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Concise Communication   **Transplantation**   **Virology**

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High multiplicity of infection following transplantation of hepatitis C virus–positive organs

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Introduction

Individuals with end-stage renal failure on hemodialysis experience a significant morbidity and mortality benefit with renal transplantation (1). The wait time for kidney transplantation, however, exceeds 3 to 5 years in many parts of the United States, and new approaches to decreasing that wait time are needed. Many high-quality kidneys from deceased donors with hepatitis C virus (HCV) infection are discarded annually. Direct-acting antiviral agents (DAAs) that have high HCV cure rates and limited side effects have created the opportunity to transplant organs from HCV-infected donors into HCV-uninfected recipients.

The Transplanting Hepatitis C Kidneys into Negative Kidney Recipients [THINKER] trial (ClinicalTrials.gov identifier NCT02743897) is an open-label, single group, pilot trial testing the safety and efficacy of transplantation of kidneys from HCV genotype 1–viremic donors into HCV-uninfected individuals, followed by HCV plasma VL testing and elbasvir-grazoprevir (Zepatier) anti-HCV treatment at day 3 after transplantation. Initial results from the first 20 participants of this trial demonstrated excellent clinical outcomes, with few serious adverse events and maintenance of renal allograft function after transplantation (2, 3). Virologically, all participants had detectable HCV viremia after transplantation and responded well to anti-HCV therapy with sustained virologic

response 12 weeks after therapy. At 6 months after transplantation, all recipients remained cured of HCV and maintained renal function comparable to matched recipients of HCV-negative kidneys (3). Thus, HCV was universally transmitted via transplantation and effectively eliminated by anti-HCV therapy. The concurrent USing Hepatitis c positive hearts for nEgative Recipients (USHER) clinical trial assessing cardiac transplantation from HCV-positive donors (ClinicalTrials.gov, NCT03146741; ref. 4) demonstrated similarly positive clinical outcomes and HCV cure rates. Sustained virologic response (SVR) was achieved in 9 of 10 participants; 1 participant with a positive cross-match at transplantation experienced antibody-mediated rejection and multi-organ failure, and died. Reports from other centers performing organ transplantation from HCV-positive to HCV-negative recipients have shown similarly positive clinical outcomes with effective HCV clearance (5–7). The underlying mechanisms and viral kinetics of HCV transmission from donor to recipient in this setting, however, are unknown.

Transmission of HCV in settings other than transplantation occurs largely through parenteral exposure to blood or blood products from an HCV-infected individual, with sexual transmission accounting for a smaller but important component of the pandemic (8). Despite the variability in mode of acquisition, studies employing modern sequencing techniques, including single genome sequencing (SGS), have shown that in sexual and injection-related infection, HCV transmission from donor to recipient is generally characterized by a stringent genetic bottleneck that reflects the passage of a very limited number of viruses from donor to recipient (9–13).

Here, we characterized the plasma virus populations from 4 genotype 1a HCV-positive donors and their organ recipients to determine the multiplicity of infection and assess the stringency of the HCV transmission process. In contrast to sexual and injec-
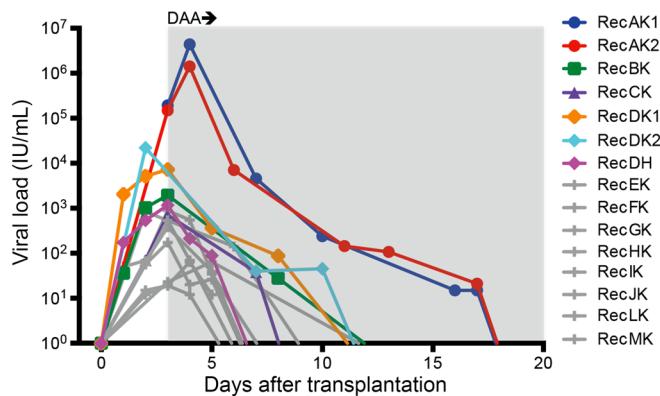
Figure 1. Plasma HCV kinetics in organ transplant recipients. Anti-HCV DAA therapy was initiated at day 3 after transplantation in all participants, as shown with gray shaded area. The 7 organ recipients described in the present study are shown with solid colored symbols; additional participants are shown in gray.

tion-related infection, we found a highly permissive genetic bottleneck with multiple viruses transmitted from donor to recipient during the organ transplant process.

