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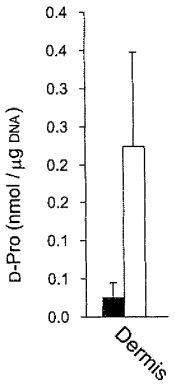
(54) **EVALUATION/SCREENING METHOD FOR DISEASES ASSOCIATED WITH D-AMINO ACID UTILIZING DAO1-/- MOUSE**

(57) Disclosed is an evaluation method which can rapidly discriminate a $Dao^{-/-}$ homozygote from a large number of animals produced in a mating experiment between a DAO enzyme deficient mouse and other disease model mice, to rapidly perform a quantitative measurement of the D-amino acids contained in a large number of samples. The invention provides a method for evaluating the effect of a test condition on a mouse tissue, or cultured tissue cells derived from the tissue. The method comprises the steps of: providing a $Dao1^{-/-}$ mouse or the like; exposing the tissue from the $Dao1^{-/-}$ mouse or the like, to the test condition; and analyzing the effect of exposing the tissue from the $Dao1^{-/-}$ mouse or the like, to the test condition.

FIG.7-2



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Description

TECHNICAL FIELD

[0001] The present invention relates to a method for evaluating of the effect of a test condition on a mouse tissue, or cultured tissue cells derived from the tissue, an evaluation system for carrying out the evaluation method, and a method for screening medicinal and/or cosmetic candidate substances by using the evaluation system.

BACKGROUND ART

[0002] All amino acids other than glycine have two optical isomers, referred to as D-type and L-type. The L-amino acids are used in protein synthesis in organisms, and the amino acids contained in proteins are mostly L-amino acids. On the other hand, although D-amino acids are contained in some of the biologically active peptides of lower forms of life, such D-amino acid-containing peptides are often biosynthesized during a process of post-translation modification. That is to say, amino acids, which constitute proteins and peptides, are predominantly L-amino acids, and D-amino acids are an exceptional presence.

[0003] D-amino acids are structural constituents of peptide glycans of the cell walls of bacteria. Furthermore, it has been reported that free D-amino acids that do not constitute the peptides are present in lower animals, such as aquatic animals and insects,. However, there was a time when it was believed that the amino acids present in higher animals are of the L-type, and that the D-type was present only in trace quantities (Non-Patent Document 1).

[Non-Patent Document 1] Corrigan J.J., Science 164:142-149 (1969).

[0004] However, the presence, and the role thereof, of D-amino acids in mammals, including humans, is only just becoming clear in recent years, due to advances in analytical methods such as optical resolution methods (Non-Patent Document 2). In regard to D-aspartate, as a result of double staining methods using an anti-D-aspartate antibody, or the like, it became clear that it is localized in the prolactin producing cells in rat pituitary glands. Furthermore, administration of D-aspartate to cells of a rat pituitary gland-derived cell line that produces and secretes prolactin increases dose-dependently the prolactin secretion. Therefore, it is considered that D-aspartate controls the secretion of prolactin in prolactin producing cells (Non-Patent Document 3).

[Non-Patent Document 2] Hamase K, Morikawa A, and Zaitsu K., J Chromatogr B 781: 73-91 (2002).
[Non-Patent Document 3] D'Aniello A et al., FASEB J 14: 699-714 (2000).

[0005] On the other hand, it has been reported that in addition to higher concentrations of D-aspartate constantly being detected in the veins of rat testes compared to in other veinous blood, the synthesis and secretion of testosterone is dose-dependently promoted by providing D-aspartate to Leydig cells isolated and purified from rat testes (Non-Patent Document 4).

[Non-Patent Document 4] Nagata Y et al., FEBS Lett. 444:160-164 (1999).

[0006] It has been reported that D-serine selectively stimulates the glycine binding site of the NMDA receptor, which has been supposed to be associated with schizophrenia, and that neural transmission can be promoted by enhancing the function of glutamate via this receptor (Non-Patent Document 5). It has been reported that schizophrenia is actually improved by the administration of D-serine, and that schizophrenic patients have a lower D-serine concentration in the blood serum compared to healthy individuals.

[Non-Patent Document 5] Nishikawa T, Biol. Pharm. Bull. 28: 1561-1565 (2005).

[0007] With regard to the skin science, there is a report in which D-aspartate is present in tissue in which the turnover of proteins does not occur readily, such as in the eye lens (Non-Patent Document 6). Furthermore there is a report that suggests that UV exposure has a strong relationship with D-aspartate formation in the elastic fiber of the skin, since D-aspartate is contained in the elastic fiber of sunburnt skin of elderly subjects but is not contained in the elastic fiber of skin that is not sunburnt (Non-Patent Document 7).

[Non-Patent Document 6] Fujii N et al., Biol. Pharm. Bull. 28: 1585-1589 (2005).
[Non-Patent Document 7] Fujii N. et al., Biochem. Biophys. Res. Commun. 294, 1047-1051 (2002).

[0008] However, a large obstacle in exploring the presence and the role of D-amino acids in the mammal, including the human, is that D-amino acids are rapidly degraded. During the degradation of D-amino acids, firstly, the D-amino acid is oxidatively deaminated by a D-amino acid oxidase (EC 1.4.3.3, hereunder referred to as "DAO enzyme"), and is converted to the corresponding α (alpha)-keto acid. Thereafter, the α (alpha)-keto acid is converted to the corresponding L-amino acid by a transaminase. The DAO enzyme is an enzyme that specifically oxidizes D-amino acids, and is expressed in the kidneys, and other organs (Non-Patent Document 8).

[Non-Patent Document 8] Hamase K., Konno R., Morikawa A. and Zaitsu K., Biol. Pharm. Bull. 28: 1578-1584 (2005).

[0009] Although the DAO enzyme is encoded by the Daol. gene of the fifth chromosome in mouse, the missense mutant of this gene was reported in ddY mouse (Non-Patent Document 9). In this mutant gene allele (Dao^{c} or Dao^{G181R}), the Gly residue in position number 181 is substituted by an Arg residue, resulting in a protein that has lost its enzyme activity. Accordingly, the phenotype of the DAO enzyme inactivation is recessively inherited. In an individual of a recessive homozygote (hereunder referred to as a "DAO enzyme deficient mouse"), the blood serum concentration of D-alanine and D-serine rises between 5 to 8 times, and exhibits ataxia and stereotypic behavior (Non-Patent Document 10). There are no reports regarding mating experiments between a DAO enzyme deficient mouse and other disease model mice.

[Non-Patent Document 9] Konno R. and Yasumura Y. Genetics 103: 277-285 (1983).
[Non-Patent Document 10] Hashimoto A., Yoshikawa M., Niwa A. and Konno R., Brain Res. 1033:210-215 (2005).

[Problems to be Solved by the Invention]

[0010] There is a need to rapidly discriminate a Dao^{G181R}/ Dao^{G181R} homozygote from a large number of animals produced in a mating experiment between a DAO enzyme deficient mouse and other disease model mice. Furthermore, since the amount of the D-amino acid may be less than approximately 1% of the amount of L-amino acid, there is a need to separate and detect trace quantities of D-amino acids from large quantities of L-amino acids. In addition, there is a need to rapidly perform a quantitative measurement of the D-amino acids contained in samples derived from a large number of individual mice, samples derived from a number of different tissues, and samples exposed to a number of test condition.

DISCLOSURE OF INVENTION

[0011] The present invention provides method for evaluating an effect of a test condition on a mouse tissue, or cultured cells derived from the tissue. The method is comprised of the steps of: (1) providing a $Dao1^{+/+}$ mouse and a $Dao1^{-/-}$ mouse; (2) exposing a tissue of the $Dao1^{+/+}$ and $Dao1^{-/-}$ mice, or cultured tissue cells derived from the tissue to the test condition; and (3) analyzing the effect of exposing the tissue or cultured tissue cells derived from the tissue of the $Dao1^{+/+}$ and $Dao1^{-/-}$ mice to the test condition.

[0012] In the method of the present invention, the step 1 may be comprised of discriminating the $Dao1^{+/+}$ mouse and the $Dao1^{-/-}$ mouse according to a method for determining a genotype of $Dao1^{+/+}$, $^{+/-}$ and/or $^{-/-}$, comprising the steps of: identifying a $Dao1^{+/+}$, $^{+/-}$ and/or $^{-/-}$ mouse individually; extracting chromosomal DNA from the mouse; obtaining an amplified DNA fragment by amplifying the region covering the seventh exon of the extracted chromosomal DNA; digesting the amplified DNA fragments with a HpaII restriction enzyme; and analyzing the restriction enzyme-digested products of the amplified DNA fragments.

[0013] In the method of the present invention, the step of obtaining the amplified DNA fragments may be comprised of amplifying with oligonucleotide primers consisting of nucleotide sequences recited as SEQ ID NOs: 1 and 2.

[0014] In the method of the present invention, the $Dao1^{+/+}$ mouse and the $Dao1^{-/-}$ mouse may share a common combination of alleles for at least one other locus.

[0015] In the method of the present invention, the combination of the alleles for the other locus may be comprised of $Hr^{-/-}$.

[0016] In the method of the present invention, the step (3) may be comprised of: easuring the D-amino acid content within the tissue or cultured tissue cells derived from the tissue of the Dao $1^{+/+}$ mouse and the Dao $1^{-/-}$ mouse, prior to the step (2); measuring the D-amino acid content within the tissue or cultured tissue cells derived from the tissue of the $Dao1^{+/+}$ mouse and the $Dao1^{-/-}$ mouse, following the step (2) and following exposure to the test condition; and comparing between the D-amino acid content measured prior to the step (2) and the D-amino acid content measured following the step (2).

