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RESEARCH LETTER

Recommendations on single-cell RNA sequencing of skin xenografts in the study of genetic skin diseases

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Xenografts serve as an excellent model for genetic skin disease, since they allow for experimental manipulation of patient tissue in an 'in vivo' setting. Combining xenografts and single-cell RNA sequencing (scRNA-seq) has the potential to provide deep insight into the safety, efficacy and molecular mechanisms of such novel experimental therapies. For the study of treating human skin models with experimental exon skipping therapy, we transplanted human skin cells onto immunocompromised nude mice to generate human skin xenografts.[1,2]

We wanted to evaluate the applicability of single-cell (sc) sequencing on xenografts generated from recessive dystrophic epidermolysis bullosa patient's skin cells to identify the molecular effects of *COL7A1* exon skipping therapy. Skin tissue as input for scRNA-seq requires extra consideration. Performing scRNA-seq on xenografts proved to be challenging as the xenografts constitute a hybrid tissue of human and murine cells. Here, we want to share our experiences and provide recommendations for optimizing and piloting every individual step in the scRNA-seq process using human skin xenografts.

To generate human skin xenografts, we first grafted human primary fibroblasts and keratinocytes onto nude mice and treated the mice with ASOs (Figure 1A,B) as described previously by Bremer et al.[1,3] After harvesting the grafts, we enzymatically dissociated the fresh frozen tissues to obtain single cells. We used dissociation conditions optimized in our laboratory for human skin biopsies. We then followed the 10X Genomics scRNA-seq workflow which contains three quality control (QC) checks that serve as a verification of successful experiments.[4] Our samples passed these checks: cell count and viability after dissociation, cDNA quantification and QC through automated electrophoresis, and library QC before the actual sequencing step, also through automated electrophoresis. Sequencing results for these pilot samples showed a sufficient number of reads and cells as expected from cDNA quantification (Figure 1C). Closer inspection of the data showed that only mouse cells survived the protocol. Unique molecular identifier (UMI) counts and genes per cell were only sufficient in the mm10 (mouse) transcriptome (Figure 1C). While a high number of estimated cells in the GRCh38 (human) transcriptome was observed, UMI counts were low. A median of only 10 unique molecules per human cell were present, suggesting compromised cell membranes. Reads in GRCh38 were all identified as apoptotic markers and our scRNA-seq pilot samples did not contain reads mapped to our gene of interest (*COL7A1*). Hence, although all quality checks in the 10× protocol were passed, the protocol only revealed at the very end that an insufficient number of human cells had been analysed.

Peter C. van den Akker and Jeroen Bremer contributed equally to this manuscript.

