Bone Marrow Transplantation (2010) 45, 1638–1644

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ORIGINAL ARTICLE

Donor-derived DNA in hair follicles of recipients after allogeneic hematopoietic stem cell transplantation

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The hair follicles of recipients of allogeneic hematopoietic SCT (HSCT) constitute the tissue with the greatest need for regeneration after high-dose chemotherapy. Previous studies have shown a lack of donor-derived DNA in the hair follicles of recipients. Therefore, we carried out a study to determine whether male donor-derived genetic material can be found in female recpients' hair follicles after HSCT. Fluorescent-based PCR with analyses of Y-chromosome STR (Y-STR) and RQ-PCR with the sex-determining region Y (SRY) were used independently to evaluate chimerism status. Our results proved the existence of donor-derived stem DNA in the recipients' hair follicle cells. This report undermines the validity of data indicating that hair follicle cells maintain 100% of recipient origin.
Bone Marrow Transplantation (2010) **45,** 1638–1644; doi:10.1038/bmt.2010.27; published online 22 February 2010
Keywords: HSCT; hair follicles chimerism; sex-mismatched allografts; Y-STR; SRY

Introduction

Experimental and clinical studies have identified donor cells in almost all the tissues of recipients after allo-SCT.[1–5] Blood, buccal swabs with epithelial cells, as well as hair follicles, are among the most commonly investigated materials in forensic genetics.[6,7] It is a well documented fact that blood that comes from a person who has had a succesful allogeneic hematopoietic SCT (HSCT) is not suitable for personal identification and kinship analysis because of its conversion to a complete donor type.[8,9] As revealed by Hong *et al.*,[10] in buccal swabs taken from the majority of the patients examined, there exists donor chimerism ranging from 10 to 96%. Similarly, Thiede *et al.*[11] substantiated the admixture of a donor pattern in the same material in the range of 5–63%. Tran *et al.*[12] were the first to report the non-hematopoietic origin of male donor-derived cells in the internal cheek surface of female recipients of HSCT. The other commonly used cellular sources for forensic purposes are fingernails and hair.[13,14] Imanishi *et al.*[15] revealed that in the fingernails of 9 out of 21 investigated recipients HSCT mixed chimerism ranged from 8.9 to 72.9% of donor cells. Similarly, Pearce *et al.*,[16] in four out of eight patients examined, found a contribution of donor-derived cells in fingernails in the range of 14–58%. Hair has been regarded as constituting only the recipient type and therefore as an entirely safe material to use in DNA forensic investigations. Up to now, nobody has documented the existence of donor genetic material in the hair of recipients after HSCT, although intensive research efforts toward this aim have been undertaken.[10,17] This fact is difficult to explain as the origin of both nail and hair is from ectoderm and their biological features are similar.

As we had extensive experience in the study of DNA polymorphism and with two highly sensitive methods of chimerism analysis, that is fluorescent-based PCR of STR (STR-PCR) and RQ-PCR with TaqMan technology at our disposal, we decided to verify the existing thesis that hair follicles are devoid of transdifferentiation and maintain 100% of their recipient origin.

Patients and methods

Ten female patients, who had had received allogeneic hematopoietic stem cell engraftment from male donors (nine from their brothers, one from her father) were recruited to the study from the Department of Haematology, Poznan University of Medical Sciences. The Bioethics Committees of the Medical Universities of Lodz and Poznan gave their approval for the study and informed consent for the investigation was received from all the

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Received 16 June 2009; revised 16 December 2009; accepted 14 January 2010; published online 22 February 2010

subjects. The preparatory regimen before transplantation included the use of the specific cytotoxic drugs recommended by the European Group for Blood and Bone Marrow Transplantation (EBMT).[18] Myeloablative conditioning involved the use of BuCy4 in one patient, dexaBEAM in one patient and TBI/Cy in two patients. Non-myeloablative conditioning included MelFlu in one patient, TreoFlu in one patient, TreoCy in two patients, FluBu + ATG in one patient and FLAMSA in one patient. Acute GVHD was observed in 3 out of the 10 investigated patients, whereas chronic GVHD was present in 5 of them. Acute GVHD preceded the onset of chronic GVHD in only one case and transient skin GVHD was observed in three patients (grade I). At the time of analysis, no signs of skin or mucous membrane GVHD were observed. The patients' characteristics are presented in Table 1.