Results and Discussion

Viral kinetics from the day of transplantation until virus became undetectable on anti-HCV therapy are shown in Figure 1. In the 3 days between transplantation and anti-HCV therapy initiation, plasma HCV levels increased markedly in each participant, indicating productive de novo infection of HCV with ongoing virus replication in the recipient host prior to therapy. Peak viremia ranged from 2.9–6.6 $\log_{10}$ IU/mL at day 3 to 5 after transplantation. Upon initiation of anti-HCV therapy, plasma viral load (VL) dropped quickly, reaching a level undetectable by clinical assay in a median of 9 days (range 5–18 days).

To molecularly characterize the transmission process, we analyzed a donor-recipient group with higher HCV VLs: a HCV-infected deceased organ donor (Donor A) and 2 kidney recipients (RecAK1 and RecAK2). Donor A had plasma HCV levels of 7.3 $\log_{10}$ IU/mL at the time of kidney procurement. RecAK1 and RecAK2 had plasma HCV levels of 5.3 $\log_{10}$ IU/mL and 5.2 $\log_{10}$ IU/mL, respectively, at day 3 after transplantation (Figure 2). In both recipients, plasma HCV levels increased more than 1 log between day 3 and 5, then fell until undetectable at day 18 after transplantation (Figure 2E). We performed SGS of 5′ half genomes (Core through NS4), from time of organ procurement (donor) and day 3 after transplantation (recipients), to obtain 113 sequences (median 37 in each individual). SGS is a method of end-point dilution PCR that precludes recombination between heterologous templates, minimizes in vitro sequencing errors, and proportionally represents the composition of complex virus populations (9, 12, 13). Power calculations (14) predict that 30 or greater sequences per sample provides 90% likelihood of identifying variants that represent at least 10% of the population.

Maximum likelihood (ML) phylogenetic trees representing the sequences from each individual are shown in Figure 2, A–C. The ML tree of Donor A demonstrates a maximum diversity of 1.8%, consistent with chronic HCV infection (9). Donor A's diverse

sequences cluster into 4 larger lineages with high bootstrap support, as indicated in Figure 2A. Similar maximum within-participant diversity was observed in each recipient's sequences (1.8% and 1.7% for RecAK1 and RecAK2, respectively), with similar clustering of the 4 distinct lineages. In the combined tree (Figure 2D) and highlighter plot (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI127203DS1), sequences from the donor and recipients are interspersed and without segregation within the 4 clusters, with a small number of additional sequences from both recipients falling outside of these lineages.

Recipient sequences were derived from samples obtained 3 days after transplantation, when transmitted variants would have had few replication cycles with which to accrue diversity within the recipient (15). The limited time for within-recipient virus evolution and the similarity of the diversity and tree structure between the donor and recipient phylogenies suggest that the many distinct viruses sampled in the recipients represent unique transmitted/ founder (TF) viruses, or viruses that were transmitted from the donor and founded productive infection in the recipient (9, 14, 16). The fact that no 2 sequences were identical suggests that the number of transmitted viruses from Donor A to both recipients far exceeds the number of sequences depicted in the recipient phylogenies (14). Thus, innumerable distinct TF viruses representing the full range of viruses circulating in the donor established productive infection in each transplant recipient, demonstrating a highly permissive transmission process. This contrasts with sexual and injection-related transmission, in which acutely infected individuals are routinely infected with one or few viruses and early phylogenetic trees represent a single or few lineages of identical and near-identical sequences (9, 12).

