



- (51) International Patent Classification:
C12Q 1/68 (2006.01)
- (21) International Application Number:
PCT/US2016/035041
- (22) International Filing Date:
31 May 2016 (31.05.2016)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
62/169,280 1 June 2015 (01.06.2015) US
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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHODS AND COMPOSITIONS FOR PROGNOSTICATIONS AND/OR CLINICAL MANAGEMENT OF GRAFT-VERSUS-HOST DISEASE AND TRANSPLANT REJECTION

(57) Abstract: Compositions and methods are provided to determine the likelihood of a subject to develop a complication resulting from an adaptive immune response upon receiving an organ or tissue transplantation. The likelihood is based on expression or activity levels of suppressor of fused (SUFU) mRNA and/or SUFU protein. Such methods and compositions find use in enabling personalized prophylactic or therapeutic treatment regimens for subject based on assessed risk.



METHODS AND COMPOSITIONS FOR PROGNOSTICATIONS AND/OR CLINICAL
MANAGEMENT OF GRAFT-VERSUS-HOST DISEASE AND TRANSPLANT REJECTION

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The invention was made with government support under grant numbers NIH P30 CA-21765-364 24 and CA-21765 awarded by the National Institutes of Health. The government has certain rights in the invention.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The official copy of the sequence listing is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file named 447938SEQLIST.txt, created on June 1, 2015 and having a size of 163 KB and is filed concurrently with the specification. The sequence listing contained in this ASCII formatted document is part of the specification and is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to the field of transplant biology. More specifically, the invention relates to determining the risk for transplant rejections and graft-versus-host disease and to personalizing prophylactic regimens based on the assessed risk.

BACKGROUND OF THE INVENTION

Tissue and organ transplantations such as hematopoietic stem cell transplantation (HSCT) are used to treat a variety of malignant and non-malignant diseases. However, serious complications can occur, including transplant rejection and graft-versus-host disease (GVHD). Several factors, related to both the donor and recipient, have been identified as potential risk factors for transplant rejection and GVHD. Among these risk factors is genetic disparity between donor and recipient in human leukocyte antigen (HLA). However, relatively little is known about non-HLA genetic factors that may contribute to transplant rejections or GVHD. Identifying such factors would allow for improved risk stratification and individualized treatment and prophylaxis in patients experiencing or at risk for transplant rejection or GVHD.

SUMMARY OF THE INVENTION

Compositions and methods are provided to determine the likelihood of a subject developing a complication (*e.g.*, graft versus host disease (GVHD)) resulting from an adaptive immune response upon receiving an organ or tissue transplantation, such as a hematopoietic stem cell transplantation (HSCT). Methods comprise assaying a biological sample from the subject for expression or activity levels of *suppressor of fused (SUFU)* mRNA and/or SUFU protein. Decreased *SUFU* mRNA and/or SUFU protein expression or activity indicates an increased risk for a complication (*e.g.*, GVHD), whereas increased *SUFU* mRNA and/or SUFU protein expression or activity indicates a decreased risk for a complication. In specific methods, assaying for expression or activity levels of *SUFU* mRNA and/or SUFU protein in the biological sample comprises detecting in the genome of the subject a marker locus that is associated with increased or decreased risk of the complication (*e.g.*, GVHD), wherein the marker locus comprises rs17114808 or a marker locus site that is in complete disequilibrium with rs17114808.

Methods are further provided for screening for sonic hedgehog (SHH) signaling pathway antagonists. Specific methods comprise contacting an antigen-presenting cell (APC) with a candidate compound and determining if the compound inhibits regulation of transplant rejection or graft-versus-host disease by the SHH pathway.

Various compositions are further provided comprising kits that allow for the level and/or activity of *SUFU* mRNA and/or SUFU protein to be determined.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the results of genome-wide screening of single-nucleotide polymorphisms (SNPs) associated with acute graft-versus-host disease (GVHD) in patients who underwent hematopoietic stem cell transplantation (HSCT). Figure 1A shows a Manhattan plot of p-values from the genome-wide analyses. The horizontal axis indicates each SNP's chromosomal location, while the vertical axis indicates the degree of SNP association with acute GVHD. Figure 1B shows the percentage and number (n) of subjects with and without acute GVHD stratified by rs17114808 SNP genotype in the discovery cohort. Figure 1C shows cumulative incidence of acute GVHD in the discovery cohort. CC indicates patients homozygous for *SUFU* allele with cytosine at the rs17114808 position; CT indicates heterozygous with cytosine and thymine at the same position.

Figure 2 shows validation of *SUFU* SNPs in association with acute GVHD. Figure 2A shows a SNP assay that can distinguish different *SUFU* genotypes in the validation cohort. The upper left cluster represents allele CC, the center cluster of two points represents allele CT, and the

point circled at the bottom right represents allele TT. A representative typing output is shown. Figure 2B shows the percentage and number (n) of subjects with and without acute GVHD stratified by rs17114808 SNP genotype in the validation cohort. Figure 2C shows cumulative incidence of acute GVHD in the validation cohort. The CC/TT line shows no increase from 4 weeks forward, while the CC line increases until about 16 weeks. CC indicates patients homozygous for *SUFU* allele with cytosine at rs17114808 position; CT indicates heterozygous with cytosine and thymine at the same position; and TT indicates homozygosity with thymine in that position.

Figure 3 shows *SUFU* polymorphisms in healthy population. Figure 3A shows the relative abundance of *SUFU* mRNA in peripheral blood mononuclear cells (PBMCs) from six representative healthy individuals with homozygous (CC or TT) or heterozygous (CT) alleles at the rs17114808 position. Data was normalized to GAPDH mRNA levels and are presented as fold change relative to the expression of GAPDH. Figure 3B shows *SUFU* protein production in PBMCs from the same six healthy individuals as determined by Western blot. α -tubulin was used as a loading control.

Figure 4 shows that *SUFU* suppresses allogeneic T-cell proliferation by reducing HLA-DR expression in dendritic cells. Figure 4A shows expression of *SUFU* transcripts in myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs), and peripheral blood mononuclear cells (PBMCs). Plasmid DNA of *SUFU* was used as positive control and water as a negative control. Figure 4B shows data from three independent experiments in which DCs were isolated from *SUFU*-CC- and *SUFU*-CT-positive donors and used in allogeneic mixed lymphocyte reaction (MLR) assays. Figure 4C shows HLA-DR expression as determined by flow cytometry in mDCs (upper left) and pDCs (upper right) that were isolated from the blood of healthy volunteers with different groups of *SUFU* alleles. Mean fluorescence intensity (MFI) of HLA-DR expression in mDCs (bottom left) and pDCs (bottom right) from 4 to 5 volunteers of each *SUFU* allelic group is plotted. Figure 4D shows mean fluorescence intensity (MFI) of MHC class I expression on myeloid (upper left) and plasmacytoid DC (upper right) from individuals with different *SUFU* allele are shown. Average MFI of MHC class I expression from 3 individuals from each group of *SUFU* alleles are shown at the bottom. * indicates p-value <0.05; ** indicates p-value <0.01; and *** indicates p-value <0.001.

Figure 5 shows that overexpression of *SUFU* reduces whereas silencing of *SUFU* increases allogeneic T-cell proliferation in MLR assays using THP-1 cells induced into antigen-presenting cells (APCs). *SUFU* was either ectopically expressed in myeloid cell line THP-1 (Figure 5A) or

silenced in THP-1 cells by siRNA (Figure 5B). *SUFU* overexpressed and silenced THP-1 cells were then induced into APCs and used for MLR (Figure 5C). Figure 5D shows surface expression of HLA-DR (upper) and MFI (bottom). Mock indicates THP-1 cells without genetic manipulation; *SUFU*⁺ indicates ectopically *SUFU*-expressing THP-1 cells; *SUFU*⁻¹ and *SUFU*⁻² indicate two
5 separate siRNAs used to silence *SUFU* in THP-1 cells; * indicates p-value <0.05; and ** indicates p-value <0.01.

Figure 6 shows validation of the SNP assay. The SNP assay was validated by sequencing the assay products. The underlined nucleotides and corresponding arrows indicate the SNP position. Alleles CC (homozygous for C-allele), CT (heterozygous for the C-allele and the T-allele),
10 and TT (homozygous for the T-allele) are showing with surrounding genomic sequence (SEQ ID NOS: 3, 5, and 4 respectively

Figure 7 shows induction of the THP-1 cell line into APCs. The THP-1 cell line was cultured in RPMI1640 medium (left) or in combination with cytokines that induced them into APCs (right) (Figure 7A). Figure 7B shows expression of dendritic cell (DC) markers in THP-1
15 cells and THP-1 cells induced into APCs.

Figure 8 shows that *SUFU* has no effect on expression of DC markers. *SUFU* gene was overexpressed or silenced in THP-1 cell lines, which were then induced into APCs. Expression of different DC makers was determined by flow cytometric analysis. THP indicates THP-1 cell line; THP-*SUFU*⁺ indicates *SUFU* gene ectopically expressed in THP-1 cell lines; and THP-*SUFU*⁻
20 indicates *SUFU* was silenced in THP-1 cell lines by siRNA.

DETAILED DESCRIPTION OF THE INVENTION

The present inventions now will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the inventions are shown.
25 Indeed, these inventions may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Like numbers refer to like elements throughout.

Many modifications and other embodiments of the inventions set forth herein will come to
30 mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended

claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

I. Overview

5 Methods and compositions are provided to identify subjects that will have an increased or decreased likelihood to develop a complication resulting from an adaptive immune response (e.g., graft-versus-host disease (GVHD)) upon receiving an organ or tissue transplantation (e.g., hematopoietic stem cell transplantation (HSCT)). The various methods and compositions provided herein employ detecting the level and/or activity of *suppressor of fused (SUFU)* mRNA and/or
10 SUFU protein to assess risk for such complications. Based on the assessed risk, personalized prophylaxis or treatment regimens can be administered to the subjects.

 In one non-limiting embodiment, a single-nucleotide polymorphism (SNP) is detected that is linked to risk for complications resulting from an adaptive immune response upon receiving an organ or tissue transplantation. This SNP, denoted herein as rs17114808, is in the 3' UTR region
15 of the *SUFU* gene and correlates with expression levels and/or activity of the SUFU protein.

II. Determining Likelihood of Complications Resulting from an Immune Response upon Receiving an Organ or Tissue Transplantation

 Methods and compositions are provided for determining the likelihood of a subject to
20 develop a complication resulting from an immune response (e.g., an adaptive immune response) upon receiving an organ or tissue transplantation. Such methods comprise assaying a biological sample from the subject for *SUFU* mRNA and/or SUFU protein expression or activity. Decreased *SUFU* mRNA and/or SUFU protein expression or activity indicates an increased risk for the complication, whereas increased *SUFU* mRNA and/or SUFU protein expression or activity
25 indicates a decreased risk for the complication. Assessment of risk for the complication can further be based on the degree of human leukocyte antigen (HLA) mismatch between the subject and the organ or tissue transplanted, as described in further detail below. Based on the assessed risk, a personalized prophylaxis or treatment regimen can be administered to the subject.

 As used herein, an "increased risk" of developing a complication resulting from an immune
30 response (e.g., an adaptive immune response) upon receiving an organ or tissue transplantation comprises a statistically significant increase in the risk of developing the complication. The risk can be based on the presence of a particular risk factor relative to risk in the absence of that risk factor. The increased risk can include, for example, a risk that is at least about 10% higher, 15%

higher, 20% higher, 25% higher, 30% higher, 35% higher, 40% higher, 45% higher, 50% higher, 55% higher, 60% higher, 65% higher, 70% higher, 75% higher, 80% higher, 85% higher, 90% higher, 95% higher, 100% higher, 110% higher, 120% higher, 130% higher, 140% higher, 150% higher, 160% higher, 170% higher, 180% higher, 190% higher, 200% higher, or greater. Statistical significance means $p \leq 0.05$.

As used herein, a “decreased risk” of developing a complication resulting from an immune response (e.g., an adaptive immune response) upon receiving an organ or tissue transplantation comprises a statistically significant decrease in the risk of developing the complication, including, for example, a risk that is at least about 10% lower, 15% lower, 20% lower, 25% lower, 30% lower, 35% lower, 40% lower, 45% lower, 50% lower, 55% lower, 60% lower, 65% lower, 70% lower, 75% lower, 80% lower, 85% lower, 90% lower, 95% lower, 99% lower, or greater. Statistical significance means $p \leq 0.05$. The risk can be based on the presence of a particular risk factor relative to risk in the absence of that risk factor.

Complications resulting from an immune response (e.g., an adaptive immune response) upon receiving an organ or tissue transplantation, and methods for detecting them, are known. Such complications can include, for example, transplant rejections and graft-versus-host disease (GVHD). Characteristics of these complications are described below.

A. Organ or Tissue Transplantations and Complications Arising Therefrom

i. Types of Transplantations

The methods and compositions disclosed herein relate to subjects who receive an organ or tissue transplantation. As used herein, the term “transplantation” refers to the process of taking an organ or tissue (called a “transplant” or “graft”) from one individual (i.e., a donor) and placing it into the same individual or a different individual (i.e., a recipient). The transplant may optionally undergo treatment *ex vivo* prior to introduction into the recipient.

A “tissue” is any biological entity derived from an organism that is comprised of one or more nucleated cells. The biological entity can be derived from an organism directly or via an isolated progenitor cell or population. For example, a tissue could include bone marrow, peripheral blood, umbilical cord blood, or hematopoietic stem cells derived therefrom. In some cases, a tissue can be a group or collection of similar cells and the intercellular substance which act together to perform a particular function. For example, a tissue can be a whole or partial organ. Examples of tissues commonly transplanted are bone marrow, hematopoietic stem cells, and organs such as

liver, heart, skin, bladder, lung, kidney, cornea, pancreas, pancreatic islets, brain tissue, bone, and intestine.

An "organ" is any part of the body exercising a specific function. For example, an organ can be a group of several tissue types that perform a given function. Exemplary organs include heart, kidney, liver, pancreas, and lung.

The individual who provides the transplant is called the "donor," and the individual who received the transplant is called the "host" or "recipient." As used herein, the term "donor" refers to a vertebrate organism from which an organ or tissue is removed (or otherwise derived, for example, by tissue culturing techniques) prior to introduction into the recipient organism. The donor may be of the same (allograft) or different (xenograft) species. Examples of donor types include identical twin donors, matched related donors, matched unrelated donors, mismatched related donors, haploidentical donors, and umbilical cord blood donors. As used herein, the term "recipient" refers to a vertebrate organism which receives a donor organ or tissue.

Transplantations can be, for example, autologous, syngeneic, allogeneic, or xenogeneic. An "autologous transplantation" is a transplantation in which a recipient's own organ or tissue is returned to the recipient (i.e., the donor and recipient are the same). For example, autologous transplantation is typically used as a method of returning a patient's own stem cells as a rescue therapy after high-dose myeloablative therapy. Immunosuppression is typically not required after autologous transplantation, because the immune system that is reconstituted is that of the original host. Because the native immune system returns after autologous transplant, this technique is not used for correction of immunodeficiencies. Examples of conditions that can be treated by autologous transplantation include multiple myeloma, non-Hodgkin lymphoma, Hodgkin disease, acute myeloid leukemia, neuroblastoma, germ cell tumors, autoimmune disorders (e.g., systemic lupus erythematosus, systemic sclerosis), and amyloidosis.

An "allogeneic transplantation" or "allograft" is a transplantation of an organ or tissue from a donor source other than the recipient but from the same species. The donor and recipient may be genetically related or unrelated. This type of transplant is used in the context of many malignant and nonmalignant disorders to replace a defective host marrow or immune system with a normal donor marrow and immune system. The degree of HLA match between the donor and the recipient can be an important factor in these transplants; well-matched transplants decrease the risk of graft rejection and GVHD, both of which are among the most serious complications of transplantation. Examples of conditions that can be treated by allogeneic transplantation include acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia,

myeloproliferative disorders, myelodysplastic syndromes, multiple myeloma, non-Hodgkin lymphoma, Hodgkin disease, aplastic anemia, pure red-cell aplasia, paroxysmal nocturnal hemoglobinuria, fanconi anemia, thalassemia major, sickle cell anemia, severe combined immunodeficiency (SCID), Wiskott-Aldrich syndrome, hemophagocytic lymphohistiocytosis, inborn errors of metabolism (e.g., mucopolysaccharidosis), Gaucher disease, metachromatic leukodystrophies, adrenoleukodystrophies, epidermolysis bullosa, severe congenital neutropenia, Shwachman-Diamond syndrome, Diamond-Blackfan anemia, and leukocyte adhesion deficiency.

A "syngeneic transplantation" is a transplantation of an organ or tissue from a donor source who is an identical twin of the recipient.

A "xenogeneic transplantation" is a transplantation of an organ or tissue from one species to another (e.g., transplantation of a heart from a transgenic pig into a human).

In some embodiments, the transplantation is hematopoietic stem cell transplantation (HSCT). As used herein, the term "hematopoietic stem cell transplantation" or "HSCT" refers to transplantation of a blood progenitor/stem cell from any source. HSCT can include, for example, the intravenous infusion of autologous or allogeneic stem cells collected from bone marrow, peripheral blood, or umbilical cord blood to reestablish hematopoietic function in patients whose bone marrow or immune system is damaged or defective. In specific embodiments, the transplantation is an allogeneic HSCT. See, e.g., Welniak *et al.* (2007) *Annu. Rev. Immunol.* 25:139-170.

ii. Types of Complications

Several types of complications can occur in a subject upon receiving an organ or tissue transplantation. In some instances, such complications result from an immune response, such as an autoimmune response or an adaptive immune response. The immune response can be directed against the donor organ or tissue by the recipient. Alternatively, it can be directed against the recipient by the donor organ or tissue.

In some embodiments, the complication is an organ or tissue transplant rejection. The term "organ or tissue transplant rejection" as used herein refers to a consequence of organ or tissue transplantation caused by the recipient's or host's immune system in response to the transplanted organ or tissue, which can damage or destroy it. Thus, "organ or tissue transplant rejection" is controlled by the host subject.

The antigens responsible for rejection of genetically disparate tissues are called histocompatibility antigens and are products of histocompatibility genes. Histocompatibility

antigens are encoded on more than 40 loci, but the loci responsible for the most vigorous allograft rejection reactions are located on the major histocompatibility complex (MHC).

In humans, the MHC is called the human leukocyte antigen (HLA) system and is located on the short arm of chromosome 6, near the complement genes. Other antigens cause only weaker reactions, but combinations of several minor antigens can elicit strong rejection responses. The MHC genes are codominantly expressed, meaning that each individual expresses these genes from both the alleles on the cell surface. Furthermore, they are inherited as haplotypes or two half sets (one from each parent). This makes a person half identical to each of his or her parents with respect to the MHC complex. This also leads to a 25% chance that an individual might have a sibling who is HLA identical.

The MHC molecules are divided into two classes. The class I molecules are normally expressed on all nucleated cells, whereas the class II molecules are expressed only on the professional antigen-presenting cells (APCs), such as dendritic cells, activated macrophages, and B cells. The physiological function of the MHC molecules is to present antigenic peptides to T cells, since the T lymphocytes only recognize antigen when presented in a complex with an MHC molecule. The class I molecules are responsible for presenting antigenic peptides from within the cell (e.g., antigens from the intracellular viruses, tumor antigens, self-antigens) to CD8 T cells. The class II molecules present extracellular antigens such as extracellular bacteria to CD4 T cells.

The immune response to a transplanted organ consists of both cellular (lymphocyte mediated) and humoral (antibody mediated) mechanisms. Although other cell types are also involved, the T cells are central in the rejection of grafts. The degree of immune response to a graft depends partly on the degree of genetic disparity between the grafted organ or tissue and the host. Xenografts have the most disparity and elicit the maximal immune response, undergoing rapid rejection. Autografts (e.g., grafts from one part of the body to another, such as skin grafts), are not foreign tissue and, therefore, do not elicit rejection. Isografts, which are grafts between genetically identical individuals (e.g., monozygotic twins), also undergo no rejection. For allografts (i.e., grafts between members of the same species that differ genetically), the degree to which they undergo rejection depends partly on the degree of similarity or histocompatibility between the donor and the recipient. The degree and type of response also vary with the type of the transplant. Some sites, such as the eye and the brain, are immunologically privileged (i.e., they have minimal or no immune system cells and can tolerate even mismatched grafts). Skin grafts are not initially vascularized and so do not manifest rejection until the blood supply develops. The heart, kidneys, and liver are highly vascular organs and lead to a vigorous cell mediated response in the host.

In various embodiments, the transplant rejection can be hyperacute, acute, or chronic. In hyperacute rejection, the transplanted tissue is rejected within minutes to hours because vascularization is rapidly destroyed. Hyperacute rejection is humorally mediated and occurs because the recipient has preexisting antibodies against the graft, which can be induced by prior blood transfusions, multiple pregnancies, prior transplantation, or xenografts against which humans already have antibodies. The antigen-antibody complexes activate the complement system, causing massive thrombosis in the capillaries, which prevents the vascularization of the graft.

Acute rejection manifests commonly in the first six months after transplantation. Acute cellular rejection is mediated by lymphocytes that have been activated against donor antigens, primarily in the lymphoid tissues of the recipient. The donor dendritic cells (also called passenger leukocytes) enter the circulation and function as APCs. Humoral rejection is form of allograft injury and subsequent dysfunction, primarily mediated by antibody and complement. It can occur immediately post-transplantation (hyperacute) or during the first week. The antibodies are either preformed antibodies or represent antidonor antibodies that develop after transplantation.

Chronic rejection develops months to years after acute rejection episodes have subsided. Chronic rejections are both antibody- and cell-mediated. Chronic rejection can appear as fibrosis and scarring in all transplanted organs, with the specific histopathological picture depending on the organ or tissue transplanted.

In other embodiments, the complication is graft-versus-host disease (GVHD). See, e.g., Ferrara *et al.* (2009) *Lancet* 373:1550-1561. In specific embodiments, the GVHD is acute GVHD. The term "graft-versus-host-disease" or "GVHD" refers to a pathological reaction that occurs between the host and the grafted organ or tissue. It is an immune-mediated disease resulting from a complex interaction between donor and recipient adaptive immunity. The grafted or donor tissue dominates the pathological reaction. GVHD can be seen following stem cell and/or solid organ transplantation. GVHD often occurs in immunocompromised subjects such as those who are immunocompromised due to receiving immunosuppressive therapy prior to transplant of the graft. When transplanted, such subjects can receive "passenger" lymphocytes in the transplanted stem cells or solid organ. These lymphocytes recognize the recipient's tissue as foreign and attack and mount an inflammatory and destructive response in the recipient. GVHD has a predilection for epithelial tissues, especially skin, liver, and mucosa of the gastrointestinal tract.