Donor and pretransplant recipient blood samples to serve as the reference material were collected in EDTA-coated tubes. Post transplant peripheral blood and post transplant buccal swabs from every recipient were collected, in duplicate, and frozen until DNA extraction. From four to eight post transplant hairs with roots were plucked using disposable gloves to avoid contamination. The recipient's hairs that included the roots and follicles were briefly rinsed in saline solution as described earlier by Hong *et al*.[10] The liquid after hair rinsing was spun, the supernatant was discarded and the remaining sediment was smeared on a slide. The preparations were fixed with 96% ethanol and stained with hematoxylin and eosin. The same staining procedure was performed for one hair from each patient. The above-mentioned preparations were examined by both a pathomorphologist and a cytologist independently for numerical image analysis with the use of a Jenamed light microscope and MultiScan adapter. Genomic DNA from all the investigated pretransplant and post transplant sample sources was isolated by the same procedure, that is by the Sherlock AX kit (A&A Biotechnology, Gdynia, Poland). The total concentration of each DNA sample was evaluated with a Qubit fluorometer and Quant-iT dsDNA HS kit (Invitrogen, Paisley, UK).

To observe whether the male donor's cells are present among the recipient's and, if so, to estimate the amount of male DNA in the samples, the Quantifiler Y Human Male DNA Quantification kit (Applied Biosystems, Foster City, CA, USA) was used. This kit uses a 5′ nuclease assay and TaqMan probe-based technology to detect the sex-determining region Y (SRY). The samples were run on a 96-well optical reaction plate with the set of DNA quantification standards in the Applied Biosystems 7900HT real-time PCR System with SDS Software v2.0, according to the manufacturer's protocol.

Fluorescent-based PCR was conducted with the use of an AmpFlSTR Yfiler PCR Amplification kit (Applied Biosystems), which allows for the co-amplification and multi-dye detection of 16 STR loci along the Y-chromosome (Y-STR): DYS456, DYS389I, DYS390, DYS389II (6-FAM dye-labeled), DYS458, DYS19, DYS385a/b (VIC dye-labeled), DYS393, DYS391, DYS439, DYS635, DYS392 (NED dye-labeled), GATA H4, DYS437, DYS438 and DYS448 (PET dye-labeled) markers. Amplification was performed following the manufacturer's instructions. PCR products were separated on 5% polyacrylamide denaturing gels on an ABI Prism 377 sequencer (Applied Biosystems). Allele detection was performed with GeneScan-500 LIZ as internal lane standard using Gene Scan Software v3.7. Genotyper Software v3.7 and the Allelic Ladder were used to mark the allele number of the analyzed samples (Applied Biosystems). The sensitivity evaluation of the applied multiplex was performed based on the DNA 007 male genomic control included in the kit, which was added to the reaction mix in known amounts. As with the previous step, the co-amplification of the 15 autosomal unlinked loci and gender-determining amelogenin marker included in the AmpFlSTR Identifiler PCR Amplification kit (Applied Biosystems) was performed (data not presented). The donor chimerism state was assessed according to Nollet *et al*.[19] and is included in Table 1.

Results

In the hair root preparations, fragments of hair follicles and cells with nuclei were visible. In the sediment smears obtained after the rinsing of the patients' hairs, single

Table 1 Characteristics of patients and of chimerism studies in their post transplant materials: blood, buccal swab and hair follicle

Patient no.	*Age—years at HSCT*	*Diagnosis*	*Graft source*	*Time—days post HSCT*	*Percentage of donor area autosomal STR/Y-STR*[a]		
					Blood	*Buccal swab*	*Hair follicle*
1	24	BAL	BM	329	100/100	22/100	0/100
2	24	CML	BM	516	100/100	67/100	0/100
3	53	AML	PB	103	100/100	0/100	0/100
4	48	AML	PB	607	100/100	53/100	0/100
5	48	ALL	BM	603	100/100	54/100	0/100
6	29	AML	PB	218	100/100	45/100	0/100
7	51	AML	PB	781	100/100	0/100	0/100
8	51	AML	PB	2764	100/100	0/100	0/100
9	22	Lymphoma	PB	2291	100/100	0/100	0/100
10	28	CML	BM	2738	100/100	20/100	0/100

Abbreviations: BAL = biphenotypic acute leukemia; HSCT = hematopoietic SCT; PB = peripheral blood.
[a]Exclusively donor type chimerism as a result of selective amplification of male Y-STR sequences.