To compare the relatedness of the donor and recipient virus populations more objectively, we determined genealogical sorting indices (GSIs; Supplemental Table 1). The GSI tests the phylogenetic similarities of 2 sequence groups with the null hypothesis that the virus populations were the same. GSI values range from 0 (complete interspersion) to 1 (complete monophyly), giving a value for each compartment being compared, with statistical significance indicating greater-than-random segregation between groups. The low GSI values without statistical significance ($P >$ 0.95 for all comparison) between viruses of Donor A and RecAK1 and RecAK2 further support that there was minimal to no impedance of HCV transmission between donor and recipient.

We next asked whether a permissive transmission process also occurred in transmissions where recipients had lower VLs. Donor B's VL was unknown; Donor C had a plasma VL of 5.2 $\log_{10}$ IU/mL (Figure 3A). RecBK and RecCK had VLs of 2.9 $\log_{10}$ IU/mL and 3.3 $\log_{10}$ IU/mL, respectively, at day 3 after transplantation. We performed SGS of 5′ half genomes in these participants ($N =$ 129 total sequences, median 38). The ML phylogeny of sequences from the Donor B–RecBK pair, shown in Figure 3B, demonstrates that the donor and participant had similar maximum within-participant diversities (0.7% and 0.6%, respectively), and complete interspersion of sequences. As seen in Donor A's recipients, viruses resembling essentially all of the sampled donor lineages are detected in the recipient. GSI values were also low and statistically nonsignificant (Supplemental Table 1). For RecCK, we were only
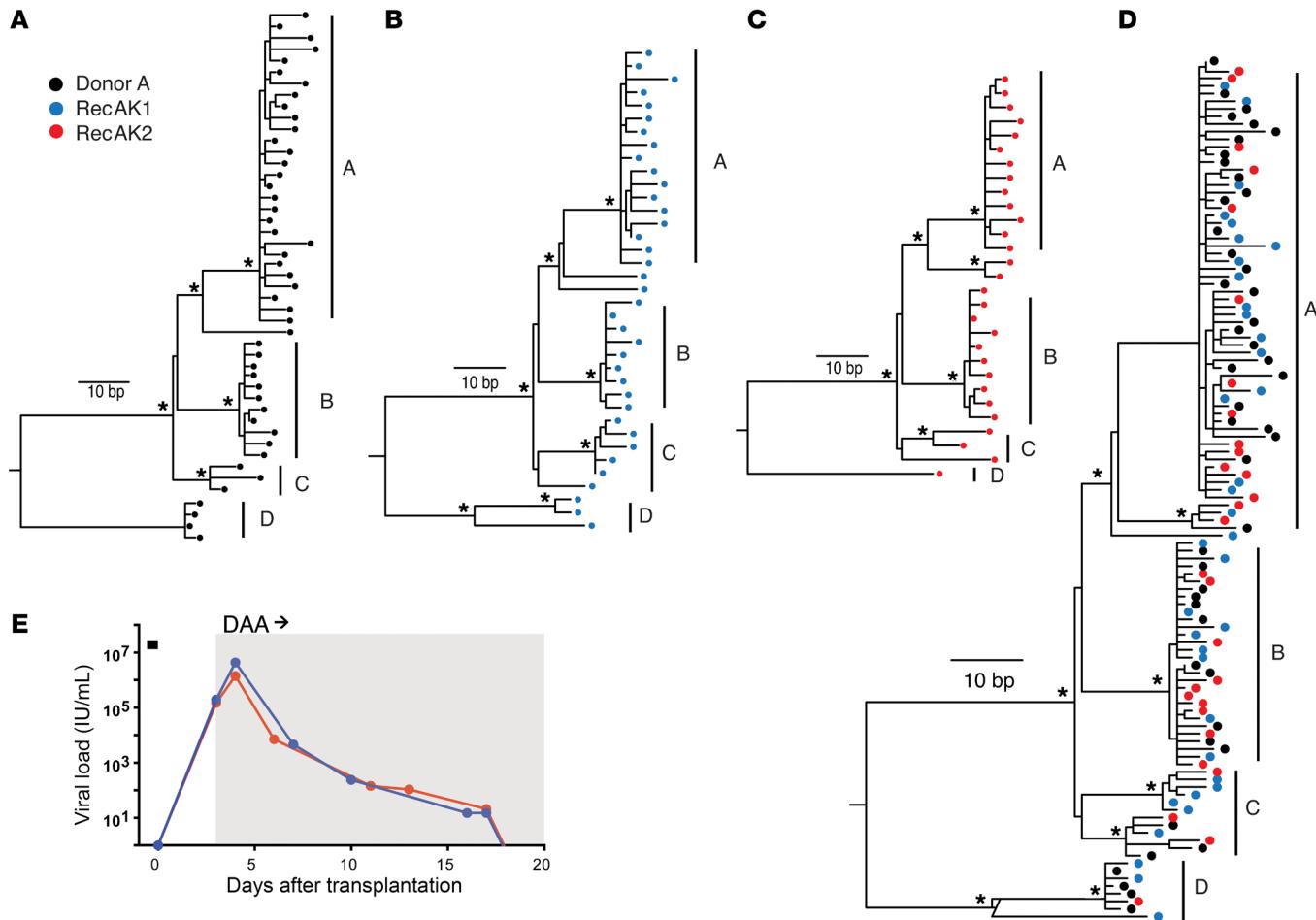
Figure 2. Transplantation of kidneys from an HCV-positive donor with high plasma VL. Maximum likelihood phylogenetic trees of 5′ half genome sequences from (**A**) Donor A, (**B**) RecAK1, and (**C**) RecAK2. (**D**) Donor A and RecAK1 and RecAK2's sequences are displayed together in a combined ML phylogeny. The genetic distance is shown by scale bar; bootstrap values over 90 are indicated with asterisks. In each phylogeny, the same 4 clusters, identified as A–D, are identifiable. (**E**) Viral kinetics of Donor A at the time of organ procurement and RecAK1 and RecAK2 in the days after transplantation (as shown in Figure 1).