GVHD occurs frequently in the allograft setting but rarely occurs in the autologous setting. The disease may cause significant morbidity and mortality and has been divided into acute and chronic forms. Acute GVHD is a common complication of allogeneic transplantation; it occurs

within the first 100 days after the procedure. Acute GVHD involves skin, mucosal surfaces, gut, and liver. It starts as an erythematous, macular skin rash, and as it progresses, blistering of the skin similar to severe burns, severe abdominal pain, profound diarrhea, and hyperbilirubinemia develop. Acute GVHD is graded as per Glucksberg criteria. Grade I disease is confined to the skin and is mild; grade II-IV have systemic involvement. Grade III and IV acute GVHD carry a grave prognosis. Risk factors for acute GVHD include HLA-mismatched grafts, matched unrelated donors (MUD) grafts, grafts from a parous female donor, and advanced patient age.

Chronic GVHD develops 2-12 months after transplantation (typically after day 100) and involves the skin, eyes, mouth, liver, fascia, and almost any organ in the body. Patients with chronic GVHD present with chronic lichenoid skin changes, dryness of the eyes and mouth, and lichenoid skin changes in the oral mucosa, with ulceration and oral pain. Impaired range of motion occurs from fibrosis of the dermis and fascia. Hyperbilirubinemia and elevated alkaline phosphatase can occur. Although the clinical presentation of chronic GVHD mostly resembles scleroderma, it can mimic any other autoimmune disease. In addition to allogeneic HSCT, procedures associated with high risk of GVHD include transplantation of solid organs containing lymphoid tissue and transfusion of unirradiated blood products. Another risk factor is advanced patient age. Yet other risk factors include peripheral blood stem cell transplants, mismatched or unrelated donors, second transplant, and donor leukocyte infusions (DLIs). The greatest risk for chronic GVHD is acute GVHD.

B. Subjects and Biological Samples

By "subject" is intended a vertebrate. Preferably, subjects are mammals, e.g., primates, humans, rodents, or agricultural and domesticated animals such as dogs, cats, cattle, horses, pigs, sheep, and the like. Preferably, the subject is a human. Preferably, the subject is a candidate for an organ or tissue transplantation. In certain embodiments, the subject can be a young adult or a pediatric subject. A young adult is 18-21 years. A pediatric subject is 0-18 years.

As used herein, the term "biological sample" refers to a sample of biological material, within or obtainable from a subject, from which a nucleic acid or protein is recoverable. The term biological sample can also encompass any material derived by processing the sample, such as cells or their progeny. Processing of the biological sample may involve one or more of filtration, distillation, extraction, concentration, fixation, inactivation of interfering components, and the like. A biological sample can comprise any sample in which one desires to determine the level and/or activity of the *SUFU* gene, *SUFU* mRNA, or *SUFU* protein. In some embodiments, a biological

sample comprises a nucleic acid, such as genomic DNA, cDNA, or mRNA. In some embodiments, a biological sample comprises a protein. A biological sample can comprise a sample from any organism, including a mammal, such as a human, a primate, a rodent, a domestic animal (e.g., a feline or canine), or an agricultural animal (e.g., a ruminant, horse, swine, or sheep). The biological sample can be derived from any cell, tissue, or biological fluid from the organism of interest. The sample may comprise any clinically relevant tissue, such as a bone marrow sample, a tumor biopsy, a fine needle aspirate, or a sample of bodily fluid, such as blood, plasma, serum, lymph, ascitic fluid, cystic fluid, or urine. In some embodiments, the sample comprises a buccal swab. The sample used in the methods disclosed herein will vary based on the assay format, nature of the detection method, and the tissues, cells, or extracts that are used as the sample.

In some specific embodiments, the biological sample is a peripheral blood mononuclear cell (PBMC) or is derived from a PBMC. A PBMC is any blood cell having a round nucleus, such as a lymphocyte, a monocyte, or a macrophage. In specific embodiments, the biological sample is an antigen-presenting cell (APC) or is derived from an APC. An APC is a cell that displays foreign antigens complexed with major histocompatibility complexes (MHCs) on their surfaces (i.e., the process of antigen presentation). In more specific embodiments, the biological sample is a dendritic cell (DC) or is derived from a DC. The dendritic cell can be, for example, a myeloid dendritic cell (mDC) or a plasmacytoid dendritic cell (pDC).

C. Detecting Level or Activity of SUFU

Various methods and compositions can be used to assay for the level and/or activity of *SUFU* mRNA and/or *SUFU* protein. “*SUFU*” or “Suppressor of Fused” (also known as Suppressor of Fused Homolog or *SUFUH*) is a component of the Sonic hedgehog (SHH) signaling pathway. More specifically, it is a negative regulator of the SHH signaling pathway. SHH signaling activity is governed by the balance of GLI activators and repressors (see, e.g., Hui & Angers (2011) *Annu. Rev. Cell. Dev. Biol.* 27:513-527). In the absence of SHH, Patched (PTCH) inhibits Smoothened (SMO) to repress signaling through GLI3. SHH binding to PTCH alleviates SMO inhibition and initiates signaling to promote GLI-dependent transcription. GLI2 is the main transcriptional activator, whereas GLI1 potentiates SHH signaling as a secondary activator. *SUFU* and *KIF7* are two key conserved regulators of GLI proteins (see, e.g., Cooper *et al.* (2005) *Development* 132:4407-4417; Cheung *et al.* (2009) *Sci. Signal.* 2:ra29; Endoh-Yamagami *et al.* (2009) *Curr. Biol.* 19:1320-1326; Liem *et al.* (2009) *Proc. Natl. Acad. Sci. USA* 106:13377-13382; and Svard *et al.* (2006) *Dev. Cell* 10:187-197). They interact directly with GLI proteins and control their

processing, stabilization, as well as subcellular distribution (see, e.g., Kogerman *et al.* (1999) *Nat. Cell Biol.* 1:312-319; Barnfield *et al.* (2005) *Differentiation* 73:397-405; Chen *et al.* (2009) *Genes Dev.* 23:1910-1928; Wang *et al.* (2010) *Development* 137:2001-2009; Tukachinsky *et al.* (2010) *J. Cell. Biol.* 191:415-428; and Humke *et al.* (2010) *Genes Dev.* 24:670-682. The SUFU polypeptide is involved in downregulating GLII-mediated transcription of target genes. It can be part of a corepressor complex that acts on DNA-bound GLI1. It can also sequester GLI1, GLI2, and GLI3 in the cytoplasm, and it may also act by targeting GLI1 to degradation. It is ubiquitous in adult tissues, and is present in the nucleus and cytoplasm. See, e.g., Stone *et al.* (1999) *J. Cell. Sci.* 112:4437-4448; Kogerman *et al.* (1999) *Nat. Cell Biol.* 1:312-319; Murone *et al.*, (2000) *Nat. Cell Biol.* 2:310-312; and Chi *et al.* (2012) *Cell. Signal.* 24:1222-1228. A summary of SHH signaling in humans is provided in Villavicencio *et al.* (2000) *Am. J. Hum. Genet.* 67:1047-1054.

The human genomic sequence of *SUFU* is set forth in SEQ ID NO: 1. An exemplary coding region of *SUFU* is set forth in SEQ ID NO: 2, and the amino acid sequence of *SUFU* is set forth in SEQ ID NO: 6. It will be appreciated by those skilled in the art that DNA sequence polymorphisms may exist within a population (e.g., the human population). Such genetic polymorphisms in a polynucleotide comprising the *SUFU* gene as set forth in SEQ ID NO: 3, 4, and 5 may exist among individuals within a population due to natural allelic variation. As used herein, reference to a *SUFU* gene, genomic sequence, mRNA, or polynucleotide encompasses such natural variations, as does reference to a SUFU protein or polypeptide. Likewise, it will be appreciated by those skilled in the art that various isoforms of SUFU can exist. As used herein, reference to a *SUFU* gene, genomic sequence, mRNA, or polynucleotide encompasses such isoforms, as does reference to a SUFU protein or polypeptide.

As used herein, a decrease in *SUFU* mRNA expression or activity or a decrease in SUFU protein expression or activity refers to any statistically significant reduction in expression or activity levels when compared to an appropriate control. Such a decrease includes a reduction of at least about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or greater when compared to an appropriate control. Statistical significance means $p \leq 0.05$. A decrease in expression or activity can occur via any mechanism at any stage. For example, a decrease in expression could occur via events or regulation that occurs during transcription, post-transcription, during translation, or post-translation.

As used herein, an increase in *SUFU* mRNA expression or activity or an increase in SUFU protein expression or activity refers to any statistically significant increase in expression or activity levels when compared to an appropriate control. Such an increase includes an increase of at least

about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, 100%, 200%, or greater when compared to an appropriate control. Statistical significance means $p \leq 0.05$. An increase in expression or activity can occur via any mechanism at any stage. For example, an increase in expression could occur via events or regulation that occurs during transcription, post-transcription, during translation, or post-translation.

The level and/or activity of *SUFU* mRNA and/or SUFU protein in a given subject is compared to a reference level. The term "reference level" is intended to mean a threshold expression or activity level in a control subject or control subject population. A reference level will depend on the assay performed and can be determined by one of ordinary skill in the art. A reference level may be a baseline level. In some embodiments, a reference level is the average expression or activity level of *SUFU* mRNA or SUFU protein in a random population of subjects. In some embodiments, multiple reference levels can be used. For example, a first reference level can be based on the average expression or activity level of *SUFU* mRNA or SUFU protein in one or more subjects with increased risk for developing a particular complication upon receiving an organ or tissue transplantation, and a second reference level can be based on the average expression or activity level in one or more subjects with decreased risk.

Although any method can be used to assay for a decreased expression or activity level, the expression or activity level of *SUFU* mRNA and/or SUFU protein is assayed in some specific embodiments by detecting in the genome of a subject the rs17114808 marker locus. "Marker" or "molecular marker" or "marker locus" is a term used to denote a nucleic acid or amino acid sequence that is sufficiently unique to characterize a specific locus in the genome. Any detectable polymorphic trait can be used as a marker so long as it is inherited differentially and exhibits linkage disequilibrium with a phenotypic trait of interest. The rs17114808 can be referred to as a single-nucleotide polymorphism (SNP). The term "polymorphism" refers to the occurrence of two or more alternative genomic sequences or alleles between or among different genomes or individuals. The term "single-nucleotide polymorphism" refers to a site of one nucleotide that varies between alleles.

An individual is "homozygous" if the individual has only one type of allele at a given locus (e.g., a diploid individual has a copy of the same allele at a locus in each of two homologous chromosomes). An individual is "heterozygous" if more than one allele type is present at a given locus (e.g., a diploid individual with one copy each of two different alleles).

"Allele" means any of one or more alternative forms of a genetic sequence. In a diploid cell or organism, the two alleles of a given sequence typically occupy corresponding loci on a pair of

homologous chromosomes. With regard to a SNP marker, allele refers to the specific nucleotide base present at that SNP locus in an individual.

As demonstrated herein, the rs17114808 marker locus is associated with susceptibility to GVHD that can arise following an organ or tissue transplantation. Subjects who are homozygous for a C-allele at rs17114808

(ACTGTTACAATACTTCAAGATCACTCTTTACACCTCTTCAAAGCAAAGTCAT)(SEQ ID NO: 4) have lower *SUFU* mRNA and *SUFU* protein levels and an increased risk for acute GVHD.

In contrast, subjects who are homozygous for a T-allele at rs17114808

(ACTGTTATAATACTTCAAGATCACTCTTTACACCTCTTCAAAGCAAAGTCAT) (SEQ ID NO: 3) and subjects who are heterozygous (T-allele and C-allele) have higher levels of *SUFU* mRNA and *SUFU* protein and a decreased risk for acute GVHD.

“Linkage” refers to a phenomenon wherein alleles on the same chromosome tend to segregate together more often than expected by chance if their transmission was independent. Genetic recombination occurs with an assumed random frequency over the entire genome. Genetic maps are constructed by measuring the frequency of recombination between pairs of traits or markers. The closer the traits or markers are to each other on the chromosome, the lower the frequency of recombination, and the greater the degree of linkage. Traits or markers are considered herein to be linked if they generally co-segregate. A 1/100 probability of recombination per generation is defined as a map distance of 1.0 centiMorgan (1.0 cM). The genetic elements or genes located on a single chromosome segment are physically linked. Two loci can be located in close proximity such that recombination between homologous chromosome pairs does not occur between the two loci during meiosis with high frequency, *e.g.*, such that linked loci co-segregate at least about 90% of the time, *e.g.*, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.75%, or more of the time. The genetic elements located within a chromosome segment are also genetically linked, typically within a genetic recombination distance of less than or equal to 50 centiMorgans (cM), *e.g.*, about 49, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.75, 0.5, or 0.25 cM or less. That is, two genetic elements within a single chromosome segment undergo recombination during meiosis with each other at a frequency of less than or equal to about 50%, *e.g.*, about 49%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1%, 0.75%, 0.5%, or 0.25% or less. “Closely linked” markers display a cross over frequency with a given marker of about 10% or less (the given marker is within about 10cM of a closely linked marker). Put another way, closely linked loci co-segregate at least about 90% of the time. Genetic linkage as evaluated by recombination frequency is impacted by the chromatin structure of the region comprising the loci. Typically, the region is assumed to have a

euchromatin structure during initial evaluations. However, some regions, such as regions closer to centrosomes, have a heterochromatin structure. Without further information, the predicted physical distance between genetic map positions is based on the assumption that the region is euchromatic, however if the region comprises heterochromatin the markers may be physically closer together.

5 With regard to physical position on a chromosome, closely linked markers can be separated, for example, by about 1 megabase (Mb; 1 million nucleotides), about 500 kilobases (Kb; 1000 nucleotides), about 400 Kb, about 300 Kb, about 200 Kb, about 100 Kb, about 50 Kb, about 25 Kb, about 10 Kb, about 5 Kb, about 2 Kb, about 1 Kb, about 500 nucleotides, about 250 nucleotides, or less.

10 In other specific embodiments, expression or activity level of *SUFU* mRNA and/or *SUFU* protein is assayed by detecting in the genome of a subject a marker locus that is closely linked to the rs17114804 marker locus or a marker locus that is in complete linkage disequilibrium with the rs17114808 marker locus. The term "linkage disequilibrium" refers to the nonrandom association between two or more alleles at two or more loci such that certain combinations of alleles are more
15 likely to occur together on a chromosome than other combinations of alleles. In other words, it refers to the occurrence in a population of two linked alleles at a frequency higher or lower than expected relative to the allele frequencies at the corresponding loci. For example, linked loci can co-segregate more than 50% of the time or up to 100% of the time. A second marker locus that is in "complete linkage disequilibrium" with a first marker locus is one that cannot be distinguished
20 from the first marker locus (i.e., the markers have not been separated by recombination and are transmitted/co-inherited together approximately 100% of the time).

An example of a marker locus in complete linkage disequilibrium with the rs17114808 marker locus is the rs17114803 marker locus. The rs17114803 marker locus is located on chromosome 10 and is a coding synonymous SNP in the *SUFU* gene.

25
i. Detecting *SUFU* Activity

Various methods and compositions for detecting *SUFU* activity are provided. Such methods can include assaying for known functions and capabilities of *SUFU*, such as those within the SHH signaling pathway. Such functions (examples of which are described above) are known,
30 as are assays for testing them. For example, one readout of *SUFU* activity could be a reporter assay measuring *SUFU*-mediated inhibition of *GLI*-mediated transcription.

Other methods of detecting *SUFU* activity can rely on assaying for *SUFU* regulation of SHH-mediated regulation of GVHD, as demonstrated in the Examples. For example, decreasing

expression of SUFU through silencing increases the expression of HLA-DR in antigen-presenting cells, whereas increasing expression of SUFU through ectopic expression decreases the expression of HLA-DR. Similarly, SUFU overexpression inhibits allogeneic T-cell proliferation, whereas silencing of SUFU increases it. Assays measuring these functional readouts are examples of methods of detecting SUFU activity.

ii. Detecting SUFU Polynucleotides

Various methods and compositions for detecting the mRNA levels of *SUFU* or for detecting the rs17114808 marker locus in the genomic DNA of a subject are provided.

In one embodiment, a method is provided for assaying a biological sample from a subject for the rs17114808 marker locus or a marker locus in complete linkage disequilibrium with the rs17114808 marker locus. Homozygosity with cytosine at rs17114808 indicates an increased risk for transplant rejection or GVHD, and heterozygosity with cytosine and thymine at rs17114808 or homozygosity with thymine at rs17114808 indicates decreased risk for transplant rejection or GVHD.

It is further recognized that the rs17114808 marker locus may be detected along with other markers in a multiplex or panel format. Markers are selected for their predictive value alone or in combination with the rs17114808 marker locus. Ultimately, the information provided by the methods disclosed herein will assist a physician in choosing the best course of treatment for a particular patient.

As used herein, the use of the term "polynucleotide" is not intended to limit the present invention to polynucleotides comprising DNA. Those of ordinary skill in the art will recognize that polynucleotides, can comprise ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The polynucleotides of the invention also encompass all forms of sequences including, for example, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

The term "gene" refers to a nucleic acid (e.g., DNA) sequence that comprises coding sequences necessary for the production of a polypeptide, precursor, or RNA (e.g., rRNA, tRNA). The term also encompasses the coding region of a structural gene and the sequences located adjacent to the coding region on both the 5' and 3' end which allow for the expression of the sequence. Sequences located 5' of the coding region and present on the mRNA are referred to as 5' non-translated sequences. Sequences located 3' or downstream of the coding region and present on

the mRNA are referred to as 3' non- translated sequences. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene that are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the mature messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

As used herein, the "nucleic acid complement" of a sample comprises any polynucleotide contained in the sample. The nucleic acid complement that is employed in the methods and compositions disclosed herein can include all of the polynucleotides contained in the sample or any fraction thereof. For example, the nucleic acid complement could comprise the genomic DNA and/or the mRNA and/or cDNAs of a given biological sample. Thus, the rs17114808 marker locus can be detected in the genomic DNA or, alternatively, the level and/or activity of *SUFU* mRNA or *SUFU* protein can be detected through the transcribed products thereof.

It is recognized that a biological sample can be processed differently depending on the assay being employed to detect the level and/or activity of *SUFU* mRNA or *SUFU* protein. For example, when detecting the rs17114808 marker locus, preliminary processing designed to isolate or enrich the sample for the genomic DNA can be employed. A variety of techniques known to those of ordinary skill in the art may be used for this purpose. When detecting the level of *SUFU* mRNA, different techniques can be used enrich the biological sample with mRNA. Various methods to detect the level of mRNA or the presence of the rs17114808 locus can be used.

As used herein, a "probe" is an isolated polynucleotide attached to a conventional detectable label or reporter molecule, such as a radioactive isotope, ligand, chemiluminescent agent, enzyme, or the like. Such a probe is complementary to a strand of a target polynucleotide, such as a polynucleotide comprising the rs17114808 marker locus or a polynucleotide that can detect *SUFU* mRNA. Deoxyribonucleic acid probes may include those generated by PCR using *SUFU* mRNA/cDNA specific primers or rs17114808 markers, oligonucleotide probes synthesized *in vitro*, or DNA obtained from bacterial artificial chromosome, fosmid, or cosmid libraries. Probes include not only deoxyribonucleic or ribonucleic acids but also polyamides and other probe materials that can specifically detect the presence of a target DNA sequence. For nucleic acid probes, examples of detection reagents include, for example, radiolabeled probes, enzymatic labeled probes (e.g., horse radish peroxidase and alkaline phosphatase), affinity labeled probes (e.g., biotin, avidin, and streptavidin), and fluorescent labeled probes (e.g., 6-FAM, VIC, TAMRA, MGB, fluorescein,

rhodamine, and texas red [for BAC/fosmids]). One skilled in the art will readily recognize that the nucleic acid probes described in the present invention can readily be incorporated into one of the established kit formats which are well known in the art.

As used herein, “primers” are isolated polynucleotides that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand and then be extended along the target DNA strand by a polymerase (e.g., a DNA polymerase). Primer pairs of the invention refer to their use for amplification of a target polynucleotide (e.g., by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods). “PCR” or “polymerase chain reaction” is a technique used for the amplification of specific DNA segments (see U.S. Pat. Nos. 4,683,195 and 4,800,159, herein incorporated by reference).

Probes and primers are of sufficient nucleotide length to bind to the target DNA sequence and specifically detect and/or identify a polynucleotide comprising the rs17114808 marker locus or comprising *SUFU* mRNA. It is recognized that the hybridization conditions or reaction conditions can be determined by the operator to achieve this result. This length may be of any length that is of sufficient length to be useful in a detection method of choice. Generally, 8, 11, 14, 16, 18, 20, 22, 24, 26, 28, 30, 40, 50, 75, 100, 200, 300, 400, 500, 600, or 700 nucleotides or more, or between about 11-20, 20-30, 30-40, 40-50, 50-100, 100-200, 200-300, 300-400, 400-500, 500-600, 600-700, 700-800, or more nucleotides in length are used. Such probes and primers can hybridize specifically to a target sequence under high stringency hybridization conditions. Probes and primers according to specific embodiments may have complete DNA sequence identity of contiguous nucleotides with the target sequence, although probes differing from the target DNA sequence and that retain the ability to specifically detect and/or identify a target DNA sequence may be designed by conventional methods. Accordingly, probes and primers can share about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity or complementarity to the target polynucleotide. Probes can be used as primers, but are generally designed to bind to the target DNA or RNA and are not used in an amplification process.

Specific primers can be used to amplify the rs17114808 marker locus and or *SUFU* mRNA to produce an amplicon that can be used as a “specific probe” or can itself be detected for identifying the rs17114808 marker locus or for determining the level of *SUFU* mRNA in a biological sample. When the probe is hybridized with the polynucleotides of a biological sample under conditions that allow for the binding of the probe to the sample, this binding can be detected and thus allow for an indication of the presence of the level of the *SUFU* expression in the

biological sample. Such identification of a bound probe has been described in the art. The specific probe may comprise a sequence of at least 80%, between 80% and 85%, between 85% and 90%, between 90% and 95%, and between 95% and 100% identical (or complementary) to a specific region of the *SUFU* gene, mRNA, or cDNA.