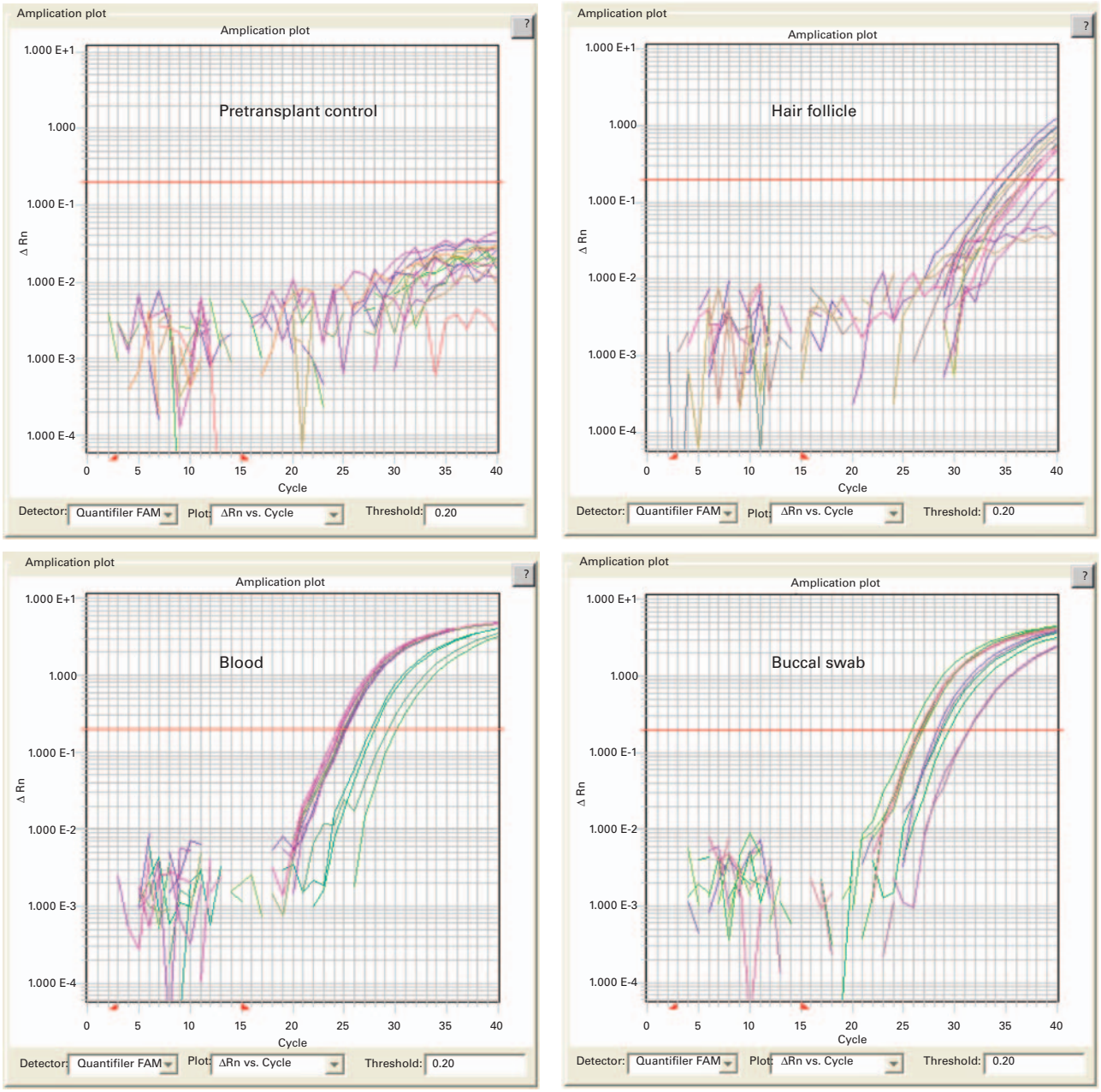

Figure 1 Amplification plots from a male-specific RQ-PCR assay with SRY sequence (Quantifiler Y Human Male DNA Quantification kit, 7900HT SDS). Pretransplant recipients' control samples, male DNA undetected in all 10 women; post transplant recipients' hair follicles, male DNA detected in 8 out of 10 women in a range of 10–170 pg/μl; post transplant recipients' blood, male DNA detected in all 10 women in a range of 2.57–51.18 ng/μl; post transplant recipients' buccal swabs, male DNA detected in all 10 women in a range of 0.96–19.16 ng/μl.

epithelial cells containing the nuclei and numerous keratinized cellular fragments without nuclei or organelles were visible. On the basis of independent microscopic examinations performed by two researchers no morphological elements of blood were found in any of the slides of the recipient's hair studied nor in any of the sediment smears remaining after hair rinsing.

