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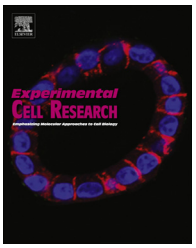

Research Article

Strategies for rapidly mapping proviral integration sites and assessing cardiogenic potential of nascent human induced pluripotent stem cell clones

Cheryl Dambrot[a,b], *Henk P.J. Buermans*[c], *Eszter Varga*[d,e], *Georgios Kosmidis*[b], *Karin Langenberg*[f], *Simona Casini*[b], *David A. Elliott*[g], *Andras Dinnyes*[d,e], *Douwe E. Atsma*[a], *Christine L. Mummery*[b,h], *Stefan R. Braam*[f,*], *Richard P. Davis*[b,h,**]

[a]*Department of Cardiology, Leiden University Medical Center, Leiden, The Netherlands*
[b]*Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands*
[c]*Leiden Genome Technology Center, Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands*
[d]*BioTalentum Ltd., Gödöllö, Hungary*
[e]*Molecular Animal Biotechnology Laboratory, Szent Istvan University, Gödöllö, Hungary*
[f]*Pluriomics B.V., Leiden, The Netherlands*
[g]*Murdoch Childrens Research Institute, The Royal Children's Hospital, Parkville, VIC, Australia*
[h]*Netherlands Proteomics Institute, Utrecht, The Netherlands*

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ABSTRACT

Recent methodological advances have improved the ease and efficiency of generating human induced pluripotent stem cells (hiPSCs), but this now typically results in a greater number of hiPSC clones being derived than can be wholly characterized. It is therefore imperative that methods are developed which facilitate rapid selection of hiPSC clones most suited for the downstream research aims. Here we describe a combination of procedures enabling the simultaneous screening of multiple clones to determine their genomic integrity as well as their cardiac differentiation potential within two weeks of the putative reprogrammed colonies initially appearing. By coupling splinkerette-PCR with Ion Torrent sequencing, we could ascertain the number and map the proviral integration sites in lentiviral-reprogrammed hiPSCs. In parallel, we developed an effective cardiac differentiation protocol that generated functional cardiomyocytes within 10 days without requiring line-specific optimization for any of the six independent human pluripotent stem cell lines tested. Finally, to demonstrate the scalable potential of these procedures, we picked 20 nascent iPSC clones and performed these independent assays concurrently. Before the clones required passaging, we were able to identify clones with a single integrated copy of the reprogramming vector and robust cardiac differentiation potential for further analysis.

*Correspondence to: Pluriomics B.V. Galileiweg 8, 2333BD Leiden, The Netherlands.
**Correspondence to: Department of Anatomy and Embryology, Leiden University Medical Center, 2300RC Leiden, The Netherlands.
E-mail addresses: stefan.braam@pluriomics.com (S.R. Braam), r.p.davis@lumc.nl (R.P. Davis).

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Introduction

Since the initial reports that human fibroblasts could be reprogrammed to a self-renewing, pluripotent state [1,2], interest in induced pluripotent stem cells (iPSC) has grown exponentially as their many applications in biomedical research and regenerative medicine have become evident. However, a remaining challenge for the field is to address the technical issues that contribute to individual cell line differences and thereby confound our ability to distinguish disease-associated variability from background noise. These include methods for rapidly assessing the quality and genomic integrity of the reprogrammed cell lines, and robust protocols for differentiation that are completely defined and readily transferable across multiple iPSC lines.

Most reprogramming methods generate a large number of putative iPSC clones that appear morphologically similar to embryonic stem cells (ESCs); however only a subset of these have comparable molecular and functional features [3]. To distinguish *bona fide* iPSCs from those that are only partially reprogrammed, detailed characterization of the clones is required, which is often lengthy and expensive. This typically limits the number of clones that are examined to fewer than six, with the selection of clones for further analysis predominantly based on subjective criteria like morphology and culture characteristics [4,5]. While this is generally sufficient to identify at least one genuine iPSC clone that can indefinitely self-renew and is pluripotent, it does not assess the clonal variability in differentiation efficiency that is often detected when pluripotent stem cells (PSCs) undergo further guided differentiation to functional cell subtypes [6–9]. One potential source of this clonal variation if integrative reprogramming methods have been used might be insertional mutagenesis caused by the reprogramming factors [10,11]. However, the genomic integrity of iPSCs is frequently not assessed, despite studies demonstrating that the integration of viral vectors can result in the dysregulation of adjacent genes [12,13]. This altered gene expression could lead to perturbed signalling pathways that may alter the pluripotency or differentiation potential of the iPSCs, similar to that observed in some virally-transduced hematopoietic stem cell lines [14].