5 As used herein, “amplified DNA” or “amplicon” refers to the product of polynucleotide amplification of a target polynucleotide that is part of a nucleic acid template. For example, to determine whether the nucleic acid complement of a biological sample comprises the C-allele or the T-allele of the rs17114808 marker locus, the nucleic acid complement of the biological sample may be subjected to a polynucleotide amplification method using a primer pair that includes a first
10 primer derived from the 5' flanking sequence adjacent to the rs17114808 marker locus and a second primer derived from the 3' flanking sequence adjacent to the rs17114808 marker locus to produce an amplicon that is diagnostic for the presence of the C-allele or the T-allele of the rs17114808 marker locus. By “diagnostic” for the rs17114808 marker locus is intended the use of any method or assay which discriminates between the T-allele and the C-allele of the rs17114808 marker locus
15 in a biological sample. The amplicon is of a length and has a sequence that is also diagnostic for the rs17114808 marker locus. In some cases, the amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair to any length of amplicon producible by a DNA amplification protocol. A member of a primer pair derived from the flanking sequence may be located a distance from the junction or breakpoint. This distance can range from one nucleotide
20 base pair up to the limits of the amplification reaction, or about twenty thousand nucleotide base pairs. The use of the term “amplicon” specifically excludes primer dimers that may be formed in the DNA thermal amplification reaction.

Methods for preparing and using probes and primers are described, for example, in
Molecular Cloning: A Laboratory Manual, 2.sup.nd ed, vol. 1-3, ed. Sambrook *et al.*, Cold Spring
25 Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989 (hereinafter, “Sambrook *et al.*, 1989”);
Current Protocols in Molecular Biology, ed. Ausubel *et al.*, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates) (hereinafter, “Ausubel *et al.*, 1992”); and
Innis *et al.*, *PCR Protocols: A Guide to Methods and Applications*, Academic Press: San Diego, 1990. PCR primer pairs can be derived from a known sequence, for example, by using computer
30 programs intended for that purpose such as the PCR primer analysis tool in Vector NTI version 10 (Informax Inc., Bethesda Md.); PrimerSelect (DNASTAR Inc., Madison, Wis.); and Primer3 (Version 0.4.0.COPYRGT., 1991, Whitehead Institute for Biomedical Research, Cambridge,

Mass.). Additionally, the sequence can be visually scanned and primers manually identified using guidelines known to one of skill in the art.

As outline in further detail below, any conventional nucleic acid hybridization or amplification or sequencing method can be used to specifically detect the presence of the rs17114808 marker locus and/or the level of the *SUFU* mRNA. By “specifically detect” is intended that the polynucleotide can be used either as a primer to amplify a region of the *SUFU* polynucleotide or the polynucleotide can be used as a probe that hybridizes under stringent conditions to a polynucleotide comprising the rs17114808 marker locus or a polynucleotide comprising the *SUFU* mRNA or cDNA. By “shares sufficient sequence identity or complementarity to allow for the amplification of a rs17114808 marker locus or a *SUFU* polynucleotide” is intended the sequence shares at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity or complementarity to a fragment or across the full length of the rs17114808 marker locus or to the *SUFU* polynucleotide.

A variety of nucleic acid techniques known to those of ordinary skill in the art, including, for example, nucleic acid sequencing, nucleic acid hybridization, and nucleic acid amplification. Nucleic acid hybridization includes methods using labeled probes directed against purified DNA, amplified DNA, and fixed leukemia cell preparations (fluorescence *in situ* hybridization). Illustrative examples of nucleic acid sequencing techniques include, for example, chain terminator (Sanger) sequencing and dye terminator sequencing. Chain terminator sequencing uses sequence-specific termination of a DNA synthesis reaction using modified nucleotide substrates. Extension is initiated at a specific site on the template DNA by using a short radioactive (or otherwise labeled) oligonucleotide primer complementary to the template at that region. The oligonucleotide primer is extended using a DNA polymerase, standard four deoxynucleotide bases, and a low concentration of one chain terminating nucleotide, most commonly a di-deoxynucleotide. This reaction is repeated in four separate tubes with each of the bases taking turns as the di-deoxynucleotide. Limited incorporation of the chain terminating nucleotide by the DNA polymerase results in a series of related DNA fragments that are terminated only at positions where that particular di-deoxynucleotide is used. For each reaction tube, the fragments are size-separated by electrophoresis in a slab polyacrylamide gel or a capillary tube filled with a viscous polymer. The sequence is determined by reading which lane produces a visualized mark from the labeled primer as you scan from the top of the gel to the bottom. Dye terminator sequencing alternatively labels the terminators. Complete sequencing can be performed in a single reaction by labeling each of the

di-deoxynucleotide chain-terminators with a separate fluorescent dye, which fluoresces at a different wavelength.

The present invention further provides methods for identifying nucleic acids which do not necessarily require sequence amplification and are based on, for example, the known methods of Southern (DNA:DNA) blot hybridizations, *in situ* hybridization, and FISH of chromosomal material, using appropriate probes.

In situ hybridization (ISH) is a type of hybridization that uses a labeled complementary DNA or RNA strand as a probe to localize a specific DNA or RNA sequence in a portion or section of tissue (*in situ*), or, if the tissue is small enough, the entire tissue (whole mount ISH). DNA ISH can be used to determine the structure of chromosomes. Sample cells and tissues are usually treated to fix the target transcripts in place and to increase access of the probe. The probe hybridizes to the target sequence at elevated temperature, and then the excess probe is washed away. The probe that was labeled with either radio-, fluorescent- or antigen-labeled bases is localized and quantitated in the tissue using autoradiography, fluorescence microscopy, or immunohistochemistry, respectively. ISH can also use two or more probes, labeled with radioactive or non-radioactive labels, to simultaneously detect two or more transcripts. In some embodiments, fluorescence *in situ* hybridization (FISH) is employed.

In specific embodiments, probes for detecting the rs17114808 marker locus or *SUFU* polynucleotides are labeled with appropriate fluorescent or other markers and then used in hybridizations. The Examples section provided herein sets forth various protocols that are effective for detecting the genomic abnormalities, but one of skill in the art will recognize that many variations of these assays can also be used. Specific protocols are well known in the art and can be readily adapted for the present invention. Guidance regarding methodology may be obtained from many references including: *In situ Hybridization: Medical Applications* (eds. G. R. Coulton and J. de Belleruche), Kluwer Academic Publishers, Boston (1992); *In situ Hybridization: hi Neurobiology; Advances in Methodology* (eds. J. H. Eberwine, K. L. Valentino, and J. D. Barchas), Oxford University Press Inc., England (1994); *In situ Hybridization: A Practical Approach* (ed. D. G. Wilkinson), Oxford University Press Inc., England (1992)); Kuo *et al.* (1991) *Am. J. Hum. Genet.* 42:112-119; Klinger *et al.* (1992) *Am. J. Hum. Genet.* 51:55-65; and Ward *et al.* (1993) *Am. J. Hum. Genet.* 52:854-865). There are also kits that are commercially available and that provide protocols for performing FISH assays (available from e.g., Oncor, Inc., Gaithersburg, MD). Patents providing guidance on methodology include U.S. 5,225,326; 5,545,524; 6,121,489 and 6,573,043. All of these references are hereby incorporated by reference in their entirety and may be used along

with similar references in the art and with the information provided in the Examples section herein to establish procedural steps convenient for a particular laboratory.

Southern blotting can be used to detect specific DNA sequences. In such methods, DNA that is extracted from a sample is fragmented, electrophoretically separated on a matrix gel, and transferred to a membrane filter. The filter bound DNA is subject to hybridization with a labeled probe complementary to the sequence of interest. Hybridized probe bound to the filter is detected.

In hybridization techniques, all or part of a polynucleotide that selectively hybridizes to a target polynucleotide comprising the *SUFU* polynucleotide or the rs17114808 marker locus is employed. "Stringent conditions" or "stringent hybridization conditions," when referring to a polynucleotide probe, is intended to refer to conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of identity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length or less than 500 nucleotides in length.

As used herein, a substantially identical or complementary sequence is a polynucleotide that will specifically hybridize to the complement of the nucleic acid molecule to which it is being compared under high stringency conditions. Appropriate stringency conditions which promote DNA hybridization, for example, 6X sodium chloride/sodium citrate (SSC) at about 45° C., followed by a wash of 2X SSC at 50° C., are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Typically, stringent conditions for hybridization and detection will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50%

formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours. The duration of the wash time will be at least a length of time sufficient to reach equilibrium.

5 In hybridization reactions, specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\% \text{ GC}) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine
10 nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if
15 sequences with $\geq 90\%$ identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1°C, 2°C, 3°C, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6°C,
20 7°C, 8°C, 9°C, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11°C, 12°C, 13°C, 14°C, 15°C, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less
25 than 45°C (aqueous solution) or 32°C (formamide solution), it is optimal to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A
30 Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York) and Haymes *et al.* (1985) In: *Nucleic Acid Hybridization, a Practical Approach*, IRL Press, Washington, D.C.

A polynucleotide is said to be the “complement” of another polynucleotide if they exhibit complementarity. As used herein, molecules are said to exhibit “complete complementarity” when every nucleotide of one of the polynucleotide molecules is complementary to a nucleotide of the other. Two molecules are said to be “minimally complementary” if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional “low-stringency” conditions. Similarly, the molecules are said to be “complementary” if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional “high-stringency” conditions.

Regarding the amplification of a target polynucleotide (e.g., by PCR) using a particular amplification primer pair, “stringent conditions” are conditions that permit the primer pair to hybridize to the target polynucleotide to which a primer having the corresponding sequence (or its complement) would bind and preferably to produce an identifiable amplification product (the amplicon) having a region of a *SUFU* polynucleotide or the rs17114808 marker locus in a DNA thermal amplification reaction. In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify a region of the *SUFU* polynucleotide or the rs17114808 marker locus. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Methods of amplification are further described in US Patent No. 4,683,195, 4,683,202 and Chen *et al.* (1994) *PNAS* 91:5695-5699. These methods as well as other methods known in the art of DNA amplification may be used in the practice of the embodiments of the present invention. It is understood that a number of parameters in a specific PCR protocol may need to be adjusted to specific laboratory conditions and may be slightly modified and yet allow for the collection of similar results. These adjustments will be apparent to a person skilled in the art.

The amplified polynucleotide (amplicon) can be of any length that allows for the detection of the rs17114808 marker locus or for the detection of the *SUFU* polynucleotide. For example, the amplicon can be about 10, 50, 100, 200, 300, 500, 700, 100, 2000, 3000, 4000, or 5000 nucleotides in length or longer.

Any primer can be employed in the methods of the invention that allows the rs17114808 marker locus or a region of the *SUFU* polynucleotide to be amplified and/or detected. Methods for

designing PCR primers are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Other known methods of PCR that can be used in the methods of the invention include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, mixed DNA/RNA primers, vector-specific primers, partially mismatched primers, and the like.

Thus, in specific embodiments, a method of detecting the presence of the rs17114808 marker locus in a biological sample is provided. The method comprises: (a) providing a sample comprising the nucleic acid complement of a subject; (b) providing a pair of DNA primer molecules that can amplify an amplicon having the rs17114808 marker locus; (c) providing DNA amplification reaction conditions; (d) performing the DNA amplification reaction, thereby producing a DNA amplicon molecule; and (e) detecting the DNA amplicon molecule. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

In other embodiments, a method of detecting the level of the *SUFU* polynucleotide in a biological sample is provided. The method comprises: (a) providing a sample comprising the nucleic acid complement of a subject; (b) providing a pair of DNA primer molecules that can amplify an amplicon having the *SUFU* polynucleotide; (c) providing DNA amplification reaction conditions; (d) performing the DNA amplification reaction, thereby producing a DNA amplicon molecule; and (e) detecting the DNA amplicon molecule. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

In still other embodiments, the rs17114808 marker locus or the *SUFU* polynucleotide may be amplified prior to or simultaneous with detection. Illustrative examples of nucleic acid amplification techniques include, for example, polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), and nucleic acid sequence based amplification (NASBA). The polymerase chain reaction (U.S. Pat. Nos. 4,683,195, 4,683,202, 4,800,159 and 4,965,188, each of which is herein incorporated by reference in its entirety),

commonly referred to as PCR, uses multiple cycles of denaturation, annealing of primer pairs to opposite strands, and primer extension to exponentially increase copy numbers of a target nucleic acid sequence. For other various permutations of PCR see, e.g., U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,800,159; Mullis *et al.*, (1987) *Meth. Enzymol.* 155: 335; and, Murakawa *et al.*, (1988) *DNA* 7: 287, each of which is herein incorporated by reference in its entirety.

The ligase chain reaction (Weiss (1991) *Science* 254: 1292, herein incorporated by reference in its entirety), strand displacement amplification (Walker *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89: 392-396; U.S. Pat. No. 5,270,184; and U.S. Pat. No. 5,455,166; each of which is herein incorporated by reference in its entirety), and thermophilic SDA (tSDA) (EP Pat. No. 0 684 315, incorporated by reference in its entirety) are known methods which can be employed in detecting the level and/or activity of *SUFU*.

Any method can be used for detecting either the non-amplified or amplified polynucleotides including, for example, Hybridization Protection Assay (HPA) (U.S. Pat. No. 5,283,174 and Nelson *et al.* (1995) *Nonisotopic Probing, Blotting, and Sequencing*, ch. 17 (Larry J. Kricka ed., 2d ed.), each of which is herein incorporated by reference in its entirety); quantitative evaluation of the amplification process in real-time (U.S. Pat. Nos. 6,303,305 and 6,541,205, each of which is herein incorporated by reference in its entirety); and determining the quantity of target sequence initially present in a sample, but which is not based on a real-time amplification (U.S. Pat. No. 5,710,029, herein incorporated by reference in its entirety).

Amplification products may be detected in real-time through the use of various self-hybridizing probes, most of which have a stem-loop structure. Such self-hybridizing probes are labeled so that they emit differently detectable signals, depending on whether the probes are in a self-hybridized state or an altered state through hybridization to a target sequence. For example, "molecular torches" are a type of self-hybridizing probe that includes distinct regions of self-complementarity (referred to as "the target binding domain" and "the target closing domain") which are connected by a joining region (e.g., non-nucleotide linker) and which hybridize to each other under predetermined hybridization assay conditions. In a preferred embodiment, molecular torches contain single-stranded base regions in the target binding domain that are from 1 to about 20 bases in length and are accessible for hybridization to a target sequence present in an amplification reaction under strand displacement conditions. Under strand displacement conditions, hybridization of the two complementary regions, which may be fully or partially complementary, of the molecular torch is favored, except in the presence of the target sequence, which will bind to the single-stranded region present in the target binding domain and displace all or a portion of the

target closing domain. The target binding domain and the target closing domain of a molecular torch include a detectable label or a pair of interacting labels (e.g., luminescent/quencher) positioned so that a different signal is produced when the molecular torch is self-hybridized than when the molecular torch is hybridized to the target sequence, thereby permitting detection of probe:target duplexes in a test sample in the presence of unhybridized molecular torches. Molecular torches and a variety of types of interacting label pairs are disclosed in U.S. Pat. No. 6,534,274, herein incorporated by reference in its entirety.

Another example of a detection probe having self-complementarity is a "molecular beacon." Molecular beacons include nucleic acid molecules having a target complementary sequence, an affinity pair (or nucleic acid arms) holding the probe in a closed conformation in the absence of a target sequence present in an amplification reaction, and a label pair that interacts when the probe is in a closed conformation. Hybridization of the target sequence and the target complementary sequence separates the members of the affinity pair, thereby shifting the probe to an open conformation. The shift to the open conformation is detectable due to reduced interaction of the label pair, which may be, for example, a fluorophore and a quencher (e.g., DABCYL and EDANS). Molecular beacons are disclosed in U.S. Pat. Nos. 5,925,517 and 6,150,097, herein incorporated by reference in their entireties.

Other self-hybridizing probes are well known to those of ordinary skill in the art. By way of non-limiting example, probe binding pairs having interacting labels, such as those disclosed in U.S. Pat. No. 5,928,862 (herein incorporated by reference in its entirety) might be adapted for use in the present invention. Probe systems used to detect single nucleotide polymorphisms (SNPs) might also be utilized in the present invention. Additional detection systems include "molecular switches," as disclosed in U.S. Publ. No. 20050042638, herein incorporated by reference in its entirety. Other probes, such as those comprising intercalating dyes and/or fluorochromes, are also useful for detection of amplification products in the present invention. See, e.g., U.S. Pat. No. 5,814,447 (herein incorporated by reference in its entirety).

Various methods can be used to detect the polynucleotide of interest, including, for example, Genetic Bit Analysis (Nikiforov *et al.* (1994) *Nucleic Acid Res.* 22: 4167-4175) where a DNA oligonucleotide is designed which overlaps both the adjacent flanking DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking sequence) a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA

polymerase and labeled ddNTPs specific for the expected next base. Readout may be fluorescent or ELISA-based. A signal indicates presence of the insert/flanking sequence due to successful amplification, hybridization, and single base extension.

Another detection method is the Pyrosequencing technique as described by Winge ((2000) *Innov. Pharma. Tech.* 00: 18-24). In this method, an oligonucleotide is designed that overlaps the junction. The oligonucleotide is hybridized to a single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. dNTPs are added individually and the incorporation results in a light signal which is measured. A light signal indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single or multi-base extension.

Fluorescence Polarization as described by Chen *et al.* ((1999) *Genome Res.* 9: 492-498) is also a method that can be used to detect an amplicon of the invention. Using this method, an oligonucleotide is designed which overlaps the inserted DNA junction. The oligonucleotide is hybridized to a single-stranded PCR product from the region of interest (one primer in the inserted DNA and one in the flanking DNA sequence) and incubated in the presence of a DNA polymerase and a fluorescent-labeled ddNTP. Single base extension results in incorporation of the ddNTP. Incorporation can be measured as a change in polarization using a fluorometer. A change in polarization indicates the presence of the genomic abnormality sequence due to successful amplification, hybridization, and single base extension.

Taqman® (PE Applied Biosystems, Foster City, Calif.) is a method of detecting and quantifying the presence of a DNA sequence and is fully understood in the instructions provided by the manufacturer. Briefly, a FRET oligonucleotide probe is designed which overlaps the junction. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Hybridization of the FRET probe results in cleavage and release of the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

In one embodiment, the method of detecting a rs17114808 marker locus or the *SUFU* polynucleotide comprises: (a) contacting the biological sample with a polynucleotide probe that hybridizes under stringent hybridization conditions with a *SUFU* polynucleotide and specifically detects the *SUFU* polynucleotide; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the polynucleotide, wherein detection of

hybridization indicates the level of the *SUFU* polynucleotide or the presence of the C-allele or the T-allele of the rs17114808 marker locus.

iii. Detecting *SUFU* Polypeptides

5 The level and/or activity of the *SUFU* polypeptide may be detected using a variety of protein techniques known to those of ordinary skill in the art, including, for example, protein sequencing and immunoassays.

10 Illustrative non-limiting examples of protein sequencing techniques include, for example, mass spectrometry and Edman degradation. Mass spectrometry can, in principle, sequence any size protein but becomes computationally more difficult as size increases. A protein is digested by an endoprotease, and the resulting solution is passed through a high pressure liquid chromatography column. At the end of this column, the solution is sprayed out of a narrow nozzle charged to a high positive potential into the mass spectrometer. The charge on the droplets causes them to fragment until only single ions remain. The peptides are then fragmented and the mass-charge ratios of the
15 fragments measured. The mass spectrum is analyzed by computer and often compared against a database of previously sequenced proteins in order to determine the sequences of the fragments. The process is then repeated with a different digestion enzyme, and the overlaps in sequences are used to construct a sequence for the protein.

20 In the Edman degradation reaction, the peptide to be sequenced is adsorbed onto a solid surface (e.g., a glass fiber coated with polybrene). The Edman reagent, phenylisothiocyanate (PTC), is added to the adsorbed peptide, together with a mildly basic buffer solution of 12% trimethylamine, and reacts with the amine group of the C-terminal amino acid. The terminal amino acid derivative can then be selectively detached by the addition of anhydrous acid. The derivative isomerizes to give a substituted phenylthiohydantoin, which can be washed off and identified by
25 chromatography, and the cycle can be repeated. The efficiency of each step is about 98%, which allows about 50 amino acids to be reliably determined.

30 Illustrative examples of immunoassays include, for example, immunoprecipitation, Western blot, ELISA, immunohistochemistry, immunocytochemistry, flow cytometry, and immuno-PCR. Polyclonal or monoclonal antibodies detectably labeled using various techniques known to those of ordinary skill in the art (e.g., calorimetric, fluorescent, chemiluminescent or radioactive) are suitable for use in the immunoassays. Antibodies against *SUFU* are known in the art.

 Immunoprecipitation is a technique of precipitating an antigen out of solution using an antibody specific to that antigen. The process can be used to identify protein complexes present in

cell extracts by targeting a protein believed to be in the complex. The complexes are brought out of solution by insoluble antibody-binding proteins isolated initially from bacteria, such as Protein A and Protein G. The antibodies can also be coupled to sepharose beads that can easily be isolated out of solution. After washing, the precipitate can be analyzed using mass spectrometry, Western blotting, or any number of other methods for identifying constituents in the complex.

A Western blot, or immunoblot, is a method to detect protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate denatured proteins by mass. The proteins are then transferred out of the gel and onto a membrane, typically polyvinylidene difluoride or nitrocellulose, where they are probed using antibodies specific to the protein of interest. As a result, researchers can examine the amount of protein in a given sample and compare levels between several groups.

An ELISA, short for Enzyme-Linked Immunosorbent Assay, is a biochemical technique to detect the presence of an antibody or an antigen in a sample. It utilizes a minimum of two antibodies, one of which is specific to the antigen and the other of which is coupled to an enzyme. The second antibody will cause a chromogenic or fluorogenic substrate to produce a signal. Variations of ELISA include sandwich ELISA, competitive ELISA, and ELISPOT. Because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a useful tool both for determining serum antibody concentrations and also for detecting the presence of antigen.

Immunohistochemistry and immunocytochemistry refer to the process of localizing proteins in a tissue section or cell, respectively, via the principle of antigens in tissue or cells binding to their respective antibodies. Visualization is enabled by tagging the antibody with, for example, color producing or fluorescent tags. Typical examples of color tags include, for example, horseradish peroxidase and alkaline phosphatase. Typical examples of fluorophore tags include, for example, fluorescein isothiocyanate (FITC) or phycoerythrin (PE).

