Video Article

Generation of Integration-free Human Induced Pluripotent Stem Cells Using Hair-derived Keratinocytes

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URL: https://www.jove.com/video/53174
DOI: doi:10.3791/53174

Keywords: Developmental Biology, Issue 102, Keratinocytes, human induced pluripotent stem cells, hair, integration-free reprogramming, regenerative medicine, episomal vectors

Date Published: 8/20/2015

Citation: Hung, S.S., Pébay, A., Wong, R.C. Generation of Integration-free Human Induced Pluripotent Stem Cells Using Hair-derived Keratinocytes. *J. Vis. Exp.* (102), e53174, doi:10.3791/53174 (2015).

Abstract

Recent advances in reprogramming allow us to turn somatic cells into human induced pluripotent stem cells (hiPSCs). Disease modeling using patient-specific hiPSCs allows the study of the underlying mechanism for pathogenesis, also providing a platform for the development of *in vitro* drug screening and gene therapy to improve treatment options. The promising potential of hiPSCs for regenerative medicine is also evident from the increasing number of publications (>7000) on iPSCs in recent years. Various cell types from distinct lineages have been successfully used for hiPSC generation, including skin fibroblasts, hematopoietic cells and epidermal keratinocytes. While skin biopsies and blood collection are routinely performed in many labs as a source of somatic cells for the generation of hiPSCs, the collection and subsequent derivation of hair keratinocytes are less commonly used. Hair-derived keratinocytes represent a non-invasive approach to obtain cell samples from patients. Here we outline a simple non-invasive method for the derivation of keratinocytes from plucked hair. We also provide instructions for maintenance of keratinocytes and subsequent reprogramming to generate integration-free hiPSC using episomal vectors.

Video Link

The video component of this article can be found at https://www.jove.com/video/53174/

Introduction

The discovery of human induced pluripotent stem cells (hiPSCs) has revolutionized the field of regenerative medicine, providing a feasible method for generation of patient-specific stem cells [1-3]. hiPSCs have been successfully generated from various somatic cell types, including fibroblasts [4,5], hematopoietic cells [6,7], renal epithelial cells from urine [8] and keratinocytes [9,10]. To date, skin fibroblasts and hematopoietic cells represent the most commonly used cell sources for generating patient-specific iPSCs. Arguably, this is due to the fact that skin biopsies and blood collection are routine medical procedures and large biobanks of patient blood or skin samples have been established in many countries.

In contrast to blood cells and skin fibroblast which require invasive extraction methods, keratinocytes represent an easily accessible cell type for hiPSC generation. Keratinocytes are keratin-rich epithelial cells that form the exterior epidermal barrier of the skin and are also found in nails and hair [11]. In particular, keratinocytes can be found on the outer root sheath (ORS) of hair follicles, an external cellular layer that covered the hair shaft together with the inner root sheath (IRS) cells ([12], **Figure 1**). As hair collection is a simple procedure that does not require the assistance of medical personnel, it provides an opportunity for patients to collect and send their own hair samples to laboratories, which would greatly facilitate the collection of patient samples for hiPSC generation. Epidermal keratinocytes also have a higher reprogramming efficiency and faster reprogramming kinetics compared to fibroblasts, adding to the advantages of using keratinocytes as the starting cells for hiPSC generation [9,13]. Furthermore, hiPSCs can also be generated using other cell populations within the hair follicle, including the dermal papilla cells located at the base of the hair follicle [14,15].

Previous reports of iPSC generation using hair-derived cells often utilize retroviral or lentiviral-based reprogramming methods [9,14,15]. However, these viral methods introduce undesirable genomic integration of foreign transgenes during reprogramming. In comparison, the use of episomal vectors represents a feasible, non-viral reprogramming method to generate integration-free iPSCs [4]. We have previously developed a simple, cost-effective and non-viral method to efficiently reprogram keratinocyte into hiPSCs using episomal vectors [13]. Here we provide a detailed protocol for the generation of keratinocyte-derived hiPSCs, including the derivation of keratinocytes from plucked hair, expansion and maintenance of the keratinocytes and subsequent reprogramming to generate hiPSCs.

