A proposal of a novel experimental procedure to genetically identify disease gene loci in humans

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Abstract: Forward genetics in humans is beneficial in terms of diagnosis and treatment of genetic diseases, and discovery of gene functions. However, experimental mating is not possible among humans. In order to overcome this problem, I propose a novel experimental procedure to genetically identify human disease gene loci. To accomplish this, somatic cells from patients or their parents are reprogrammed to the pluripotent state, oogenesis is induced, the oocytes are parthenogenetically activated in the presence of cytochalasin, and embryonic stem cells are established from the parthenogenetic blastocysts. This protocol produces a set of diploid pluripotent stem cell clones having maternal and paternal chromosomes in different manners to each other. The genetic loci for the disease genes are determined through the conventional processes of positional cloning. Thus, taking advantage of the strategy proposed here, if the abnormality is reproducible using patient-derived pluripotent stem cells, a single carrier of the genetic mutations would be adequate to identify the disease gene loci.

Keywords: genetic disease, human genetics, iPS cell, meiosis, parthenogenesis

Introduction

There are many types of human genetic diseases. Identification of the mutations responsible for these diseases is beneficial in terms of diagnosis and treatment of genetic diseases, and discovery of gene functions. However, experimental mating to identify the responsible gene loci is not possible in humans. In addition, the generation time of humans is so long that only a few persons received a certain genetic mutation. These restrictions make it difficult to identify disease genes in humans.

Therefore, in human genetic studies, data on symptoms and genotypes need to be collected from a number of patients and controls. This takes much cost to identify the disease genes. In addition, mutations in various genes cause similar symptoms if the affected genes contribute to the same functions (locus heterogeneity). Thus, the method for conventional human genetics that may together analyze many patients who carry different mutations in different genes makes the problems difficult.

Recently, the whole-genome sequence of patients in a family was determined to identify the candidate genes of Mendelian disorders using “next generation sequencing” technology.1,2) Because there are millions of genetic polymorphisms in each human genome, one have to hypothesize (e.g., homozygous mutations, nonsynonymous mutations, mutations in functional gene units, genes known to function in the abnormal tissues of diseases, and so on) to pick out the responsible mutations of the diseases.1,2) In addition, each person has different sensitivities to certain disease genes because of the diversity of the genetic background in humans. In fact, there are many mutations, including both heterozygous dominant mutations and homozygous recessive mutations, responsible for certain diseases in the genomes of people not affected by the diseases.1) Thus, it may be difficult to accurately identify the disease-responsible mutations out of the known
biological mechanisms) or the susceptibility genes of heterozygous polygenic disorders using whole-genome sequencing technology if only a few patients are available.

Because of these technical limitations in human genetics, the genes responsible for many diseases have still not been identified. In particular, it is difficult to identify susceptible genes for polygenic diseases. In the case of idiopathic generalized epilepsy (IGE), combinations of several susceptibility genes are thought to be risk factors for the disease. Each of the susceptibility genes is supposed to have a small effect and be neither necessary nor sufficient to cause epilepsy. This results in high heterogeneity of the susceptible gene loci in patients with IGE. Thus, despite hundreds of genetic association studies on epilepsy, the mutations responsible for IGE have not been definitively identified.

In order to overcome the abovementioned difficulties, I propose a novel experimental procedure to genetically identify the gene loci responsible for human genetic disorders. Taking advantage of the strategy proposed here, if the abnormality of the disease is reproducible using patient-derived pluripotent stem cells, a single carrier of the genetic mutations would be enough to identify the disease gene loci.

Method

The gene loci responsible for human genetic disorders are experimentally determined in the order described below (Figs. 1, 2). This method is applicable to many types of inheritance, such as dominant, recessive, and multigenic traits.

1. **Induction of pluripotent stem cells.** Somatic cells from the patients and controls are...
reprogrammed to induced pluripotent stem (iPS) cells. Reprogramming factors should be introduced into the cells so as not to alter the genomic sequences. Plasmid vector, transposons, and recombinant proteins can be used to introduce these factors into the cells. Chemical reagents are also useful to increase the efficiency of reprogramming.

