Donor Fibroblast Chimerism in the Pathogenic Fibrotic Lesion of Human Chronic Graft-Versus-Host Disease

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Purpose. Tissue atrophy and excessive fibrosis are prominent histologic features of chronic graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation, but the underlying mechanism remains unknown. The current study was undertaken to investigate whether the increase in fibroblasts at the site of pathogenic fibrosis originated from transplanted donor cells in patients with chronic GVHD.

Methods. Lacrimal gland biopsy specimens were obtained from nine patients with chronic GVHD. The male-specific sequences detected by fluorescein in situ hybridization (FISH) and in situ hybridization (ISH) were used as markers for the donor cells in seven female patients who had received a transplant from male donors. Primary fibroblast cultures were generated from lacrimal gland biopsy specimens and examined for mismatched genetic markers between recipients and donors.

Results. In lacrimal gland specimens obtained from seven female patients who received a sex-mismatched transplant, 13.4% to 26.7% of $CD34^+$ fibroblasts that accumulated in the fibrotic lesion were donor derived, as determined by FISH for the Y-chromosome. The male-specific mRNA was also detected in the lacrimal gland fibroblasts by ISH. Primary lacrimal gland fibroblast cultures were generated from four patients with chronic GVHD and further examined for mismatched genetic markers between recipients and donors. As a result, the presence of donor origin of the fibroblasts was demonstrated by detecting the Y-chromosome sequence and donor-specific microsatellite genetic markers.

Conclusions. These findings together indicate the chimeric status of accumulated $CD34^+$ fibroblasts in the lacrimal gland of patients with chronic GVHD. Fibroblasts originating from circulating donor-derived precursors may participate in the excessive fibrosis in these patients. (*Invest Ophthalmol Vis Sci.* 2005;46:4519–4527) DOI:10.1167/iovs.05-0227

Graft-versus-host disease (GVHD) is a major cause of morbidity and mortality in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) for hematologic malignancies.[1] Acute GVHD is a distinctive syndrome of dermatitis, hepatitis, and enteritis that occurs soon after HSCT.[1] The pathogenic process of acute GVHD is explained as an alloimmune response of donor lymphocytes to recipient cells, followed by dysregulated cytokine production and the recruitment of additional effector cells.[2] In contrast, chronic GVHD is a more pleiotropic syndrome that develops several months after transplantation and frequently affects the skin, lung, liver, gastrointestinal tract, mouth, and eye.[1] The main histologic feature of chronic GVHD is widespread tissue atrophy and fibrosis with lymphocytic infiltration,[3] but less is understood about the pathophysiology of the condition.[1,4]

Dry eye has been recognized as a major complication in patients in whom chronic GVHD develops after allogeneic HSCT.[5–7] In most cases, severe dry eye progresses rapidly after the onset of symptoms and sometimes leads to blindness. Because of the increasing number of long-lived survivors who have received allogeneic HSCT, dry eye has a significant impact on these patients' quality of life. However, the pathogenic process of dry eye associated with chronic GVHD remains largely unknown.

We have been examining the pathogenesis of chronic GVHD by focusing on lacrimal gland involvement, one of the most frequent complications in the disease.[7–9] The main histologic findings in the affected lacrimal gland are marked fibrosis of the interstitium and a prominent increase in the number of $CD34^+$ fibroblasts accompanied by mild lymphocytic infiltration. Clinically, the severity of the dry eye correlates with the degree of fibrotic changes, rather than of lymphocytic infiltration, indicating that excessive extracellular matrix accumulation primarily contributes to the exocrine dysfunction. The $CD34^+$ fibroblasts at the interstitium also play a role in inflammation, in which the fibroblasts attach to lymphocytes and express human leukocyte antigen class II and costimulatory molecules. These findings together indicate that $CD34^+$ fibroblasts play an important role in the pathogenesis of lacrimal gland chronic GVHD.

Recently, several studies have reported the presence of donor-derived nonhematopoietic cells in various organs of HSCT recipients. These include neurons of the central nervous system,[10] gastric and intestinal epithelium,[11,12] subepithelial myofibroblasts of the intestine,[13] hepatocytes, keratinocytes,[12] and buccal epithelial cells.[14] These donor-derived cells are thought to play a role in regenerating the damaged tissues, but their proportion is very small. Several in vivo studies involving animal models, however, indicate that bone marrow–derived fibroblasts contribute to wound repair[15] and even to the development of pathogenic fibrosis.[16] These observations led us to hypothesize that a subset of the fibroblasts increased at the chronic GVHD lesion originated from transplanted donor cells. To test this hypothesis, we evaluated the origin of the $CD34^+$ fibroblasts that accumulate in the chronic GVHD lacrimal gland by detecting mismatched genetic markers in tissue specimens as well as in primary cultures of fibroblasts.