able to generate 4 sequences due to low plasma VL and minimal plasma availability. Nevertheless, RecCK's sequences are genetically distinct and distributed throughout Donor C's plasma virus population, suggesting a high multiplicity of infection (Figure 3C). Thus, even lower VL transmission is associated with a highly permissive process.

Finally, we aimed to molecularly characterize HCV transmission in both kidney and heart transplant processes. Donor D had a plasma VL of 7.3 $\log_{10}$ IU/mL and donated 2 kidneys and his heart to separate recipients. HCV VLs from day 1 through virus suppression are shown in Figure 4A, demonstrating that the heart recipient had more than 1 log lower peak plasma viremia than the kidney recipients. We generated 141 total 5′ sequences (median 35 per participant). Donor D's plasma virus had maximum pairwise diversity of 2.0%, consistent with chronic HCV infection. The combined ML phylogenetic tree (Figure 4B) demonstrates interspersion, with interdigitated sequences from the donor, kidney, and heart recipients in all but one branch of the tree.

Notably, one major branch of the tree contains only a single recipient sequence among donor sequences. This lineage, labeled "E1E2-deleted lineage" in Figure 4B, is a substantial component

of the donor population (37% of sequences; Figure 4C), but was sampled only as a minor variant in RecDK2 and was not found in the other 2 recipients. Analysis of donor sequences in this lineage reveals closely related sequences with a large deletion within the E1, E2, and p7 encoding regions, as shown in the Highlighter plot in Supplemental Figure 2A. The single RecDK2 sequence aligning within this lineage encodes a full 5′ genome with intact open reading frames in all genes (Supplemental Figure 2B). This type of mutation has been previously described in chronically HCV-infected individuals and is prevalent in patients with end-stage liver disease (17, 18). These deleted viruses have not been reported in acutely HCV-infected individuals. The deleted viruses retain genes essential for autonomous replication and have been shown to be capable of in vitro replication when coexpressed with full-length viral genomes, but have unclear fitness in vivo (19). While deleted viruses represent a substantial proportion of viruses circulating in the donor plasma, they did not establish infection in any recipients.