(2) Detection of abnormalities. Assay systems are established to distinguish the affected iPS cells from the normal control (Fig. 1A). Nonketotic hyperglycinemia (NKH) is a genetic disorder affecting glycine metabolism. In the NKH patients examined in a previous study, glycine cleavage activity in liver homogenates was severely defective. In most cases, NKH is caused by an abnormality in one of three components of the glycine cleavage multi-enzyme system (GLDC, AMT, and GCSH). However, the disease-responsible genes have not yet been identified in a few exceptional patients with NKH. In NKH, the affected iPS cells might be distinguished from the normal controls after differentiation into hepatocytes and quantification of their glycine metabolic activities.

As another example, in the case of IGE, expression levels of GLT1, a glial glutamate transporter, decrease and Iba1, an actin-crosslinking molecule in microglia, increases in the brains of EL mice, an animal model of IGE. Even in the case of a systemic disease such as IGE, the patient’s iPS cells may also be distinguished from the normal controls, if the expression level of the human counterpart of GTL1 or Iba1 is abnormal in glial cells differentiated from patient-derived iPS cells.

(3) Induction of meiosis. Meiosis is induced in patient-derived iPS cells (Figs. 1B and 2B). A heterozygote of responsible mutations is used. In the case of genetic disorders in an autosomal recessive trait, cells from the parents of the patients are analyzed. Oogenesis is chosen for the meiosis of iPS cells because of its compatibility with the following procedures.

Creating functional germ line cells from iPS cells is a significant problem. However, when chimeric
mice containing mouse ES/iPS cells are created, ES/iPS cell-derived germ cells are known to be formed in the body. The following describes my thoughts on the ethically unproblematic application of that method to human iPS cells.

Human ES cells are characteristically similar to mouse epiblast stem cells, which are epiblast-derived pluripotent stem cells, rather than to ES cells derived from mouse blastocyst inner cell mass. Human ES cells tend to be more prone to become primordial germ cells (PGCs) in response to bone morphogenetic proteins (BMPs) than mouse ES/iPS cells in vitro. In the case of mouse system, when a tissue fragment containing PGCs is transplanted into a space at the junction of the allantois with the root of the amnion of a primitive streak-stage mouse embryo, the exogenous PGCs can start to migrate toward the genital ridge in the same way as the endogenous PGCs as the embryo develops. E7.5 mouse embryos (containing primitive streak-stage mouse embryo) can be developed into E10.5 equivalent embryos by 3 days of whole embryo culture. In addition, an E10.5 embryo urogenital ridge region can be organ cultured and differentiated into an ovarian-like tissue, but not a testis. Further, ovaries derived from E12.5 (the time point when ovary structures appear in female embryos) or later mouse fetuses can be transplanted into ovariectomized female mice and matured in the mouse’s body. In addition, male PGCs, as well as female PGCs, can develop as oocytes in female embryos.

Using these techniques together, oocytes may be generated from patient-derived iPS cells in the fetus-derived ovaries as depicted in Fig. 2; however, this will need to be confirmed experimentally in the future.

The production of gametes from pluripotent stem cells in vitro has been studied extensively. Thus, meiotic induction, including normal homologous recombination of chromosomes, may be applied to iPS cells in vitro in future.

In the ovaries of mice, oogonia enter the meiotic phase on embryonic day 13.5, and the immature oocytes are arrested after the diplotene of the first meiotic prophase. In immature oocytes at the diplotene stage, the number of the chromosomes is 4n, and recombination between the homologous chromosomes is complete. The meiotic process of the oocytes proceeds again shortly before ovulation and stops at the second meiotic metaphase. At this point, the number of chromosomes in the oocytes is 2n, and each pair of homologous chromosomes consists of those of maternal and paternal origin in various ratios (Fig. 1B). Therefore, some oocytes have homozygous genetic mutations, while others have heterozygous or no mutations (Fig. 1C).

(4) Parthenogenesis of oocytes. Oocytes that are arrested at the first meiotic prophase or second meiotic metaphase are collected and cultured in vitro. Next, these are stimulated to induce parthenogenesis (Fig. 2C). If the oocytes are treated with cytochalasin when parthenogenetically activated to prevent extrusion of the polar bodies during meiosis, the parthenogenetic embryos keep diploidy and possess maternal and paternal chromosomes in various ratios (Figs. 1C, 3A). Parthenogenetic embryonic stem (pES) cell clones are collected from the resultant blastocysts of the parthenogenetic embryos and cultured separately in wells of culture plates (Fig. 2C).

Parthenogenetic embryos of wild-type mice cannot complete ontogeny. However, pES cells of mice differentiate into many types of cells if injected into normal embryos or cultured in vitro. In the case of humans, parthenogenetic cells are also known to differentiate into many types of cells as ovarian teratomas.