Protocol

The collection of human hair sample from individuals requires ethical approval by the human research ethics committee in the host institutions and should be done in compliance with the institutional guidelines.

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1. Isolation of Keratinocytes from Plucked Hair

1. Thaw Extracellular Matrix (ECM) solution (*i.e.,* Matrigel) on ice O/N.
2. Using pre-chilled pipette tips, add 200 µl ECM solution to 12 ml of chilled DMEM/F12 medium. Coat a 12-well plate with 1 ml of diluted ECM solution into each well. Incubate the coated plate O/N at 37 °C. Wash the coated plate with pre-warmed DMEM/F12 medium before use.
3. Collect primary human hair from the individual's head by placing fingers close to the root of the hair and plucking the hair in one quick and smooth motion. Check to confirm that each collected hair contains an intact hair follicle and determine the growth phase of the hair under a dissecting microscope (**Figure 1**).
    1. Collect 5-10 anagen hairs from each individual to extract keratinocytes. Process plucked hairs as soon as possible to ensure successful derivation of keratinocytes.
    Note: **Figure 1** illustrates the morphology of hairs in different phases of the growth cycle. Anagen hair is in the growth phase, with a visible outer layer of epithelium surrounding the length of the hair, known as the ORS. On the other hand, telogen hair represents hair in the resting phase and does not have a visible ORS, but has a clear ball of cells covering the root of the hair, known as the telogen bulge.
4. Place the hair samples into a petri dish containing 10 ml of Antibiotic Mix to wash for 5 min at RT.
5. Using sterilized scissors and forceps, cut off excessive hair shaft, leaving a hair fragment with an intact hair follicle and around 0.5 - 1 cm of hair shaft.
6. Carefully transfer 1 hair fragment into each well of the ECM-coated plate using forceps.
7. Using a 1 ml pipette, add approximately 3 drops of Knockout Serum Replacement (KSR) medium (~100 - 200 µl) to the hair samples.
Note: Keep the hair fragments in close contact to the plate surface to allow for attachment and subsequent keratinocyte outgrowth. Adding excessive media at this step may causes the hair fragments to float and decreases the chance of successful hair attachment.
8. Incubate the hair samples in a 37 °C incubator with 5% CO_2 and allow the hair follicles to attach O/N. On the following day, gently add another 3 drops of KSR medium (~100 - 200 µl) to keep the hair samples moist.
9. Observe the hair samples daily to confirm that the hair follicles have attached successfully. Generally attachment of hair follicles is apparent within 1-3 days. Thick hairs are usually easier to attach compared to fine hairs, as fine hairs have higher tendency to float which hinder the attachment process.
    1. Once the hair have attached to the coated plate, add 500 µl of KSR medium to the well. Take extra care when adding the medium as hair samples may not have attached firmly. From this point, change media every 2 days.
    Note: Keratinocyte outgrowths should be visible from the ORS region of the hair fragment after 3-7 days (**Figure 2A-B**). Allow the keratinocytes to grow up to 14 days before passaging the cells as described in the next section.