GSI values comparing Donor D and the 2 kidney recipients' sequences further reveal the distinction between donor and recipient viruses. If the deleted sequence lineage is included in the analyses, GSI values demonstrate statistically significant differ-
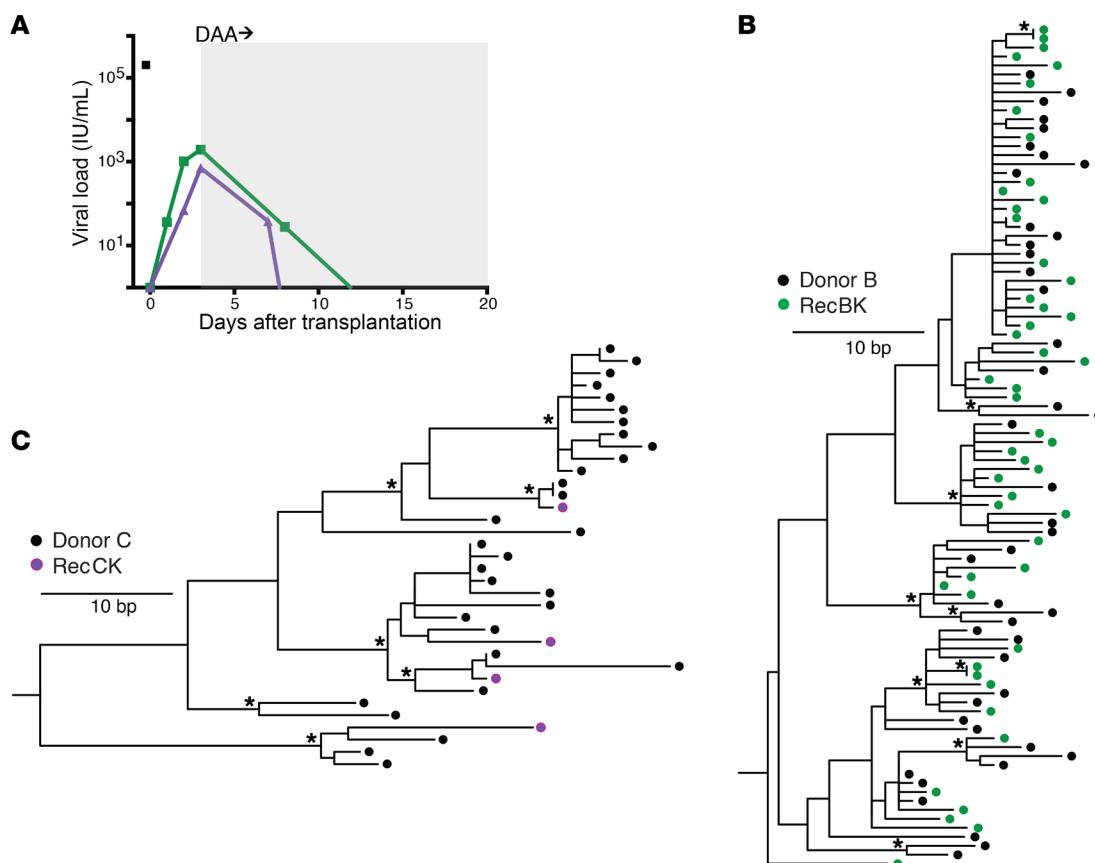
Figure 3. Transplantation of kidneys from an HCV-positive donor with lower plasma VL. (**A**) Viral kinetics of Donor C and RecBK and RecCK (as shown in Figure 1). Donor B's VL is unknown. Combined ML tree of 5′ half genome sequences from (**B**) Donor B and RecBK and (**C**) Donor C and RecCK. In both donor-recipient pairs, recipient virus sequences fall throughout the donor phylogeny. The genetic distance is shown by scale bar; bootstrap values over 90 are indicated with asterisks.

ences between donor and recipient sequences ($P < 0.02$ for all). If the deleted lineages are excluded from the analysis, GSI values indicate insignificant differences between donor and recipient virus populations ($P > 0.5$ for all). Thus, with the exception of the deleted virus lineage, viruses were permissively transmitted via both renal and cardiac transplantation.