To address these issues, we developed a novel combination of methods to aid in selecting hiPSC clones for studying cardiogenesis. Furthermore, both procedures are scalable, allowing many potential iPSC clones to be screened simultaneously. To demonstrate this, the number and location of the proviral integrations, as well as the cardiac differentiation potential of 20 putative iPSC clones was determined within 2 weeks following their emergence during reprogramming. These methods require minimal amounts of starting material and can assist in identifying the optimal iPSC clones for further characterization. Furthermore, these procedures potentially could be applied to aid in the selection of transgenic human PSCs in which, for example, overexpression constructs, therapeutic genes or ectopic reporters have been randomly integrated.

Materials and methods

Ethics statement

Human skin biopsies were obtained from patients after individual written permission using standard informed consent procedures, and following approval for use in this study by the Leiden University Medical Center's medical ethics committee. Control skin samples and milk teeth were obtained as waste tissue from donors in accordance with the Dutch Federation of Biomedical Scientific Societies "Use of human tissue for scientific research" and "Code of good use" directives. All samples were collected and anonymized by the treating physician.

Human iPSC derivation

Human dermal fibroblasts or dental pulp cells were reprogrammed to iPSCs using the pRRL.PPT.SF.hOKSMidTomato-preFRT polycistronic lentiviral vector as previously published [15]. Based on morphology, human (h) ESC-like colonies were manually picked 30 days after transduction. All human samples were obtained after informed consent.

PSC culture and differentiation

PSCs were cultured on Matrigel (BD Biosciences)-coated tissue culture dishes in mTeSR1 according to the manufacturer's protocol (Stem Cell Technologies). The control hESC and dermal fibroblast (DF)-derived hiPSC lines used in this study are listed in Table S1. To initiate differentiation to cardiomyocytes, the PSCs were dissociated into small clusters of cells and seeded onto a Matrigel-coated cell culture dish in mTeSR1. Three days later (differentiation day(d) 0), the medium was replaced with low-insulin (1 mg/l), (LI)-BPEL medium [16] and supplemented with BMP4, Activin A, CHIR99021 and XAV939 as indicated. If the cultures were to be maintained for more than 16 days, the cells were overlaid with additional Matrigel (1:100) on differentiation d3 to prevent detachment from the dishes. From differentiation d6, the LI-BPEL medium was refreshed twice a week.

Southern blot

Genomic DNA was digested with SphI (Promega) overnight, resolved by gel electrophoresis and transferred to Hybond-N+ Nylon membrane (GE Healthcare). Hybridization and detection of the probe, encompassing the coding sequences of *c-Myc*, was performed as previously described [17].

Amplification and sequencing of vector integration sites

To determine the lentiviral integration sites in either the DF hiPSC lines or the putative iPSC clones, genomic DNA was isolated with the DNAeasy Blood and Tissue Kit (Qiagen) and splinkerette PCR performed [18–20]. Oligonucleotides and PCR amplification conditions used are listed in Tables S2 and S3. To fragment the DNA, either 2 μg was sheared for a target peak of 200 bp using a Covaris S2 sonicator according to the manufacturer's protocol, or 5 μg was digested with the restriction enzymes CviQI, AseI and BclI (all from New England Biolabs (NEB)). The resulting DNA fragments were concentrated, blunt-ended using the NEBNext End Repair module (NEB) and modified at the 3′ ends with the addition of an adenosine (NEBNext dA-Tailing Module; NEB). Splinkerette adaptors were ligated to the DNA fragments using Quick T4 DNA Ligase (NEB). To prevent amplification of internal lentiviral sequences, samples were digested overnight with BglII. Primary amplification of the adaptor-ligated splinkerette products