[0017] In the method of the present invention, the D-amino acid content is measured by column chromatography using an optical resolution column system.

[0018] In the method of the present invention, the D-amino acid content may be measured by an immunological technique using a monoclonal antibody that discriminates optical isomers.

[0019] According to the method of the present invention, the D-amino acid which is evaluated for the effect of the test condition on the mouse tissue or cultured tissue cells derived from the tissue may be D-proline.

[0020] In the method of the present invention, the tissue or cultured tissue cell derived from the tissue, for which the D-amino acid content is measured, is derived from one or more tissues selected from a group consisting of epidermis, dermis, kidney, pancreas, testis, adrenal gland, cerebellum, pituitary gland, and blood serum.

[0021] In the method of the present invention, the D-amino acid which is evaluated for the effect of the test condition on a mouse tissue or cultured tissue cells derived from the tissue may be D-proline, wherein the D-proline may be measured by column chromatography using an optical resolution column, and the tissue may be epidermis or dermis.

[0022] The present invention provides an evaluation system for carrying out the method of the present invention. The evaluation system is comprised of: a $Dao1^{+/+}$ and $Hr^{-/-}$ mouse; a $Dao1^{-/-}$ and $Hr^{-/-}$ mouse; oligonucleotide primers consisting of nucleotide sequences recited as SEQ ID NOs: 1 and 2; and an optical resolution column system that discriminates the optical isomers of proline

[0023] The present invention provides a method for screening medicinal and/or cosmetic candidate substances that is characterized by evaluating the medicinal and/or cosmetic candidate substances by using the evaluation system of the present invention.

[0024] In the present specification, the gene denoted as Dao 1 is a mouse gene referred to as D-amino acid oxidase 1, and a detailed explanation is disclosed on the homepage of the Mouse Genome Informatics project at the Jackson Laboratories in the US (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=markerDetail&key=7803) . As a mutant of the Dao 1 gene, Dao 1^{G181R} has been known (http://www.informatics.jax.org/searches/allele_report.cgi?_Marker_key=7803&int:_Set_ key=847160). In the present invention, the $Dao1^{-}$ genotype represents any genotype of enzyme activity-deficient mutant allele of D-amino acid oxidase, including, but not limited to, the Dao 1^{G181R}. The Dao 1^{+} genotype of the present invention represents an allele in which, with regard to the D-amino acid oxidase 1 enzyme, is a wild type, that is to say, it is the same, or is substantially the same, as the enzyme in which the amino acid residue at position number 181 of the protein encoded by the Dao1 gene is glycine, and the degradation of the D-amino acid is carried out at the same rate, or substantially the same rate, as the wild type.

[0025] The mouse with the Dao^{+} and Dao^{-} genotypes in the present invention, may have any allele of other genes. Some of the genes may manifest a specific phenotype in a homozygote, and others may manifest a specific phenotype in a heterozygote. Thus, in the present invention, an evaluation of the effect of the test condition on the D-amino acid content in the mouse tissue or cultured tissue cells derived from the tissue may be performed with a mouse with the $Dao1^{+/+}$ genotype (a $Dao1^{+/+}$ mouse) and a mouse with the $Dao1^{-/-}$ genotype (a $Dao1^{-/-}$ mouse) under a condition in which the combination of the alleles is the same with regard to at least one of the other genes. The effect of the test condition may be the effect of the test condition on the difference of Dao1 action to the phenotype of the first combination of alleles of at least one of the other genes, as compared with Dao1 action to the phenotype of the second combination of alleles of the at least one of the other genes.

[0026] In addition to a disease model phenotype, the other genes may be a gene involved in the synthesis of the D-amino acid or the metabolic pathway of degradation, any gene that relates to the health of all organs or the whole body of the mouse such as aging, immunity, stress reactions, nutrition, movement, sensing, memory, behavior, blood circulation, digestion, excretion, reproduction, etc. The other gene may be a causative gene of a disease model mouse that includes, but not limited to, obese mouse (Lep^{ob} / Lep^{ob}), thymus dependent immunodeficient mouse ($Foxn1^{nu}$ / $Foxn1^{nu}$), senescence accelerated mouse (SAMP1/TaSlc, SAMP6/TaSlc, SAMP8/TaSlc and/or SAMP10/TaSlc), osteoarthritic mouse ($Laq1^{MRL}$/$Laq1^{MRL}$), and hairless mouse (Hr^{hr}/ Hr^{hr}). According to the homepage entitled Online Inheritance of Man of John Hopkins University, the products of the Hr gene are considered to be a transcription factor that is involved in hair formation (http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=602302). According to the homepage of the Mouse Genome Informatics project, in a mouse, in addition to spontaneous mutants resulting from the insertion of MLV proviruses, such as Hr^{hr}, a number of alleles, such as mutants that are accidentally created as a result of the insertion of a transgene, or the like, and knockout mouse, have been reported (http://www.informatics.j ax.org/imsr/fetch?page=imsrSummary&op:gsymname=%3D&gs ymname=Hr&gsymnameBreadth=C). The Hr^{-} genotype of the present invention includes Hr^{hr}, although it is not limited to this, and represents a genotype in which an adult of an Hr^{hr} homozygote does not have hair as a result of loss of function of any Hr gene product.

[0027] In the present invention, when the evaluation of the effect of the test condition on the D-amino acid content is performed under a condition in which the combination of the alleles of at least one of the other genes is same, the step (1) of the method of the present invention, may comprise determining the genotype of at least one of the other genes, in addition to the method for determining the Dao $1^{+/+}$, $^{+/-}$ and/or $^{-/-}$ genotypes.

[0028] When the step (1) of the method of the present invention is carried out by selecting by way of the method for determining the $Dao1^{+/+}$, $^{+/-}$ and/or$^{-/-}$ genotypes, the individual identification of the mouse is performed by labeling the breeding cage of the mouse and/or the body of the mouse. Labeling the mouse may involve using ear punching, embedding of a high-frequency or other labeling-purpose chip into the body, or any other method that is well-known to those skilled in the art.

[0029] The determination of the Dao1$^{+/+}$, $^{+/-}$ and/or $^{-/-}$ genotypes in the step (1) of the method of the present invention may be performed by any procedure. The determination of the Dao1 genotype is carried out by collecting a small quantity of tissue or other biological material from each individual mouse, and analyzing the Dao1 gene or gene products thereof contained therein. The preferable method for determining the Dao1 genotype is a method that is able to discriminate the Dao1 gene sequence that encodes the amino acid residue of position number 181 of the Dao1 gene product. The chromosomal DNA collection from the individual mouse and the analysis of the Dao1 genotype may be carried out by using any method that is well-known to those skilled in the art. In the analysis of the Dao1 genotype, an amplification method, such as the PCR method, the SMAP method, the LAMP method, or the like, may be used. The different genotypes of the Dao1 gene may be discriminated on the basis of the successful amplification or not with different oligonucleotide primers, in addition to the presence or absence of cleavage site of a specific restriction enzyme on the chromosomal DNA or amplified DNA. The presence or absence of cleavage site of the specific restriction enzyme and/or the presence or absence of amplification may be performed by obtaining an electrophoretic band pattern by the Southern blotting with a probe which can detect the presence or absence of cleavage site of the specific restriction enzyme, following the separation of the amplified DNA, separation of the chromosomal DNA after restriction enzyme digestion, or the like. The preferable method for discriminating the different genotypes of the Dao1 gene is the gene amplification method explained below.

[0030] The differentiation of the different genotypes of the Dao1 gene by the gene amplification method in the method for evaluating of the present invention utilizes the fact that the restriction enzyme HpaaII recognizes the mutation site of the Dao1 gene. FIG. 1 is an alignment diagram of the nucleotide sequence related to the method for determining the Dao1 genotype of the present invention. FIG. 1 shows the alignment results of the Dao1 gene chromosomal DNA sequence (Dao1_genomic), a wild type sequence between base number 15111 and base number 15460 from the transcription initiation point of the Dao1 gene (sequence number 4 in the sequence listing), a wild type sequence of cDNA of the 7th exon section that includes a point mutation (the guanine at number 15223 from the transcription initiation point is substituted by adenine) in the Dao1^{G181R} mutant (base number 625 to number 726 of sequence number 3 of the sequence listing), gene amplification-purpose primers (a Forward primer (sequence number 1) and a Reverse primer (sequence number 2)) used in an embodiment of the present invention, and the recognition sequence of the restriction enzyme HpaII. The inverse complementary sequence of the reverse primer sequence (Reverse primers*) is recited as sequence identification number 5 in the sequence listing. FIG. 2 is a HpaII restriction enzyme map of the DNA region used in the method for determining the Dao1 genotype of the present invention. In the 7th exon of the wild type chromosomal DNA, a 3-position HpaII cleavage site (downward facing arrows) is present. Among these, the HpaII recognition sequence (CCGG) nearest the 5' end side becomes CCAG in the Dao1^{G181R}mutant mouse chromosomal DNA, and it is not recognized by HpaII (*). The 7th exon region is amplified by a forward primer (Forward) located in the 6th intron, and a reverse primer (Reverse) positioned in the 7th intron. When the amplification products derived from the wild type mouse genomic DNA (Wild type), and the amplification products derived from the mutant mouse genomic DNA (Dao1^{G181R}) are digested by HpaII, they are cleaved in 3 places and 2 places, respectively.