The inclusion of a donor with E1E2-deleted viruses circulating at the time of organ procurement allowed for interesting discoveries. While a substantial portion of Donor D's virus population was deleted viruses, none of the deleted forms established productive infection in organ recipients. These findings suggest that E1E2-deleted viruses are less fit in the context of virus transmission and may require conditions of chronic infection to persist. Further, the lack of transmission of deleted forms is the only example in these 4 donor-recipient groups where a donor lineage was not transmitted to the recipient. Thus, this exception reinforces the central observation of a highly permissive transmission process, in which a large number of the potential replication-competent viruses establish infection in each organ recipient.

We note several important limitations to this study. First, analyses are limited to 4 donors and 7 recipients with genotype 1a infection from a single center (donor clinical data included in Supplemental Table 2). Next, limited plasma availability for Donor B and RecCK prevented determination of plasma VL and

limited the depth of sequencing of recipient TF viruses. Additionally, we cannot definitively ascribe the source of inoculum virus. Given the high number of TF viruses seen across recipients, we can infer that the virus inoculum is large, but all potential sources of virus are unclear. We assume much of the transmitted virus arises from free virus in donor blood retained within transplanted organs, but other sources, including infected blood cells or extrahepatic tissue, are possible. Further exploration into the source of transmitted virus and the effects of intra-transplant procedures is needed to define the origin of transmitted HCV. Accordingly, this molecular characterization can serve as a baseline from which to measure the effects of modifications to surgical and medical procedures.

In summary, we have demonstrated a highly permissive HCV transmission process in recipients of organs from HCV-positive donors, with infection founded by multiple genetically distinct viruses from throughout the range of donor viruses. The high multiplicity of infection was true in both renal and cardiac transplantation and in recipients with a range of peak plasma VLs. Notably, all participants achieved clinical cure from HCV infection with SVR at week 12 after therapy and maintenance of HCV clearance since (3, 4). Given the clinical success of transplantation of organs from HCV-positive donors to HCV-negative recipients, it is likely that this procedure will become more common (3, 14, 20). The
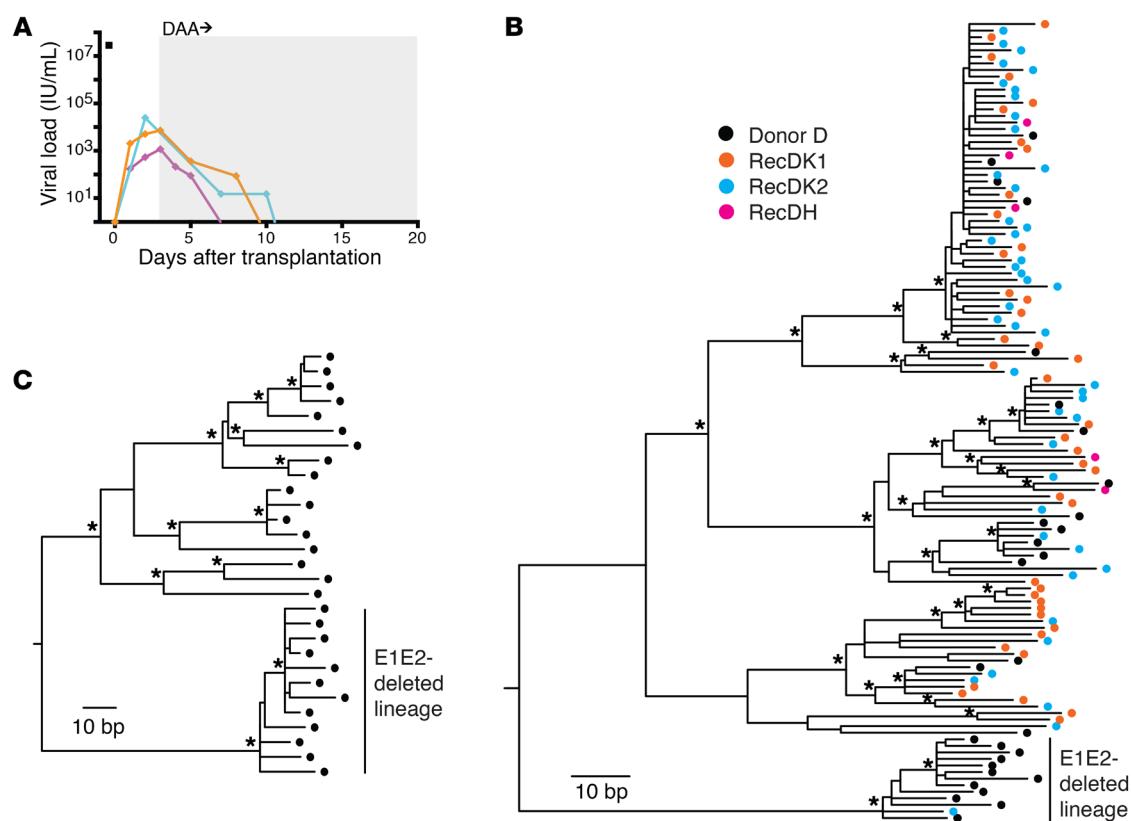
Figure 4. Transplantation of kidneys and a heart from one HCV-positive donor. (**A**) Viral kinetics of Donor D and RecDK1, RecDK2, and RecDH (as shown in Figure 1). (**B**) Combined ML phylogenetic tree of 5′ half sequences from Donor D and the 3 organ recipients. Sequences are distributed throughout the phylogeny, with the exception of the lowest cluster, labeled E1E2-deleted lineage, in which only 1 recipient sequence aligns. (**C**) ML phylogenetic tree of Donor D sequences demonstrates the substantial proportion of E1E2-deleted viruses in the donor virus population. The genetic distance is shown by scale bar; bootstrap values over 90 are indicated with asterisks.