(5) Phenotyping of the clones. The pES cell clones in each well are induced to differentiate into appropriate cells, and the cellular functions are examined (Figs. 1C and 2C) using the assay systems established in the previous step (Fig. 1A). The clones carrying the mutations responsible for the diseases show abnormality in their functions, while those without the mutations have normal phenotypes (Fig. 1C).

(6) Genetic analysis. Genomic DNA is obtained from each clone (Fig. 2C). The responsible or susceptible gene loci for the diseases are identified by standard procedures of genetic analysis, integrating the information of the phenotype and genotype of each clone (Fig. 2C). The availability of parthenogenetic embryos for the genetic mapping of certain genes in mice and humans has been discussed previously.

Hotspots for meiotic homologous recombination exist in every 10–100 kb on human chromosomes. Therefore, the resolution of this method to identify the genetic loci may be 10–100 kb.

Mutations responsible for the diseases are identified by comparing the nucleotide sequences of the responsible loci of the patients and controls.

Although the number of samples necessary to determine disease gene loci depends on the complex-
ity of the disease, given that hundred samples from persons are necessary to identify the loci of a certain disease within a 1cM-region, hundred oocytes would give a similar result using experimental procedure proposed. To save the cost of genotyping, a following strategy is available. A part of the hundred clones is subjected to first genome-wide analysis to roughly narrow down the loci in a 20cM-region, for example, and only the clones having recombination points within it (approximately 20 clones out of a hundred) are secondly analyzed intensively for fine mapping of the loci.

(7) Functional analysis. To confirm the responsible mutations experimentally, iPS cells from patients are genetically modified and rescued from the symptoms. Transfection of the wild-type genes, knockdown of the disease genes using RNA interference, and introduction of mutations into genomic DNA\textsuperscript{(51,52)} can be used to rescue the patient-derived iPS cells. The assay system mentioned in (2) is available to examine abnormalities of the cells.

Discussion

Bioethical practicability. The experimental method currently proposed will (i) contribute to the development of new diagnostic methods, and (ii) require highly efficient homologous recombination of chromosomes during meiosis. Therefore, creation of
germ cells from human iPS cells for implementation of this technique is ethically permissible. (Article 4 of the “Policy on Research Involving the Creation of Germ Cells from Human iPS Cells or Human Tissue Stem Cells,” brought into effect on May 20, 2010 by the Ministry of Education, Culture, Sports, Science and Technology. In this policy, PGCs are defined as germ cells.)

In addition, as the human parthenogenetic embryos created by this study are not human embryos as defined in the Act on Regulation of Human Cloning Techniques (Act No. 146, December 6, 2000) (A cell or a cell group that has the potential to grow into an individual through the process of development in utero of a human or an Animal), their creation is possible.

Also, while injecting PGC into an animal embryo corresponds to the creation of an “Animal-Human Chimeric Embryo” as defined in the Act on Regulation of Human Cloning Techniques (in this Act, PGCs are defined as somatic cells), the Act does not place any restrictions on the handling of animal-human chimeric embryos. (Even human-animal chimeric embryos do not infringe the Act provided they are not transplanted into a human or animal uterus.) In addition, the urogenital ridges and ovaries extracted from that animal chimeric embryo are not embryos, and there are no problems even if they are transplanted into an animal and matured.

As a strategy to prevent the accidental fertilization of human germ cells induced from human iPS cells, one of the options in this study is to focus only on oogenesis. By transplanting iPS-derived PGCs into gonads derived from females, both male and female-derived iPS cells are advanced to oogenesis, and all analysis should be by oocyte parthenogenetic development.

In the case of mice, if the E10.5-derived urogenital ridge is organ cultured under certain conditions, it will differentiate into ovaries but not testicles. Therefore, there is no need to be concerned over the accidental production of human sperm or the subsequent human fertilized embryos in the mouse, provided the method given in Fig. 2 is used, i.e., introducing iPS cell-derived PGC into genital primordia at this time and differentiating the genital primordia that differentiate into ovaries by organ culture. Also, when the ovaries that have been organ cultured are matured in a female mouse, by selecting transplantation into the renal capsule (not the location of the ovaries), it is thought that fertilization of human ooctyes by mouse sperm by unforeseen mating can be prevented in advance.

As described above, no ethical problems are assumed to arise in the implementation of this proposal, but it goes without saying that the approval of ethics committees at research institutions must be obtained before actual implementation.