[0031] In regard to the measurement of the D-amino acid content in the method for evaluating of the present invention, it is acceptable if it is carried out using any method that is well-known to those skilled in the art. For example, a method in which the D- and L-amino acids are stereospecifically derivatized beforehand by o-phthalaldehyde (OPA), N-tert-butyloxycarbonyl-L-cystine (Boc-L-Cys), or another modifying reagent, and thereafter, performs the separation by using an analytical column such as ODS-80TsQA with a gradient solution of a 100 mM acetate buffer solution (pH 6.0) and an acetonitrile mixed solution, can be used for the simultaneous measurement of the D-type and L-type of aspartate, serine, and alanine. Furthermore, a method in which the D- and L-amino acid is derivatized beforehand by a fluorescence reagent such as 4-fluoro-7-nitro-2,1,3-benzoxydiazole (NBD-F), and thereafter, following stereospecific separation of the amino acids using an analytical column such as ODS-80TsQA, Mightysil RP-18GP, or the like, stereospecifically performs the separation by optical resolution using a Pirkle type chiral stationary phase column (for example, Sumichiral OA-2500 S or R), can be used in the microdetermination of proline, leucine, and other amino acids (Kenji Hamase and Kiyoshi Zaitsu, Bunseki Kagaku, Volume 53, 677-690 (2004)). The optical resolution column system in the present specification refers to, at the very least, a separation analysis system using an optical resolution column, and it may include separation analysis by an analytical column other than an optical resolution column. Alternatively, it is possible to determine the quantity of a D-amino acid by an immunological method using a monoclonal antibody that discriminates between the optical isomers of amino acids, for example, a monoclonal antibody that specifically binds to D-leucine, D-aspartate, or the like (Japanese Patent Application No. 2008-27650 Specification).

[0032] The test condition in the present invention refer to physical, chemical, and/or biological processing conditions applied systemically or locally to the test animal. The physical processing includes; light rays including ultraviolet rays and infrared rays, or electromagnetic waves, acceleration including sound, vibrations, and zero gravity, temperature, bathing in warm water or cold water, drying, or humidifying, although it is not limited to these. The chemical processing includes protons, and the application of inorganic substances and/or organic substances, although it is not limited to these. The biological processing includes consumption of diet, water, or the like, light-dark cycle, cage area, cage type,

the number of individual animals bred in the same cage, other breeding conditions, and administration of medicines, although it is not limited to these. In regard to the test condition in the present invention, these may be one of the aforementioned processes, or a combination thereof, and each process may be carried out continuously and/or intermittently.

[0033] In the determination of the D-amino acid content in the method for evaluating of the present invention, "measuring the D-amino acid content within the tissue or cultured tissue cells derived from the tissue of the mouse with the $Dao1^{+/+}$ genotype and the mouse with the $Dao1^{-/-}$ genotype following step (2) and following exposure to the test condition" includes a case where the D-amino acid content is determined following the end of the exposure to the test condition, and a case where the D-amino acid content is determined during the exposure to the test condition.

[0034] According to the method for evaluating of the present invention, the effect of the test condition with respect to the various characteristics derived from the tissue of the mouse with the $Dao1^{+/+}$ genotype and the mouse with the $Dao1^{-/-}$ genotype, or cultured tissue cells derived from the tissue, can be evaluated. Such characteristics can be evaluated for the effect of the test condition with respect to: changes in the D-amino acid content; fluctuations in the content of other substances related to the metabolic pathway of the D-amino acid, for example, L-amino acids, α (alpha)-keto acids, or the like; changes in the characteristics related to immunity, including biological and/or pathological characteristics of the digestive system, the liver, the kidneys, the cardiovascular system, or the like, related to the metabolism, digestion and absorption, and degradation and excretion of these substances, as well as allergies with respect to pollen, house dust, and the like, eczema, transplantation immunity of skin and other organs, although it is not limited to these; changes in the characteristics related to the infection by microorganisms or the coexistence with microorganisms; changes in behavior, memory, sensing, and other neurobiological characteristics; changes in the characteristics related to the enhancement and/or suppression of cancers and/or cell proliferation; characteristics related to the aging of skin and other organs, for example, wrinkling, loss of hair, or the like; and characteristics related to the health and/or beauty of skin, such as moisture retention, barrier characteristics, or the like.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035]

FIG. 1 is an alignment diagram of the nucleotide sequence related to the method for determining the Dao1 genotype of the present invention.

FIG. 2 is a HpaII restriction enzyme map of the DNA region used in the method for determining the Dao1 genotype of the present invention.

FIG. 3-1 is electrophoretic patterns of the HpaII degradation fragments of the amplification products derived from the chromosomal DNA of a $Dao1^{+/+}$ homozygote mouse (first lane), a $Dao1^{G181R/R181R}$ homozygote mouse (fourth lane), and $Dao^{+/G181R}$ F1 generation heterozygote mice (second and third lane) confirmed from the Dao1 enzyme activity.

FIG. 3-2 is electrophoretic patterns of the HpaII degradation fragments of the amplification products derived from the chromosomal DNA of 12 F2 generation individual mice that are an F2 generation of a cross between a hairless mouse (Hr^{hr} / Hr^{Hr}, $Dao1^{+/+}$) and a Dao1 enzyme deficient mouse ($Hr^{+/+}$, $Dao1^{G181R/G181R}$) that possess a hairless phenotype.

FIG. 4-1 are the optical isomers of proline and 4-hydroxyproline.

FIG. 4-2 is a column flow path diagram of a system that simultaneously analyzes the optical isomers of proline and 4-hydroxyproline.

FIG. 5 are waveform diagrams (D1 and D2) of the elution pattern of the first and the second columns of the system that simultaneously analyzes the optical isomers of proline and 4-hydroxyproline.

FIG. 6-1 is an elution pattern of an optical resolution column of L-type and D-type proline in the blood serum of a Dao1 gene wild type homozygote mouse ($Dao1^{+/+}$).

FIG. 6-2 is an elution pattern of an optical resolution column of L-type and D-type proline in the blood serum of a Dao1 enzyme activity deficient type homozygote mouse ($Dao1^{G181R/G181R}$).

FIG. 7-1 is a bar graph showing the D-proline abundance in various organs of individuals in which the genotype was determined as $Dao^{+/+}$ and $Dao1^{G181R/G181R}$.

FIG. 7-2 is a bar graph comparing the D-proline abundance in the dermis.

FIG. 8 is a bar graph examining the growth of tumor cells in Dao1 enzyme deficient mice.

BEST MODE FOR CARRYING OUT THE INVENTION

[0036] Hereunder is a detailed description of the present invention. The technical scope of the present invention is limited by the description of the claims, and the embodiments of the present invention are only exemplary.

[Embodiment 1]

Development of a Method for Determining the $Dao1^{+/+}$, $^{+/-}$, and/or $^{-/-}$ Genotypes

[0037] $Dao1^{G181R}$ is a mutant in which, of the cDNA nucleotide sequence of the Dao1 gene recited as sequence identification number 3, the guanine of number 661 is substituted by adenine. As a result, in contrast to the wild type, in which the cleavage sequence of the restriction enzyme HpaII becomes (C↓CGG), it becomes CCAG in the $Dao1^{G181R}$ mutant and is not cleaved. Here, since the nucleotide numbers 625-726, which includes the site of mutation, includes the 7th exon (GenBank entry number NM_010018.2), it is possible to discriminate between a wild type and a mutant even with chromosomal DNA, according to the presence or absence of the HpaII cleavage site.

[0038] Following individual identification of postweaned mice, chromosomal DNA was extracted from the tail of each individual mouse and purified using a commercial mammalian genome DNA mini-prep kit (Sigma, G1N70-1 KT). An oligonucleotide consisting of the nucleotide sequence of sequence number 1 located in the 6th intron was used as the forward primer, and an oligonucleotide consisting of the nucleotide sequence of sequence number 2 located in the 7th intron was used as the reverse primer. The mouse chromosomal DNA was amplified by using a commercial reaction mixture (Promega, M7122) and thermal cycling settings of (1) 94°C, 4 minutes, 1 cycle, (2) 94°C, 30 seconds, 55°C, 30 seconds, 72°C , 30 seconds, 40 cycles, (3) 72°C, 10 minutes, 1 cycle, and (4) 4°C, storage. The PCR reaction products were purified using a commercial kit (Qiagen, 28104), and restriction enzyme processing was performed under the presence of HpaII (TOYOBO, HPA201) and incubated at 37°C for 3 hours. Following deactivation of the restriction enzyme by heating at 70°C for 5 minutes, the length of the DNA fragments was analyzed by a commercial electrophoresis microchip (Agilent, 2100 bioanalyzer).