Flow cytometry is a technique for counting, examining, and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical/electronic detection apparatus. A beam of light (e.g., a laser) of a single frequency or color is directed onto a hydrodynamically focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). Each suspended particle passing through the beam scatters the light in some way, and fluorescent

chemicals in the particle may be excited into emitting light at a lower frequency than the light source. The combination of scattered and fluorescent light is picked up by the detectors, and by analyzing fluctuations in brightness at each detector, one for each fluorescent emission peak, it is possible to deduce various facts about the physical and chemical structure of each individual particle. FSC correlates with the cell volume and SSC correlates with the density or inner complexity of the particle (e.g., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness).

Immuno-polymerase chain reaction (IPCR) utilizes nucleic acid amplification techniques to increase signal generation in antibody-based immunoassays. Because no protein equivalence of PCR exists, that is, proteins cannot be replicated in the same manner that nucleic acid is replicated during PCR, the only way to increase detection sensitivity is by signal amplification. The target proteins are bound to antibodies which are directly or indirectly conjugated to oligonucleotides. Unbound antibodies are washed away and the remaining bound antibodies have their oligonucleotides amplified. Protein detection occurs via detection of amplified oligonucleotides using standard nucleic acid detection methods, including real-time methods. Methods to assay for SUFU polypeptide activity are known. See, for example, Oshimori *et al.* (2009) *The EMBO Journal* 28: 2066–2076, which describes methods to assay for maintenance of microtubule-organizing activity and structural integrity of the centrosome.

D. Personalized Prophylaxis or Treatment Regimens

Methods are provided for administering a personalized prophylaxis or treatment regimen to a subject based on an assessed risk for developing a complication upon receiving an organ or tissue transplantation. Such complications can result, for example, from an adaptive immune response. The assessed risk is based on *SUFU* mRNA and/or *SUFU* protein expression or activity, as described in detail above.

As used herein, “personalized prophylaxis or treatment regimen” refers to a prophylaxis or treatment regimen that is tailored to a subject’s risk assessment. The risk assessment is based on *SUFU* mRNA and/or *SUFU* protein expression or activity. However, it can further be based on other factors. For example, it can further be based on the degree of human leukocyte antigen (HLA) mismatch between the recipient subject and the donor providing the organ or tissue to be transplanted.

Prophylactic strategies to reduce organ or tissue transplant rejection or GVHD can involve selection of the optimal available donor and graft type and post-transplantation

immunosuppression, and therapeutic strategies to treat organ or tissue transplant rejection or GVHD can involve immunosuppression strategies.

i. Donor/Recipient Matching

In some embodiments, a personalized prophylaxis regimen can be based on donor/recipient matching. For example, a subject with increased risk for a complication (e.g., transplant rejection or GVHD) can receive a more perfectly matched donor, whereas a subject with decreased risk for a complication can receive a less perfectly matched donor. Donor/recipient matching is “more perfectly matched” when the parameters used for matching are more stringent than they would be without a risk assessment based on *SUFU* mRNA and/or *SUFU* protein expression or activity. In some embodiments, more perfectly matched means that fewer HLA mismatches as described below are tolerated (e.g., 1 fewer, 2 fewer, 3 fewer, or more). Donor/recipient matching is “less perfectly matched” when the parameters used for matching are less stringent than they would be without a risk assessment based on *SUFU* mRNA and/or *SUFU* protein expression or activity. In some embodiments, less perfectly matched means that more HLA mismatches as described below are tolerated (e.g., 1 more, 2 more, 3 more, or more). In other embodiments, a donor that is “more perfectly matched” or “less perfectly matched” is based on other donor/recipient crossmatching parameters.

Some form of tissue typing or crossmatching is performed prior to organ or tissue transplantation to assess, for example, donor-recipient compatibility for human leukocyte antigen (HLA) and ABO blood group. See, e.g., Mullally & Ritz (2007) *Blood* 109:1355-1362; Warren *et al.* (2012) *Blood* 120:2796-2806. These tests can include, for example, the following: (1) testing ABO blood group compatibility; (2) testing of recipient sera for reactivity with donor lymphocytes (a positive crossmatch can be a contraindication to transplantation); (3) panel-reactive antibody (PRA) screening of the serum of a recipient for lymphocytic antibodies against a random cell panel; and/or (5) mixed lymphocyte reaction (MLR) testing to assess the degree of major histocompatibility complex (MHC) class I and class II compatibility.

In addition, for HSCT, the relative risk for GVHD increases from bone marrow-derived HSCT to peripheral blood HSCT to umbilical cord HSCT. HLA mismatching increases the incidence of GVHD.

Donor/recipient matching is largely based on human leukocyte antigen (HLA) typing. HLAs are expressed on the surface of various cells, in particular white blood cells (WBCs). These antigens are also known as the major histocompatibility complex (MHC) and occupy the short arm of chromosome 6. See, e.g., Tiercy (2002) *Transpl Immunol.* 9:173-180. This genetic region has

been divided into chromosomal regions, called classes. Classes I, II, and III have been defined. Class I is made up of HLA-A, HLA-B, and HLA-C, as well as HLA-E, HLA-F, and HLA-G. Class II is made up of HLA-DR, HLA-DP, and HLA-DQ, as well as variations on these genes. Traditionally, the loci critical for matching have been HLA-A, HLA-B, and HLA-DR. HLA-C and HLA-DQ are also now considered when determining the appropriateness of a donor.

The relative importance of various HLA loci has been addressed in various studies. For example, one study considered matching for HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1, HLA-DQA1, HLA-DPB1, and HLA-DPA1 in the context of HSCT. See, e.g., Lee *et al.* (2007) *Blood* 110:4576-4582. The minimum level of matching that was reported to be associated with the highest survival was no mismatches. A single mismatch at HLA-A, HLA-B, HLA-C, or HLA-DRB1 was reported to be associated with higher mortality. Single mismatches at HLA-B and HLA-C were reported to be better tolerated than mismatches at HLA-A or HLA-DRB1. Mismatches at HLA-DP or HLA-DQ loci were reported to not be associated with survival. Thus, matching for the HLA-A, HLA-B, HLA-C, and HLA-DRB1 alleles appeared to be most critical.

In the context of HSCT, a completely matched sibling donor is generally considered the ideal donor. For unrelated donors, a complete match or a single mismatch is considered acceptable for most transplantation, although in certain circumstances, a greater mismatch is tolerated. Umbilical cord HSCT cell sources have historically been thought of as “immunologically naive” and matched for HLA-A, HLA-B, and HLA-DRB1, without consideration of HLA-C. Some recent data suggest that mismatch of HLA-C is an independent risk factor for transplant-related mortality, and this area is under investigation. See, e.g., Eapen *et al.* (2011) *Lancet Oncol.* 12:1214-1221.

Other genetic loci currently being studied include the ligands for natural killer cells known as killer immunoglobulin-like receptors (KIRs). A KIR, along with its HLA-C ligand, is part of the biological process to prime natural killer cells to attack non-self-intruders such as leukemia cells. KIR and HLA-C mismatching between donor and HSCT recipient has been associated with reduced post-transplantation relapse of leukemia. See, e.g., Venstrom *et al.* (2012) *N. Engl. J. Med.* 367:805-816.

The selection of the donor graft is multifactorial and can include, for example, the following: (1) availability of a matched sibling donor; (2) survival and disease control data for specific illnesses with different graft sources; (3) the urgency to move ahead with the transplantation (e.g., an urgent transplantation may preclude a matched unrelated donor, which can take time to identify and procure a donor); (4) speed of engraftment; (5) risk of GVHD; (6) need for

a subsequent graft from the same donor; (7) transplant center preference; and (8) availability of identical twin donors.

In rare instances, patients who are candidates for transplantation have an identical twin who can serve as a donor. These patients do not require post-transplantation immunosuppressive therapy and do not develop GVHD.

Matched, related donors are usually siblings, because they have the opportunity to inherit the same HLA genes located on chromosome 6. A given sibling has a 25% chance of being HLA matched at the A, B, and DRB1 loci (a 6-antigen match, because each complex is inherited from each parent and expressed codominantly). Finding matched, related donors other than siblings is unlikely unless the patient's parents happen to have very common haplotypes or intermarrying among families has occurred such that first cousins are fully HLA matched.

If a donor and recipient are not related, serologic typing alone does not ensure that the individuals share the same HLA genes. This is evident clinically by the higher risk of GVHD in recipients of unrelated donor grafts. DNA-based techniques for molecular typing have demonstrated that only 55% of serologically identical donor and recipient pairs (i.e., antigen matched) are highly matched by molecular typing (i.e., allele matched). Patients who are truly highly matched appear to have better outcomes. As a result, most transplantation centers now require complete serologic and molecular matching at the class II region before using a donor for a given transplantation procedure.

One study reported that when nonmalignant diseases are treated with HSCT using unrelated donors, HLA mismatches are associated with graft failure but not with GVHD. Reviewing 663 HSCTs that used bone marrow or peripheral blood stem cells from donors who were not related to the transplant recipients, the investigators reported a link between patient mortality and HLA-A, HLA-B, HLA-C, and HLA-DRB1, but not HLA-DQB1 or HLA-DPB1, mismatches. See, e.g., Horan *et al.* (2012) *Blood* 120:2918-2924.

Although most centers require a complete match at the HLA-A, HLA-B, and HLA-DRB1 loci for an individual to be used as a transplant donor, some centers consider the use of single antigen-mismatched siblings. As expected, transplants from such donors are associated with a higher risk of GVHD, although the overall survival rate may not differ from that observed with fully matched siblings.

Transplantation centers have been exploring the use of donors who are only haploidentical and are therefore mismatched at all 3 loci. These grafts must be manipulated *in vitro* to reduce the number of immunocompetent T cells and, therefore, to lessen the likelihood and severity of GVHD.

One potential advantage of using haploidentical donors is that multiple individuals are usually available who could serve as potential donors within a given family, including parents, siblings, and children. Another advantage is an equal availability of donors for all ethnic and racial groups, in contrast to the availability of matched, unrelated donors. In haploidentical transplantations, mismatching of maternal antigens, rather than paternal antigens, seems to be better tolerated, presumably because of exposure to maternal HLA antigens during the prenatal and perinatal period.

HSCT studies in acute myeloid leukemia have been reported to show comparable survival in matched sibling transplantation and matched unrelated transplantation. The 7/8 matched unrelated donor HSCTs were reported to show higher early mortality, but comparable long-term survival compared with matched related donors and matched unrelated donors. It was reported that matched related HSCTs have the lowest frequency of GVHD. See, e.g., Saber *et al.* (2012) *Blood* 119:3908-3916.

Cord blood transplantation can also be performed. Owing to the relative immaturity of the immune system in cord samples, stem cells from this source allow the crossing of immunologic barriers that would otherwise be prohibitive. As a result, the degree of tolerable HLA disparity is much greater in cord blood transplants. A match of 3-4 out of the 6 HLA-A, HLA-B and HLA-DRB1 antigens is sufficient for transplantation. For the same reason, the degree and severity of GVHD are low following cord blood transplants.

The advantages of cord blood transplant include the fact that it is readily available, carries less risk of transmission of blood-borne infections, and is transplantable across HLA barriers with diminished risk of GVHD, compared with similarly mismatched stem cells from peripheral blood or bone marrow. See, e.g., Koh (2004) *Ann. Acad. Med. Singapore* 33:559-569. A major limitation, however, is the relatively small volume obtained from cord blood collections. This makes using this approach difficult for transplantation in adults, since the small volume results in delayed engraftment and increased risk of infections and mortality. In addition, some evidence suggests increased occurrence of engraftment failure in umbilical cord HSCT.

In one retrospective analysis of 1525 adults with leukemia treated with primarily 4/6 matched umbilical cord HSCT versus peripheral blood or bone marrow 8/8 or 7/8 matched unrelated HSCT, no difference was reported in leukemia-free survival in all arms. Umbilical cord transplants were reported to be associated with later engraftment and higher treatment-related mortality, despite having lower incidents of GVHD. See, e.g., Eapen *et al.* (2010) *Lancet Oncol.* 11:653-660. Most of the umbilical cord transplants were 4/6 matches, and there is evidence that closer HLA matching in umbilical cord transplants improves outcomes.

ii. Prophylaxis and Treatment Regimens

In some embodiments, a personalized prophylaxis or treatment regimen can be based on prophylaxis or treatment regimens that are typically received when receiving an organ or tissue transplantation. A prophylaxis regimen refers to a measure or procedure for which the purpose is to prevent rather than to treat a disease or complication. An example of such a measure is using more stringent donor/recipient matching parameters, as described above. Thus, in a prophylactic application, a prophylaxis regimen is administered to a subject susceptible to or otherwise at risk of developing a complication resulting upon receiving an organ or tissue transplantation. A therapeutic regimen or treatment regimen refers to a measure or procedure for which the purpose is to treat a disease or complication, e.g., by arresting or slowing the progression of the disease or reducing the manifestation, extent, or severity of one or more clinical symptoms. Thus, in a treatment application, a treatment regimen is administered to a subject suspected of having, or already having, a complication resulting from an organ or tissue transplantation.

In specific embodiments, the personalized prophylaxis or treatment regimen can be based on immunosuppression regimens. For example, a subject with increased risk for a complication (e.g., transplant rejection or GVHD) can receive a more intensive prophylaxis or treatment regimen (e.g., an immunosuppression regimen), whereas a subject with decreased risk for a complication can receive a less intensive prophylaxis or treatment regimen.

A prophylaxis or treatment regimen or is “more intensive” when it is designed to provide a stronger prophylactic or therapeutic effect than the prophylaxis or treatment regimen that would have been provided to the subject without a risk assessment based on *SUFU* mRNA and/or *SUFU* protein expression or activity. Likewise, an immunosuppression regimen or is “more intensive” when it is designed to provide a stronger immunosuppressive effect than the immunosuppression regimen that would have been provided to the subject without a risk assessment based on *SUFU* mRNA and/or *SUFU* protein expression or activity.

In some embodiments, a more intensive regimen is one that is provided over a longer period of time, or with increased dosages, and/or at an increased frequency. In some embodiments, a more intensive regimen is one that includes stronger prophylactic, therapeutic, or immunosuppressive agents. In some embodiments, a more intensive regimen is one that includes a greater number of prophylactic, therapeutic, or immunosuppressive agents.

A prophylaxis or treatment regimen is “less intensive” when it is designed to provide a weaker prophylactic or therapeutic effect than the prophylaxis or treatment regimen that would

have been provided to the subject without a risk assessment based on *SUFU* mRNA and/or *SUFU* protein expression or activity. Likewise, an immunosuppression regimen or is “less intensive” when it is designed to provide a weaker immunosuppressive effect than the immunosuppression regimen that would have been provided to the subject without a risk assessment based on *SUFU* mRNA and/or *SUFU* protein expression or activity.

In some embodiments, a less intensive regimen is one that is provided over a shorter period of time, or with decreased dosages, and/or at a decreased frequency. In some embodiments, a less intensive regimen is one that includes weaker prophylactic, therapeutic, or immunosuppressive agents. In some embodiments, a less intensive regimen is one that includes a smaller number of prophylactic, therapeutic, or immunosuppressive agents.

Prophylactic and treatment regimens for transplant rejection and GVHD are known to those of ordinary skill in the art. See, e.g., *Graft Versus Host Disease Treatment & Management* on emedicine.medscape.com; *Immunology of Transplant Rejection* on emedicine.medscape.com; Pavletic & Fowler (2012) *Hematology Am. Soc. Hematol. Educ. Program* 2012:251-264. Post-transplantation immunosuppressive drugs can be used in two phases: an initial induction phase, which requires much higher doses of drugs, and a later maintenance phase. Examples of immunosuppressive agents in current use include immunophilin-binding agents, mammalian target of rapamycin (mTOR) inhibitors, antiproliferative agents, antibodies, and corticosteroids.

Immunophilin-binding agents include, for example, cyclosporine and tacrolimus. These agents are calcineurin inhibitors; they primarily suppress the activation of T lymphocytes by inhibiting the production of cytokines, specifically IL-2.

Mammalian target of rapamycin (mTOR) inhibitors include sirolimus, which is a macrocyclic antibiotic that presumably modulates the activity of the mTOR inhibitor, which inhibits IL-2-mediated signal transduction and results in T- and B-cell cycle arrest in the G1-S phase.

Antiproliferative agents include azathioprine and mycophenolate mofetil (MMF). Other antiproliferative agents, such as cyclophosphamide and leflunomide, can also be used. Antiproliferative agents inhibit DNA replication and suppress B- and T-cell proliferation.

Antibodies include IL-2 receptor antagonists (basiliximab and daclizumab), which are FDA-approved for kidney transplantation induction. Antilymphocyte globulin, including monoclonal antibodies (e.g., muromonab-CD3) and polyclonal antibodies (e.g., antithymocyte globulins derived from either equine or rabbit sources), are also approved for the treatment of rejection. They also have been used as induction agents at some transplantation centers. Antibodies interact with

lymphocyte surface antigens, depleting circulating thymus-derived lymphocytes and interfering with cell-mediated and humoral immune responses. Lymphocyte depletion also occurs either by complement-dependent lysis in the intravascular space or by opsonization and subsequent phagocytosis by macrophages.

5 Corticosteroids are also used. However, the newer regimens are trying to minimize the use of steroids and thereby avoid the adverse effects that are associated with them. Steroids are still important in treating episodes of acute rejection.

 Common types of immunosuppression to optimize the balance between stable engraftment and reduction of risk and severity of GVHD include non-pharmacogenetic methods such as total
10 body irradiation and pharmacogenetic methods such as treatment with steroids, vitamin D, DNA alkylating agents (e.g., cyclophosphamide), inhibitors of de novo pyrimidine synthesis (e.g., Brequinar, leflunomide), inhibitors of de novo purine synthesis (e.g., 6-mercaptopurine, azathioprine, mizoribine, mycophenolate mofetil); kinase and phosphatase inhibitors (e.g., Cyclosporin A, FK-506 (tacrolimus), sirolimus), and lymphocidal agents (e.g., Rituximab,
15 alemtuzumab (Campath)).

 Prophylaxis of GVHD can be achieved either by T-cell depletion of the graft or by using immunosuppressive agents against donor cytotoxic lymphocytes. T-cell depletion results in a significant reduction in GVHD but is accompanied by an increased risk of engraftment failure and rate of relapse due to the loss of the graft-versus-tumor effect.

20 A common regimen used to prevent acute GVHD consists of cyclosporine or tacrolimus along with a few days of methotrexate. However, cyclosporine and tacrolimus are each associated with renal toxicity, and methotrexate is associated with severe mucositis. Sirolimus (Rapamycin) and mycophenolate mofetil are alternatives with lower toxicity. Other measures to decrease acute GVHD include gut decontamination with metronidazole, the administration of intravenous
25 immunoglobulin, and the use of a less intense preparative regimen.

 The criterion standard for primary prophylaxis of acute GVHD is cyclosporin A (CSP A) for 6 months and short-course methotrexate in T-cell-replete allogeneic HSCT (criterion standard). Cyclosporin A levels are typically kept above 200 ng/mL.

 Substitution of tacrolimus for CSP A is frequently used, especially in unrelated-donor
30 transplantation, because it may improve the control of GVHD. The addition of prednisone to the prophylactic regimen also reduces the incidence of GVHD. See, e.g., Ratanatharathorn *et al.* (1998) *Blood* 92:2303-2314.

Antithymocyte globulin (ATG) given before HSCT can significantly reduce the risk of grade III or IV acute GVHD and extensive chronic GVHD. See, e.g., Mollee *et al.* (2001) *Br. J. Haematol.* 113:217-223.

Other agents that have been studied for GVHD prophylaxis include combinations with or substitutions by other agents such as mycophenolate mofetil, sirolimus, pentostatin, Campath-1H, keratinocyte growth factor (KGF), and suberoylanilide hydroxamic acid (SAHA). See, e.g., Lopez *et al.* (2005) *Biol. Blood Marrow Transplant.* 11:307-313; Antin *et al.* (2003) *Blood* 102:1601-1605; and Kottaridis *et al.* (2000) *Blood* 96:2419-2425. See also Blazar *et al.* (2012) *Nat. Rev. Immunol.* 12:443-458; Pavletic & Fowler (2012) *Hematology Am. Soc. Hematol. Educ. Program* 2012:251-264.

Extracorporeal photopheresis (ECP) is an immunomodulatory procedure that collects lymphocytes and mixes them with 8-methoxypsoralen (which intercalates into the DNA of the lymphocytes), rendering them susceptible to ultraviolet light radiation effects that cause apoptosis. The lymphocytes are then returned to the patient. ECP has been used as part of a conditioning regimen together with pentostatin and total body irradiation with promising results.

Primary systemic treatment for acute GVHD typically consists of continuing the original immunosuppressive prophylaxis (CSP A or tacrolimus) and adding methylprednisolone. Exemplary doses can be in the range of 1-60 mg/kg, and the most common starting dose is 2 mg/kg/d given in 2 divided doses. Median time to resolution of acute GVHD is 30-42 days. In subjects who respond to initial therapy, short-term tapering treatment with prednisone to a cumulative dose of 2000 mg/m² is effective and expected to minimize steroid-related complications. Other therapies are ATG, CSP alone, mycophenolate mofetil, daclizumab, anti-IL-2 receptor, anti-CD5-specific immunotoxin, and a pan T-cell ricin A-chain immunotoxin (XomaZyme). These agents can be used alone or in combination. Additional therapies can include the addition of *ex vivo* cultured mesenchymal cells derived from unrelated donors or conventional steroid therapy. See, e.g., Ferrara & Yanik (2005) *Clin. Adv. Hematol. Oncol.* 3:415-419, 428.

Secondary therapy can be initiated upon failure of the initial therapy. Examples of secondary therapies include: ATG or multiple pulses of methylprednisolone (at doses higher than those used in initial therapy); addition of mycophenolate mofetil (MMF) at 2g daily; Muromomab-CD3 (Orthoclone OKT3) monoclonal antibody; humanized anti-Tac antibody to the IL-2 receptor; IL-1 receptor or IL-1 receptor antagonists; monoclonal antibodies against tumor necrosis factor- α (TNF- α), psoralen and ultraviolet A irradiation (PUVA), and conversion of CSP to tacrolimus.