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Materials and Methods

Study Subjects

We studied nine allogeneic HSCT recipients who had clinically significant dry eye[17] and who had a diagnosis of chronic GVHD.[18] Lacrimal gland biopsy specimens that had been obtained for diagnostic purposes were available in all patients. Seven patients were women who had received sex-mismatched HSCTs from male donors (male-to-female recipients) with full donor lymphohematologic engraftment. A male patient who had received an HSCT from a male donor (male-to-male recipient) and a female patient who had received an HSCT from a female donor (female-to-female recipient) principally served as control subjects. All recipients underwent a myeloablative regimen consisting of total body irradiation in combination with cytosine arabinoside or cyclophosphamide, or a combination of fludarabine and melphalan. Peripheral blood mononuclear cells, bone marrow cells, and Epstein-Barr virus (EBV)-transformed B-cell line (BCL) obtained from recipients before and after HSCT as well as donor-derived peripheral blood mononuclear cells and bone marrow cells were used as a source of genomic DNA. Dry eye was diagnosed according to the diagnostic criteria proposed by Lemp,[17] with some modifications: Any sign of tear film instability (tear break-up time [BUT] $\leq$5 seconds, Schirmer test $\leq$5 mm, or cotton thread test $\leq$10 mm), and any abnormality of the ocular surface (rose-bengal score $\geq$3, fluorescein score $\geq$1), and/or symptoms of ocular irritation. Severe dry eye was defined as reduced reflex tearing (Schirmer test with nasal stimulation $\leq$10 mm) and ocular surface abnormality (rose-bengal score $\geq$3 and/or fluorescein score $\geq$1).[7,19] In some experiments, the lacrimal gland tissue obtained from a female patient with Sjögren's syndrome was used as a control. Written informed consent was obtained in advance from all patients in accordance with the principles expressed in the Declaration of Helsinki. This study was approved by the Keio University Institutional Review Boards.

Lacrimal Gland Specimens

Lacrimal gland tissues obtained by biopsy were divided into pieces and subjected to histologic analysis and primary culturing of fibroblasts. For culture, a portion of the tissue was placed on fibronectin-coated plastic plates and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS), 50 U/mL penicillin, and 50 μg/mL streptomycin without any exogenous growth factor. Proliferating adherent cells were passaged and expanded, and the cells at the third to fifth passage were used as cultured fibroblasts. In some experiments, a primary culture of fibroblasts generated from the skin biopsy of a female volunteer was used as a control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens were subjected to hematoxylin and eosin staining or CD34 immunostaining. For diaminobenzidine staining, anti-CD34 monoclonal antibody (mAb; NU-4A1; Nichirei, Tokyo, Japan) and peroxidase-conjugated secondary Ab (En Vision+; Dako, Glostrup, Denmark) were used in combination with nuclear staining with hematoxylin. For fluorescent staining, a fluorescein isocyanate (FITC)-conjugated anti-CD34 mAb (43A1; Calbiochem, San Diego, CA) was used in combination with TO-PRO-3 nuclear staining (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Double staining of frozen sections was also performed with FITC-conjugated anti-CD34 mAb in combination with mouse anti-CD45 (PD7/26; Dako) or anti-CD90 mAb (AS02; Dianova, Hamburg, Germany) followed by inoculation with Alexa 568–conjugated rabbit anti-mouse IgG antibody (Molecular Probes) for 1 hour at room temperature. Isotype-matched mAbs to irrelevant antigen were used as controls in all tissue staining. The sections were mounted and examined with a confocal microscope (LSM5 Pascal; Carl-Zeiss Meditec, Göttingen, Germany).

Cultured fibroblasts were fixed with 4% paraformaldehyde for 10 minutes at room temperature and incubated with FITC-conjugated anti-CD34 mAb for 1 hour at room temperature. The cells were also incubated with mouse mAb to CD90, vimentin (V9; Dako), collagen type I (I-8H5; Daiichi Fine Chemical, Takaoka City, Japan), CD45, or pancytokeratin (mAb cocktail; Triton Bioscience, Alameda, CA), or rabbit polyclonal Ab to factor VIII (Dako) in combination with a tetramethylrhodamine isothiocyanate isomer R (TRITC)-conjugated anti-mouse or anti-rabbit IgG antibodies (Dako) for 1 hour at room temperature. Isotype-matched antibodies to the irrelevant antigen were used in control experiments. Fluorescent images were obtained with a confocal laser fluorescence microscope.

Fluorescein In Situ Hybridization Analysis for the Y-Chromosome

Fluorescein in situ hybridization analysis (FISH) for the Y-chromosome was performed principally according to the published method.[12,20] Briefly, we performed a two-step procedure on 6-μm-thick paraffin-embedded sections: immunostaining with FITC-conjugated anti-CD34 mAb and TO-PRO-3, followed by FISH of the same section using a DNA probe for the Y-chromosome (Vysis, Downers Grove, IL) and restaining with TO-PRO-3. The slides were observed under a confocal laser fluorescence microscope. The fields of the immunostaining and FISH images were matched according to the location and architecture of the tissue, especially the nuclear distribution, to be as close to each other as possible. $CD34^+$ spindle-shaped cells with an oval nucleus residing in the interstitium were regarded as fibroblasts, whereas cells with a single Y-FISH signal in the nucleus were regarded as donor-derived male cells. Three observers blindly evaluated at least 100 nonoverlapping fibroblasts. The frequency (%) of donor-derived fibroblasts was calculated according to the after following formula: the number of $Y\text{-}FISH^+$ fibroblasts divided by the number of fibroblasts. At least three different fields of three to four sections were analyzed by three independent observers and the results expressed as the mean $\pm$ SD. Cultured fibroblasts were also analyzed with a combination of X- and Y-specific probes (Vysis).

In Situ Hybridization for Detection of Male-Specific mRNA

Donor-derived male cells were further detected by in situ hybridization (ISH; GenPoint System; Dako).[21] The *Smcy* gene was used as a male-specific marker,[22] and an antisense probe (5′-TTGTCTTAGTGCAGGGCTCTAGGCTCAAGTGCTGCT-3′) and the corresponding sense probe were labeled with digoxigenin at the 5′ end and incubated with consecutive sections.

Detection of the Y-Chromosome by PCR

Genomic DNA was extracted from freshly isolated cells or cultured cells by proteinase K digestion, followed by phenol extraction and ethanol precipitation. The Y-chromosome-specific *TSPY* gene[23] was amplified by PCR with a sense primer (5′-CTTCCACCTTCAGCCACCGCCTCCTCT-3′) and an antisense primer (5′-CTGTTGTGCGCTGCCTTGACGACCCAG-3′). The PCR condition was 40 cycles of 30 seconds at 95°C, 30 seconds at 66°C, and 1 minute at 72°C.[24] The PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide. The PCR procedure was repeated at least two times.

