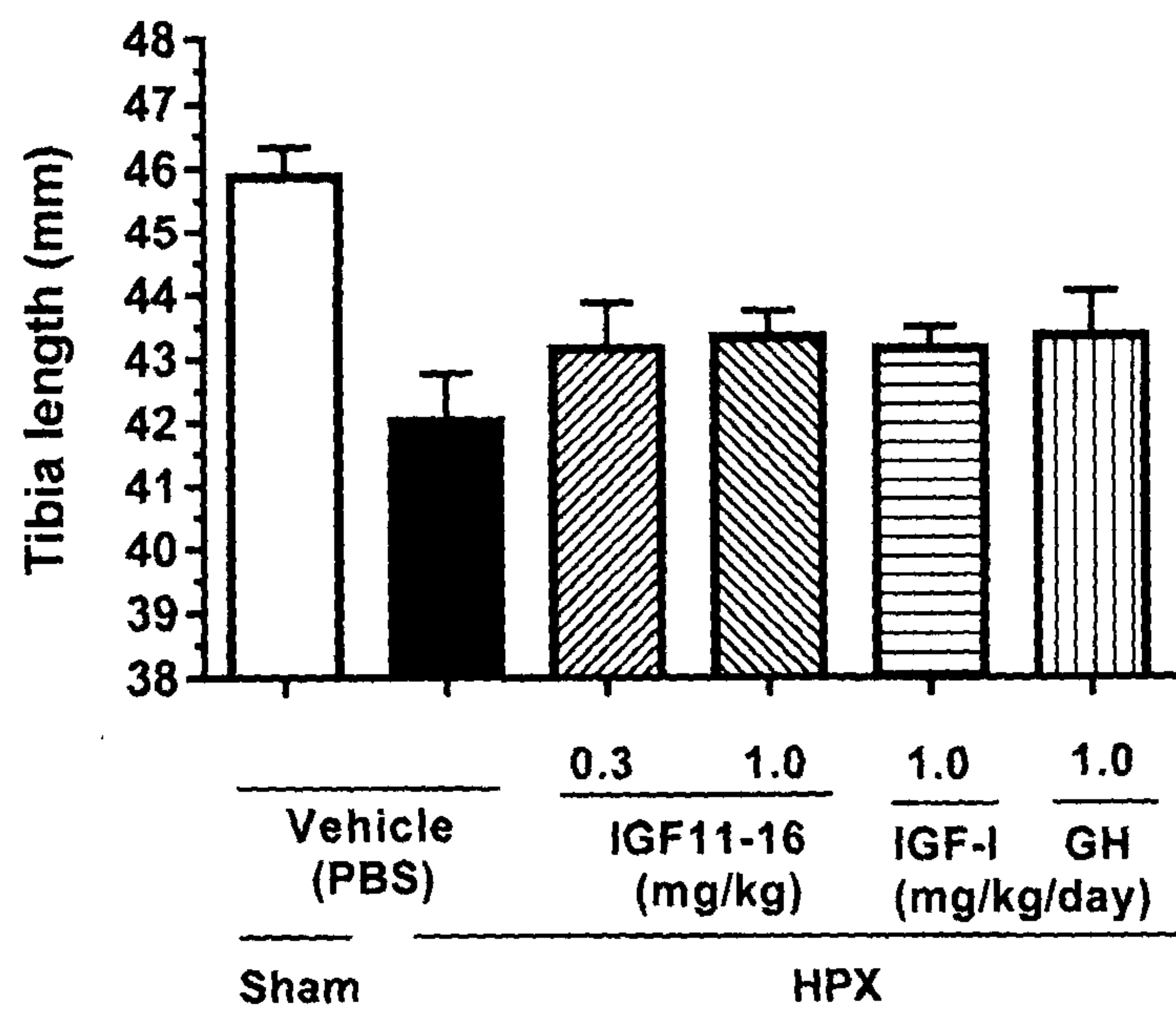


FIG. 10



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