The amplification plots from a male-specific RQ-PCR assay with SRY sequence for the post transplant recipient's materials, as well as pretransplant recipients' control samples, are presented in Figure 1. RQ-PCR assay of the post transplant samples of blood and epithelial cells from buccal swabs from all the investigated women revealed donor-derived male DNA in the range of 2.57–51.18 ng/μl and 0.96–19.16 ng/μl, respectively. In the hair follicles of 8 out of 10 of the investigated women the SRY gene sequence, which corresponds with male DNA, was detected in the range of 10–170 pg. This amount is equivalent to the range 1–28 of male target sequences or male cells that were detected in a background of a few hundred to a few thousand cells present in the sample. In addition, we found no SRY sequence in any of the 10 female pretransplant

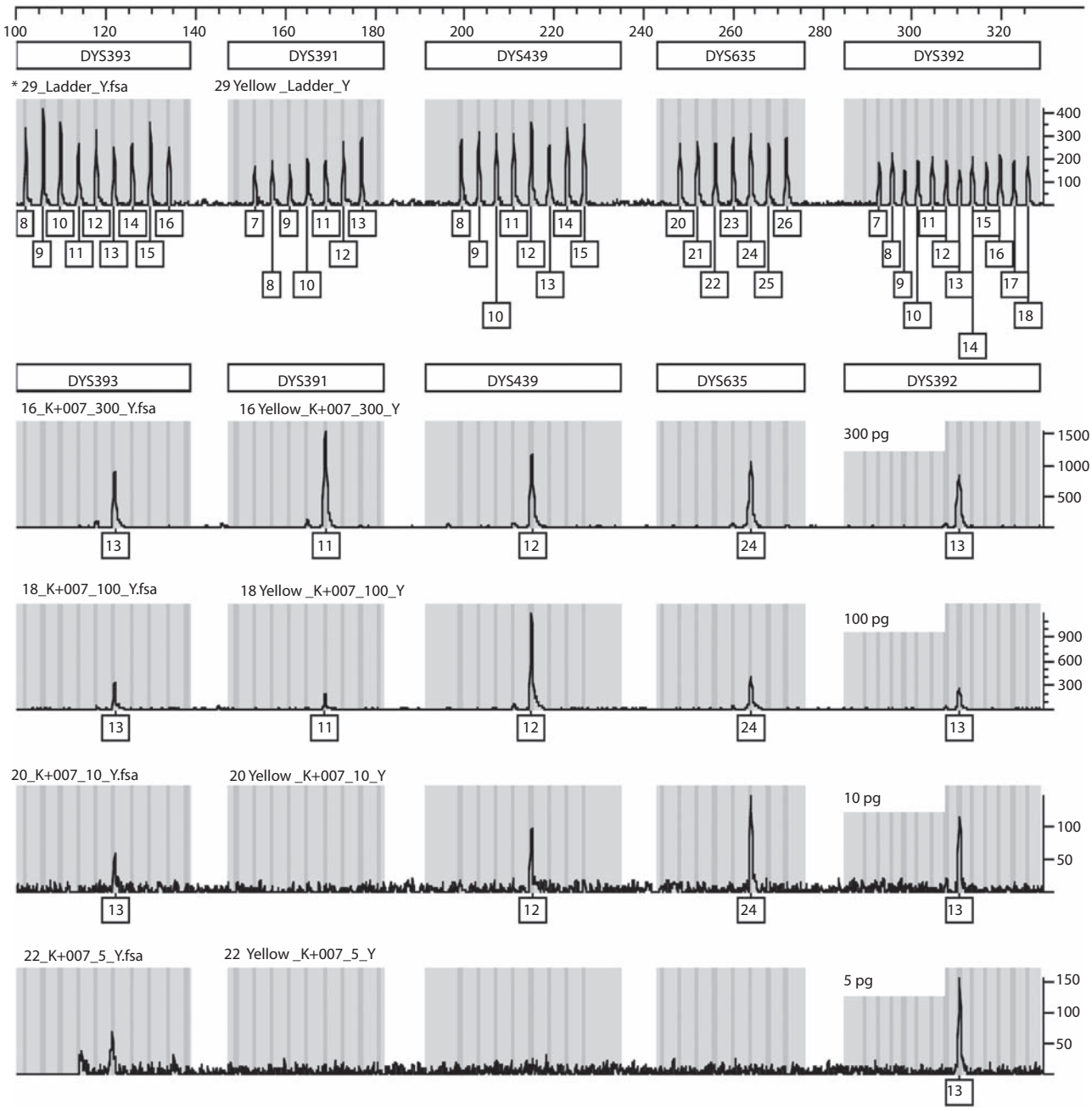

Figure 2 AmpFlSTR Yfiler kit results of amplification of male Control DNA 007 on the Genotyper v3.7 software plot. The Y-STR haplotype is shown with the allele number displayed underneath each peak. From top to bottom: AmpFlSTR Yfiler Allelic Ladder, then input quantities of male DNA template—300, 100, 10, 5 pg, respectively. The panels correspond with the following NED dye-labeled markers: DYS393, DYS391, DYS439, DYS635, DYS392.

control samples. Thus, the possibility that the male fraction detected in the post transplant female's hair was derived from their sons or brothers, whose cells had passed into their circulation because of fetomaternal transfer, has been ruled out.

To evaluate the sensitivity of applying Y-STR multiplex we subsequently added control male DNA to the PCR reaction mix in quantities of: 300, 100, 10 and 5 pg, which are approximately equal to 50, 17, 2 and 1 genome cells, respectively. It seemed that the full 16 locus Y-haplotype was received for a DNA quantity input of 300 and 100 pg. For the 10 and 5 pg of input DNA template a partial haplotype was observed with low copy number features, that is allele dropout and peak imbalance. The results are presented in Figure 2 (NED dye-labeled markers) and Supplementary Figure 2 (6-FAM,VIC,PET dye-labeled markers). The study involving post transplant blood and buccal swabs from women recipients with a male donor revealed a complete donor Y-haplotype. Similarly, a male haplotype of donor origin was observed in hair follicles of all 10 women after HSCT. A full 16-loci donor's haplotype profile was observed in 8 patients. For the two remaining patients, where the RQ SRY-TaqMan assay method did not detect male DNA, a partial Y-STR haplotype with 6–8