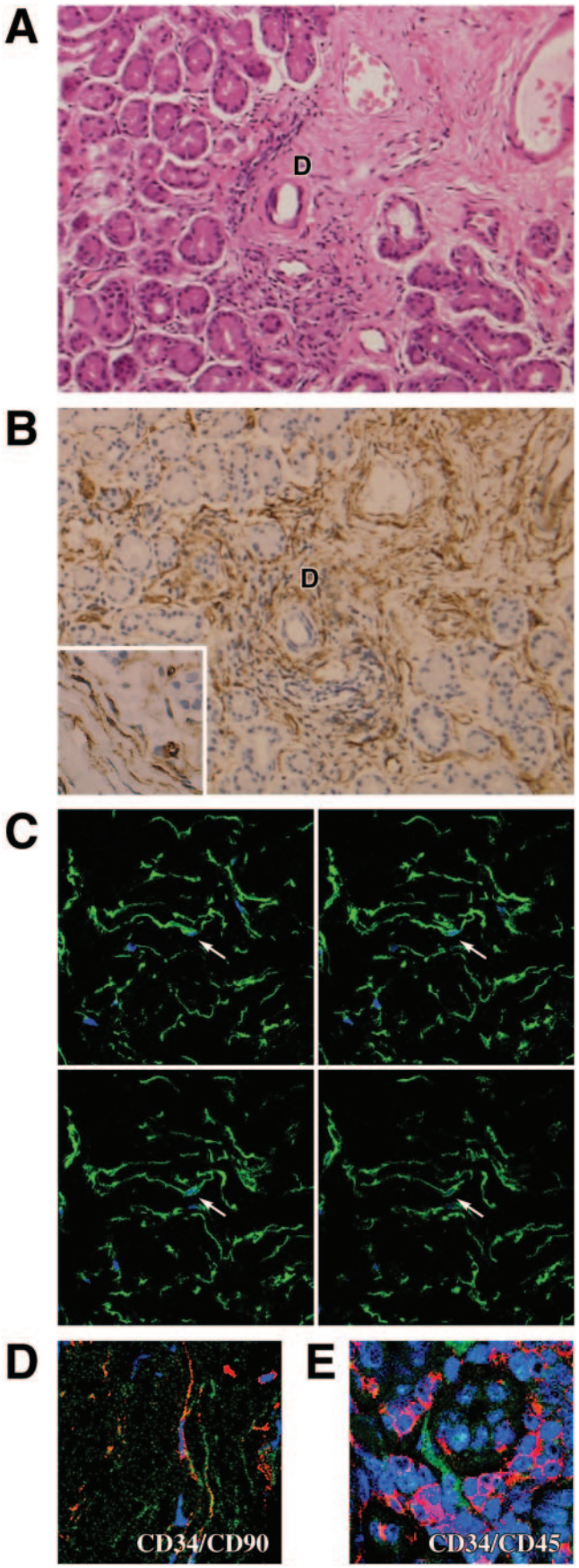
Detection of Mismatched Microsatellite Markers

Microsatellite polymorphic markers were determined by PCR using fluorescein-labeled primers followed by analysis of the length of the products using a capillary automated sequencer (Prism 310; Applied Biosystems, Inc. [ABI], Foster City, CA).[25] The polymorphic microsatellite markers evaluated were *D10S674*, *D10S518*, *D10S466*, *chr10.fa.O7frz.20604262*, *AL157895.3_20342*, *D10S595*, *D10S211*, and *D10S1228* on chromosome 10.[25] Mismatched polymorphic mark-

TABLE 1. Demographic and Clinical Characteristics of Patients with Chronic GVHD

Case	Age at Biopsy	Recipient Gender	Donor Gender	Hematopoietic Stem Cell Source	Underlying Disease	Donor Type	TBI	Interval between HSCT and Biopsy (mo)	Interval between Dry Eye Onset and Biopsy (mo)	FS (points)	RB (points)	BUT (seconds)	Degree of Dry eye	Clinically Affected Chronic GVHD Organs
1	32	Female	Male	Bone marrow	APL	Related	+	36	18	3	3	3	Mild	Eye, liver
3	35	Female	Male	Bone marrow	CML	Unrelated	+	14	7	6	5	0	Severe	Eye, mouth, liver, lung, skin
11	22	Female	Male	Bone marrow	APL	Unrelated	+	29	18	5	7	2	Severe	Eye, mouth, liver
13	44	Female	Male	Bone marrow	MDS	Unrelated	+	11	5	6	5	3	Severe	Eye, mouth, intestine, skin
14	39	Female	Male	Peripheral blood	CML	Related	+	13	2	1	3	5	Mild	Eye
16	42	Female	Male	Bone marrow	ALL	Related	+	23	12	5	3	3	Severe	Eye, mouth, liver, lung
17	36	Female	Male	Peripheral blood	MM	Related	−	11	9	3	5	3	Severe	Eye, mouth, liver
15	38	Male	Male	Bone marrow	MDS	Unrelated	+	9	2	3	5	4	Mild	Eye
2	47	Female	Female	Bone marrow	AML	Unrelated	+	5	0	3	3	5	Mild	Eye, liver

APL, acute promyelocytic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; MM, multiple myeloma; AML, acute myelogenous leukemia; TBI, total body irradiation; FS, fluorescein score; RB, rose-bengal score; BUT, tear break-up time.



ers were identified by comparing the peaks generated from the samples with those obtained using control donor and recipient-derived cells. All microsatellite analysis was repeated at least two times.