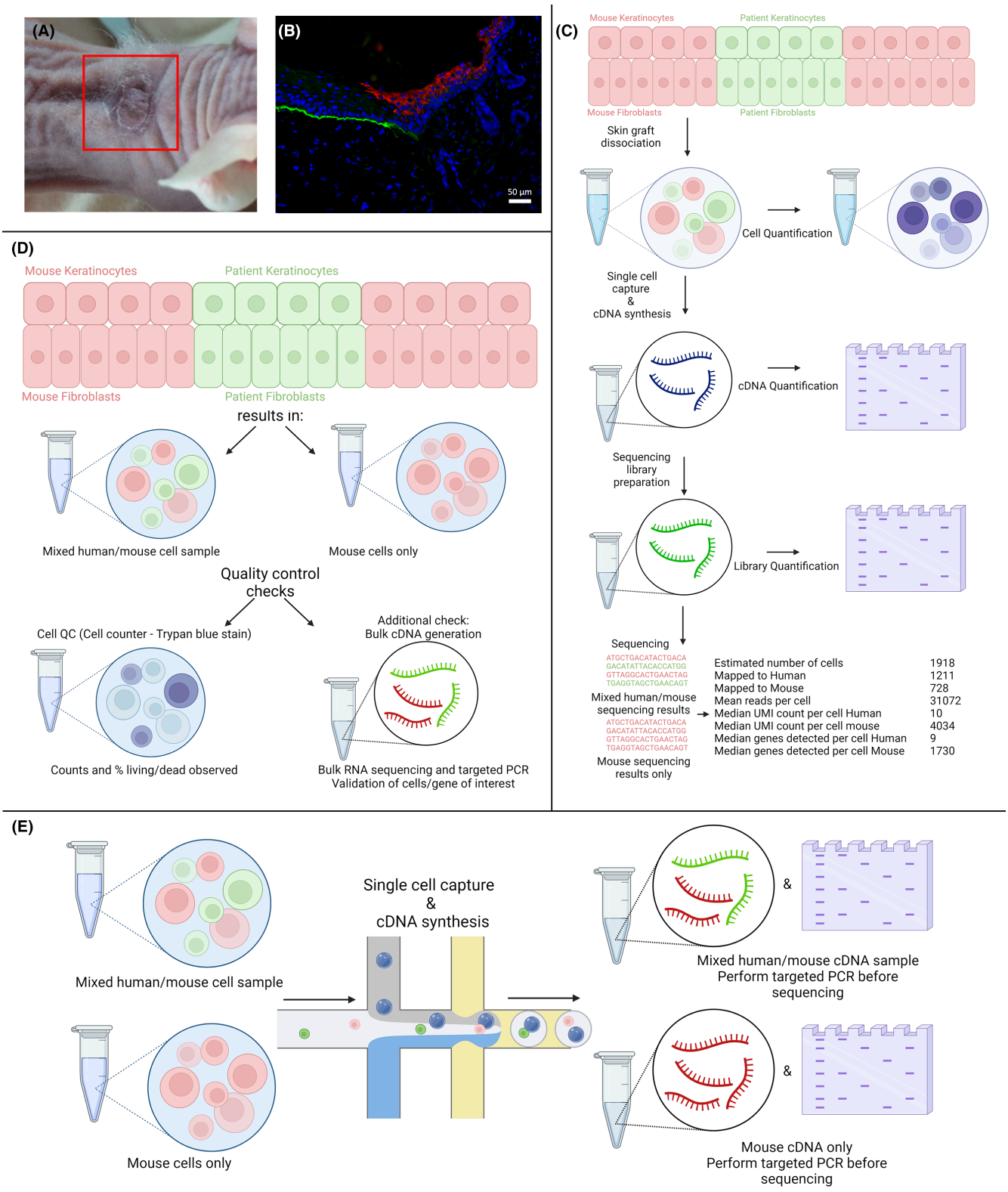

FIGURE 1 Workflow for performing single-cell sequencing on xenograft tissue. (A) Human skin xenograft transplanted on the back of nude mice, as described before.[1] Grafting area marked. (B) 20× magnification cryosection of the human xenograft 4 weeks after grafting procedure. Stained for human *COL7A1* (Green - LH7.2) and mouse keratin 1 (Red). (C) Standard workflow for scRNA-seq including all the QC steps that are performed in our own experiment. After sequencing, numbers of cells in both human and mouse transcriptomes seem sufficient. The human partition of the reads observed only counts a median of 9 genes per cell, with a median of 10 cDNA unique molecules (UMI) found per human cell. Therefore, unlike the mouse partition, human cells sequenced were not adequate for proper analysis. (D) After skin dissociation a trypan blue stain is used to count living and dead cells. As cell type is not identified at this point, we recommend adding an extra cDNA isolation step before continuing with sequencing of the final single-cell barcoded library to test for presence of sequence of interest. (E) During single-cell capture cDNA is generated, this captured cDNA can be evaluated using PCR with primers specific to target and the barcode. With this method, the presence of the target of interest in the sequencing library can be confirmed.

Aiming to understand where in the process the human cells were lost, we introduced additional QC steps since the included QC checks are of a quantifying nature only. In a parallel experiment, we isolated bulk mRNA from xenografts and verified the presence of *COL7A1* with bulk sequencing (Figure 1D). We were also not able to detect human *COL7A1* in cDNA resulting from the single-cell pipeline using PCRs with primers specific for the barcoded cDNA (Figure 1E). Only mouse *COL7A1* was detected in PCR and sequencing results. Hence, most likely human cells of interest were compromised during dissociation, despite this step having been optimized on healthy human skin biopsies.

It has been shown previously that, during xenograft development, mouse fibroblasts infiltrate into the new human dermis leading to mixed cell populations.[5] Precise excision of the human graft can minimize mouse cell presence during dissociation, but the graft will always be hybrid.[2] Dissociation of hybrid tissues requires separate optimization to obtain enough viable human cells, as dissociation conditions differ between xenografts and human skin biopsies. This most likely led to preferential dissociation of one cell type leading to a bias of observed cells towards one species. Longer dissociation (i.e. overnight) will lead to increased dissociation but will also impact transcriptomic profile. The 10× scRNA-seq workflow incorporates both dead and living cells. Potential ways to remedy these problems include dead cell removal kits and FACS. However, these steps decrease yield substantially and will impact the observed mRNA profile due to added stressors.[6,7] Since our interest lay in mRNA profiles of ASO treated cells, we chose not to perform these extra steps. The three QC steps incorporated in the 10X workflow show only whether cell and cDNA content are sufficient for sequencing. For xenografts, including additional QC steps that verify the presence of information of interest in the sample is necessary. In conclusion, we advise researchers interested in combining the use of xenograft with scRNA-seq experiments to independently pilot each step of the protocol and test for the presence of information of interest where possible to ensure generation of valuable data and avoid waste of valuable resources.

AUTHOR CONTRIBUTIONS

Study design prepared by PCA and JB. Data collection performed by FCV and JB. Data analysis and interpretation was performed by FCV. Study supervised by JB and PCA. Manuscript prepared by FCV. Manuscript reviewed by NK, MCB, PCA and JB.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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