[0039] FIG. 3-1 is an electrophoretic pattern of the HpaII degradation fragments of the amplification products derived from the chromosomal DNA of a $Dao1^{+/+}$ homozygote mouse (first lane), a $Dao1^{G181R/G181R}$ homozygote mouse (fourth lane), and $Dao^{+/G181R}$ F1 generation heterozygote mice (second and third lane) confirmed from the Dao1 enzyme activity. As can be understood from FIG. 3-1, since a DNA fragment of 95 bp can be obtained from the chromosomal DNA of the $Dao1^{+}$ gene, and a DNA fragment of 107 bp can be obtained from the chromosomal DNA of the $Dao1^{G181R}$ gene, a wild type homozygote, and a mutant homozygote and heterozygote were able to be clearly discriminated. FIG. 3-2 is the result of performing the Dao1 gene determination on 12 F2 generation individual mice that are an F2 generation of a cross between a hairless mouse (Hr^{hr} / Hr^{Hr}, $Dao1^{+/+}$) and a Dao1 enzyme deficient mouse ($Hr^{+/+}$, $Dao1^{G181R/G181R}$), which possess a hairless phenotype. Table 1 is a table in which the Dao1 genotype and sex of 138 F2 generation individual mice, which possess a hairless phenotype, have been compiled.

[0040]

[Table 1]

F2 Generation Hairless Mouse ($Hr^{-/-}$)					
$Dao1^{+/+}$		Dao 1 $^{+/-}$		$Dao1^{-/-}$	
39		55		44	
F	M	F	M	F	M
18	21	31	24	22	22
F is female, and M is male.					

[0041] As discussed above, as a result of the present determination method, it became possible to rapidly determine the Dao1 genotype of a large number of individual mice.

[Embodiment 2]

Development of a Method for Quantitative Analysis of the Optical Isomers of Proline and 4-Hydroxyproline

[0042] Proline and 4-hydroxyproline are high in content in skin collagen. Therefore, a method that can simultaneously separate and quantitatively analyze all of the optical isomers of both proline and 4-hydroxyproline was developed.

[0043] FIG. 4-1 shows the optical isomers of proline and 4-hydroxyproline. Although there are just 2 types of optical isomers, the L-type or D-type, for proline, in regard to the optical isomers of 4-hydroxyproline, there are 4 types as a result of the difference in the trans-type and the cis-type in addition to the difference in the L-type or the D-type. Firstly, fluorescence labeling was performed by derivatizing the amino acid with a fluorescence reagent NBD-F. Thereafter, as

shown in FIG. 4-2, reverse phase separation chromatography was performed in a first column, and the respective peaks for trans-4-hydroxyproline, cis-4-hydroxyproline, and proline were detected. Then, the fractions of the respective peaks were divided using a column switching valve, introduced into a second column, and optical resolution chromatography was performed.

[0044] The waveform diagram 1D of FIG. 5 is one that was detected at a fluorescence emission of 530 nm by irradiating the elution pattern of a monolithic ODS column using a solvent with a flow rate of 40 μ (micro) L/min with an excitation light of 470 nm. The waveform diagram 2D is the result of automatically detecting the respective peaks of trans-4-hydroxyproline, cis-4-hydroxyproline, and proline of the elution pattern of 1D, and performing optical resolution chromatography by performing valve switching and introducing only the fractions of the respective peaks to a QN-2-AX column.

[0045] The waveform diagram of FIG. 6-1 is an elution pattern of an optical resolution column of L-type and D-type proline in the blood serum of a Dao1 gene wild type homozygote mouse ($Dao1^{+/+}$), and the waveform diagram of FIG. 6-2 is an elution pattern of an optical resolution column of L-type and D-type proline in the blood serum of a Dao1 enzyme activity deficient type homozygote mouse ($Dao1^{G181R/G181R}$). Although D-proline could be barely detected in a Dao1 wild type homozygote mouse, it could be clearly detected in a Dao1 enzyme activity deficient type homozygote mouse.

[0046] FIG. 7-1 is the result of comparing the D-proline abundance in various organs of individuals for which the genotypes were determined as $Dao1^{+/+}$ and $Dao1^{G181R/G181R}$ among F2 generation hairless mice of a cross between a hairless mouse and a Dao1 enzyme activity deficient type mouse as determined in Embodiment 1, and FIG. 7-2 is the result of comparing the D-proline abundance in the dermis. The units of the vertical axis of the graph of FIG. 7-1 are μmol/μ (micro) L with respect to the blood serum, and pmol/mg in other tissue. The units of the vertical axis of the graph of FIG. 7-2 are nmol/μ (micro) g DNA. In both, the average and standard error of the measured quantity in the mouse organs for the 5 respective mice are displayed. In addition to the blood serum, several times or more D-proline was present in the Dao1 enzyme activity deficient mouse than the wild type mouse in the pituitary gland, adrenal gland, pancreas, and dermis. Furthermore, although D-proline was barely detected in the cerebellum, kidneys, and liver of a Dao1 enzyme activity wild type mouse, D-proline was clearly detected in a Dao1 enzyme activity deficient mouse. The D-proline quantity in the testis of a Dao1 enzyme activity deficient mouse is just slightly more than in a wild type mouse, and a marked difference was not observed. Also, in regard to the skin, it has become clear for the first time that the D-amino acid content in a Dao1 enzyme activity deficient mouse is greater than in a wild type. Hereafter, the effect of ultraviolet light irradiation and aging on the D-amino acid content in the skin will be made clear. In this analysis, with regard to the content of D-4-hydroxyproline, both the cis-isomer and the trans-isomer were below the detection limit in all tissues.

[Embodiment 3]

Tumor Growth in Dao1 Enzyme Deficient Mouse

[0047] Sarcoma cells of the Swiss Webster Sarcoma 180 line were cultured using a 10% fetal bovine serum (Irvine Scientific, Lot #300A80601) added DMEM (Sigma) medium under humidified conditions with 5% CO_2 at 37°C. A suspension of 1 x 10^7 units/mL was prepared, and 0.05 mL each of the footpad of the right hind leg of a Dao1 enzyme deficient mouse or a wild type mouse were transplanted by endermic injection. The major axis, minor axis, and thickness of the tumor was measured with a caliper every week following the transplant, and the tumor volume was calculated by the formula below.

$$\text{Tumor volume } (mm^3) = \text{major axis } (mm) \times \text{minor axis } (mm) \times (\text{thickness } (mm) - 3)$$

Here, in regard to the thickness, the original thickness of the foot was made 3 mm, and the difference thereof was made the thickness of the tumor.

[0048] The result is shown in FIG. 8. FIG. 8 is a bar graph comparing the change in the tumor volume for a Dao1 enzyme deficient mouse and a control mouse after 1, 2, and 3 weeks elapsed following the transplant. In contrast to the control mouse, in which a tumor volume that was already 60 mm^3 1 week after the transplant increased 2-fold in 3 weeks, in the Dao 1 enzyme deficient mouse, the tumor volume, which was only approximately 20 mm^3 1 week after the transplant, decreased to approximately 5 mm^3 at 2 weeks, and was completely eliminated at 3 weeks. From this result, it became clear that in a Dao1 enzyme deficient mouse, there is activity that suppresses the growth of the transplanted tumor and eliminates the tumor.

[0049] The crossing experiment system established this time between a hairless mouse and a Dao1 enzyme deficient mouse is anticipated to be greatly utilized hereafter for elucidating the role performed by D-amino acids with respect to collagen diseases or collagen disease-related diseases, such as erythematodes, skin bound disease, dermatomyositis,

Sjoegren syndrome, polyarteritis nodosum, Bechet's disease, rheumatoid arthritis, or the like, diabetes, photodermatitis, contact dermatitis, decubitus ulcers, and dermal observations such as stains, subfusc, wrinkling, sagging, or the like.

SEQUENCE LISTING

<110> Kyushu University, National University Corporation
Shiseido Company Ltd

<120> Method for Evaluating and Screening D-amino acid Related Diseases Using Dao1-/- Mouse

<130> 37427

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Claims

1. A method for evaluating an effect of a test condition on a mouse tissue, or cultured cells derived from the tissue, said method comprising the steps of:

   (1) providing a Dao1$^{+/+}$ mouse and a Dao1$^{-/-}$ mouse;
   (2) exposing a tissue of the Dao1$^{+/+}$ and Dao1$^{-/-}$ mice, or cultured tissue cells derived from the tissue to the test condition; and
   (3) analyzing the effect of exposing the tissue or cultured tissue cells derived from the tissue of the Dao1$^{+/+}$ and Dao1$^{-/-}$ mice to the test condition.

2. The method according to claim 1,
   wherein the step (1) is comprised of discriminating the Dao1$^{+/+}$ mouse and the Dao1$^{-/-}$ mouse according to a method for determining a genotype of Dao1$^{+/+}$, $^{+/-}$ and/or $^{-/-}$, comprising the steps of:

   identifying a Dao1$^{+/+}$, $^{+/-}$ and/or $^{-/-}$ mouse individually;
   extracting chromosomal DNA from the mouse;
   obtaining an amplified DNA fragment by amplifying the region covering the seventh exon of the extracted chromosomal DNA;
   digesting the amplified DNA fragments with a HpaII restriction enzyme; and
   analyzing the restriction enzyme-digested products of the amplified DNA fragments.

3. The method according to claim 2,
   wherein said step of obtaining the amplified DNA fragments, is comprised of amplifying with oligonucleotide primers consisting of nucleotide sequences recited as SEQ ID NOs: 1 and 2.

4. The method according to any one of claims 1 - 3, wherein the Dao1$^{+/+}$ mouse and the Dao1$^{-/-}$ mouse share a common combination of alleles for at least one other locus.

5. The method according to claim 4, wherein the combination of the alleles for the other locus is comprised of Hr$^{-/-}$.