Other therapies include ABX-CBL (an immunoglobulin (Ig) M (IgM) murine monoclonal antibody that recognizes CD147), visilizumab (a humanized anti-CD3 monoclonal antibody with a mutated IgG2 isotype), daclizumab (a humanized anti-interleukin-2 receptor alpha chain antibody), infliximab (a genetically constructed IgG1 murine-human chimeric monoclonal antibody that binds the soluble subunit and the membrane-bound precursor of TNF- α), etanercept (a soluble dimeric TNF- α receptor 2), denileukin diftitox (a recombinant protein composed of IL-2 fused to diphtheria toxin), and pentostatin at 1.5 mg/m².

III. Kits

The materials used in the above assay methods are ideally suited for the preparation of a kit. Various detection reagents can be developed and used to assay the level and/or activity *SUFU* mRNA or *SUFU* protein. The terms “kits” and “systems,” as used herein are intended to refer to one or more *SUFU* mRNA or *SUFU* protein level and/or activity detection reagents in combination with one or more other types of elements or components (e.g., other types of biochemical reagents, containers, packages, such as packaging intended for commercial sale, substrates to which SNP detection reagents are attached, electronic hardware components, and the like). Accordingly, the present invention further provides *SUFU* mRNA and/or *SUFU* protein expression and/or activity detection kits and systems, including packaged probe and primer sets (e.g., TaqMan probe/primer sets), arrays/microarrays of nucleic acid molecules, and beads that contain one or more probes, primers, antibodies, or other detection reagents for detecting of the level and/or activity of *SUFU* mRNA and/or *SUFU* protein. The kits/systems can optionally include various electronic hardware components. For example, arrays (e.g., DNA chips) and microfluidic systems (e.g., lab-on-a-chip systems) provided by various manufacturers typically include hardware components. Other kits/systems (e.g., probe/primer sets) may not include electronic hardware components, but can include, for example, one or more *SUFU* mRNA or *SUFU* protein level and/or activity detection reagents along with other biochemical reagents packaged in one or more containers.

In some embodiments, a *SUFU* mRNA or *SUFU* protein level and/or activity detection kit typically contains one or more detection reagents and other components (e.g., a buffer, enzymes, such as DNA polymerases or ligases, chain extension nucleotides, such as deoxynucleotide triphosphates, positive control sequences, negative control sequences, and the like) necessary to carry out an assay or reaction, such as amplification and/or detection of a polynucleotide comprising a region of the *SUFU* cDNA or comprising the rs17114808 marker locus. A kit can further contain means for determining the amount of the target polynucleotide and means for

comparing with an appropriate standard, and can include instructions for using the kit to detect the level and/or activity of the *SUFU* polypeptide.

In specific embodiments, a kit for identifying the level of the *SUFU* mRNA and/or *SUFU* protein in a biological sample is provided. The kit comprises a first and a second primer, wherein
5 the first and second primer amplify a polynucleotide comprising the rs17114808 marker locus.

Further provided are polynucleotide detection kits comprising at least one polynucleotide that can specifically detect the *SUFU* polynucleotide. In specific embodiments, the polynucleotide comprises at least one polynucleotide molecule of a sufficient length of contiguous nucleotides homologous or complementary to SEQ ID NO: 1, 2, 3, 4, 5, 7, 8, 9 or 10 or a variant thereof to
10 allow for the detection of the level and/or activity of *SUFU* mRNA and/or *SUFU* protein.

Kits can also be used to detect *SUFU* protein. For antibody based detection systems, the present invention provides a kit which comprises an antibody capable of specifically binding to the *SUFU* protein and one or more of the following: wash reagents and reagents capable of detecting the presence of bound antibodies of the kit.

In specific embodiments, the kit comprises a compartmentalized kit and includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers, or strips of plastic or paper. Such containers allow one to efficiently transfer reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated, and the agents or solutions of each container can be added in a
15 quantitative fashion from one compartment to another. Such containers may include a container which will accept the test sample, a container which contains the antibodies or probes used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, etc.), and containers which contain the reagents used to detect the bound antibody or the hybridized probe. Any detection reagents known in the art can be used including, but not limited to those
20 described supra.

IV. Methods of Screening for SHH Pathway Antagonists

Methods are provided for identifying binding and/or modulating agents of the SHH pathway (also referred to herein as a "screening assay"). In particular, identification of various SHH
30 pathway antagonists is of interest. In specific embodiments, SHH antagonists that inhibit SHH pathway regulation of GVHD are of particular interest.

The candidate compounds employed in the various screening assays can include any candidate compound, including, for example, peptides, peptidomimetics, small molecules,

antibodies, siRNAs, miRNAs, shRNAs, or other drugs. Such candidate compounds can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, nonpeptide oligomer, or small molecule libraries of compounds (Lam (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Bio/Techniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (U.S. Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:1865-1869), or phage (Scott and Smith (1990) *Science* 249:386-390; Devlin (1990) *Science* 249:404-406; Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382; and Felici (1991) *J. Mol. Biol.* 222:301-310).

In some embodiments, an assay to screen for SHH pathway antagonists is a cell-free assay comprising contacting a SHH pathway polypeptide or biologically active fragment or variant thereof with a test compound and determining the ability of the test compound to bind to a SHH pathway polypeptide or the biologically active variant or fragment thereof. Binding of the test compound to a SHH pathway polypeptide can be determined either directly or indirectly. In a further embodiment, the test or candidate compound specifically binds to or selectively binds to a SHH pathway polypeptide.

In other embodiments, an assay comprises contacting a biological sample comprising a SHH pathway polypeptide with a candidate compound and determining the ability of the candidate compound to decrease the activity of a SHH pathway polypeptide. The biological sample can be tissues, cells, or biological fluids isolated from a subject (and optionally subsequently processed), as well as tissues, cells, or fluids present within a subject. In some embodiments the biological sample is from lymph nodes, spleen, bone marrow, blood, or primary tumor. Determining the

ability of the candidate compound to decrease the activity of a SHH pathway polypeptide can be accomplished, for example, by determining SHH pathway activity.

In some embodiments, an assay comprises contacting an antigen-presenting cell (APC) with a candidate compound and determining if the candidate compound inhibits SHH pathway regulation of GVHD. The APC can be, for example, a dendritic cell. In some embodiments, the assessing comprises assessing the antigen presentation capability of the APC. For example, expression of HLA-DR can be assessed. In some embodiments, the assessing comprises assessing stimulation or activation of donor T-cells by APCs. For example, T-cell proliferation can be assessed. In some embodiments, an allogeneic mixed leukocyte response (MLR) assay can be used. In some embodiments, SHH pathway activity can be assessed by assessing expression or activity SHH pathway components. For example, SHH pathway activity can be assessed by assessing expression or activity of *SUFU*, *GLI1*, and/or *GLI2*.

Further provided are novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

V. Sequence Identity

As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

An "isolated" or "purified" polynucleotide or polypeptide or biologically active fragment or variant thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For purposes of the invention, "isolated" when used to refer to nucleic acid molecules excludes isolated chromosomes. For example, in various embodiments, the isolated nucleic acid molecules can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived.

By "fragment" is intended a portion of the polynucleotide or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a polynucleotide may encode protein fragments that retain the biological activity of the SUFU polypeptide. Alternatively, fragments of a *SUFU* polynucleotide or gene need not encode an active SUFU polypeptide. Instead, such

polynucleotide fragments can comprise a sufficient length of the *SUFU* polynucleotide to be used as a probe or a primer that allows for the specific detection of the *SUFU* polynucleotide. In specific embodiments, the *SUFU* polynucleotide comprises the T-allele of rs17114808 (SEQ ID NO: 3) or the C-allele of rs17114808 (SEQ ID NO: 4) or a primer that will amplify the rs17114808 marker locus or a probe that will specifically hybridize to the rs17114808 marker locus. Thus, fragments of a nucleotide sequence may range from at least about 10 nucleotides, 15 nucleotides, 20 nucleotides, 50 nucleotides, 75 nucleotides, 100 nucleotides, 200 nucleotides, 300 nucleotides, 400 nucleotides, 500 nucleotides, 600 nucleotides, 700 nucleotides and up to the full-length polynucleotide employed in the invention. Methods to assay for the activity of a desired polynucleotide or polypeptide are described elsewhere herein.

"Variants" is intended to mean substantially similar sequences. For polynucleotides, a variant comprises a deletion and/or addition of one or more nucleotides at one or more internal sites within the native polynucleotide and/or a substitution of one or more nucleotides at one or more sites in the native polynucleotide. As used herein, a "native" polynucleotide or polypeptide comprises a naturally occurring nucleotide sequence or amino acid sequence, respectively. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the polypeptides employed in the invention. Variant polynucleotides also include synthetically derived polynucleotide, such as those generated, for example, by using site-directed mutagenesis, but continue to retain the desired activity. Generally, variants of a particular polynucleotide of the invention having the desired activity will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters described elsewhere herein.

Variants of a particular polynucleotide of the invention (i.e., the reference polynucleotide) can also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant polynucleotide and the polypeptide encoded by the reference polynucleotide. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs and parameters described elsewhere herein. Where any given pair of polynucleotides employed in the invention is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

"Variant" protein is intended to mean a protein derived from the subject polypeptide by deletion or addition of one or more amino acids at one or more internal sites in the native protein and/or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of protein, as discussed elsewhere herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native protein will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Graft-versus-host disease (GVHD) is a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). To identify recipient risk factors, a genome-wide study was performed including 481,820 single-nucleotide polymorphisms (SNPs). Two GVHD susceptibility loci (rs17114803 and rs17114808) within the *SUFU* gene were identified in the discovery cohort ($p=2.85 \times 10^{-5}$). The incidence of acute GVHD among patients homozygous for CC at *SUFU* rs17114808 was 69%, which was significantly higher ($p = 0.0002$) than the 8% rate observed in CT heterozygous patients. In an independent validation cohort of 100 patients, 50% of the patients with the CC genotype developed GVHD compared to 8% of the patients with either CT or TT genotype ($p = 0.008$). In comparison to CC dendritic cells, those from CT expressed higher levels of *SUFU* mRNA and protein, had lower levels of surface HLA-DR, and induced less allogeneic mixed leukocyte response (MLR). Ectopic expression of *SUFU* in THP-1 derived DCs reduced HLA-DR expression and suppressed MLR, whereas silencing of *SUFU* enhanced HLA-DR expression and increased MLR. Thus our findings provide novel evidence that recipient *SUFU* germline polymorphism is associated with acute GVHD and is a novel molecular target for GVHD prevention and treatment.

Introduction

Hematopoietic stem cell transplantation (HSCT) is used to treat a variety of malignant and non-malignant diseases. Successful allogeneic HSCT involves intensive immunosuppression of the recipient, followed by infusion of the donor stem cell graft. In addition to hematopoietic stem cells, the graft also contains CD4⁺ and CD8⁺ $\alpha\beta$ T-cells. One of the main benefits of allogeneic HSCT is the alloreactivity of the donor T-lymphocytes toward recipient malignant cells, leading to the beneficial graft-versus-malignancy effect.¹ However, this non-specific alloreactivity may also direct toward normal tissues in the recipient, resulting in graft-versus-host disease (GVHD).^{2,3}

Many factors, related to both the donor and the recipient, have been identified as potential risk factors for the development of GVHD. The most important risk factor is the genetic disparity between the donor and recipient in human leukocyte antigen (HLA).⁴ The frequency of acute GVHD is directly related to the degree of HLA mismatch between the donor and recipient.⁵ Furthermore, about 40% of recipients of HLA-identical grafts experience acute GVHD triggered by disparity in minor antigens.⁶ Relatively little is known about non-HLA genetic factors in the recipient that may contribute to the development of GVHD.^{7,8} Identifying such factors is useful because it will allow development of novel molecular targeted therapy, improved risk stratification, and individualized GVHD prophylaxis and treatment. Patients at low risk for the development of acute GVHD may have immunosuppression decreased to safely allow a stronger graft-versus-leukemia effect, while those at high risk for GVHD may require a more intensive or prolonged immunosuppression regimen to prevent GVHD mortality.

In this study, we investigated the role of recipient germline SNPs in the development of acute GVHD in a group of pediatric patients who received allogeneic HSCT at a single institution for treatment of either acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML). We identified two SNPs in *suppressor of fused (SUFU)* that were associated with acute GVHD and elucidated the mechanisms of action.

METHODS

Patients, donors, and transplant regimen

For the discovery cohort, germline samples were available for 38 patients with ALL and 30 with AML who underwent HSCT at St. Jude Children's Research Hospital between 1995 and 2007. For the validation cohort, samples were available from an additional 100 patients who underwent HSCT also at St. Jude. Details of the patient and transplant characteristics are included in Table 1. All patients in the discovery cohort were

treated with a myeloablative conditioning regimen which included total body irradiation (doses 1200-1400 cGY) in the majority of patients. The validation cohort included patients with both malignant and nonmalignant diseases and a wide variety of treatment regimens, including some reduced intensity regimens, thus allowing the evaluation of the generalizability of the SUFU SNP effects in various transplant settings.

Table 1. Patient and transplant characteristics of the discovery and validation cohort

			Discovery cohort (n = 68)		Validation cohort (n = 100)	
	CC	CT	p-value	CC	CT/TT	P-value
Diagnosis			0.09			0.94
AML	28	10		29	6	
ALL	27	3		27	3	
Other	0	0		32	3	
Age at HSCT-mean years	9.7	9.26	0.29	10.5	10.36	0.89
Sex			0.83			0.53
Male	32	8		56	9	
Female	23	5		32	3	
Race			0.14			0.55
White	44	8		58	6	
Black	9	4		14	3	
Asian	0	1		2	0	
Other	2	0		14	3	
Donor			0.79			0.46
MRD	12	4		21	1	
MMRD	5	1		47	7	
MUD	38	8		20	4	
HLA Mismatch			0.66			1
3 antigen	2	1		33	5	
2 antigen	2	0		8	1	
1 antigen	10	1		7	1	
0 antigen	41	11		37	5	
Stem cell source			0.99			1
Marrow	50	12		41	6	
PBSC	4	1		46	6	
Cord blood	1	0		1	0	
TBI			0.99			0.35
Yes	51	13		50	9	
No	4	0		38	3	
GVHD prophylaxis [#]						
CNI	55	12	0.19	67	8	0.49
MTX	16	3	1	20	2	1
MMF	11	5	0.17	38	3	0.35
Survival at last follow up			0.86			0.35
Alive	26	6		35	7	
Dead	29	7		53	5	

Subject may have received more than one drug for GVHD prophylaxis.

CC, homozygous for cytosine at position rs17114808; CT, heterozygous with cytosine and thymine at the same position; TT, homozygous with thymine at the same position; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MRD, matched related donor; MMRD, mismatched related donor; MUD, matched unrelated donor; HLA, human leukocyte antigen; PBSC, peripheral blood stem cells; TBI, total body irradiation; GVHD, graft-versus-host disease; CNI, calcineurin inhibitor; MTX, methotrexate; MMF, mycophenolate.

Table 2. Genotype, patient and transplant characteristics of discovery and validation cohorts according to development of aGVHD.

	Discovery Cohort (n = 68)			Validation Cohort n=100)		
N	29 (no aGVHD)	39 (aGVHD)	p-value	55 (no aGVHD)	45 (aGVHD)	p-value
SNP rs 17114808			6.70E-05*			0.011*
CC	17	38		44	44	
CT/TT	12	1		11	1	
Diagnosis			1			0.77
ALL	16	22		19	13	
AML	13	17		21	17	
Other				15	15	
Age at HSCT (mean years)			0.21			0.029
	10.76	8.91		9.12	12.15	
Gender			0.46			0.29
Female	10	18		22	13	
Male	19	21		33	32	
Race			0.37			0.85
White	21	31		34	30	
Black	7	6		9	8	
Asian	1	0		1	1	
Other	0	2		11	6	
HLA Mismatch (of 6 Antigens)			0.12			0.6
0 Ag MM	25	27		20	22	
1 Ag MM	2	9		4	4	
2 Ag MM	0	2		6	3	
3 Ag MM	2	1		23	15	
Stem Cell Source			0.8			0.37
Cord	0	1		0	1	
Marrow	26	36		24	23	
PBSC	3	2		31	21	
T cell depleted graft			0.8			0.16
No	18	22		21	24	

	Discovery Cohort (n = 68)			Validation Cohort n=100)		
N	29 (no aGVHD)	39 (aGvHD)	p-value	55 (no aGVHD)	45 (aGVHD)	p-value
Yes	11	17		34	21	
TBI			0.31			0.84
No	3	1		22	19	
Yes	26	38		33	26	
Myeloablative Conditioning						1
No	0	0		17	13	
Yes	29	39		38	32	
ATG			0.28			0.16
No	11	9		25	27	
Yes	18	30		30	18	
CNI			0.43			0.0009
No	1	0		21	4	
Yes	28	39		34	41	
MMF			0.57			0.0024
No	21	31		40	19	
Yes	8	8		15	26	
MTX			0.6			0.63
No	22	27		44	34	
Yes	7	12		11	11	
Survival Status			0.81			1
Alive	13	19		23	19	
Expired	16	20		32	26	

* Fisher's exact test

aGVHD, acute graft-versus-host disease; CC, homozygous for cytosine at position rs17114808; CT, heterozygous with cytosine and thymine at the same position; TT, homozygous with thymine at the same position; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; HLA, human leukocyte antigen; MM, mismatch; PBSC, peripheral blood stem cells; TBI, total body irradiation; ATG, antithymocyte globulin; CNI, calcineurin inhibitor; MMF, mycophenolate; MTX, methotrexate.

Genome-wide screening and statistical analysis

Germline DNA was extracted from patient samples obtained before HSCT and the discovery cohort was genotyped using the Affymetrix GeneChip Human Mapping 500K set or the Affymetrix Genome-wide Human SNP Array 6.0 (Affymetrix) as previously described.^{10,25,26} The 68 patients that belong to the discovery cohort were evaluated for a

total of 481,820 SNPs. Each SNP was coded as 0 (AA), 1 (AB), or 2 (BB). The complication of acute GVHD was defined as any stage of acute GVHD in any organ (skin, liver, or gastrointestinal) and coded as 1 (yes) or 0 (no).

To test the associations between each SNP and acute GVHD, the Spearman rank correlation test was used.^{10,27} Due to the small sample size and to provide effective control of the type I error rate, p-values were obtained by a hybrid-permutation method with 2000 permutations.¹⁰ The profile information threshold method²⁸ was used to select SNPs significantly associated with acute GVHD and estimate the false discovery rate for the corresponding p-value cutoff.

For both the discovery and validation cohorts, continuous variables for patient and transplant characteristics between different SNP groups were compared using the Wilcoxon rank-sum test. Categorical variables were compared using the Pearson's Chi-square test, or Fisher's exact test if appropriate. The survival probabilities after HSCT were estimated using Kaplan-Meier method and compared using the Mantel-Haenszel statistic²⁹. Cumulative incidences of GVHD were estimated using the methods of Kalbfleisch and Prentice³⁰ and compared using the methods of Gray³¹, with adjustment for competing risk of death. Univariate and multivariate Fine and Gray's and logistic regression models were used to test associations between clinical and genetic factors and acute GVHD. Factors with p-values less than 0.1 were included in the multivariate analysis. Backward stepwise regression was also performed for Fine and Gray's regression model based on BIC to select the final model using R package crrstep found at the website located at cran.r-project.org/web/packages/crrstep/index.html.

Cell line, culture, isolation of blood dendritic cells, and generation of THP-1-derived dendritic cells

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers with informed consent under a protocol approved by our institutional review board in accordance with the Declaration of Helsinki. Peripheral blood myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) were isolated from PBMCs cultured overnight using the Myeloid or Plasmacytoid Dendritic Cell Isolation Kit following the manufacturer's instructions (Miltenyi Biotec).³² Myeloid cell line THP-1 was purchased from American Type Culture Collection. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin

(Invitrogen Life Technologies) and were maintained at 37°C in a humidified atmosphere with 5% CO₂. THP-1–derived dendritic cells (DCs) were generated as previously described.³³ Briefly, THP-1 cells at a density of 1×10^5 per well were cultured in the presence of granulocyte-macrophage colony-stimulating factor (2500 U/mL; Miltenyi) and interleukin-4 (250 U/mL; R&D Systems Inc.) for 5 days at 37°C under 5% CO₂. On day 3, 90% of the medium was replaced with fresh medium and cytokines. On day 5, DC growth medium was replaced with medium containing maturation cytokines, including interleukin-1 β (10 ng/mL), interleukin-6 (10 ng/mL), tumor necrosis factor α (10 ng/mL), and prostaglandin E₂ (1 μ g/mL). DCs were then harvested on day 7 and washed for further assays.

SUFU SNP assay

To detect the presence of various alleles of *SUFU*, a single-nucleotide mismatch detection assay was developed as described previously.³⁴ Briefly, primers for the assay were designed in such a way that they amplified all alleles of the *SUFU* gene as well as the amplicon containing the polymorphic region of interest. The forward primer was 5'-CCCCTTTCCTGCCTTCTTACC-3' (**SEQ ID NO: 7**) and the reverse primer was 5'-TCATGACTTTGCTTTGAAGAGGTGTA-3' (**SEQ ID NO: 8**). The probe for *SUFU* alleles with a thymine at position rs17114808 was 6Fam ATGGGACTGTTATAATACT (**SEQ ID NO: 9**)-MGBNFQ (**Molecular-Groove Binding Non-Fluorescence Quencher**) and for those with a cytosine at the same position was VIC TGGGACTGTTACAATACT (**SEQ ID NO: 10**)-MGBNFQ. Each assay reaction mix contained a 250 nM probe concentration and 100 ng of genomic DNA in 1 $\times$ TaqMan genotyping master mix (Applied Biosystems). The assay was performed on an HT7900 Sequence Detection System (Applied Biosystems) following the allelic discrimination assay protocol provided by the manufacturer.

RT-qPCR and siRNA gene silencing

Total RNA was extracted from PBMCs or cell lines using RNA extraction kits (Qiagen). cDNA was generated from the total RNA using SuperScript Reverse Transcriptase (Invitrogen). *SUFU* transcript was quantified using a *SUFU*-specific QuantiTect Primer Assay (Qiagen) according to the manufacturer's instructions. *SUFU*

expression in THP-1 cells was silenced using siRNA (Open Biosystems) following the manufacturer's instructions.

***SUFU* cloning, expression, and Western blot**

5 Total RNA was extracted from PBMCs using RNA extraction kits (Qiagen). cDNA was generated from the total RNA using SuperScript Reverse Transcriptase (Invitrogen), and *SUFU* was amplified by PCR and cloned into mammalian expression vector pcDNA3 (Invitrogen). The identity of *SUFU* was confirmed by sequencing. THP-1 cells were transfected with pcDNA3 vector containing *SUFU* by electroporation (Gene
10 Pulser II; Bio-Rad). Stable cell lines were generated by selection in Geneticin (Invitrogen).