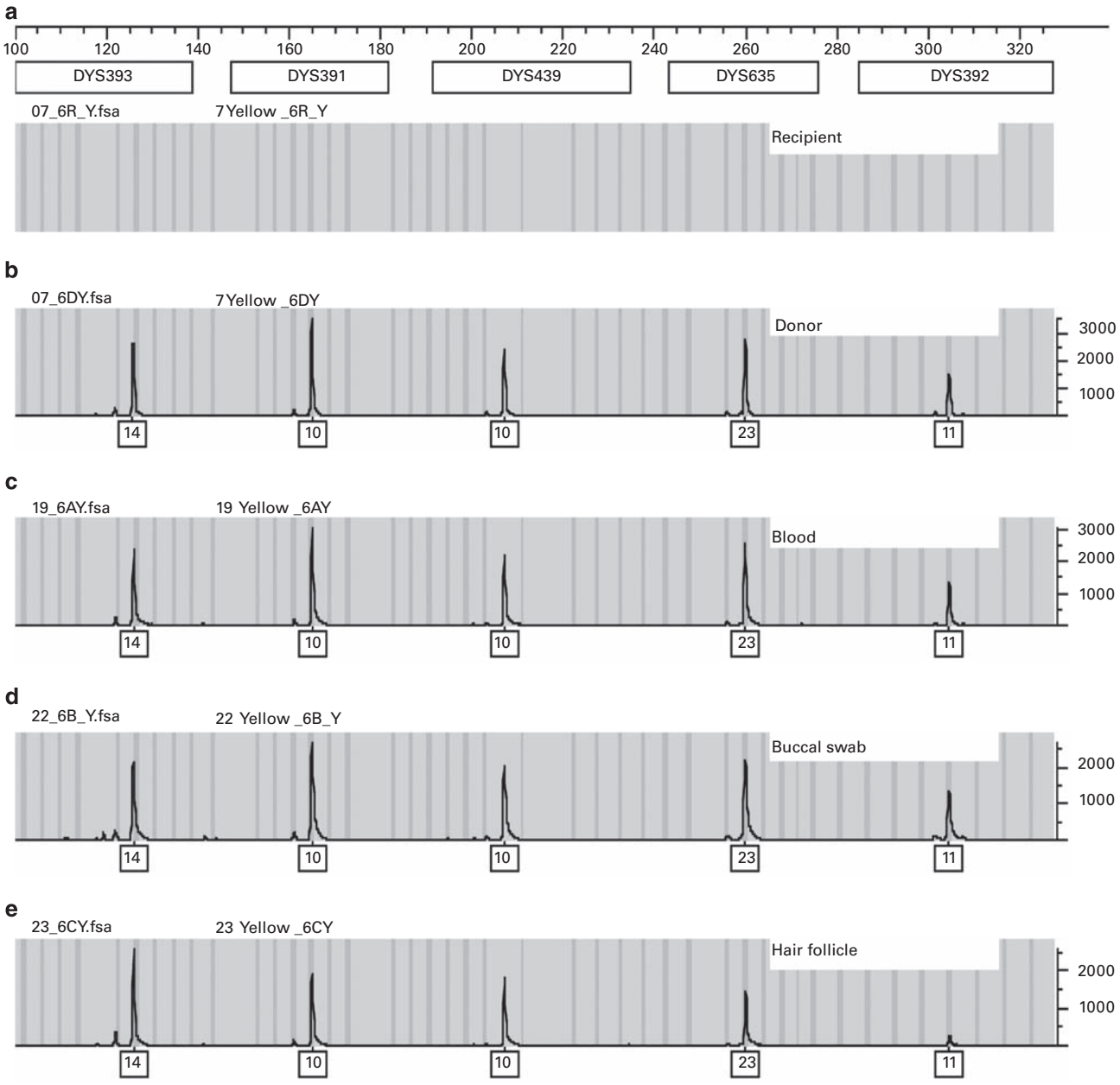

Figure 3 AmpFlSTR Yfiler kit results of the amplification of samples from representative patient No 6 (a female receiving HSCT from a male) on the Genotyper v3.7 software plot. The profile is shown from top to bottom as follows: (**a**) pretransplant recipient's control sample, (**b**) donors' sample, (**c**) post transplant recipient's blood, (**d**) post transplant recipient's buccal swab and (**e**) post transplant recipient's hair follicle. The panels correspond with the following NED dye-labeled Y-STR markers: DYS393, DYS391, DYS439, DYS635, DYS392 with the allele number displayed underneath each peak.

loci was revealed. A representative female patient (No 6), who underwent successful HSCT is presented in Figure 3 (NED dye-labeled markers) and Supplementary Figure 3 q(6-FAM,VIC,PET dye-labeled markers). These give details of the Y-STR donor's derived profile, subsequently detected in her hair follicle, blood and epithelial buccal swab.

Discussion

There have been numerous reports that adult stem cells can cross lineage barriers and differentiate into cells outside their own tissue.[20–23] Donor-derived cells have been revealed in almost all tissues of recipients after allo-SCT.[1–3,24,25] They have also been detected in cells derived from buccal swabs and in fingernails, which are among the most commonly used materials in forensic genetics.[11,12,16] Scientific efforts,[4,17] some of them recent,[10] have been undertaken to reveal the potential ability of tissue repair by donor-derived hematopoietic stem cells in a recipient's hair. As with fingernails, hair has the same ectodermal origin and regenerates continuously throughout life.

Dauber *et al*.,[4] Rovó *et al*.[17] and, more recently, Hong *et al*.,[10] applying autosomal STR-PCR analysis as their only investigatory method, documented 100% of recipient's DNA profiles without detecting any donor-origin genetic

material in hair from recipients of HSCT. It was therefore concluded that only hair follicles, but not fingernails buccal swabs nor blood, remain completely of the recipient origin.