6. The method according to any one of claims 1 - 5, wherein the step (3) is comprised of:

measuring the D-amino acid content within the tissue or cultured tissue cells derived from the tissue of the $Dao1^{+/+}$ mouse and the $Dao1^{-/-}$ mouse, prior to the step (2);
measuring the D-amino acid content within the tissue or cultured tissue cells derived from the tissue of the $Dao1^{+/+}$ mouse and the $Dao1^{-/-}$ mouse, following the step (2) and following exposure to the test condition; and
comparing between the D-amino acid content measured prior to the step (2) and the D-amino acid content measured following the step (2).

7. The method according to claim 6, wherein the D-amino acid content is measured by column chromatography using an optical resolution column system.

8. The method according to claim 6, wherein the D-amino acid content is measured by an immunological technique using a monoclonal antibody that discriminates optical isomers.

9. The method according to any one of claims 6-8, wherein the D-amino acid is D-proline.

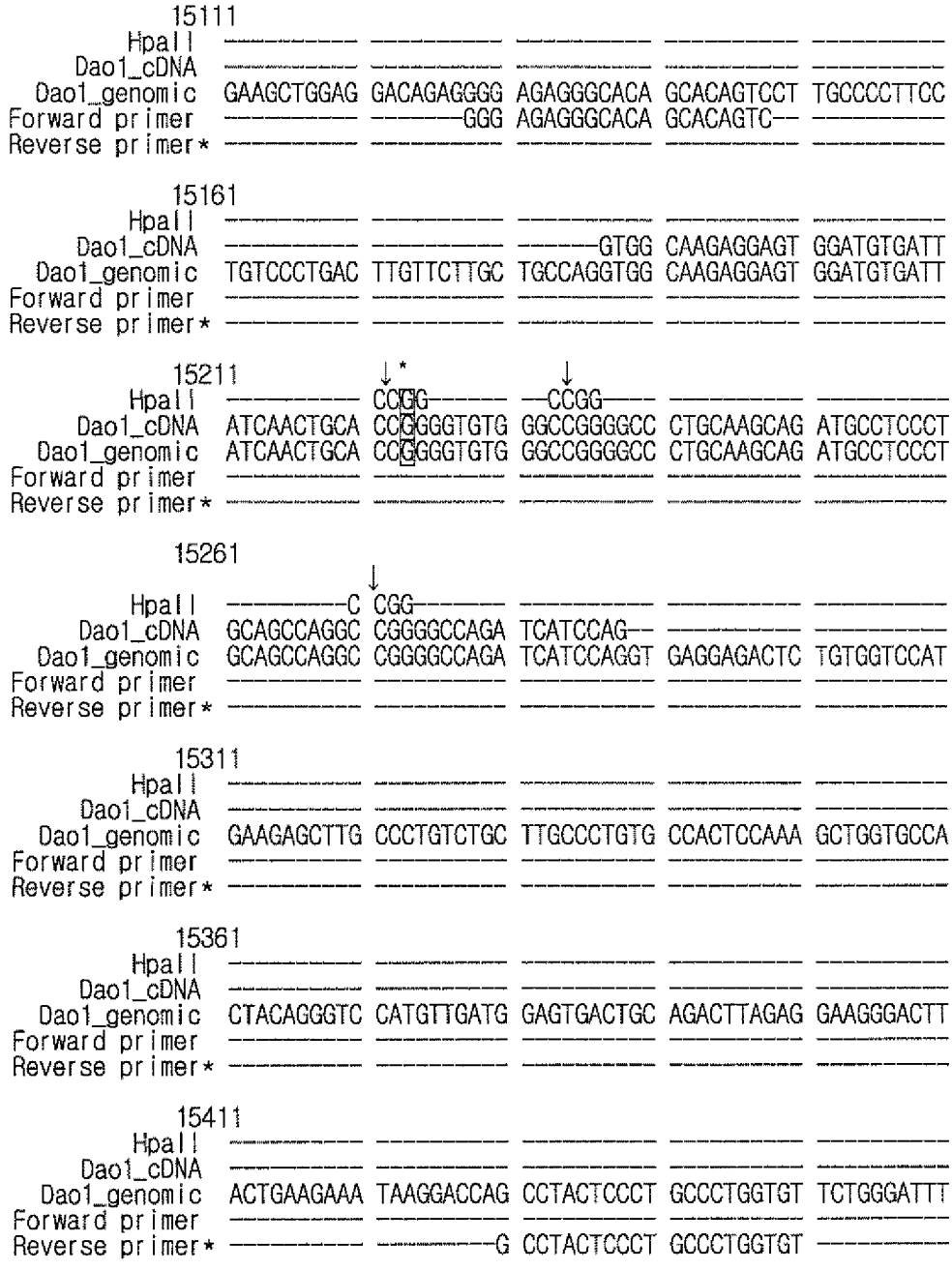
10. The method according to any one of claims 6 - 9, wherein the tissue or cultured tissue cell derived from the tissue, for which the D-amino acid content is measured, is derived from one or more tissue selected from a group consisting of epidermis, dermis, kidney, pancreas, testis, adrenal gland, cerebellum, pituitary gland, and blood serum.

11. The method according to claim 10, wherein the D-amino acid is D-proline, the D-proline is measured by column chromatography using an optical resolution column, and the tissue is epidermis or dermis.

12. An evaluation system for carrying out the method of claim 11, comprising: a $Dao1^{+/+}$ and $Hr^{-/-}$ mouse; a $Dao1^{-/-}$ and $Hr^{-/-}$ mouse; oligonucleotide primers consisting of nucleotide sequences recited as SEQ ID NOs: 1 and 2; and an optical resolution column system that discriminates the optical isomers of proline.

13. A method for screening medicinal and/or cosmetic candidate substances that is **characterized by** evaluating the medicinal and/or cosmetic candidate substances by using of the evaluation system of claim 12.

FIG.1



Dao1_cDNA: 7th exon of mouse Dao1 mRNA
Dao1_genomic: chromosomal DNA near the $Dao1^{G181R}$ mutation
Reverse primer*: corresponding reverse complement
G: A in $Dao1^{G181R}$ mutant
↓*: unable to digest in $Dao1^{G181R}$ mutant

FIG.2

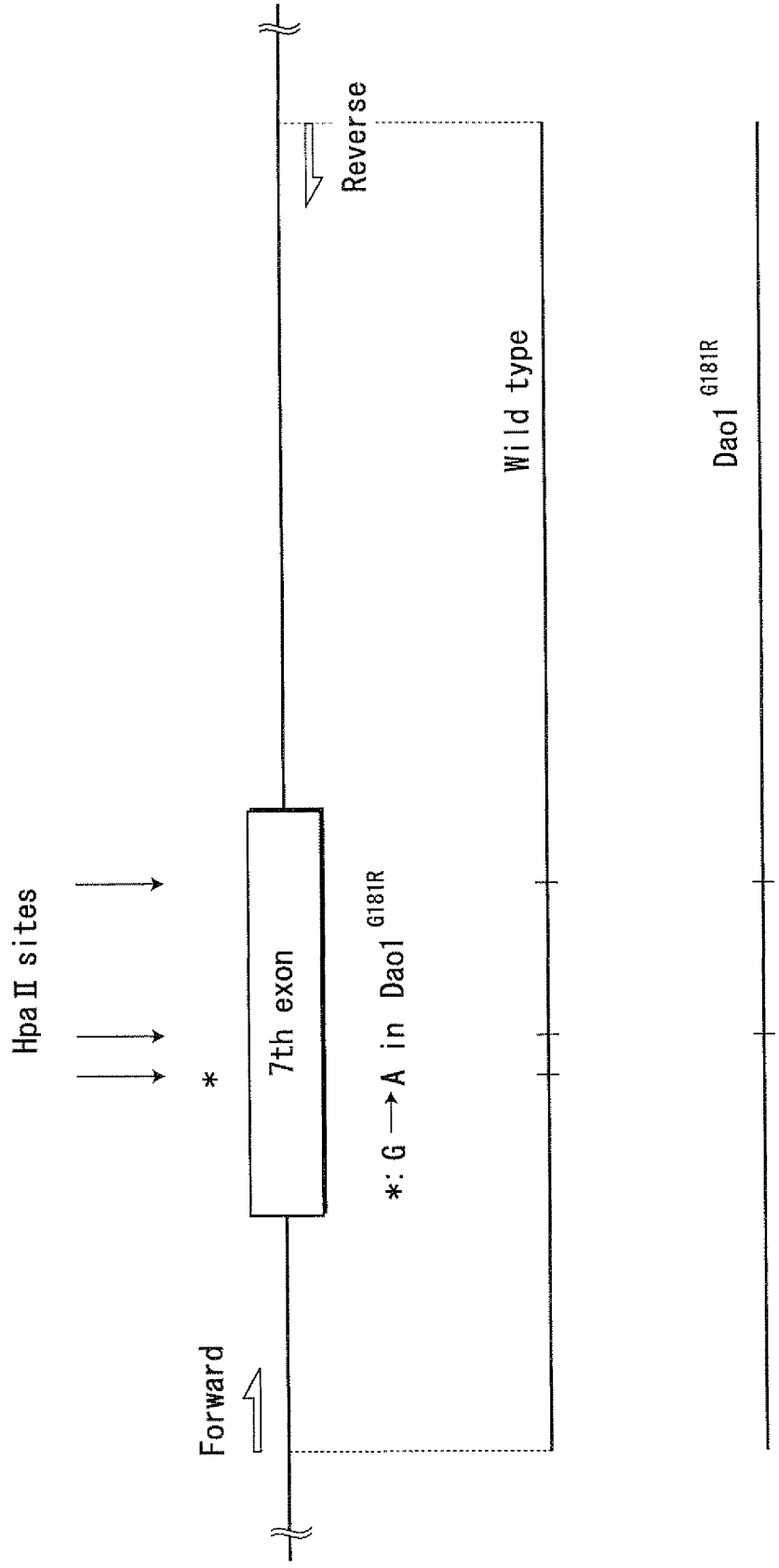

Reverse
Wild type
Dao1 G181R
Hpa II sites
*
7th exon
*: G → A in Dao1 G181R
Forward