For Western blots, the cells were lysed by adding lysis buffer (1% Triton X-100, 150 mM NaCl, and 50 mM Tris [hydroxymethyl] aminomethane-HCl, pH 7.4). Lysed cells were centrifuged, and supernatants were electrophoresed on 4% to 12% NuPAGE Bis-Tris gel (Invitrogen). Separated proteins were blotted with *SUFU*-specific antibody
15 (Open Biosystems) using a Western blotting protocol as described previously.³⁵ Pico-enhanced Chemiluminescent Substrate (Thermo Scientific) was used to detect overexpressed *SUFU* protein in THP-1 cells. The membrane was stripped with Restore Plus Western Blot Stripping Buffer (Thermo Scientific) and reblotted with anti-tubulin antibody (Sigma-Aldrich) as a loading control.

Mixed leukocyte response (MLR)

The THP-1-derived DCs, or mDCs and pDCs, were irradiated at 30 Gy and co-cultured at a ratio of 1:10 with 1×10^5 allogeneic responder CD3⁺ T-cells from a single donor in flat-bottom 96-well microtiter plates. Cell proliferation was quantified using the
25 DELFIA Cell Proliferation kit (PerkinElmer) following the manufacturer's instructions. Briefly, bromodeoxyuridine (BrdU) was added into the wells 16 h before the end of a 5-day culture. The next day, cells were fixed and spun down. The supernatant was discarded, anti-BrdU-Eu was added, and the fluorescence was measured using a Wallac Victor 2 Counter Plate Reader (both from PerkinElmer Life and Analytical Sciences).

Flow cytometry

The following antibodies were purchased from commercial suppliers and used for phenotypic analysis: FITC-conjugated anti-BDCA-1, anti-BDCA-2, anti-CD45, anti-

CD80, anti-CD83, anti-CD69, anti-CD45RA, and anti-CD11b; PE-conjugated anti-BDCA-3, anti-BDCA-4, anti-HLA-ABC, anti-CD19, anti-CD20, anti-CD25, anti-CD40, anti-CD86, anti-CCR7, and anti-HLA-DR; ECD-conjugated anti-HLA-DR, anti-CD3, and anti-CD62L; APC-conjugated anti-CD11c, anti-CD123, and anti-CD56; APC-Cy7-conjugated anti-CD14, anti-CD3, anti-CD4, and anti-CD19. Flow cytometric analyses were conducted with LSRII (BD Bioscience), and the data were analyzed with FlowJo 8.8.6 (Tree Star).

RESULTS

Genome-wide screening and validation of SNPs associated with acute GVHD

Of the 68 patients in the discovery cohort, 39 (57%) experienced acute GVHD as defined by standard criteria.³⁶ By the information profile selection criteria, 16 of the 481,820 SNPs were chosen based on the p-value of the hybrid-permutation method as being significantly associated with acute GVHD. A Manhattan plot of the chromosomal locations is shown in Figure 1A.

Among the 16 top SNPs associated with acute GVHD, two were in *SUFU*: a coding synonymous SNP at rs17114803 ($p = 2.85 \times 10^{-5}$) and another SNP in the 3' untranslated region (3' UTR), rs17114808 ($p = 2.85 \times 10^{-5}$). These SNPs are in complete linkage disequilibrium and are located on chromosome 10. Among the 68 patients in the discovery cohort, 55 (81%) were homozygous for the major allele with cytosine at rs17114808 (CC), no patient was homozygous for the minor allele thymine at this position (TT), and 13 (19%) were heterozygous with cytosine and thymine at the same position (CT). There were no clinical or demographic features that were statistically different among the different genotype groups. The cumulative incidence of acute GVHD was 69% among patients homozygous for the C allele, and only 8% among those who were heterozygous ($p = 0.0002$, Gray's test) (Figures 1B and C), suggesting a protective effect by the T allele. Furthermore, a full spectrum of GVHD severity was observed in the CC group, whereas only grade I GVHD was seen in the single case among CT recipients (Table 1). There were no clinical or demographic factors that were statistically different in patients with and without acute GVHD (Table 2). Therefore, of all factors investigated, *SUFU* SNP genotype was the only factor significantly associated with acute GVHD in the discovery cohort.

To validate our discovery cohort results, we developed a PCR-based SNP assay that can detect the presence of different *SUFU* alleles (Figure 2A). We sequenced the PCR products from each group (CC, CT, and TT) and confirmed the accuracy of the assay (Figure 6). Using the SNP assay, we genotyped another 100 patients as an independent validation cohort. Among these 100 patients, 88% were CC homozygous, 3% were TT homozygous and 9% were CT. The cumulative incidence of acute GVHD was 50% among CC homozygous, but was only 8.3% among those who were heterozygous or homozygous for the T allele ($p = 0.008$) (Figure 2B and 2C). There were no significant differences in any patient demographic or transplant-related characteristics between the allelic groups in both discovery and validation cohort (Table 1).

Table 2 demonstrates the demographic and transplant characteristics according to acute GVHD in the validation cohort. Besides *SUFU* genotype, age at HSCT, CNI use and MMF use were significantly different between those with and without acute GVHD. Univariate logistical regression analysis confirmed the associations between *SUFU* SNP genotype ($p=0.024$), age at HSCT ($p=0.032$), CNI use ($p=0.0019$) and MMF use ($p=0.0025$) and acute GVHD. Multivariate analysis showed that *SUFU* SNP genotype ($p=0.025$) was still statistically associated with acute GVHD after adjusting for age at HSCT, CNI and MMF, suggesting that *SUFU* SNP is an independent predictor of acute GVHD. The same risk factors for acute GVHD (age at HSCT, $p=0.035$; CNI, $p=0.0034$; and MMF, $p=0.0036$) were identified by time-to-event analysis using Fine and Gray's cumulative incidence model. Multivariate analysis of cumulative incidences showed that *SUFU* SNP was still statistically associated with acute GVHD ($p=0.048$) after adjusting for age at HSCT, CNI and MMF.

***SUFU* SNPs correlated with mRNA and protein level in PBMCs**

SUFU is not known to play a role in human immunology, and the biology of *SUFU* SNPs has not been elucidated. To further investigate the effect of *SUFU* allelic polymorphism, we genotyped 30 healthy subjects and found that 25 were CC (83.33%), 4 were CT (13.33%), and 1 was TT (3.33%). This distribution was similar to that of the HSCT patients in our study. Because *SUFU* rs17114808 SNP is located in the 3' UTR, we hypothesized that the SNP did not affect protein structure and function but might affect its abundance through translational control, degradation of mRNA or subcellular localization.³⁷⁻⁴⁰ We quantified the *SUFU* transcripts by RT-qPCR using *SUFU*-specific

QuantiTect RT-PCR primers and found less *SUFU* transcript in PBMCs from individuals with the CC genotype than in those with CT or TT genotypes (Figure 3A). Similarly, PBMCs from individuals who were CC homozygous produced much less SUFU protein than those from people who were heterozygous (CT) or homozygous (TT) for the minor
5 SUFU allele (Figure 3B). In contrast, there were no significant differences of HLA class I expression in both myeloid and plasmacytoid DCs from individual with different SUFU alleles (Figure 4D). These findings support the hypothesis that DCs with the T allele expressed more SUFU, which in turn suppressed HLA-DR expression specifically and reduced GVHD potential.

SUFU suppresses HLA-DR expression in blood DCs and reduces their ability to induce allogeneic T-cell proliferation

Since GVHD involves stimulation and activation of donor T-cells by recipient antigen-presenting cells (APCs), we hypothesized that SUFU might affect antigen
15 presentation by recipient APCs. Based on our laboratory findings that the T allele was associated with more *SUFU* mRNA and protein in PBMCs and on our clinical observation that CT/TT recipients had less GVHD than CC recipients, we hypothesized that SUFU inhibited GVHD by reducing antigen presentation by recipient APCs. To test our hypotheses, we first purified mDCs and pDCs from healthy individuals to confirm the
20 presence of *SUFU* transcripts in the DC populations using *SUFU*-specific RT-PCR primers (Figure 4A). We then analyzed the antigen presentation capability of mDCs and pDCs from individuals having different *SUFU* alleles by MLR assay. DCs from individuals having the *SUFU* T allele induced significantly less allogeneic T-cell proliferation than DCs from individuals with the CC genotype that produced less SUFU
25 protein (Figure 4B). DCs from CT heterozygous individuals had significantly lower expression of HLA-DR than those from CC homozygous individuals (Figure 4C). These findings support the hypothesis that patients with the T allele expressed more SUFU, which in turn reduced GVHD by decreasing antigen presentation through downregulation of HLA-DR expression in DCs.

Overexpression and silencing of *SUFU* in THP-1–derived DCs changed their HLA-DR expression and ability to induce allogeneic T-cell proliferation

To confirm the direct involvement of *SUFU* in antigen presentation by DCs, we used a myeloid cell line, THP-1, that can be induced into APCs by a combination of cytokines.^{33,41} After culturing THP-1 cells in APC induction conditions, we found higher expression of each tested DC-associated markers than in cells grown in normal growth medium (Figure 7). We then ectopically expressed (*SUFU*⁺) or silenced *SUFU* expression (*SUFU*⁻) in the THP-1 cells (Figure 5A and B) and used them for allogeneic MLR assay. Overexpression of *SUFU* inhibited allogeneic T-cell proliferation, whereas silencing of *SUFU* increased it (Figure 5C). This cell-line model confirmed our earlier observation that *SUFU* affected allogeneic T-cell proliferation induced by healthy donor DCs. Furthermore, we found that silencing *SUFU* increased specifically the expression of HLA-DR, whereas overexpression reduced it (Figure 5D), but there was no change in expression of other DC markers such as CD40, CD80, CD83, or CD86 (Figure 8). These findings directly show that *SUFU* regulates the level of HLA-DR expression specifically and thus alters allogeneic MLRs. By contrast, there were no differences in HLA-Class I or HLA-DR expression among THP-1, THP-*SUFU*⁺ and THP-*SUFU*⁻ cell lines cultured in normal growth medium without differentiating the cells into DCs (Figure 5A and 5B).

DISCUSSION

In this study, a SNP in the *SUFU* gene (rs17114808) was found to be associated with the incidence of acute GVHD in pediatric and young adult patients who underwent allogeneic HSCT. Transplant recipients who were *SUFU* CC homozygous were more susceptible to acute GVHD than recipients who had CT or TT genotypes. Remarkably, the acute GVHD in recipients with T allele was at most grade I, whereas more severe GVHD was observed in the CC group. The SNP is located in the 3' UTR of the *SUFU* gene and regulates the quantity of transcript and total protein production. DCs from individuals who are CC homozygous have less *SUFU* protein, higher level of HLA-DR expression, and stronger potential to induce alloreactive T cell response.

SUFU is a known repressor of the sonic hedgehog (SHH) signaling pathway. SHH acts as a classical morphogen during embryonic development, regulating the pattern of formation in the nervous, respiratory, and intestinal systems.⁴²⁻⁴⁵ Postnatally, SHH pathways regulate tumorigenesis by controlling gene transcription and autophagy to

maintain normal cell homeostasis.^{46,47} SHH signaling activity is governed by the balance of GLI activators and repressors.⁴⁸ SUFU is the core intracellular negative regulator of SHH signaling, interacting directly with GLI to control protein processing, stabilization, and subcellular distribution.⁴⁹⁻⁵⁴ Although the correlation between SUFU and GVHD was unknown, Pawei Zerr et al.⁵⁵ recently reported that SHH signaling is activated in human and murine chronic GVHD. They found that pharmacologic inhibition of SMO, an important co-receptor of the SHH signaling pathway, is effective for prevention and treatment of chronic GVHD. Moreover, Varas et al.⁵⁶ reported that SHH is anti-apoptotic in thymic DCs, and blockade of SHH signaling by cyclopamine abrogates the upregulation of HLA-DR expression in DCs induced by CD40 ligands; although the precise molecular mechanism was not elucidated.

Here, we found that SUFU is capable of directly reducing HLA-DR expression in both mDCs and pDCs. It is known that GVHD-associated T helper cell responses specific for minor histocompatibility antigens are mainly restricted by HLA-DR molecules. HLA-DR-silenced APCs lose their ability to induce proliferation and activation of allogeneic T-cells, which is essential for the development of GVHD. DCs from CT individuals have higher amount of SUFU, less HLA-DR expression, and reduced capacity to stimulate allogeneic T-cell proliferation as compared to CC homozygous.

The primary strength of our study is that this is the first high-density genome-wide SNP study in HSCT recipients rather than donors, identifying a statistically significant molecular determinant for GVHD development, and the only study to include functional validation. In addition to revealing this molecular marker for GVHD, the laboratory investigations showed the biological effect of the *SUFU* SNP, thereby providing the pathophysiologic mechanism for the effect of this SNP on GVHD risk. Another strength of this study is the development of a novel and simple assay for *SUFU* allele typing, which was then used to genotype the SNP in an independent cohort of patients. The SNP assay is expedient for testing patients undergoing HSCT and therefore has the potential to be useful in prognostication and in GVHD clinical management. We used the hybrid-permutation method to limit false positivity and information profile method to estimate the false discovery rate. In addition, the association between the SNP genotype and the incidence of acute GVHD was validated in an independent cohort, providing additional support. The biological mechanism elucidated for the effect of the *SUFU* SNP on GVHD further strengthens the validity of our conclusions. Future studies should assemble a larger

cohort of HSCT patients across all age groups to further examine the relationship between *SUFU* alleles and the risk of GVHD in various HSCT settings.

In summary, we identified a novel molecular determinant for acute GVHD using genome-wide analysis. Our findings are useful to individualize treatment and preventive approaches for GVHD in patients undergoing HSCT. Specifically, patients who are CC homozygous may benefit from more intensive GVHD prophylaxis, while patients with CT or TT genotypes may receive HSCT from a less-than-perfectly matched donor.

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Table 3. Summary of SEQ ID NOS.

SEQ ID NO	Type	Description	Sequence
1	DNA	Genomic SuFu sequence	See sequence listing
2	DNA	cDNA encoding SuFu	ATGGCGGAGCTGCGGCCTAGCGGCGCCCCCGGCCCCACCGCG CCCCCGGCCCTGGCCCGACTGCCCCCGGCCTTCGCTTCGC TCTTTCCCCCGGGACTGCACGCCATCTACGGAGAGTGCCGCC GCCTTTACCCTGACCAGCCGAACCCGCTCCAGGTTACCGCTAT CGTCAAGTACTGGTTGGGTGGCCAGACCCCTTGGACTATGTT AGCATGTACAGGAATGTGGGGAGCCCTTCTGCTAACATCCCC GAGCACTGGCACTACATCAGCTTCGGCCTGAGTGATCTCTATG GTGACAACAGAGTCCATGAGTTTACAGGAACAGATGGACCTA GTGGTTTTGGCTTTGAGTTGACCTTTCGTCTGAAGAGAGAAAC TGGGGAGTCTGCCCCACCAACATGGCCCGCAGAGTTAATGCA GGGCTTGGCACGATACGTGTTCCAGTCAGAGAACACCTTCTG CAGTGGGGACCATGTGTCCTGGCACAGCCCTTTGGATAACAG TGAGTCAAGAATTCAGCACATGCTGCTGACAGAGGACCCACA GATGCAGCCCGTGACAGACACCTTTGGGGTAGTTACCTTCCTC CAGATCGTTGGTGCTGCACTGAAGAGCTAACTCAGCCCAG CAGTGGAAACGGGCAGGGCATCCTGGAGCTGCTGCGGACAGTG CCTATTGCTGGCGGCCCTGGCTGATAACTGACATGCGGAGG GGAGAGACCATATTTGAGATCGATCCACACCTGCAAGAGAGA GTTGACAAAGGCATCGAGACAGATGGCTCCAACCTGAGTGGT GTCAGTGCCAAGTGTGCCTGGGATGACCTGAGCCGGCCCCC GAGGATGACGAGGACAGCCGGAGCATCTGCATCGGCACACA GCCCCGGCGACTCTCTGGCAAAGACACAGAGCAGATCCGGGA GACCCTGAGGAGAGGACTCGAGATCAACAGCAAACCTGTCT TCCACCAATCAACCTCAGCGGCAGAATGGCCTCGCCCACGA CCGGGCCCCGAGCCGCAAAGACAGCCTGGAAAGTGACAGCTC CACGGCCATCATTCCCATGAGCTGATTGCGACGCGGCAGCTT GAGAGCGTACATCTGAAATTCAACCAGGAGTCCGGAGCCCTC ATTCCTCTCTGCCTAAGGGGCAGGCTCCTGCATGGACGGCACT TTACATATAAAAGTATCACAGGTGACATGGCCATCACGTTTGT CTCCACGGGAGTGGAAGGCGCCTTTGCCACTGAGGAGCATCC TTACGCGGCTCATGGACCCTGGTTACAACCTCTGA
3	DNA	T-Allele of rs17114808	CCCAACTCTAGGGATGGGACTGTTATAATACTTCAAGATCACTCTTTACAC
4	DNA	C-allele of rs17114808	CCCAACTCTAGGGATGGGACTGTTACAATACTTCAAGATCAC TCTTTACAC
5	DNA	CT allele of rs17114808	CCCAACTCTAGGGATGGGACTGTTA[C/T]AATACTTCAAGATC ACTCTTTACAC
6	AA	SUFU protein	MAELRPSGAP GPTAPPAPGP TAPPAFASLF PPGLHAIYGE CRRLYPDQPN PLQVTAIVKY WLGGPDPLDY VSMYRNVGSP SANIPEHWHY ISFGLSDLYG DNRVHEFTGT DGPSGFGFEL TFRLKRETGE SAPPTWPAEL MQGLARYVFQ SENTFCSGDH VSWHSPLDNS ESRIQHMLLT EDPQMOPVQT PFGVVTFLQI VGVCTEELHS AQQWNGQGIL ELLRTVPIAG GPWLITDMRR GETIFEIDPH LQERVDKGIE TDGSNLSGVS AKCAWDDLRS PPEDDEDSRS ICIGTQPRRL SGKDEQIRE TLRRGLEINS KPVLPPIPNQ RQNGLAHDRA PSRKDSLESD SSTAIPHEL IRTROLESVH LKFNQESGAL IPLCLRGRLL HGRHFTYKSI TGDMAITFVS TGVEGAFATE EHPYAAHGPW LQL
7	DNA	Primer 1	CCCCCTTCCTGCCTTCTTACC
8	DNA	Primer 2	TCATGACTTTGCTTTGAAGAGGTGTA

9	DNA	Probe to detect T - allele	ATGGGACTGTTATAATACT-MGBNFQ
10	DNA	Probe to detect C-allele	TGGGACTGTTACAATACT-MGBNFQ

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present invention pertains. Furthermore, if a definition or use of a term in a reference that has been incorporated by reference herein is inconsistent or contrary to the definition or use of that term provided herein, the definition or use of that term provided herein applies, and the definition or use of that term in the reference does not apply.

Compositions or methods “comprising” or “including” one or more recited elements may include other elements not specifically recited. For example, a composition that “comprises” or “includes” a SUFU detection reagent may contain the SUFU detection reagent alone or in combination with other ingredients.

The singular forms of the articles “a,” “an,” and “the” include plural references unless the context clearly indicates otherwise. For example, the term “a compound” or “at least one compound” can include a plurality of compounds, including mixtures thereof.

Unless otherwise apparent from the context, the term “about,” when referring to a value, is meant to encompass variations of +/- 50%, +/- 20%, +/- 10, +/- 5%, +/- 1%, +/- 0.5%, or +/- 0.1% from the specified amount, as such variations are appropriate to perform the disclosed methods or employ the disclosed compositions.

Designation of a range of values includes all integers within or defining the range, and all subranges defined by integers within the range.

Any feature, step, element, embodiment, or aspect of the invention can be used in combination with any other unless specifically indicated otherwise. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED

1. A method for determining the likelihood of a subject to develop a complication resulting from an adaptive immune response upon receiving an organ or a tissue transplantation, the method comprising:

(a) assaying a biological sample from said subject for *suppressor of fused (SUFU)* mRNA and/or SUFU protein expression or activity, wherein decreased *SUFU* mRNA and/or SUFU protein expression or activity indicates an increased risk for said complication, and increased *SUFU* mRNA and/or SUFU protein expression or activity indicates a decreased risk for said complication; and

(b) administering a personalized prophylaxis or treatment regimen to said subject based on the assessed risk for said complication.

2. The method of claim 1, wherein the assessed risk for said complication is further based on the degree of human leukocyte antigen (HLA) mismatch between said subject and the organ or the tissue transplanted.

3. The method of claim 1 or 2, wherein said complication is an organ or a tissue transplant rejection.

4. The method of claim 1 or 2, wherein said complication is graft-versus-host disease (GVHD) and said organ or tissue transplantation is an allogeneic hematopoietic stem cell transplantation (HSCT).

5. The method of claim 4, wherein the GVHD is acute GVHD.

6. The method of any one of claims 1-5, wherein subjects with increased risk for said complication receive more intensive prophylaxis and subjects with decreased risk for said complication receive less intensive prophylaxis.

7. The method of claim 6, wherein the more intensive prophylaxis comprises a more intensive immunosuppression regimen and the less intensive prophylaxis comprises a less intensive immunosuppression regimen.

8. The method of any one of claims 1-7, wherein the subjects with increased risk for said complication receive said organ or tissue transplantation from a more perfectly matched donor, and subjects with decreased risk for said complication receive said organ or tissue transplantation from a less perfectly matched donor.