Results

Clinical Characteristics of Patients with Chronic GVHD

Demographic and clinical characteristics of nine HSCT recipients are summarized in Table 1. Allogeneic grafts were derived from the bone marrow in seven patients and from the G-CSF-mobilized peripheral blood in two patients. An interval between HSCT and lacrimal gland biopsy and an interval between onset of dry eye and the biopsy were various among the patients. All patients had symptomatic dry eye and ocular findings typical of chronic GVHD. The degree of dry eye was severe in five patients. Seven patients had additional chronic GVHD lesions in areas such as the mouth, intestine, liver, lung, and skin.

Lacrimal Gland Histology

The lacrimal gland of all the patients represented various degrees of fibrosis in the interstitium and irregular cell loss of the acini with mild lymphocyte infiltration (Fig. 1A). CD34 immunostaining showed marked expression in the periductal interstitium (Fig. 1B). Most $CD34^+$ cells had a spindle-shaped morphology with an oval nucleus, consistent with fibroblasts. Typical *z*-series images for CD34 expression showed that the $CD34^+$ cells had a complex morphology. They were spatially distributed but connected to each other, forming a reticular network (Fig. 1C). CD34 was originally found to be expressed on hematopoietic stem cells and endothelial cells, but was later shown also to be expressed on a subset of stromal fibroblasts in normal exocrine glands.[26] To confirm whether $CD34^+$ cells accumulated in the chronic GVHD lacrimal gland were fibroblasts, we subjected lacrimal gland tissue to double-staining for CD34 in combination with CD90 or CD45. Most $CD34^+$ cells in the interstitium coexpressed a fibroblast marker CD90,[27] but only half of $CD90^+$ fibroblasts expressed CD34 (Fig. 1D), indicating that there were $CD34^+$ and $CD34^-$ fibroblasts in the lacrimal gland from patients with chronic GVHD. In contrast, none of the $CD34^+$ cells simultaneously expressed a hematopoietic marker CD45, whereas many $CD45^+$ round cells were located around spindle-shaped $CD34^+$ cells (Fig. 1E). Taken together, these findings indicate that most $CD34^+$ spindle-shaped cells that accumulate in the interstitium of lacrimal gland are nonhematopoietic fibroblasts.

Detection of Donor-Derived $CD34^+$ Fibroblasts in the Lacrimal Gland Tissue

To evaluate the origin of the fibroblasts that accumulated in the lacrimal gland tissue, we used the Y-chromosome as a

Figure 1. Typical histologic findings of the lacrimal gland in patients with chronic GVHD. (**A**) Hematoxylin and eosin staining of a section from patient 14. D, duct. (**B**) CD34 immunostaining of the consecutive section of (**A**). *Inset*: A high-magnification view. (**C**) The *z*-series images of CD34 immunostaining on a 10-μm-thick paraffin-embedded section from patient 13. A series of 1.67-μm optical images is shown. CD34 expression was demonstrated by FITC (*green*), whereas nuclei were counterstained with TO-PRO-3 (*blue*). *Arrows*: one particular $CD34^+$ fibroblast appeared on multiple consecutive sections. (**D**) Immunofluorescent double-staining of CD34 (*green*) and CD90 (*red*) on a lacrimal gland section from patient 17. Nuclei were counterstained with TO-PRO-3 (*blue*). (**E**) Immunofluorescence double-staining of CD34 (*green*) and CD45 (*red*) on a lacrimal gland section from patient 17. Nuclei were counterstained with TO-PRO-3 (*blue*). Original magnification: (**A**, **B**) ×100; (**A**, *inset*) ×400; (**C**–**E**) ×630.