FIG.3-1

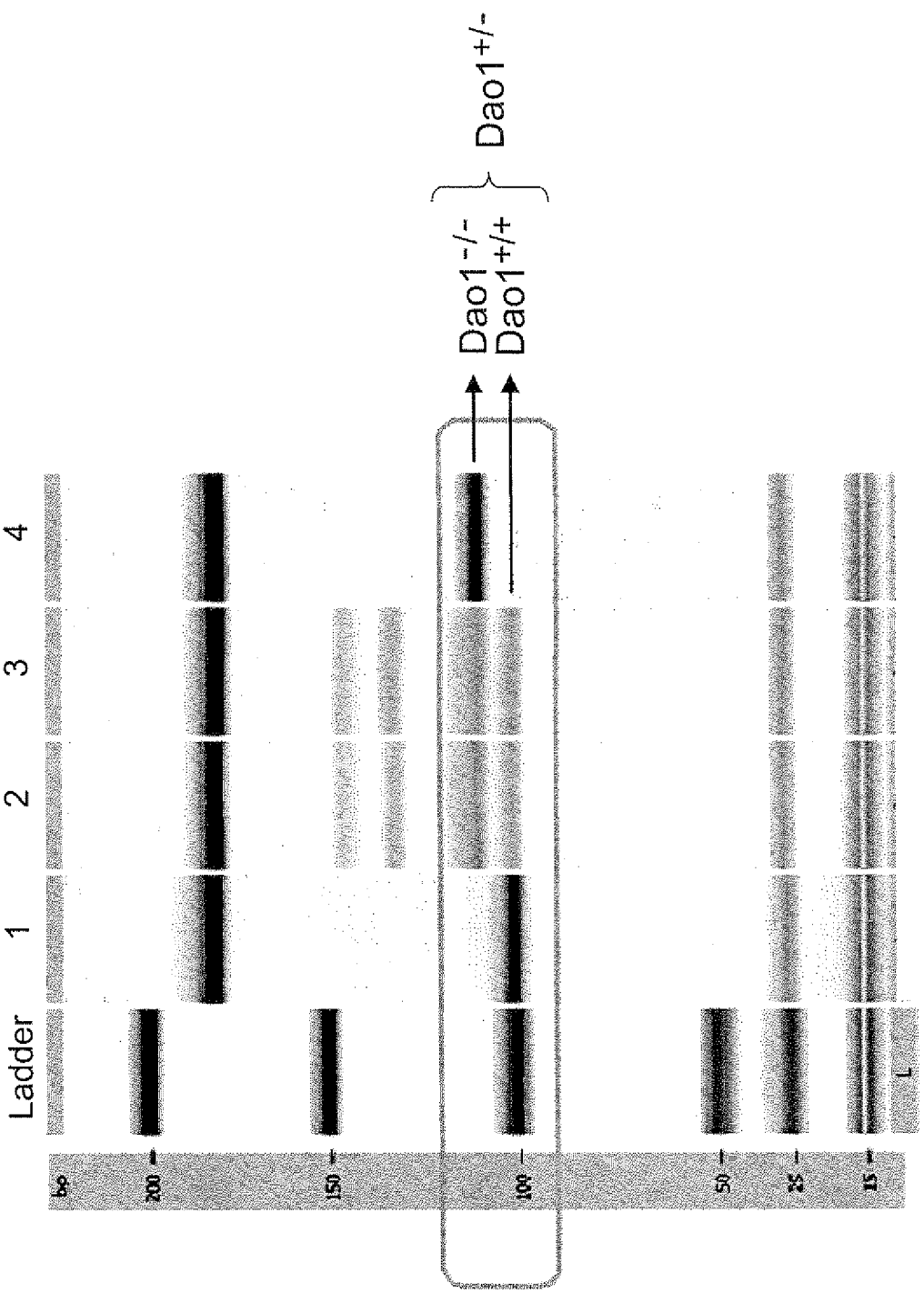
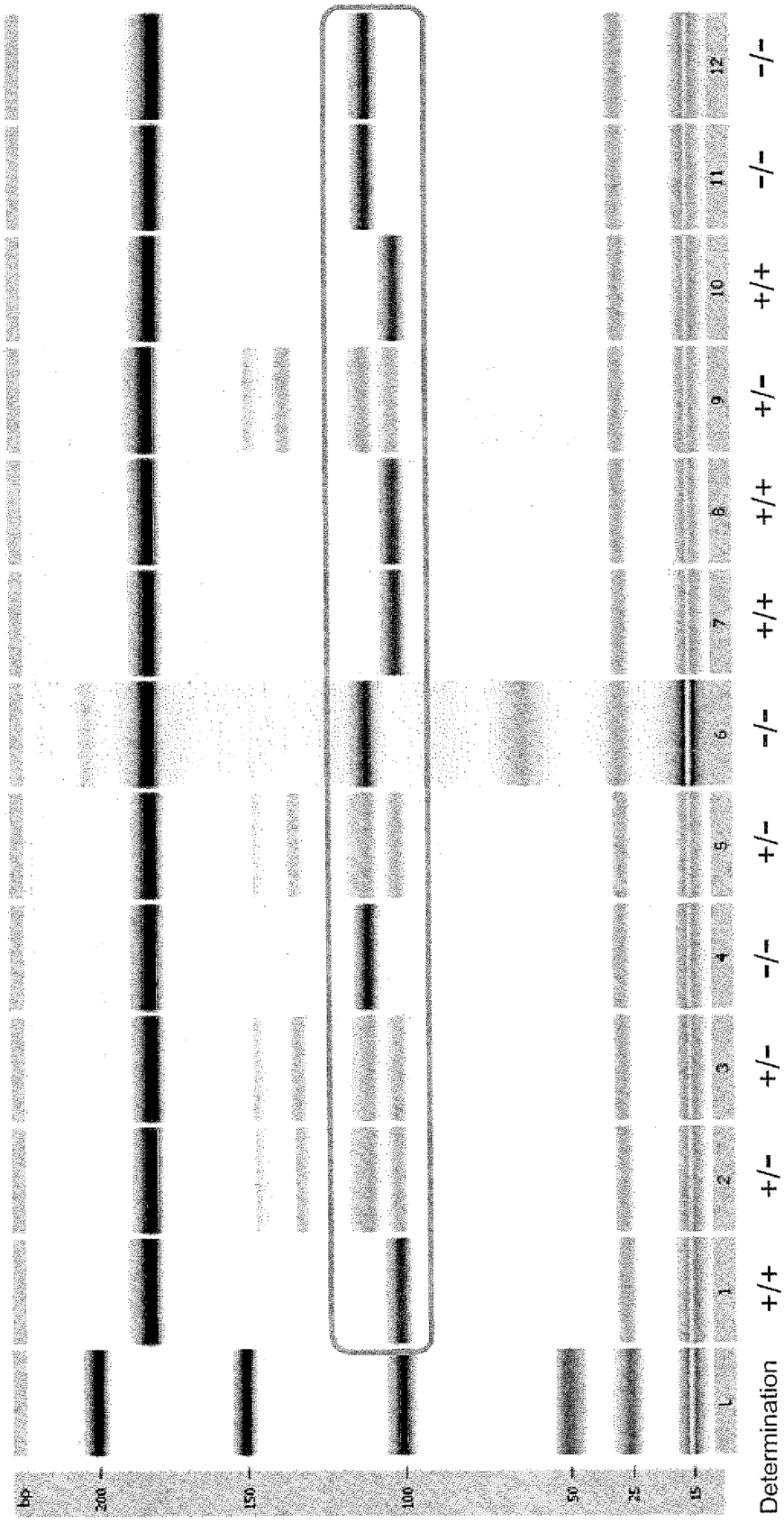


FIG.3-2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4
5
6
7
8
9
10
11
12
Determination
+/+
+/-
+/-
-/-
+/-
-/-
+/+
+/+
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FIG.4-1

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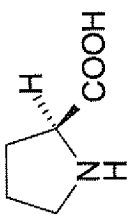
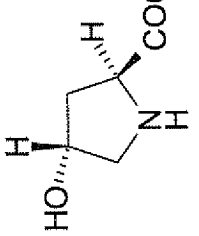
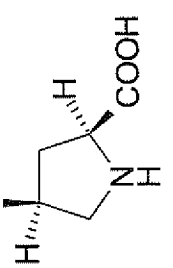
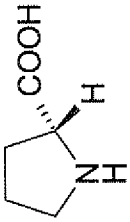
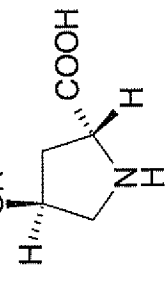
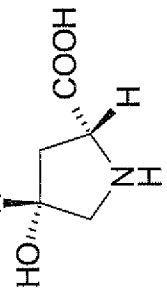
		trans - form	*cis* - form
L-enantiomer	 **L-Pro**	 ***trans*-L-Hyp**	 ***cis*-L-Hyp**
D-enantiomer	 **D-Pro**	 ***trans*-D-Hyp**	 ***cis*-D-Hyp**