It would be convenient to use a method that does not employ meiosis, which is difficult to induce artificially, for rearranging chromosomes. For example, the phenomenon of loss of heterozygosity in which chromosomes are made partially homozygous is known. Frequency of this phenomenon is increased in cells deficient in Bloom’s syndrome gene and these cells can be used as genetic tools in certain conditions. If the few cells in which such a phenomenon has occurred can be selected, and if phenotypically changed clones can be specifically recognized, it may be possible to exploit this phenomenon, because the frequency of the phenomenon is very low even in the absence of Bloom’s syndrome gene. However, it is generally difficult to collect phenotypically changed clones specifically. For example, how can one find hepatocytes which recovered their high glycine metabolic activities mixed in those with low activities when analyzing NKH? In addition, the resolution that narrows down the locus is the distance between the recombination points of the chromosome. However, the frequency of homologous recombination in mammalian somatic cells is generally extremely low. Hence, to obtain a number of recombination points that narrow down the locus, meiosis is quicker and has a track record of use in genetic analysis.

Possible application of the procedure. The experimental technique proposed here can provide analytical strategies in several research areas where analysis has previously been problematic.

1. Single-patient genetics. This method enables the identification of the disease gene loci from a single carrier of the genetic mutations. It saves costs to collect samples from many patients. In addition, this experimental procedure can be applied to cases where information on population genetics is unavailable or whole-genome sequencing cannot be applied, such as those where only a few patients are available (e.g., rare diseases or patients having exceptional mutations in major symptoms) and where each patient exhibiting the same disease has mutations on different combinations of susceptibility genes (e.g., IGE).

This procedure can be applied to Mendelian disorders inherited as dominant and recessive traits
as well as polygenic diseases. It can also be applicable to the cases that the mode of disease inheritance is unclear and whether the diseases are genetic is unknown.

This is achievable because of the following:

(a) Large sample size. The resolution of gene mapping by the classical linkage analysis depends on the sample size available. In contrast to the conventional human genetic analyses, this method would enable to extensively narrow down the candidate gene region by freely increasing the number of parthenogenetic clones.

(b) Locus homogeneity. In the study of a particular patient, abnormalities in the parthenogenetic clones are derived from identical genetic loci. This enables accurate identification of the loci.

In the case of diseases accompanied by so-called “anticipation,” even persons with a shared pedigree have mutations of different severities on the same gene, e.g., the different number of trinucleotide repeats and different severities of spinocerebellar degeneration among persons with a certain pedigree. The different severities of the disease within a pedigree sometimes lead to misjudgment of less severe patients as healthy cohorts. This makes correct determination of the responsible loci difficult using conventional genetic analyses (i.e., both linkage analysis and whole-genome sequencing strategy). The method proposed in this manuscript is also beneficial for such cases because each pES clone from one patient is thought to have identical mutations.

(c) Defined genetic background. The diversity of the genetic background of the parthenogenetic clones from one patient is defined within a certain range. This leads to a strict correlation between the responsible mutations and the phenotypes, which improves the precision of the genetic analyses.

(2) Genetic analysis of cancer cells. In addition to the usual genetic disease analysis, it is also possible to identify heterozygous mutations that cause cancer. Because cancer cells contain various mutations, there are cases where even reading the entire cancer cell genome will not reveal which mutation caused cancer. Also, cancer cells cannot, of course, be mated, so the cause of cancer cannot be genetically identified by mating experiments. When addressing these kinds of problems, if the protocols in this proposal are applied to cancer cells, it will be possible to identify the cause of canerization of particular cancer cells (if the cause is heterozygous).

The problems of cancer cells containing many mutations and mating experiments being impossible are also encountered in mouse cancer cell research. Hence, useful discoveries can be made even if this method is applied to mouse cancer research. So long as the application of this method is confined to mice, there are no technical or ethical problems.

For example, mouse cancer cells (e.g., liver cancer cells) are transformed into iPS cells, introduced into a female mouse blastocyst, and a chimeric mouse produced. The iPS-derived oocytes from that mouse are collected, parthenogenetically developed, and pES cells established. ES cells derived from parthenogenetic embryos are differentiated into the appropriate cells (e.g., liver cells). Those cells are assessed to check whether they are tumor cells (transplant experiments or examination of cell surface markers).