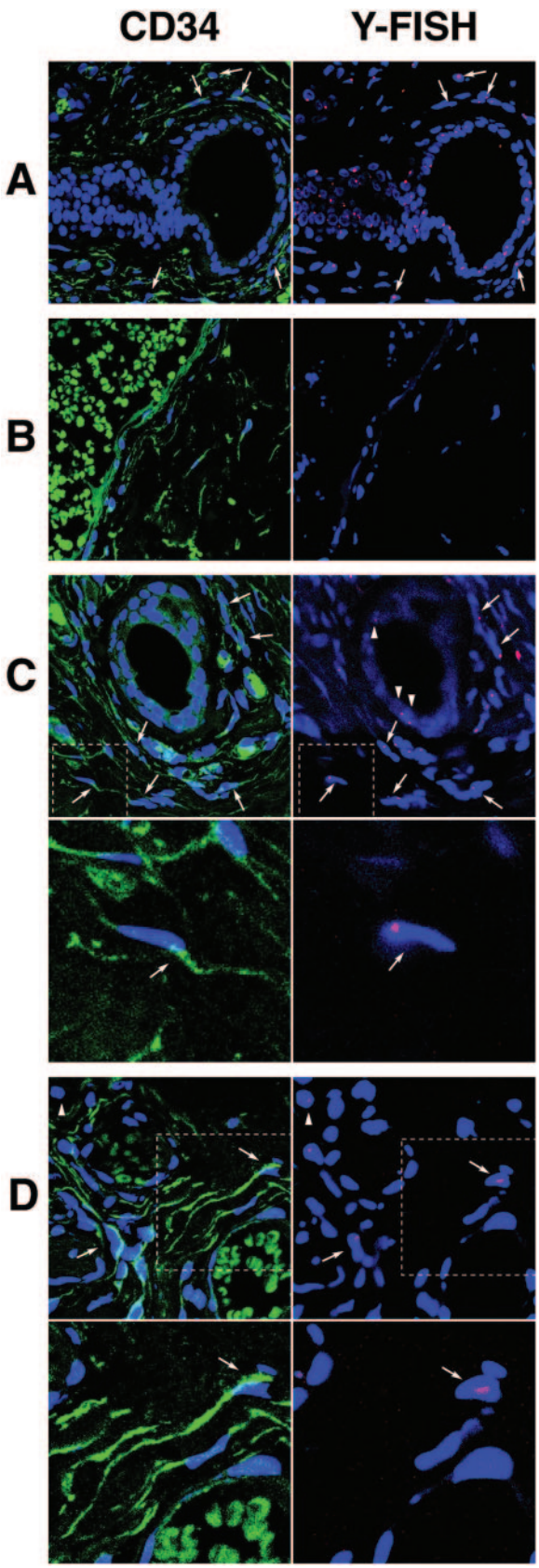


FIGURE 2. Representative CD34 immunostaining and Y-FISH images of the same lacrimal gland sections from patients with chronic GVHD. *Left*: CD34 staining (*green*); *right*: Y-FISH (*red*) with TO-PRO-3 staining (*blue*) of the same region. (**A**) Male-to-male recipient 15. (**B**) Female-to-female recipient 2. (**C**) Male-to-female recipient 17. (**D**) Male-to-female recipient 14. (**C**, **D**, *bottom*) High-magnification views of lesions shown in *dotted squares*. *Arrows*: $CD34^+$ fibroblasts with a Y-FISH signal; *arrowheads*: $Y\text{-}FISH^+$ cells negative for CD34. Red blood cells in the vascular lumen showed nonspecific autofluorescence.

marker for the donor cells in male-to-female recipients. As a marker for fibroblasts, we selected CD34 expressed by most accumulated fibroblasts. We first made sure that our procedure for identifying $Y\text{-}FISH^+$ fibroblasts was reliable by examining positive and negative control cells. Lacrimal gland sections from male-to-male recipient 15 provided the positive control and sections from female-to-female recipient 2 served as the negative control. In sections from the positive control, a Y-FISH signal was detected in nearly half of the nuclei of the $CD34^+$ fibroblasts (Fig. 2A), and the frequency of $Y\text{-}FISH^+$ fibroblasts was 45.2% ± 5.0%. The low sensitivity is probably due to truncation of the Y-chromosome sequence during sectioning and/or incomplete hybridization. The positive frequency of $Y\text{-}FISH^+$ cells in the male tissue was comparable to the value in previous reports.[13,28] In contrast, no Y-FISH signal was detected in the negative control sections (Fig. 2B). We next evaluated lacrimal gland tissue from seven male-to-female recipients. Representative images for CD34 immunostaining and Y-FISH of the same section are shown in Figures 2C and 2D. Donor-derived fibroblasts with a Y-FISH signal were found around medium-sized ducts (Fig. 2C) and blood vessels (Fig. 2D), where the number of fibroblasts was increased. The proportion of donor-derived cells in the $CD34^+$ fibroblasts ranged from 13.4% to 26.7% (Table 2), but this observed frequency was underestimated because the frequency in the male-to-male recipient was only 45.2% ± 5.0%. We also noted $Y\text{-}FISH^+$ donor-derived cells negative for CD34 in the interstitium and in the epithelia of acini and ducts. Most these cells were probably lymphocytes (Fig. 2C and Fig. 2D, arrowheads), but some may have been $CD34^-$ fibroblasts and epithelial cells.

For the seven male-to-female recipients, there was no significant association of the donor-derived fibroblast frequency with the donor type (related versus unrelated), interval between HSCT and biopsy, interval between onset of dry eye and biopsy, the degree of dry eye (severe versus mild), or the presence of other clinically affected chronic GVHD lesions. The frequency of donor-derived $CD34^+$ fibroblasts in patients 16 and 17, who received a transplant of peripheral blood stem cells, was comparable to the frequency in the other patients, who underwent transplantation of bone marrow cells.

We further examined the presence of donor-derived fibroblasts in the lacrimal gland by in situ hybridization for the male-specific *Smcy* gene. To verify the assay system, we evaluated lacrimal gland sections from male-to-male recipient 15 and a female patient with Sjögren's syndrome. As expected, a positive signal was detected for the antisense probe in the male control (Fig. 3A) but not in the female control (Fig. 3B). Representative results of male-to-female recipient 1 showed donor-derived fibroblasts (arrows) and endothelium (arrowheads) in the periductal area (Fig. 3C). The consecutive section incubated with the sense probe showed negative staining. The sections from four additional male-to-female recipients all showed positive staining specific for the antisense probe in the interstitial fibroblasts (Table 2). In consecutive sections from male-to-female recipient 17, *Smcy*-positive fibroblasts around a

TABLE 2. Identification of Donor-Derived Fibroblasts in the Chronic GVHD Lacrimal Gland Tissue

Case	Gender of Donor and Recipient	%Y-FISH$^+$ CD34$^+$ Fibroblasts*	Y-Chromosome$^+$ Fibroblasts by ISH
1	Male-to-female	13.4 ± 2.9	Present
3	Male-to-female	26.7 ± 9.4	Present
11	Male-to-female	20.0 ± 0.0	Not tested
13	Male-to-female	13.4 ± 1.2	Present
14	Male-to-female	20.1 ± 3.0	Present
16	Male-to-female	17.2 ± 6.8	Not tested
17	Male-to-female	14.7 ± 2.5	Present
15	Male-to-male	45.2 ± 5.0	Present
2	Female-to-female	0	Absent

* The mean ± SD of three independent observations of more than 100 fibroblasts.

medium-sized duct were confirmed to be positive for CD34 and Y-FISH signals (Fig. 3D).