FIG.4-2

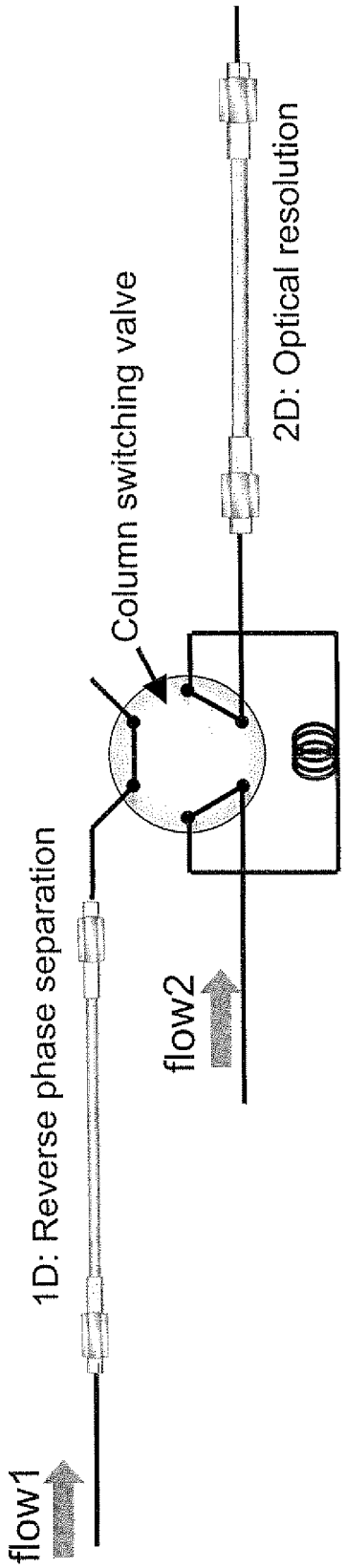

flow1
1D: Reverse phase separation
Column switching valve
flow2
2D: Optical resolution

FIG.5

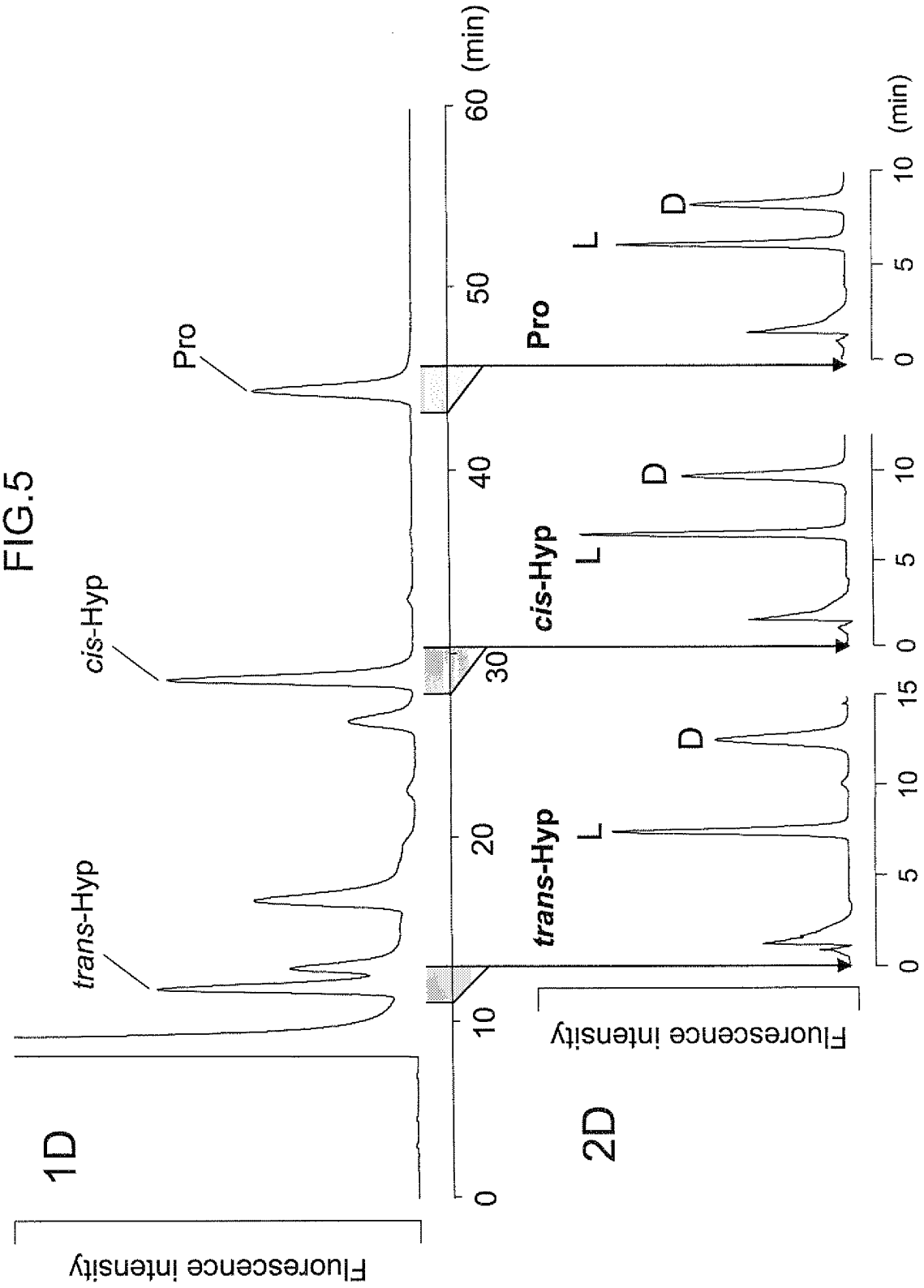

1D
Fluorescence intensity
trans-Hyp
cis-Hyp
Pro
0
10
20
30
40
50
60 (min)
2D
Fluorescence intensity
trans-Hyp
cis-Hyp
Pro
L
D
0
5
10
15
(min)

FIG.6-1

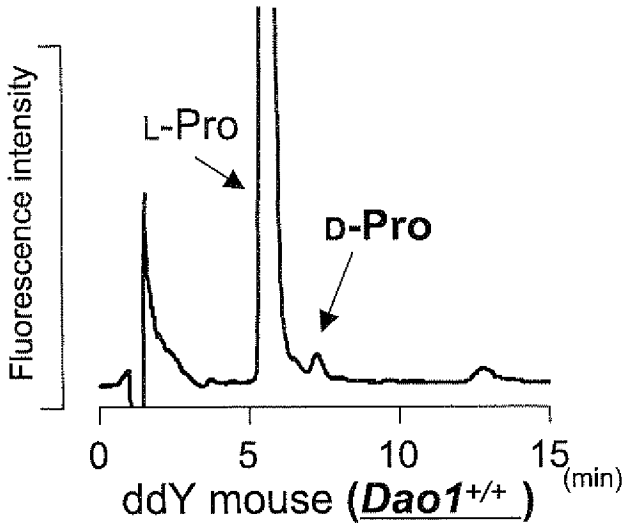

Fluorescence intensity
L-Pro
D-Pro
0
5
10
15
(min)
ddY mouse (Dao1+/+)

FIG.6-2

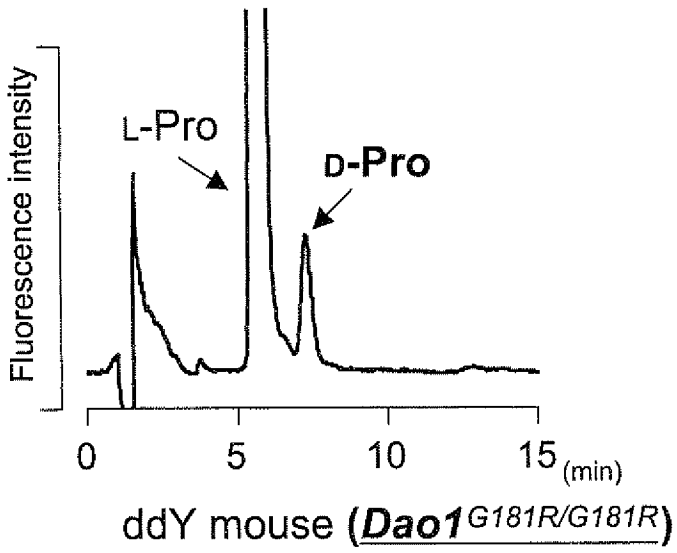

Fluorescence intensity
L-Pro
D-Pro
0
5
10
15
(min)
ddY mouse (Dao1G181R/G181R)