(3) Mammalian random mutagenesis. In forward genetics in mammals (with particular progress in research on mice), random mutations are introduced into the sperm, and the required mutants are obtained by phenotypic analysis of the offspring. In the process of searching for recessive mutations, numerous individuals need to be mated to make the randomly inserted mutations homozygous. However, the application of the research method proposed here can generate a large number of clones homozygous for the introduced mutation by, for example, carrying out oogenesis of iPS cells with randomly introduced mutations, followed by parthenogenetic development and establishing pES cells. If the pES clones with abnormal phenotypes are identified, the causative mutation can be determined by whole genome sequencing.

This strategy may be particularly effective once functional oogenesis directly from iPS cells in vitro becomes possible.

Differences from conventional human genetics. The experimental protocol proposed here differs from conventional procedures in the following ways.

(1) Reproduction of abnormalities from iPS cells. The methods used for experimental human genetics described above are applicable to genetic disorders where the symptoms are at least partially reproducible from iPS cells. The abnormalities to be reproduced need to be either the cause of the disease or the effects of the causative defects of the disease. Because reproduction of the abnormalities using patient-specific iPS cells is clinically important to establish diagnostic protocols and research drugs, more disease symptoms would be reproduced in the near future.
Because the experimental conditions for all parthenogenetic clones are the same, the proposed procedure is beneficial for analyses of multifactorial diseases that are affected by environmental factors.

In the case of slow-onset diseases, potential patients with no obvious symptoms are sometimes identified as healthy. The method proposed here is suitable for such cases because all the mutant pES clones from a patient are suspected to be “ill”, once the experimental protocol is established to reproduce the symptoms of that patient.

In vitro experimental systems are also useful for rescue experiments of the iPS cell of the patient in order to confirm that the identified mutations are the ones responsible for the disease. Moreover, the set of pES clones produced by this method can be used as a tool to analyze the functional significance of the cloned gene by comparing the phenotypes of the pES clones and expression levels or functional alteration of the gene product.

(2) Production of germ cells from iPS cells.
In the proposed experimental protocol for human genetics, the patient-derived iPS cells have to undergo meiosis. Although there are several reports describing the production of germ cells from ES/iPS cells in vitro, it is still uncertain whether functional meiosis including correct chromosomal homologous recombination occurs in vitro. However, ES/iPS cells can differentiate into gametes if they are injected into blastocysts, and the resultant chimeric embryos develop to term. Therefore, the problem is not the incapability of producing gametes from iPS cells in vitro but how to technically and ethically apply gametogenesis in chimeric embryos to human iPS cells.

One possible procedure to produce oocytes from human iPS cells is described in the Method section and depicted in Fig. 2. Because the development of PGCs into oocytes requires in vivo conditions, PGCs derived from human iPS cells have to be transplanted to nonhumans. The animal species that receives the human PGCs needs to be chosen. Human teratoma formation, reconstruction of the human adaptive immune system,55) and generation of mice with chimeric human livers56),57) are accomplished after transplantation of human cells into immune-deficient mice. Therefore, these immune-deficient mouse lines may be chosen as recipients of human iPS cell-derived PGCs.

Correct induction of a functional gamete from iPS cells is studied intensively because it is a matter of clinical importance. If functional oocytes are produced from human iPS cells in vitro in the near future, this would benefit experimental human genetics.

Meiotic arrest does not occur in the case of spermatogenesis. Therefore, meiotic arrest and reprogramming to pluripotent stem cells at the appropriate stages have to be enforced artificially, if spermatogenesis is applied to the protocol proposed in the present report. Whether this is possible remains to be determined.

(3) Genetic analysis. The experimental human genetics described here differs from the more commonly applied human genetics in terms of genetic analysis, as follows:

(a) Diseases to be studied. If the types of inheritance of certain genetic diseases are clear, familial information would have already been analyzed intensively. Thus, many patients and their families are expected to be easily accessible. In such cases, conventional genetic analysis or whole-genome sequencing strategies are the first choice to determine the disease genes, because these are easier, faster, and cheaper than the experimental procedure proposed here. Therefore, only complex diseases or the cases where only a few patients are accessible should be analyzed by the experimental human genetics described here. In such cases, the number of responsible or susceptible genes and the types of inheritance may be unclear. In order to determine the gene loci responsible for such diseases, statistical procedures utilized in quantitative trait loci analyses, nonparametric linkage analyses, or association studies are suitable. So far as the genetic analysis is applied to the parthenogenetic clones from one patient, population stratification is not a concern in contrast to conventional human genetics.