Detection of Donor-Derived Cells in Primary Fibroblast Cultures Generated from Lacrimal Gland Biopsy Specimens

Primary fibroblast cultures generated from lacrimal gland biopsies were used as a source material for confirmation of the presence of donor-derived fibroblasts. This primary culture was successfully generated from four patients with chronic GVHD, including three male-to-female recipients and one male-to-male recipient (Table 3). All the cells in these cultures had a spindle shape and were positive for vimentin, collagen type I, and CD90 (Fig. 4A), consistent with the fibroblast phenotype. None of the cells were positive for CD45, cytokeratin, or factor VIII, indicating no contamination by cells of hematopoietic, endothelial, or epithelial origin. The cultured fibroblasts were heterogeneous in terms of CD34 expression (Fig. 4A), and the proportion of CD34$^+$ cells ranged from 40% to 88% at the fifth passage in the cultures from four patients.

FISH was performed on cultured fibroblasts with probes for X- and Y-chromosomes (Fig. 4B). The culture from male-to-male recipient 15 showed that the detection sensitivity of cells positive for a set of X- and Y-FISH signals was 50%. In contrast, control dermal fibroblasts generated from a female individual exhibited one or two X-FISH signals and no Y-FISH signal in all cells. In two male-to-female recipients (patients 16 and 17), the frequency of donor-derived fibroblasts with a set of X- and Y-FISH signals in a total of 200 cells was 2% and 4%, respectively. The frequency of donor-derived fibroblasts in the primary culture was much lower than that observed in the tissue, probably because of the predominance of the residential fibroblasts of recipient origin that had grown in the in vitro culture. When Y-FISH was combined with CD34 immunostaining, donor-derived fibroblasts were detected in both CD34$^+$ and CD34$^-$ cell populations (data not shown). In situ hybridization for the *Smcy* antisense probe also demonstrated the presence of donor-derived fibroblasts in male-to-female recipient 17.

Genomic DNA was extracted from the cultured fibroblasts and used in assays to detect genetic markers that are different between recipients and donors. First, the Y-chromosome-specific *TSPY* gene was used as a marker for male cells in PCR. In a representative male-to-female recipient, patient 14, the *TSPY* gene was not amplified in the recipient before HSCT BCL, but was amplified in cultured lacrimal gland fibroblasts obtained after HSCT, donor peripheral blood and bone marrow cells, and post-HSCT recipient BCL (Fig. 4C). Analogous findings were obtained in two additional male-to-female recipients (patients 16 and 17).

Microsatellite markers were also used to test for a chimeric status based on variation in the length of the PCR products (Fig. 4D). In male-to-female recipient 14, lacrimal

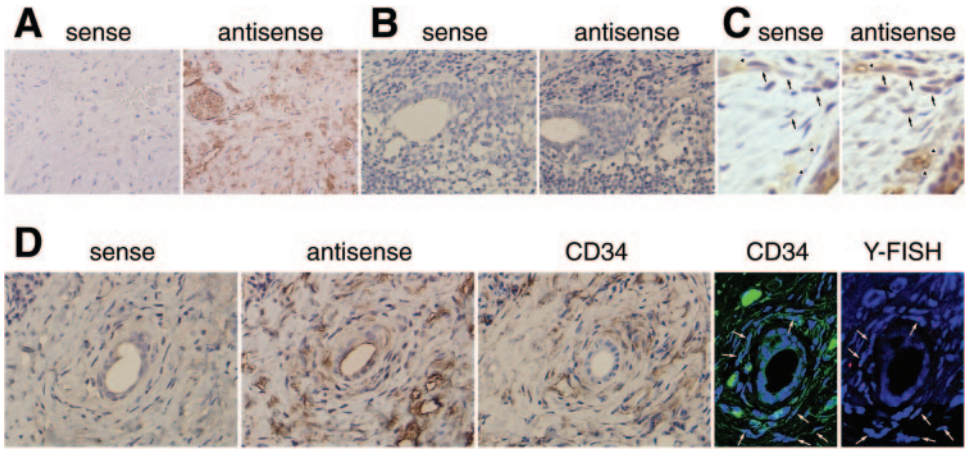


FIGURE 3. In situ hybridization for the detection of Y-chromosome-specific *Smcy* mRNA in lacrimal gland tissue sections from patients with chronic GVHD. Consecutive sections using sense and antisense probes are shown for individual specimens. (**A**) Male-to-male recipient 15. (**B**) A female patient with Sjögren's syndrome. (**C**) Male-to-female recipient 1 (diaminobenzidine staining). *Arrows*: donor-derived fibroblasts positive for the antisense signal; *arrowhead*: donor-derived endothelia of the small vessel. (**D**) Consecutive sections from male-to-female recipient 17 (immunofluorescent staining) that were subjected to in situ hybridization for *Smcy* mRNA, diaminobenzidine immunostaining for CD34, fluorescent immunostaining for CD34, and Y-FISH. Fluorescent immunostaining for CD34 and Y-FISH were performed on a single section. *Arrows*: CD34$^+$ fibroblasts with a Y-FISH signal. Original magnification: (**A**, **B**, **D**) ×200, (**C**, diaminobenzidine staining) ×400; (**D**, immunofluorescent staining) ×630.

TABLE 3. Identification of Donor-Derived Cells in Fibroblast Primary Cultures Derived from the Lacrimal Gland in Chronic GVHD

Case	Gender of Donor and Recipient	% Y-FISH$^+$ Fibroblasts*	Y-Chromosome$^+$ Fibroblasts by ISH	Y-chromosome$^+$ Fibroblasts by Y-PCR	Mismatched Microsatellite Markers†
14	Male-to-female	Not tested	Present	Present	1/8
16	Male-to-female	2	Not tested	Present	1/8
17	Male-to-female	4	Present	Present	0/8
15	Male-to-male	50	Present	Present	3/3

Y-PCR, Y-chromosome polymerase-chain reaction.