9. The method of any one of claims 1-8, wherein said subject is a human.
10. The method of any one of claims 1-9, wherein said biological sample is derived from peripheral blood mononuclear cells (PBMCs).
11. The method of any one of claims 1-10, wherein said biological sample comprises a nucleic acid.
12. The method of claim 11, wherein said nucleic acid comprises genomic DNA, mRNA, or cDNA.
13. The method of any one of claims 1-12, wherein assaying for *SUFU* mRNA and/or *SUFU* protein expression or activity comprises a nucleic acid sequencing technique or a nucleic acid amplification technique.
14. The method of claim 13, wherein said nucleic acid amplification technique is selected from the group consisting of polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), transcription-mediated amplification (TMA), ligase chain reaction (LCR), strand displacement amplification (SDA), and nucleic acid sequence based amplification (NASBA).
15. The method of claim 13 or 14, wherein assaying for *SUFU* mRNA and/or *SUFU* protein expression or activity comprises detecting in the genome of said subject a marker locus that is associated with increased or decreased risk for said complication, wherein said marker locus comprises rs17114808 or a marker locus site in complete linkage disequilibrium with rs17114808.
16. The method of claim 15, wherein detecting homozygosity with cytosine at rs17114808 indicates an increased risk for said complication.
17. The method of claim 15, wherein detecting heterozygosity with cytosine and thymine at rs17114808 or homozygosity with thymine at rs17114808 indicates decreased risk for said complication.
18. The method of any one of claims 15-17, wherein the detecting comprises amplifying the rs17114808 marker locus and detecting the resulting amplified marker amplicon.
19. The method of claim 18, wherein the amplifying comprises:
 - (a) mixing an amplification primer or amplification primer pair for the rs17114808 marker locus with said nucleic acid from said biological sample, wherein said primer or primer pair is

complementary or partially complementary to a variant or fragment of the genomic locus comprising the rs17114808 marker locus, and is capable of initiating DNA polymerization by a DNA polymerase using said nucleic acid as a template; and

(b) extending said primer or primer pair in a DNA polymerization reaction comprising said DNA polymerase and said template to generate at least one amplicon.

20. The method of claim 18 or 19, wherein the amplifying comprises amplifying a variant or fragment of one or more polynucleotides comprising SEQ ID NO: 1, 2, 3, or 4.

21. The method of any one of claims 18-20, wherein said primer or primer pair comprises a variant or fragment of one or more polynucleotides comprising SEQ ID NO: 1, 2, 3, or 4 or complements thereof.

22. The method of any one of claims 18-21, wherein said primer or primer pair comprises SEQ ID NO: 7 or 8 or variants or fragments thereof.

23. The method of claim 22, wherein said primer pair comprises SEQ ID NO: 7 and 8.

24. The method of any one of claims 18-22, further comprising providing at least one labeled nucleic acid probe suitable for detection of the rs17114808 marker locus.

25. The method of claim 24, wherein said labeled nucleic acid probe comprises a variant or fragment of one or more polynucleotides comprising SEQ ID NO: 1, 2, 3, 4, or complements thereof.

26. The method of claim 24, wherein said labeled nucleic acid probe comprises a variant or fragment of one or more polynucleotides comprising SEQ ID NO: 9 or 10 or complements thereof.

27. The method of claim 24, 25, or 26 wherein said at least one labeled nucleic acid probe comprises a first probe capable of detecting a marker locus comprising a cytosine allele at rs17114808 and a second probe capable of detecting a marker locus comprising a thymine allele at rs17114808.

28. The method of any one of claims 24-27, wherein said at least one labeled nucleic acid probe comprises SEQ ID NO: 9 or 10.

29. The method of any one of claims 13-28, wherein the detecting comprises DNA sequencing of the rs17114808 marker locus or a marker locus site in complete linkage disequilibrium with rs17114808.

30. The method of any one of claims 1-29, wherein assaying for *SUFU* mRNA and/or *SUFU* protein expression or activity comprises a nucleic acid hybridization technique.

31. The method of claim 30, wherein said nucleic acid hybridization technique is selected from the group consisting of *in situ* hybridization (ISH), microarray, and Southern blot.

32. The method of any one of claims 1-31, wherein assaying for *SUFU* mRNA and/or *SUFU* protein expression or activity comprises a polypeptide detection technique.

33. The method of any one of claims 1-32, wherein assaying for *SUFU* mRNA and/or *SUFU* protein expression or activity comprises directly assaying genomic DNA.

34. The method of any one of claims 1-33, wherein assaying for *SUFU* mRNA and/or *SUFU* protein expression or activity comprises directly assaying a transcript produced from genomic DNA.

35. A kit for determining the likelihood of a subject to develop a complication resulting from an adaptive immune response upon receiving an organ or a tissue transplantation, the kit comprising:

(a) a *SUFU* detection reagent; and

(b) instructions for using said *SUFU* detection reagent and correlating *SUFU* detection with predicted risk for said complication.

36. The kit of claim 35, wherein:

(a) said *SUFU* detection reagent comprises an anti-*SUFU* antibody; and

(b) said instructions comprise instructions for using said anti-*SUFU* antibody and correlating detected *SUFU* protein levels with predicted risk for said complication.

37. The kit of claim 35, wherein:

(a) said *SUFU* detection reagent comprises a first and a second primer and/or a first probe, wherein said first and said second primer and said probe are capable of detecting a marker locus comprising a cytosine allele or a thymine allele at rs17114808 or detecting alleles at marker loci in complete linkage disequilibrium with rs17114808; and

(b) said instructions comprise instructions for using said first and second primers and/or said first probe for detecting said marker locus and correlating the detected marker locus with predicted risk for said complication.

38. The kit of any one of claims 35-37, wherein said complication is an organ or tissue transplant rejection.

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39. The kit of any one of claims 35-37, wherein said complication is graft-versus-host disease (GVHD) and said organ or tissue transplantation is an allogeneic hematopoietic stem cell transplantation (HSCT).

40. The kit of claim 39, wherein said GVHD is acute GVHD.

41. The kit of any one of claims 37-40, wherein said first and second primer comprise SEQ ID NO: 7 and 8, and said probe comprises SEQ ID NO: 9 or 10.

42. A method of screening for a sonic hedgehog (SHH) pathway antagonist, the method comprising contacting an antigen-presenting cell (APC) with a candidate compound and determining if said candidate compound inhibits SHH pathway regulation of graft-versus-host disease (GVHD).

43. The method of claim 42, wherein said SHH pathway antagonist is a small molecule, an antibody, or a nucleic acid encoding or comprising a silencing element.

44. The method of claim 42 or 43, wherein the determining step comprises assessing the antigen presentation capability of said APC.

45. The method of claim 44, wherein the assessing comprises assessing expression of HLA-DR.

46. The method of any one of claims 42-45, wherein the determining step comprises assessing stimulation or activation of donor T-cells by said APC.

47. The method of claim 46, wherein the assessing comprises an allogeneic mixed leukocyte response (MLR) assay.

48. The method of any one of claims 42-47, wherein the APC is a dendritic cell.

49. The method of any one of claims 42-48, wherein SHH pathway activity is detected by assessing expression or activity of *SUFU*, *GLI1*, and/or *GLI2*.

FIGURE 1

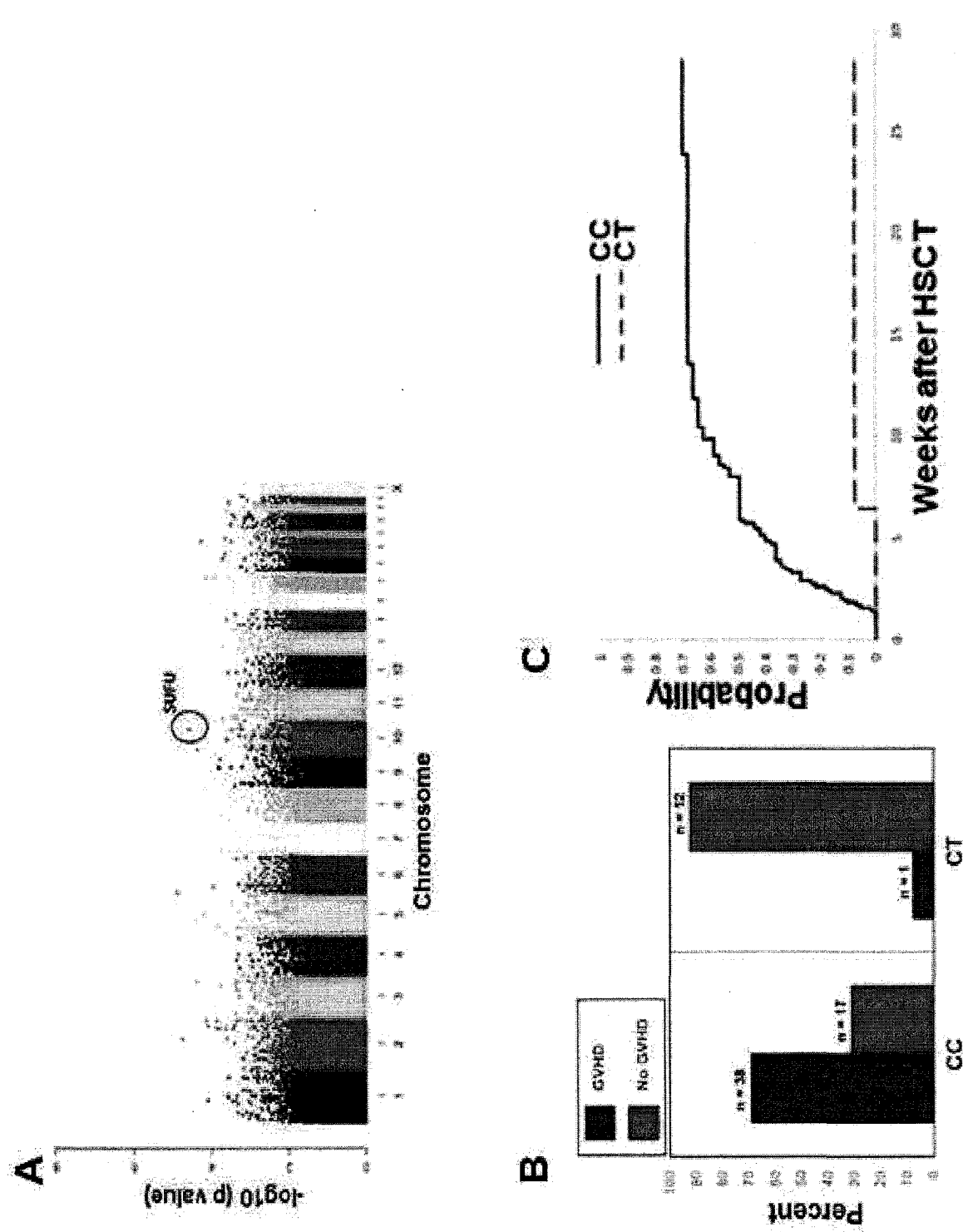
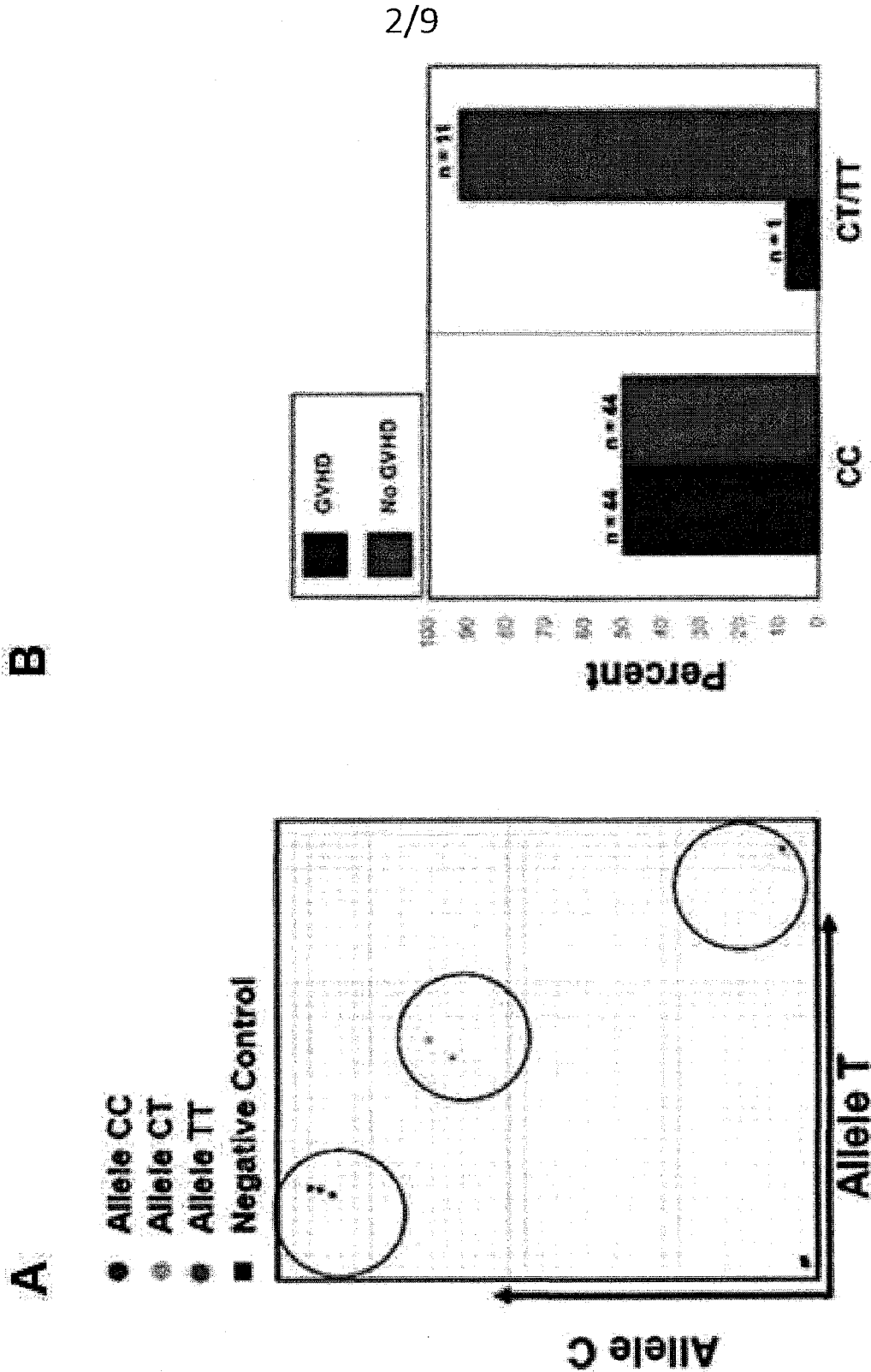