(b) Mutations to be determined. Heterozygous dominant mutation loci are determined using patient-derived iPS cells by the parthenogenetic procedure, if the dominant mutation is one component of the disease (Fig. 3B(i)). On the other hand, iPS cells from parents of the patients must be analyzed to determine the responsible gene loci, if all the mutations responsible for the diseases are homozygous recessive (Fig. 3B(ii)).

If the susceptibility genes are composed of both heterozygous dominant and homozygous recessive mutations, the homozygous recessive loci cannot be determined using either patient- or parent-derived parthenogenetic clones in some cases (Fig. 3B(iii)). However, in order to treat the patient, reversion of some of the mutations in the susceptibility genes would be adequate. (If the diseases are not restored
after reversion of a mutation at all, such mutation must not have been cloned as that in a susceptibility gene.) Therefore, it doesn’t matter in that respect, even if all the mutations of susceptible genes are not determined.

(c) Genetic bias of chromosomes in the parthenogenetic clones. In the parthenogenetic clones derived from oocytes at the second meiotic metaphase [hereafter, referred to as p(MII) clones], chromosome regions proximal to the centromere become homozygous and distal regions remain heterozygous if one recombination occurs in an arm, as shown in Figs. 1C and 3A. This kind of chromosomal segregation can be genetically analyzed by half-tetrad analysis, as well studied in attached-X chromosomes in Drosophila.58)

(d) Potential recessive mutations in the human genome. There are several disease-causing recessive mutations that are heterozygous in the human genome. In the procedure proposed here, half of the chromosomes become homozygous in the p(MII) clones and a quarter in the p(MI) clones (Fig. 3A), which results in the heterozygous recessive mutations in the human genome becoming homozygous, which may produce some disease-causing clones.

If the potential recessive mutations become homozygous, the pES cells may be affected in the following ways: (i) differentiation into cells appropriate for analysis is disturbed (e.g., differentiation into hepatocytes is disturbed in the analyses of NKH), (ii) cellular functions to be analyzed are perturbed (e.g., glycine metabolism is disturbed in the analysis of NKH), or (iii) an unrelated phenotype becomes abnormal (e.g., the mutation affects neurogenesis and the glycine metabolic activities are analyzed using pES-derived hepatocytes).

In the case of (i), affected clones that are unable to differentiate into cells appropriate for analysis should be removed from the analyses. Thereafter, the disease gene loci for the patients are determined genetically, although analyses of the loci around the potential mutations become more complicated. In the case of (ii), mutations responsible for the diseases and potential recessive mutations are determined simultaneously. Thereafter, the potential recessive mutations could be logically discriminated from the responsible mutations. The potential recessive mutation is found to affect the function of parthenogenetic cells only when it becomes homozygous, but it is heterozygous in the patient’s genome. Reversion of the potential recessive mutation does not restore the abnormality in patient cells. The effects of (iii) do not matter at all.

p(MI) clones have lower chromosomal homozygosity than p(MII) clones. p(MI) clones may have lower risk originated from the potential recessive mutations.

(4) Clonal variability of pES/iPS cells. ES/iPS cell clones do vary even if they are established from genetically identical embryos. Effect of the variability should be overcome during genetic analysis.

(a) Functional variability of differentiated pES clones. Even when pES cell lines are established from the identical parthenogenetic blastocyst, phenotypes can differ depending on the cell line of the clone. However, with regard to the phenotypic variation independent of the genetic variation, it is anticipated that the impact of the variation will be averaged out by using a large number of pES clones for genetic analysis, so that finally only the loci causing the disease phenotype will be isolated.

Furthermore, even when the severity of the symptoms is different in each patient, the causal loci in each patient can be independently identified by analyzing the pES clones produced from each patient separately.

(b) Differentiation efficiency. The efficiency with which pES cells differentiate into appropriate cells is not always 100%. In addition, the efficiency may not be same in different pES cell clones. Therefore, the cellular function to be analyzed should be compensated by the efficiency of differentiation into the appropriate cells. For example, in the case of the study with NKH, the glycine metabolism of parthenogenetic clones induced to hepatocytes should be divided by the amount of a hepatocyte marker, such as albumin, before the genetic analyses. Alternatively, specific examination of appropriate cells by FACS gating or cytological analyses may also be helpful.

The homogeneity of quality and the completeness of differentiation in ES/iPS cells are of great importance in regenerative medicine and are thus expected to be improved in future.