2. Maintenance and Passaging of Keratinocytes

1. Pre-coat a 6-well plate using the Coating Matrix Kit. Add 680 µl of the dilution medium from the kit to each well, followed by 6.8 µl of Coating Matrix solution. Shake the plate to ensure uniform distribution of the Coating Matrix solution on the plate.
2. Allow the coated plate to incubate for 30 min at RT. Remove the Coating Matrix solution prior to plating of keratinocytes.
3. Dissociate keratinocytes into a single cell suspension with 500 µl of 0.25% trypsin per well for 2-5 min at 37 °C. Inactivate the trypsin with 500 µl of medium containing serum (*e.g.,* Fetal Bovine Serum (FBS) medium) and collect into a sterile 15 ml tube. Remove the hair fragment using sterilized forceps.
4. Determine the number of harvested cells using a haemocytometer.
5. Centrifuge the cells for 3 min at 200 x g. Wash the cells with PBS and repeat centrifugation. Aspirate the PBS and resuspend the cell pellet in fresh Keratinocyte medium.
6. Plate down 4 × 10^4 keratinocytes into one well of ECM-coated plate in the presence of Keratinocyte medium. Keep the keratinocytes in a 37 °C incubator with 5% CO_2 (**Figure 2C**). Change media every day and passage cells when the culture is ~80% confluent, which may take approximately 2-7 days depending on the passage number and proliferation rate of the keratinocytes. The derived keratinocytes should be expanded to generate sufficient frozen stocks using standard slow-freezing cryopreservation methods[16].
Note: The described culture conditions support the expansion of homogenous population of keratinocytes with typical cobblestone morphology (**Figure 2C**). Occasionally, a few differentiated keratinocytes, with large and flat morphology and have lost replicative potential, may be observed in culture. Characterization of keratinocytes can be performed by immunostaining using antibodies against cytokeratin 14 as previously described [17]. Established keratinocyte cell lines should also be tested for mycoplasma after 1-3 passages using commercially available kit.

3. Generation of hiPSCs from Keratinocytes Using Episomal Vectors

1. Pre-coat a 6-well plate with 0.1% gelatin (2 ml/well). Allow gelatin to coat for at least 20 min at RT.
2. For reprogramming experiment, use keratinocytes no later than passage 6. Dissociate keratinocytes into a single cell suspension with 1 ml of 0.25% trypsin per well for 2-5 min at 37 °C. Inactivate trypsin with 1 ml of media containing serum (*e.g.,* FBS media) and collect in a 15 ml tube.
3. Centrifuge cells for 3 min at 200 x g and resuspend cells in Keratinocyte medium. Plate 1 × 10^5-1.5 × 10^5 keratinocytes per well in a gelatinized plate O/N.
4. On the following day (Day 0), check the plate to ensure cells are 50-60% confluent.
Note: If keratinocytes are less than ~50% confluent they might not grow well after transfection.
5. On Day 0, perform transfection of the episomal reprogramming vectors on the keratinocytes, using a ratio of 8 µl transfection reagent to 2 µg total plasmid DNA.
    1. Take 0.5 µg for each of the 4 plasmids (pCXLE-eGFP, pCXLE-hOct3/4-shP53F, pCXLE-hSK, pCXLE-hUL) and dilute in 100 µl of Reduced Serum medium (*e.g.,* OPTI-MEM).

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Note: The plasmid pCXLE-eGFP is used to monitor the transfection efficiencies in keratinocytes and is not required for successful reprogramming.
2. Add 8 µl of transfection reagent to the diluted DNA. Mix by flicking the tube. Allow the DNA reaction mix to rest undisturbed at RT for 15 min.
3. Replace the Keratinocyte medium with fresh medium.
4. Gently add the DNA reaction mix to keratinocyte culture and incubate in a 37 °C incubator with 5% CO_2 for 4 hr.
Note: It is not recommended to perform prolonged transfection O/N in keratinocytes as they exhibit low survival post-transfection.
5. After 4 hr post-transfection, replace the Keratinocyte medium with fresh medium.

6. On Day 1 (1 day post-transfection), change the keratinocyte medium and check transfection efficiency by estimating the percentage of eGFP positive cells under a fluorescent microscope. Typically > 60-70% transfection efficiency is observed for primary keratinocytes using this protocol.
7. On Day 2, repeat transfection as described in step 3.5. Two consecutive transfections would typically achieve > 90% transfection efficiency. Keratinocyte culture usually reaches confluence by the end of the second transfection.
1. Prepare a 10 cm dish of mouse embryonic fibroblast (MEF) feeder for each reprogramming reaction. Prepare mitotically inactivated MEF feeders as previously described [18].
2. Pre-coat a 10 cm dish with 5 ml of 0.1% gelatin for at least 20 min. Plate 4 × 10^6 mitotically inactivated MEF per 10 cm dish in FBS medium. Allow the cells to settle and attach O/N in a 37 °C incubator with 5% CO_2.