* Frequency of cells positive for both X- and Y-FISH signals in 200 cultured cells.

† The number of microsatellite markers mismatched between donor and recipient divided by the total number of markers examined.

gland fibroblasts displayed a combination of the recipient pattern observed in the recipient before HSCT BCL and the donor pattern observed in the donor peripheral blood mononuclear cells and bone marrow cells as well as in the recipient after HSCT BCL. The donor-specific peak in cultured lacrimal gland fibroblasts was lower than the recipient-specific and shared peaks, due to a low abundance of donor-derived cells in the cultured fibroblasts. Theoretically, this method should allow us to detect donor-derived cells in cultured fibroblasts, irrespective of the combination of donor–recipient genders. In fact, a combination of the donor and recipient peaks was detected in the cultured lacrimal gland fibroblasts derived from male-to-male recipient 15. Analogous findings were obtained in an additional recipient 16, but no donor–recipient-mismatch marker was detected among the markers used in another recipient (patient 17).

DISCUSSION

In this study, the chimeric status of accumulated CD34$^+$ fibroblasts in the lacrimal gland of chronic GVHD patients was clearly demonstrated by a series of experiments using lacrimal gland tissue specimens and lacrimal gland-derived cultured fibroblasts. Nearly half of the CD34$^+$ fibroblasts at the site of pathogenic fibrosis were of donor origin, and the proportion of donor-derived cells in the fibrotic chronic GVHD lesion is considerably higher than the proportions reported for other donor-derived nonhematopoietic cells engrafted in tissues of HSCT recipients.[10–14] Although both CD34$^+$ and CD34$^-$ fibroblasts accumulate in the lacrimal gland from patients with chronic GVHD, we had to use CD34 as a marker for fibroblasts because there is no reliable fibroblast marker that can be used in the tissue. This limitation should be considered on interpreting our findings. In addition, there are several other limitations in our study for ethical reasons. The study lacks specimens from non-GVHD transplant recipient control subjects and serial specimens for evaluating a potential association between influx of donor-derived fibroblasts and disease progression. The role of donor-derived fibroblasts in the pathogenesis of chronic GVHD should be further examined in animal models.

Recent studies suggest that hematopoietic stem cells may spontaneously fuse with other cells and give the appearance of differentiation.[29,30] In this regard, Tran et al.[14] reported that more than 9700 buccal epithelial cells obtained from HSCT recipients showed no evidence of fusion, with only a few exceptions, indicating that cell fusion is a rare event in vivo in humans. Given the relatively high frequency of donor-derived CD34$^+$ fibroblasts in the chronic GVHD lacrimal gland, the fusion of donor-derived hematopoietic cells with residential fibroblasts was unlikely, although we could not entirely exclude the possibility that cell fusion was partly responsible for our observations.

Although the donor-derived fibroblasts that accumulate in the lacrimal gland chronic GVHD lesions apparently originated from the transplanted donor bone marrow or mobilized peripheral blood cells, the precise origin of these cells is presently unknown. The potential cell source includes mesenchymal stem cells with a capacity for self renewal and the potential to give rise to the various mesenchymal cells identified in human bone marrow,[31] but whether such stem cells circulate in the blood is not known. In fact, two patients with the significant chimeric status of fibroblasts in the lacrimal gland received transplantation of peripheral blood cells. Alternatively, donor stem cells that are committed to differentiation primarily along the hematopoietic lineage may switch to the fibroblast lineage under the influence of signals from the local environment. In this regard, mesenchymal cells can be differentiated from a distinct hematopoietic lineage termed fibrocytes[15] and from circulating monocytes.[32]

Our findings address important questions in the pathogenesis of chronic GVHD. It was originally considered to be a later phase of acute GVHD due to allorecognition by donor T cells, but several recent studies suggest that an autoimmune-like process induced by dysfunctional immunologic recovery also plays some roles.[1] In addition, the fibrotic process is apparently accelerated and the resultant excessive fibrosis leads to functional impairment in various organs, including the lacrimal glands.[8] Our findings suggest that donor-derived fibroblasts may be involved in this process. It has been postulated that, in HSCT recipients, tissue injury caused by acute GVHD, a conditioning regimen, or in some other way facilitates the homing of circulating stem cells or precursors and their differentiation into various tissues under the signals of the local environment.[33] Therefore, it is likely that the persistent fibrotic environment in chronic inflammatory lesions promotes the recruitment and mobilization of donor-derived fibroblast precursors, which may have unlimited growth potential or may be constantly supplied from the circulation in chronic GVHD. This possibility is supported by a murine sclerodermatous GVHD showing massive fibrosis, in which infiltration of macrophages and T cells into the tissue preceded fibrosis in the skin and lung.[34] Because no specific treatment has been shown to be effective for lacrimal gland chronic GVHD, strategies that inhibit the recruitment of fibroblast precursors into the affected lesion could be a novel therapeutic