FIG. 11

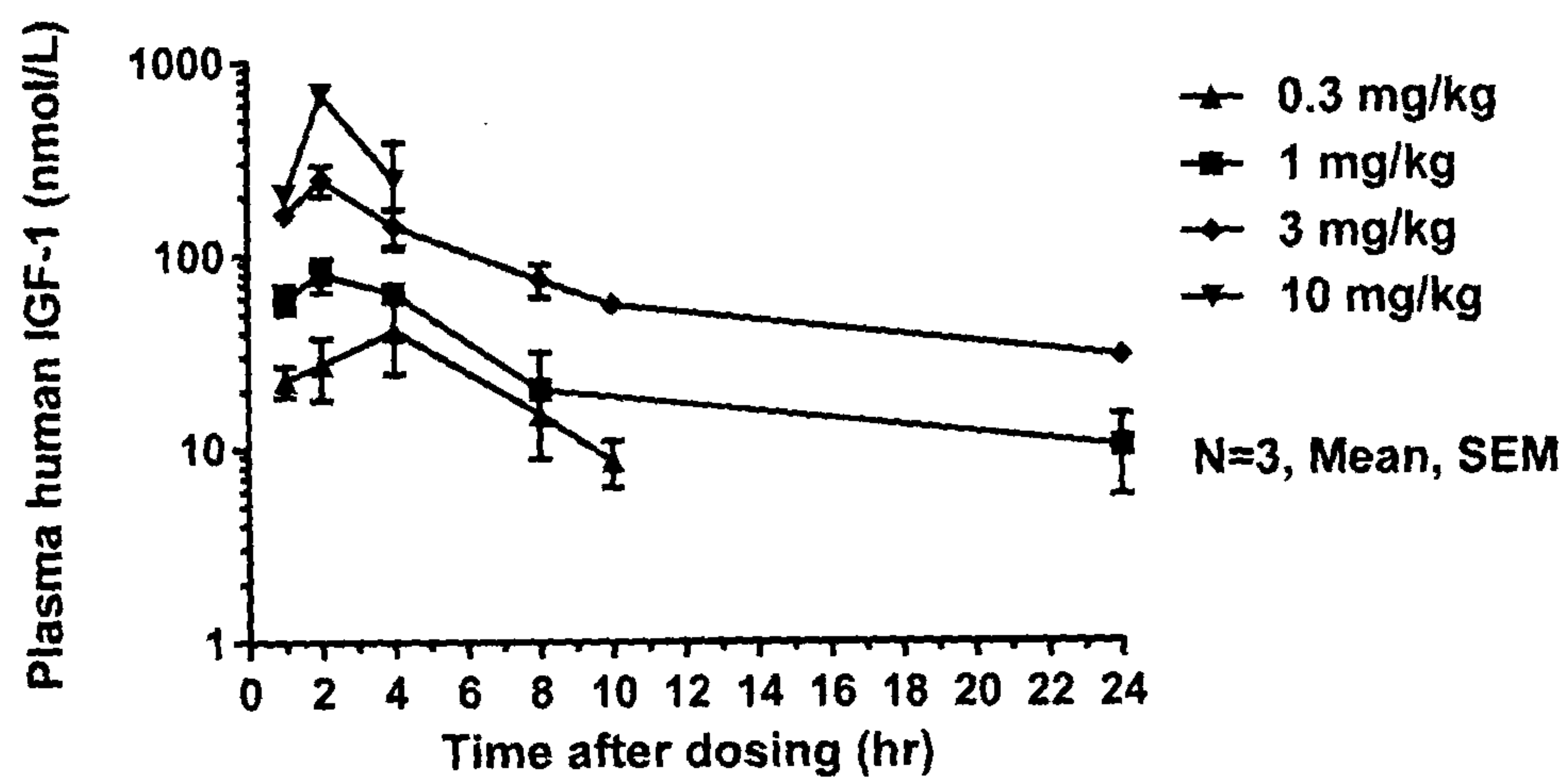


FIG. 12