FIG.7-1

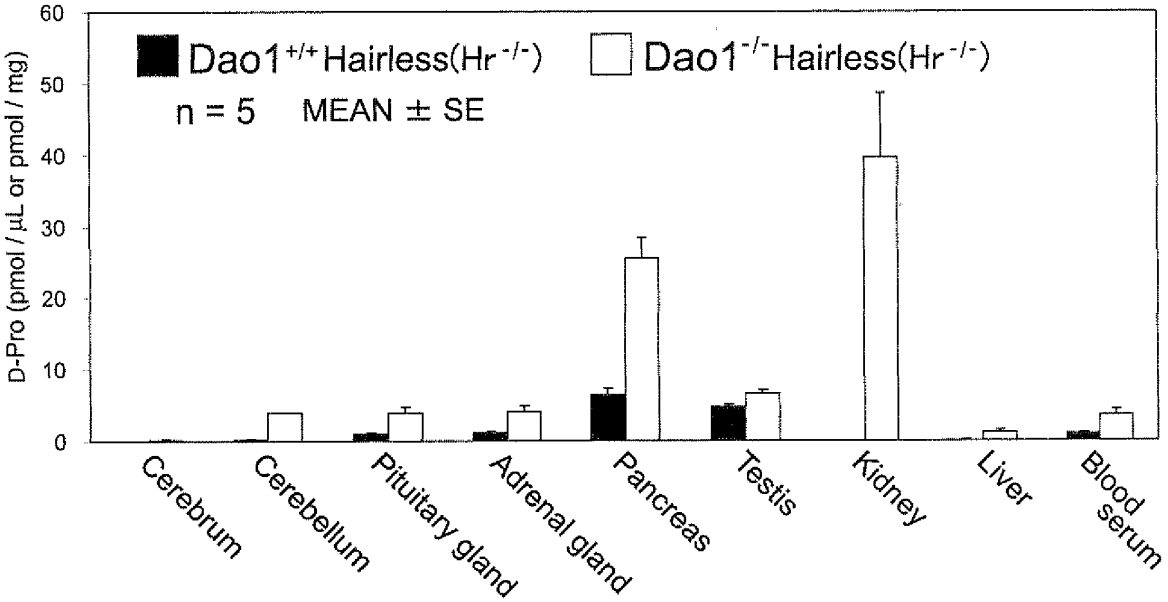

Dao1+/+Hairless(Hr-/-)
Dao1-/-Hairless(Hr-/-)
n = 5 MEAN ± SE
D-Pro (pmol / µL or pmol / mg)
60
50
40
30
20
10
0
Cerebrum
Cerebellum
Pituitary gland
Adrenal gland
Pancreas
Testis
Kidney
Liver
Blood serum

FIG.7-2

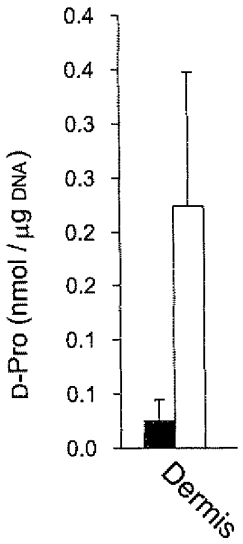
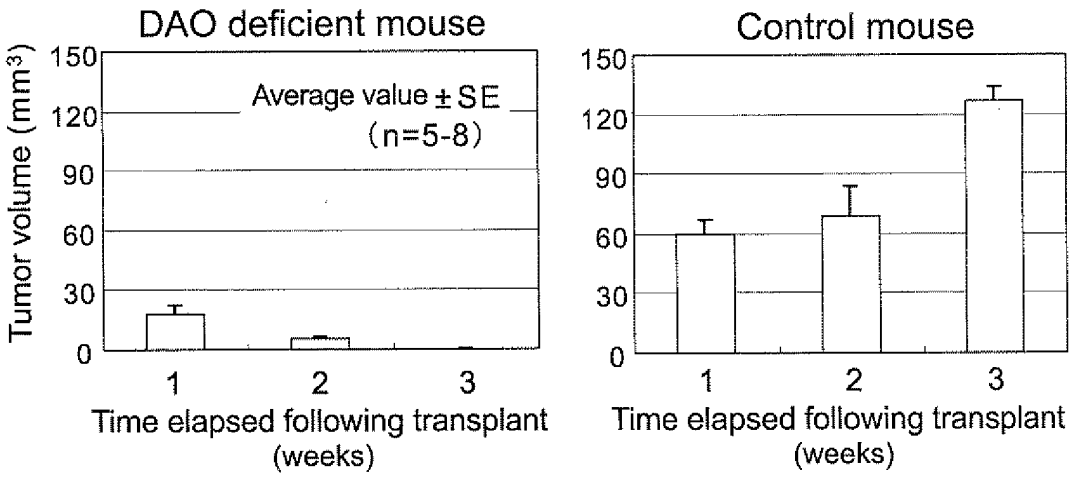

D-Pro (nmol / µg DNA)
0.4
0.4
0.3
0.3
0.2
0.2
0.1
0.1
0.0
Dermis

FIG.8


DAO deficient mouse
Tumor volume (mm3)
150
120
90
60
30
0
Average value ± SE
(n=5-8)
1
2
3
Time elapsed following transplant
(weeks)
Control mouse
150
120
90
60
30
0
1
2
3
Time elapsed following transplant
(weeks)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/063696

A. CLASSIFICATION OF SUBJECT MATTER
C12N15/00(2006.01)i, C12N15/09(2006.01)i, C12Q1/02(2006.01)i, C12Q1/68(2006.01)i, G01N30/88(2006.01)i, G01N33/15(2006.01)i, G01N33/50(2006.01)i, G01N33/53(2006.01)i, G01N33/577(2006.01)i
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N15/00, C12N15/09, C12Q1/02, C12Q1/68, G01N30/88, G01N33/15, G01N33/50, G01N33/53, G01N33/577

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2009
Kokai Jitsuyo Shinan Koho 1971-2009 Toroku Jitsuyo Shinan Koho 1994-2009

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA/BIOSIS/MEDLINE/WPIDS(STN), JSTPlus/JMEDPlus/JST7580(JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/Y/A	HASHIMOTO A.et al., Mice lacking D-amino acid oxidase activity exhibit marked reduction of methamphetamine-induced stereotypy, Eur. J. Pharmacol., 2008 May, vol.586, p.221-225	1,4,6,7, 9-11/2,3,8/ 5,12,13
X/Y/A	HAMASE K., Sensitive two-dimensional determination of small amounts of D-amino acids in mammals and the study on their functions, Chem Pharm Bull (Tokyo). 2007, vol.55, p.503-510	1,4,6,7, 9-11/2,3,8/ 5,12,13
Y	SASAKI M. et al., A single-base-pair substitution abolishes D-amino-acid oxidase activity in the mouse, Biochim. Biophys. Acta., 1992, vol.1139, p.315-318	2,3

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 September, 2009 (09.09.09)	29 September, 2009 (29.09.09)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/063696

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OHGUSU T. et al., High-throughput determination of free D-aspartic acid in mammals by enzyme immunoassay using specific monoclonal antibody, Anal Biochem. 2006, vol.357, p.15-20	8
P,X	TOJO Y. et al., Simple and rapid genotyping of D-amino acid oxidase gene recognizing a crucial variant in the ddY strain using microchip electrophoresis, J. Sep. Sci., 2009 Jan., vol.32, p.430-436	1-4,6,7,9-11

Form PCT/ISA/210 (continuation of second sheet) (April 2007)

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

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Non-patent literature cited in the description

- **Corrigan J.J.** *Science,* 1969, vol. 164, 142-149 **[0003]**
- **Hamase K ; Morikawa A ; Zaitsu K.** *J Chromatogr B,* 2002, vol. 781, 73-91 **[0004]**
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- **Kenji Hamase ; Kiyoshi Zaitsu.** *Bunseki Kagaku,* 2004, vol. 53, 677-690 **[0031]**

专利名称(译)	利用DAO1 - / - 小鼠评估/筛选与d-氨基酸相关的疾病的方法		
公开(公告)号	EP2338988A1	公开(公告)日	2011-06-29
申请号	EP2009809745	申请日	2009-07-31
[标]申请(专利权)人(译)	国立大学法人九州大学 株式会社资生堂		
申请(专利权)人(译)	KYUSHU大学，国立大学公司 SHISEIDO COMPANY，LTD.		
当前申请(专利权)人(译)	KYUSHU大学，国立大学公司 SHISEIDO COMPANY，LTD.		
[标]发明人	HAMASE KENJI ZAITSU KIYOSHI MITA MASASHI ASHIDA YUTAKA TOUJO YOUSUKE		
发明人	HAMASE, KENJI ZAITSU, KIYOSHI MITA, MASASHI ASHIDA, YUTAKA TOUJO, YOUSUKE		
IPC分类号	C12N15/00 C12N15/09 C12Q1/02 C12Q1/68 G01N30/88 G01N33/15 G01N33/50 G01N33/53 G01N33/577		
CPC分类号	G01N33/5088 A01K67/0276 A01K2217/03 A01K2217/075 A01K2217/15 A01K2227/105 A01K2267/0331 A01K2267/0356 C07K14/4702 C12N9/0024 C12N15/8509 C12Q1/26 G01N2333/90644 G01N2500/10		
优先权	2008217239 2008-08-26 JP		
其他公开文献	EP2338988A4 EP2338988B1		
外部链接	Espacenet		

摘要(译)

本发明公开了一种评估方法，该方法能够快速区分Dao - / - 纯合子与DAO酶缺陷型小鼠和其他疾病模型小鼠之间交配实验中产生的大量动物，以快速进行D-氨基酸的定量测定。包含在大量样本中。本发明提供了一种评估测试条件对小鼠组织或来自组织的培养组织细胞的影响的方法。该方法包括以下步骤：提供Dao1 - / - 小鼠等;将来自Dao1 - / - 小鼠等的组织暴露于测试条件;并且分析将来自Dao1 - / - 小鼠等的组织暴露于测试条件的效果。

FIG.7-2