highly permissive transmission process described here raises a note of caution should drug-resistant HCV variants appear within the circulating virus populations, because transmission of these variants by organ transplantation would likely be efficient, as has been described in a recent report of DAA failure after renal transplantation from a HCV-positive donor with drug-resistant virus (4). Further characterization of transplantation-associated HCV transmission in new clinical contexts, including with additional HCV genotypes in the setting of other organ transplants in communities with higher rates of drug resistance and with pretransplantation initiation of anti-HCV therapy as prophylaxis, will be needed to confirm the observed high multiplicity of infection and its clinical consequences.

Methods

Study approval. The THINKER and USHER clinical trials were conducted with the approval of the University of Pennsylvania's IRBs. Written informed consent was obtained from all participants. Study protocols and results were published (2, 4).

Viral RNA extraction and cDNA synthesis. Approximately 20,000 viral RNA copies were extracted from plasma, and cDNA was immediately synthesized as previously described (9,16). The antisense primer used for cDNA synthesis also corresponded to the first-round antisense primer (see Supplemental Methods).

Single genome amplification and direct DNA sequencing. SGS was used to generate 5′ half genome sequences (Core, E1, E2, p7, NS2, NS3, and a portion of NS4A) by nested PCR from all samples as described previously (9, 13, 16). All subjects were infected with HCV genotype 1a, so specific primers were designed (see Supplemental Methods). Amplicons were sequenced by Next Generation Sequencing using MiSeq (Illumina) after library construction by Nextera XT DNA sample preparation kit. Raw reads of each amplicon were aligned de novo and the consensus sequence of each contig was used as the final sequence for each amplicon. Inspection of the base frequency at each nucleotide position allowed for the identification of amplicons derived from multiple templates, which were excluded from further analysis.

Phylogenetic analysis. A total of 420 SGS-derived 5′ half sequences generated from 11 participants (median of 37 per participant) were analyzed phylogenetically and by direct visualization using Highlighter plots (www.HIV.lanl.gov). Phylogenetic trees were inferred using maximum likelihood/rapid bootstrapping in RAxMLv8. Evolutionary models were chosen using jModeltest (v.2.1.4) and model parameters were estimated concurrently with phylogram topology. Trees were visualized midpoint, and rooted using MEGA.v621. All sequences were deposited in GenBank (accession numbers MK289875-MK290238).

Genealogical sorting indices. GSIs were calculated as previously described. Details in the Supplemental Methods.

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