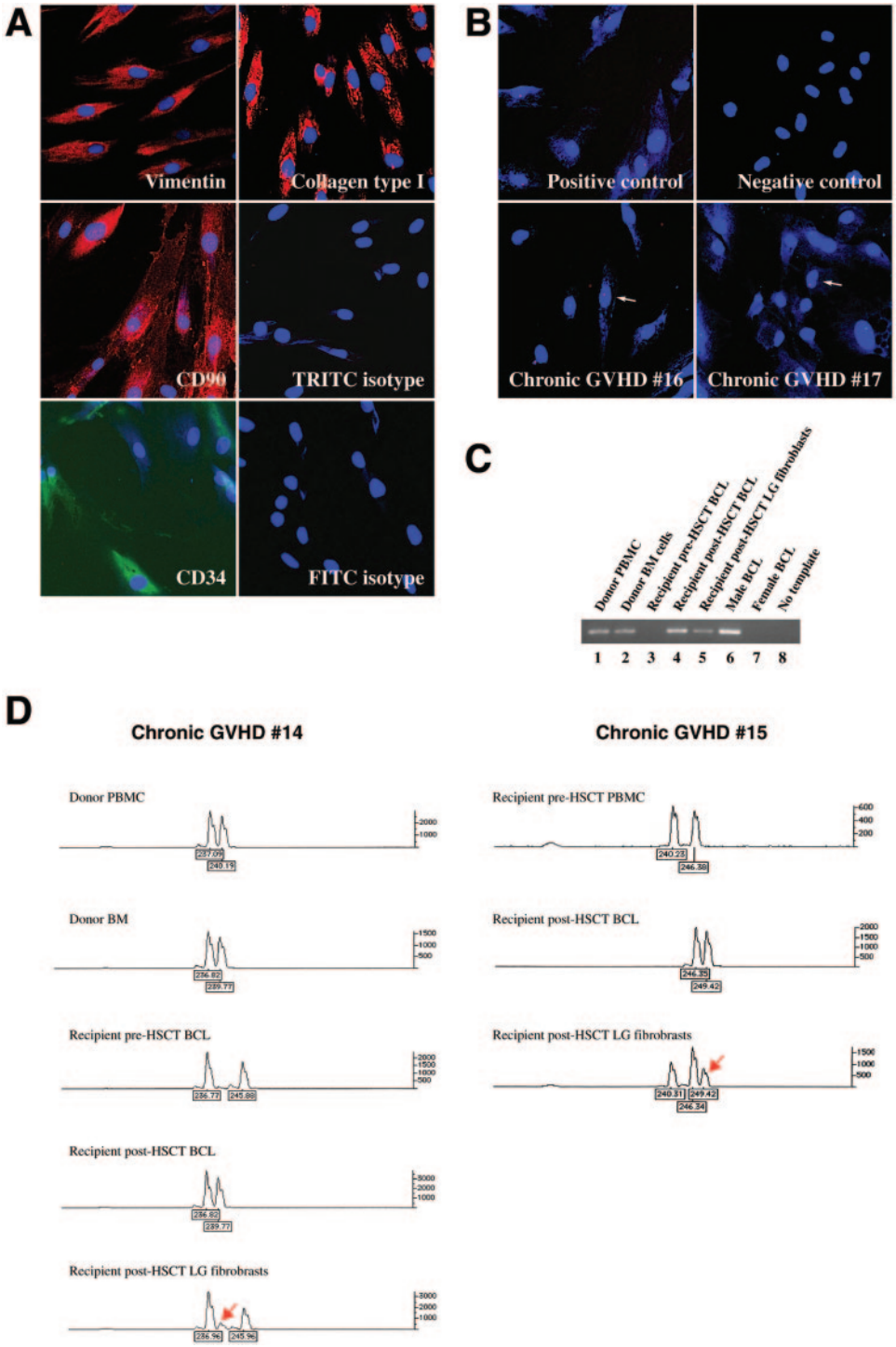


FIGURE 4. Detection of donor-derived cells in primary fibroblast cultures generated from the lacrimal gland of patients with chronic GVHD. (**A**) Cultured fibroblasts derived from patient 17 were stained for vimentin, collagen type I, CD90 (TRITC; *red*), and CD34 (FITC; *green*). Isotype-matched control mAbs to irrelevant antigens were used instead of the primary mAb as isotype controls. Nuclei were counterstained with TO-PRO-3 (*blue*). (**B**) FISH for the Y (*red*)- and X (*green*)-chromosome with TO-PRO-3 staining (*blue*). Cultured fibroblasts from male-to-male recipient 15 were used as the positive control, whereas dermal fibroblasts from a female subject were used as the negative control. Results obtained from male-to-female recipients 16 and 17 are shown. *Arrows*: fibroblasts with both X- and Y-FISH signals. (**C**) PCR for the detection of the Y-chromosome-specific *TSPY* gene in various cell sources obtained from male-to-female recipient 14 and her related donor before and after HSCT. *Lane 1*: donor PBMC; *lane 2*: donor bone marrow (BM) cells; *lane 3*, recipient pre-HSCT BCL; *lane 4*, recipient post-HSCT BCL; *lane 5*, recipient post-HSCT lacrimal gland (LG) fibroblasts; *lane 6*, male BCL as the positive control; *lane 7*, female BCL as the negative control; and *lane 8*, no-template DNA. (**D**) The analysis of polymorphic microsatellite marker *D10S1228* in various cell sources obtained from male-to-female recipient 14 and male-to-male recipient 15 before and after HSCT. The *x*- and *y*-axes show the product length in bases and signal strength, respectively. *Arrows*: indicate the donor-specific peak detected in the recipient peak pattern. Original magnification: (**A**, **B**) ×400.

approach to the fibrotic process in lacrimal gland chronic GVHD.

It has been shown recently that bone-marrow–derived precursors can serve as a source for fibroblasts in a mouse model of pulmonary fibrosis.[16] Our study demonstrates, for the first time, the presence of bone-marrow–derived fibroblasts in pathogenic fibrosis of the human disease. This finding leads us to propose a hypothesis that bone-marrow–derived fibroblasts may contribute to the pathogenesis of human fibrotic diseases, such as pulmonary fibrosis, cirrhosis of the liver, scleroderma, and ocular cicatricial pemphigoid. It would be interesting to evaluate further the chimeric status in multiple chronic GVHD tissues and potential associations between the chimeric status and response to various therapeutic regimens.

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