(c) Abnormal karyotype. The chromosome number of pES/iPS cells is sometimes disrupted during the establishment or culture of the clones. To remove clones with chromosomal abnormalities, karyotyping of the parthenogenetic clones should be performed before functional analyses. In addition, genome-wide typing of SNPs identifies the chromosomal loss of the parthenogenetic clones if all the loci on a certain chromosome have a single allele.

(d) Mutagenesis. pES/iPS cells may accumulate mutations on their genomic DNA during cell culture. However, it is not likely that the mutations may affect the genetic analyses of the parthenogenetic clones. One reason is that randomly generated
mutations rarely affect a specific phenotype to be analyzed. Even in a random mutagenesis study using a strong mutagen such as ENU, it is not easy to find appropriate mutants for the analysis of a certain phenotype. The second reason is that if the multiple clones of iPS cells are established from a patient and pES clones from distinct iPS cell clones are subject to analysis, only a small part of the clones, if any, would be affected by an identical de novo mutation, which does not matter to following genetic analysis.

(5) Uniparental genomic imprinting. Parthenogenetic embryos of mammalian species cannot develop to term. However, pES cells can differentiate into various types of cell derivatives of three germ layers. Therefore, pES cells can be used for the experimental procedure described in this report.

In the pES cells from parthenogenetic oocytes, both alleles of the genomic loci are maternally imprinted. Thus, uniparental imprinting of the pES clones may affect some cellular functions. It does not matter if the abnormalities caused by the uniparental imprinting differ from those of the patient. However, if uniparental imprinting leads to the same symptoms as those of the patient or if the mutations responsible are located near the imprinted loci, it may affect the genetic analyses, as described in the following cases.

(a) All pES clones become aberrant. If both alleles of the genetic loci necessary for the phenotype to be analyzed are suppressed by maternal genomic imprinting, then all pES clones get affected (Fig. 3C(ii)). If the functional abnormalities are restored by artificial inhibition of the DNA methylation of pES cells, genetic analyses of parthenogenetic clones may become possible.

(b) All pES clones become normal. If a disease is caused by a heterozygous mutation in an imprinted locus that leads to 50% loss of activity of the gene (e.g., 50% expression level or 50% activity of the gene product), the mutation is on an active allele, and another nonmutated allele is paternally inactive, all parthenogenetic clones become normal because both alleles of the genetic loci are maternally active in the parthenogenetic clones (Fig. 3C(iii)). In such cases, genetic analyses cannot determine the loci responsible. However, it becomes clear that the diseases could be cured by parthenogenetic processes. If the regulation of genomic imprinting restores the abnormality, chemical reagents such as DNA methyltransferase inhibitor may be candidates for therapeutic materials. If this is the case, the loci responsible for the diseases may be found near the paternally methylated loci.

(c) Some of the clones with responsible mutations become normal. In case the heterozygous recessive mutation in a differentially methylated locus is responsible for a disease and the normal allele is paternally inactivated, parthenogenetic clones carrying a heterozygous mutation become phenotypically normal because both alleles become active after parthenogenesis (Fig. 3C(iv)). In such cases, only clones carrying the mutations homozygously get affected.

Future perspectives. In future, when gene therapy would be generally accepted as a way to treat genetic disorders, all patients of the genetic diseases may try whole-genome sequencing to identify the causative mutations. These mutations, once clarified, could be treated by gene therapy. The techniques proposed could be the only way to treat patients having genetic diseases with mutations that cannot be identified by whole-genome sequencing and cross-referencing the mutations with public databases.

According to this scenario, most of the genetic disorders to which these techniques are applied would have causes beyond the known biological mechanisms. This means that researchers could study only mutations related to novel mechanisms.

In the studies of genetics, researchers never know how much scientific impact the mutants have, beforehand. Today, most genes are identified, and their functions are known or speculated from their motifs. Therefore, many geneticists may meet the cases that they clone mutations on common genes. Such cases will be more common in future. If this is the case, who would pay cost to generate and study 999 of the common mutants necessary to discover another one fruitful mutation?

In the case of human genetics, however, patients with genetic diseases come to the hospitals by themselves, and pay cost for gene diagnoses. After the happiness that 999 of the conventional mutations have been treated, the remaining one fascinating mutation beyond our knowledge would be identified experimentally. Thus, the method of the experimental human genetics proposed here is also attractive from the viewpoint of genetics as basic science.

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