Our investigation, performed using 10 sex-mismatched HSCT recipients with a male donor, proved the existence of donor-derived genetic material in recipient's hair follicles cells. We applied two highly sensitive and male-specific methods: RQ-PCR assay and multiplex STR-PCR, thereby enabling selective amplification of sequences lying on the Y-chromosome, that is the SRY region and Y-STR loci. Both of these methods allow the detection of small fractions of donor (male) sequences against a background of high amounts of recipient (female)-derived genetic material.[26,27] We also attempted, by CEP X/Y Chromosome Enumeration DNA FISH Probes (Vysis, Abbott Laboratories, Des Plaines, IL, USA), to reveal hair follicle cell origin in recipients after HSCT with the Y-chromosome. Unfortunately, these efforts were unsuccessful, because of the small number of cells (DNA) in the investigated cellular material. This observation was in line with previously published data.[28]

Ten women after HSCT from their brothers (nine) or father (one) were invited to participate in the experiment. Fluorescent PCR analysis with autosomal STR markers similar to those use by others[4,10,17] revealed the full recipient DNA pattern in hair follicles obtained from all 10 investigated women (data not presented) whereas the same analysis, with Y-STR markers, revealed a Y-haplotype of donor origin in the hair of the female recipients. The detection of a donor-derived DNA fraction based on the amplification of Y-STR markers and the absence of any donor fraction in a parallely performed analysis, based on amplification of autosomal markers in hair derived from females who had undergone transplantation with male hematopoietic stem cells, have a relatively simple explanation. Overwhelming quantities of female DNA mixed with the male fraction caused a preferential amplification of the female DNA and inhibition of the amplification of the male one.[29,30] This is a well-established fact in forensic genetics, for example in samples collected after sexual assaults.

The RQ-PCR assay with an SRY-specific sequence revealed only 1–28 donor-derived male cells in a background of several hundred to a few thousand female cells in the hair of the recipient women. Despite this, quantitative analysis seemed less effective than that from a Y-STR one. The RQ-PCR assay did not detect male DNA in the hair of two women, whereas the multiplex STR-PCR method documented markers specific for the Y-chromosome in all of them. This fact is probably the result of the different volumes of input DNA template added to the reaction, that is 2 μl in the RQ-PCR TaqMan technology vs 10 μl in the STR-PCR method. The hair from eight women gave a complete 17-loci Y-STR haplotype with 30–170 pg of male DNA. The hair from the two remaining women showed a partial Y-STR haplotype with 6–8 loci based on 0–30 pg of male DNA detected in the RQ-PCR assay.

Our exclusion of a hematopoietic origin of the donor component detected in the recipients' hair follicles is based on an elution procedure and independent microscopic examination performed by two researchers. Furthermore, our rejection of the hypothesis that the donor fraction observed in the hair of the recipients was the result of prenatal male microchimerism is based on the control investigation of pretransplant samples, presented in Figure 1 (RQ-PCR assay) and Figure 3 (Y-STR analysis). Moreover, there was no detectable correlation between either myeloablative or non-myeloablative conditioning regimens, or between the BM or peripheral blood sources of stem cells used in HSCT, and the appearence of donor-derived genetic material in the recipient's hair follicles.

In summary, hair follicle cells are not entirely devoid of donor genetic material, as has been reported to date. Our investigation proved the presence of donor-derived DNA in recipient's hair follicles, although it constitutes a minor component of the mixture. This may suggest the role of transdifferentiation in hair regeneration after HSCT. Moreover, our investigation found that hair follicles, as well as blood and buccal swabs, are not entirely safe material for forensic purposes. Analysis based exclusively on a Y-STR investigation, performed on the materials taken from sex-mismatched recipients with a male donor, showed an entirely donor haplotype. This could lead to the false identification of male gender or male haplotype in hair or other post transplant materials. The sensitivity of co-amplification and multi-color detection of 16 STR Y-chromosome loci were compared with the RQ-PCR assay. The former method proved more effective for the detection of donor chimerism. Specific amplification of Y-STR markers was able to reveal as little as one donor cell among a few thousand of the recipient ones. Therefore, the investigation of Y-STR multiplex is very useful, not only in forensic genetics, but it can also serve as a valuable tool in early diagnostics after HSCT in sex-mismatched allografts.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We are greatful to Professor Geoffrey Shaw for his language editing of this paper.

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Supplementary Information accompanies the paper on Bone Marrow Transplantation website (http://www.nature.com/bmt)