FIGURE 2



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FIGURE 2

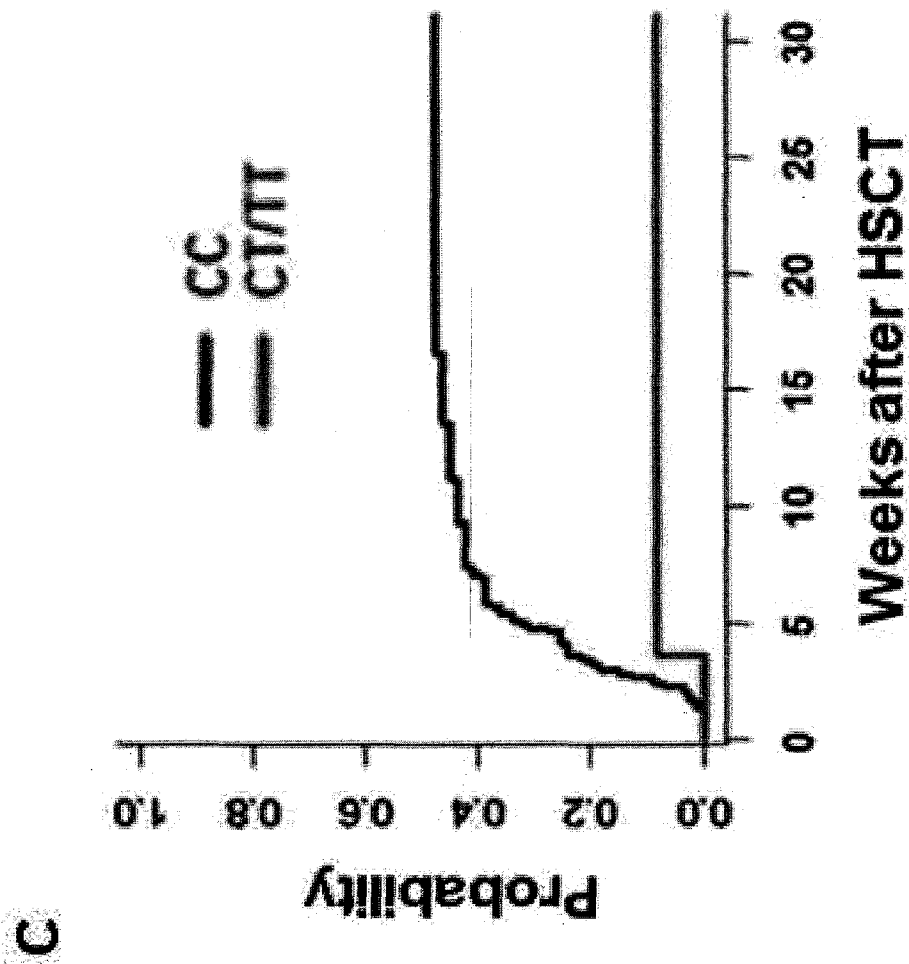


FIGURE 3

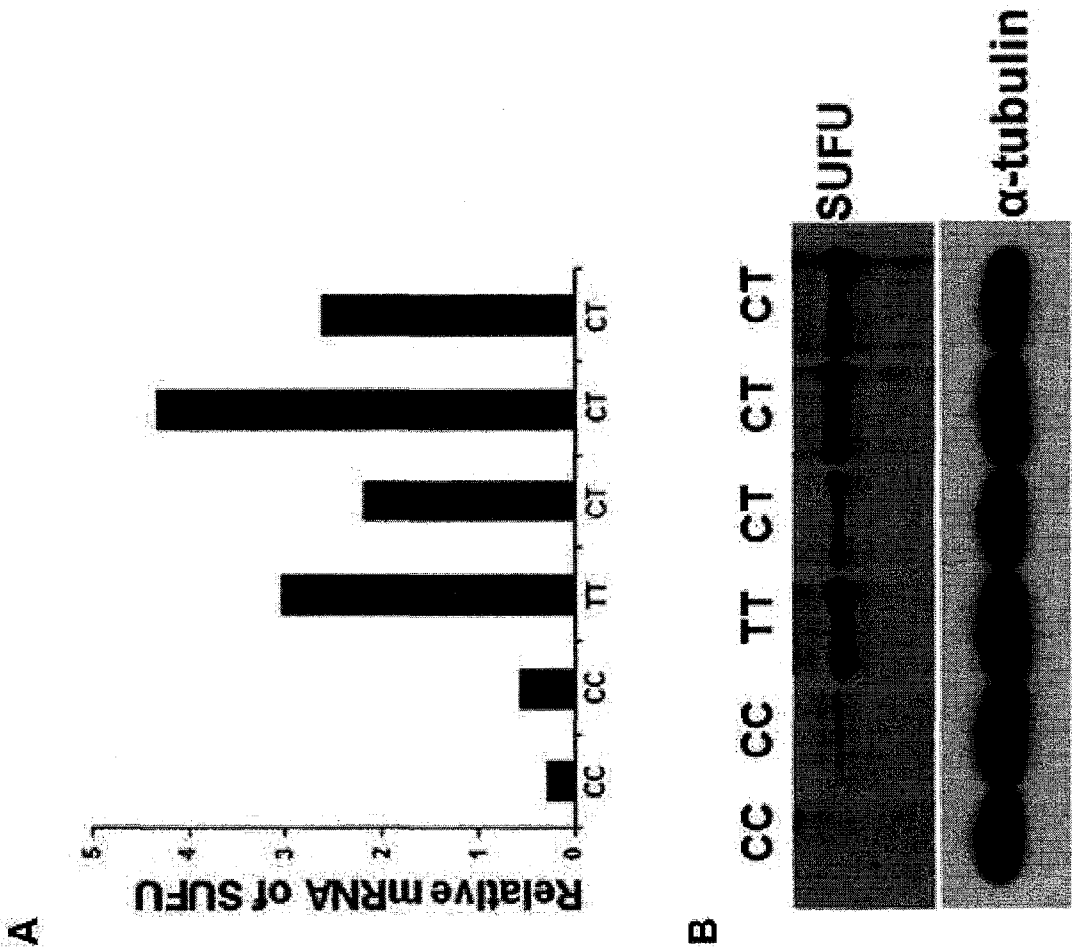


FIGURE 4

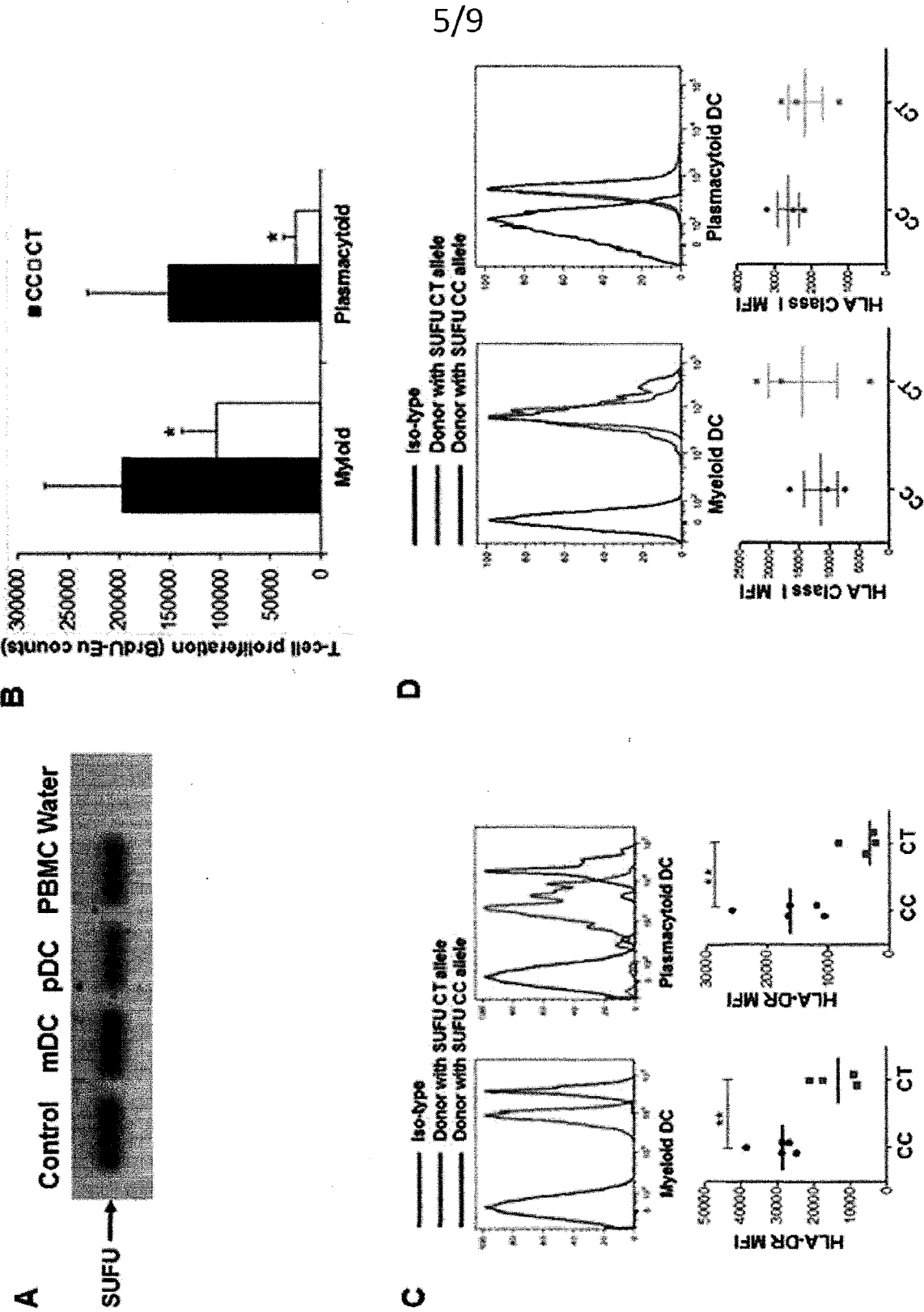
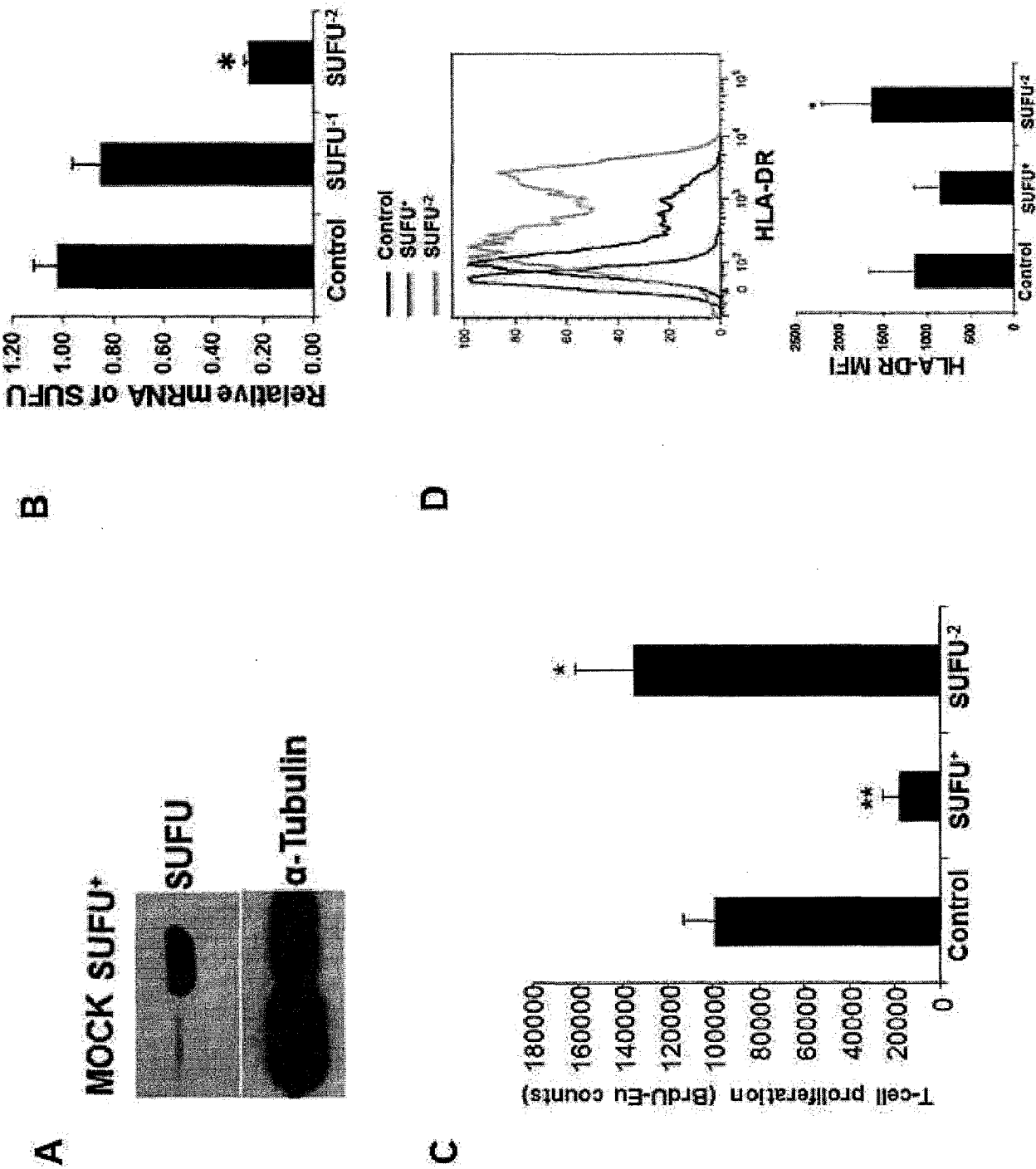


FIGURE 5



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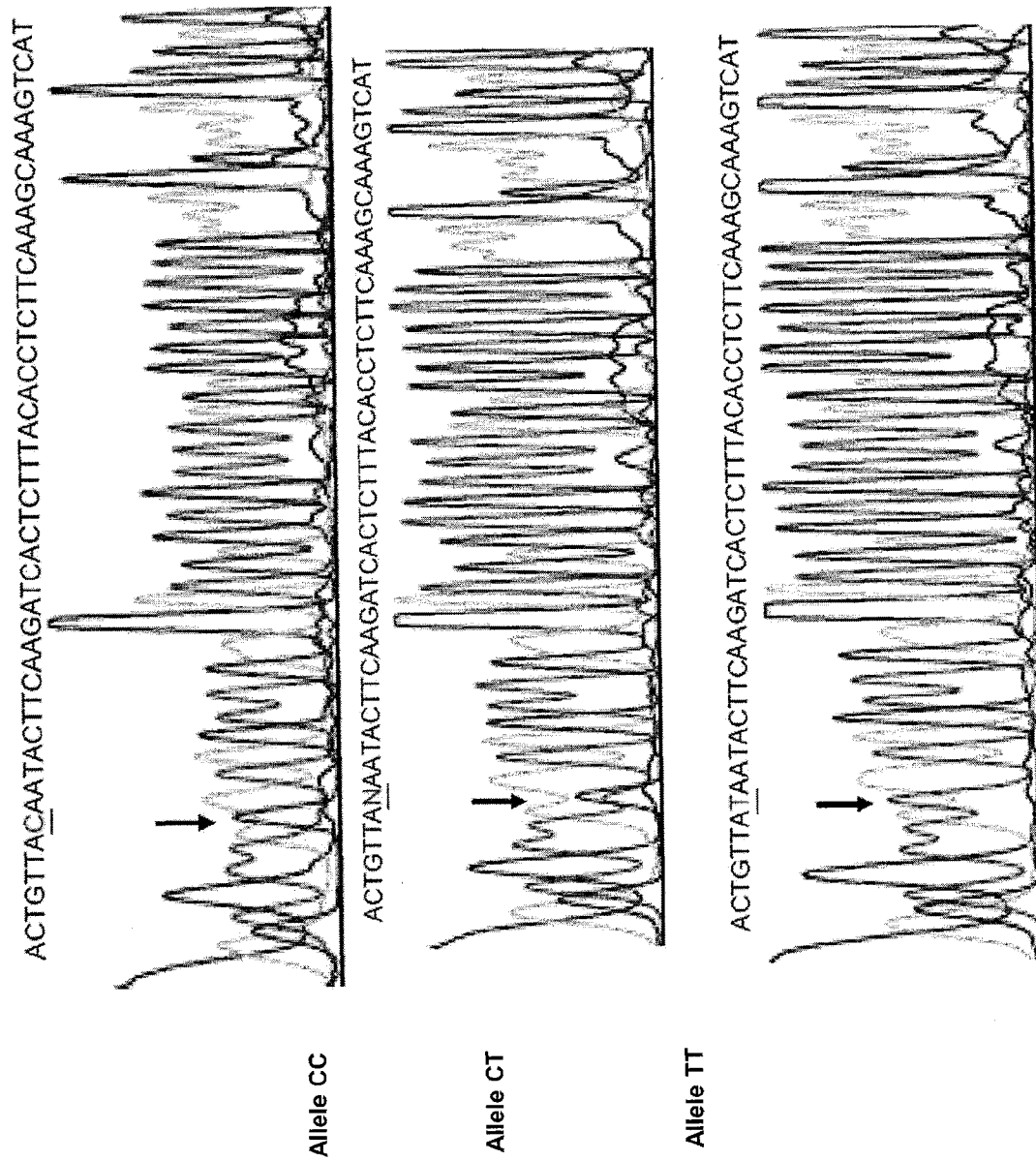
FIGURE 6

FIGURE 7

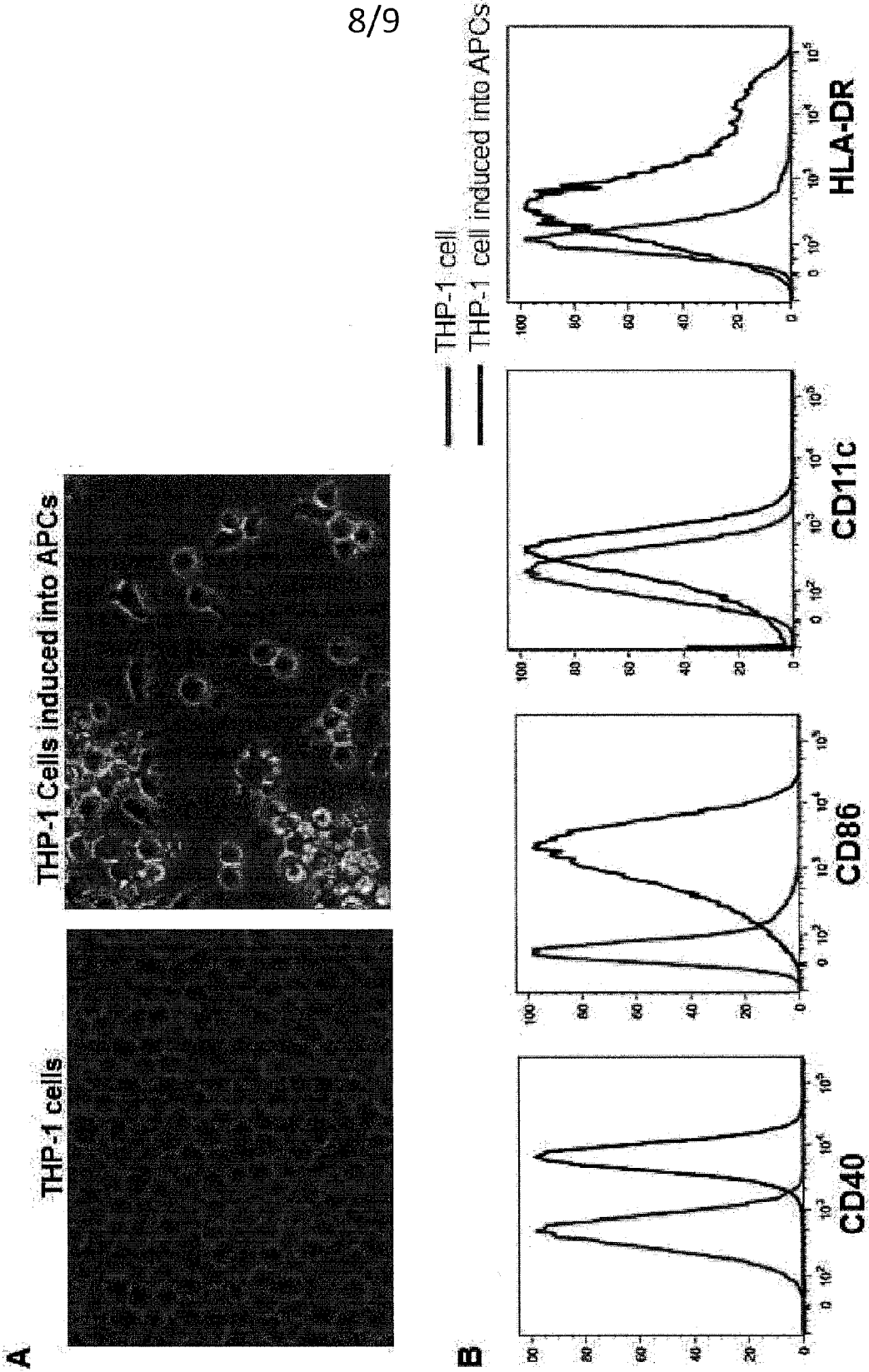
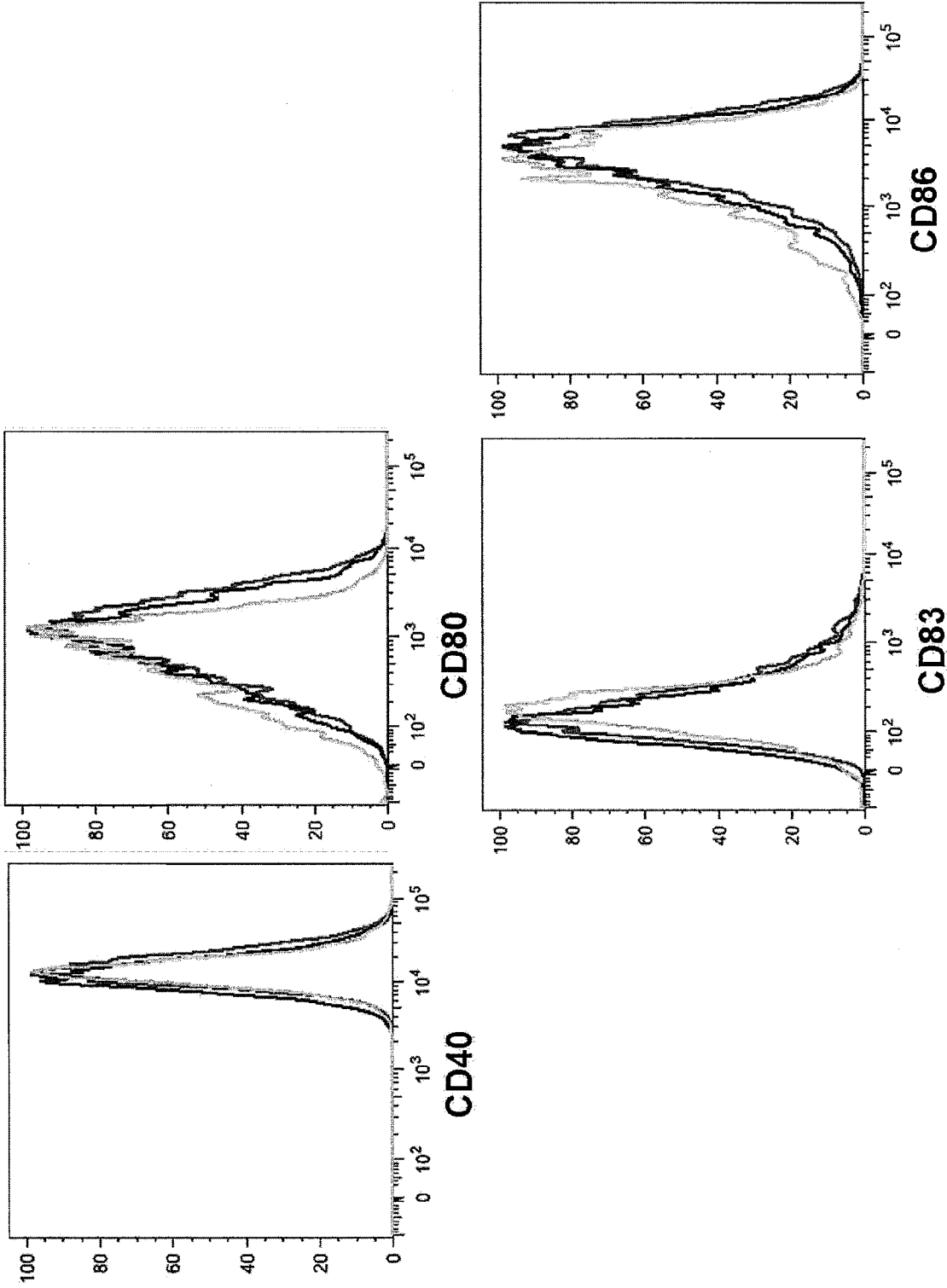


FIGURE 8

THP
THP-SUFU+
THP-SUFU-



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/035041

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

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)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XUEFENG HU ET AL: "Expression of SHH signaling molecules in the developing human primary dentition", BMC DEVELOPMENTAL BIOLOGY, BIOMED CENTRAL LTD., LONDON, GB, vol. 13, no. 1, 8 April 2013 (2013-04-08), page 11, XP021147824, ISSN: 1471-213X, DOI: 10.1186/1471-213X-13-11 p. 2, left-hand col., 2nd para; p. 7, left-hand col., 2nd para. ----- -/--	35,38-40



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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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

1 August 2016

Date of mailing of the international search report

10/08/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax: (+31-70) 340-3016

Authorized officer

Wipada, Leslie

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2016/035041

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHONG CHUAN WANG ET AL: "Aberrant expression of sonic hedgehog pathway in colon cancer and melanosis coli", JOURNAL OF DIGESTIVE DISEASES, vol. 14, no. 8, 4 July 2013 (2013-07-04), pages 417-424, XP055291979, ISSN: 1751-2972, DOI: 10.1111/1751-2980.12060 p. 418, right-hand col., 2nd para.; p. 419, table 1 -----	35,36, 38-40
X	AFFYMETRIX: "Data Sheet Affymetrix(R) Genome-Wide Human SNP Array 6.0", INTERNET CITATION, 2007, pages 1-4, XP002525407, Retrieved from the Internet: URL:http://www.affymetrix.com/support/technical/datasheets/genomewide_snp6_datasheet.pdf [retrieved on 2009-04-10] the whole document -----	35,37-40
X	DATABASE Geneseq [Online] 30 October 2008 (2008-10-30), "Influenza A virus viral replication sirna target SEQ ID NO 10512.", XP55292004, retrieved from EBI accession no. GSN:ASQ68325 Database accession no. ASQ68325 the whole document -----	35,37-41
X	ROTONDI M ET AL: "HIGH PRETRANSPLANT SERUM LEVELS OF CXCL10/IP-10 ARE RELATED TO INCREASED RISK OF RENAL ALLOGRAFT FAILURE", AMERICAN JOURNAL OF TRANSPLANTATION, BLACKWELL MUNKSGAARD, DK, vol. 4, 1 January 2004 (2004-01-01), pages 1466-1474, XP001199657, ISSN: 1600-6135, DOI: 10.1111/J.1600-6143.2004.00525.X abstract; p. 1473, left-hand col., last para. -----	1-34
X	US 2013/338035 A1 (DRESSEL RALF [DE] ET AL) 19 December 2013 (2013-12-19) para. 192-202; table 11 ----- -/--	1-34

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/035041

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XIAOBO YU ET AL: "Genetic Polymorphism of Interferon Regulatory Factor 5 (IRF5) Correlates with Allograft Acute Rejection of Liver Transplantation", PLOS ONE, vol. 9, no. 4, 30 April 2014 (2014-04-30), page e94426, XP055291948, DOI: 10.1371/journal.pone.0094426 abstract	1-34
Y	STANTON BENJAMIN Z ET AL: "Small-molecule modulators of the Sonic Hedgehog signaling pathway", MOLECULAR BIOSYSTEMS, ROYAL SOCIETY OF CHEMISTRY, GB, vol. 6, no. 1, 1 January 2010 (2010-01-01), pages 44-54, XP009137169, ISSN: 1742-206X, DOI: 10.1039/B910196A p. 48, para. bridging left- to right-hand col.; fig. 1	42-49
Y	VARAS ALBERTO ET AL: "Survival and function of human thymic dendritic cells are dependent on autocrine Hedgehog signaling", JOURNAL OF LEUKOCYTE BIOLOGY, FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY, US, vol. Journal of Leukocyte Biology, no. 6, 1 June 2008 (2008-06-01), pages 1476-1483, XP009191158, ISSN: 0741-5400, DOI: 10.1189/JLB.1107792 [retrieved on 2008-03-11] cited in the application abstract	42-49
X,P	RAFIJUL BARI ET AL: "Genome-wide single-nucleotide polymorphism analysis revealed SUFU suppression of acute graft-versus-host disease through downregulation of HLA-DR expression in recipient dendritic cells", SCIENTIFIC REPORTS, vol. 5, 11 June 2015 (2015-06-11), page 11098, XP055291727, DOI: 10.1038/srep11098 the whole document	1-49
	-/--	

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/035041

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Anonymous: "Reference SNP (refSNP) Cluster Report: rs17114808", 8 October 2004 (2004-10-08), XP055292002, Retrieved from the Internet: URL: http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=17114808 [retrieved on 2016-07-28] the whole document -----	1-49
A	ONISHI HIDEYA ET AL: "The Hedgehog inhibitor suppresses the function of monocyte-derived dendritic cells from patients with advanced cancer under hypoxia", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 436, no. 1, 22 May 2013 (2013-05-22), pages 53-59, XP028570474, ISSN: 0006-291X, DOI: 10.1016/J.BBRC.2013.05.057 p. 55, para. 3.2 -----	1-49
A	WO 2012/149653 A1 (INST RECH S CLINIQUES DE MONTREAL [CA]; CHARRON FREDERIC [CA]; IZZI LU) 8 November 2012 (2012-11-08) p. 24, l. 4-9; p. 25, l. 18-23 -----	1-49
A	KR 2014 0145669 A (MACROGEN INC [KR]; SNU R&DB FOUNDATION [KR]) 24 December 2014 (2014-12-24) the whole document -----	1-49

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2016/035041

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2013338035 A1	19-12-2013	EP 2652143 A2	23-10-2013
		EP 2738265 A2	04-06-2014
		US 2013338035 A1	19-12-2013
		WO 2012080359 A2	21-06-2012

WO 2012149653 A1	08-11-2012	US 2014079713 A1	20-03-2014
		WO 2012149653 A1	08-11-2012

KR 20140145669 A	24-12-2014	NONE	