8. On Day 3, harvest the keratinocytes with 0.25% trypsin as described in step 3.2 and 3.3. Resuspend the keratinocytes in KSR medium and plate 90% of keratinocytes from 1 well of a 6-well plate into a 10 cm MEF feeder dish with KSR medium.
9. From Day 4 onwards, change the KSR medium every day.
1. Alternatively after Day 18, replace culture medium every second day.
Note: Non-reprogrammed keratinocytes will cease to proliferate, while hiPSC colonies with defined boundary that resemble human embryonic stem cells (hESCs) will emerge from Day 14 - 21.

10. Isolate hiPSC colonies by manual dissection from Day 21 onwards. Avoid hiPSC colonies that are closely clustered together and only pick hiPSC colonies that are clearly separated from others.
Note: hiPSC colonies can be picked earlier, but the colony with defined boundaries might not be obvious at earlier time points given the small colony size. Reprogramming efficiencies may vary between different patient's keratinocytes, and is also influenced by other factors such as passage number or proliferation rate of keratinocytes.
1. For manual dissection of hiPSC colonies, use a 26G needle to cut hiPSC colonies under a dissecting microscope. Cut a hiPSC colony into small pieces around 300 - 600 µm in length. Transfer the pieces from one hiPSC colony into a new MEF feeder plate (2.5 × 10^4 MEF/cm^2) to establish a clonal line of hiPSCs. Initially, culture each hiPSC clonal line in one well of a 12 well plate or organ culture dishes, then expand to 6 well plate format.
2. Maintain established clonal lines of hiPSCs in KSR medium on MEF feeders. Once the culture reach 70 - 80% confluency with hiPSC colonies ~1.5 mm in diameter, passage hiPSCs using standard enzymatic passaging methods (Accutase, Dispase or Collagenase IV) according to the manufacturer's instruction.

11. Characterize established hiPSC lines to confirm pluripotency and their ability to differentiate into cells representative of the three germ layers *in vitro* and/or *in vivo*[13,19]. Also, routinely test established hiPSC lines for mycoplasma using commercially available kits to ensure they are pathogen-free. Routinely monitor genomic stability of hiPSC lines by karyotyping or array-based copy number variation (CNV) analysis.

Representative Results

The hair goes through 3 different phases of growth cycle: anagen (the growth phase), catagen (the regression phase) and telogen (the rest phase) [20,21]. The anagen hair follicle contains multiple layers of epithelium; these layers include the ORS, IRS and the hair shaft (**Figure 1**). Anagen hair eventually undergoes transition to the catagen phase, which is marked by apoptosis of the ORS and termination of hair shaft differentiation. Finally, catagen hair transition to the telogen phase, where apoptosis ceases and the telogen follicle becomes quiescent with a characteristic telogen bulge [20,21].

Figure 1 illustrates the morphology of a telogen hair and an anagen hair. We typically utilize anagen hair for keratinocyte derivation. Following this protocol, keratinocyte outgrowths can be observed as early as 3 days after hair attachment (**Figure 2A**) and will continue to proliferate (**Figure 2B**). In our experience, some anagen hairs may fail to attach or fail to observe keratinocyte outgrowth; thus collect at least 5 - 10 anagen hairs from each individual to ensure successful keratinocyte isolation. Subsequently, the keratinocytes can be passaged onto a new plate and maintained for multiple passages (**Figure 2C**). It is important to note that there is better growth of keratinocytes on a Coating Matrix with Keratinocyte medium as described in section 2, compared to Matrigel with KSR medium.

Following expansion, the keratinocytes can be reprogrammed to generate hiPSCs as described in section 3. **Figure 3A** shows a typical keratinocyte-derived hiPSC colony after 32 days of reprogramming. It is common to observe some differentiation at the center of the hiPSC colony. Once manually picked, the derived hiPSCs typically display high nucleus to cytoplasmic ratio and a defined colony boundary (**Figure 3B**). Established cell lines of hiPSCs can then be characterized for pluripotency as described previously [13,19], such as the expression of pluripotent markers OCT4, NANOG and TRA-160 (**Figure 3C-E**).

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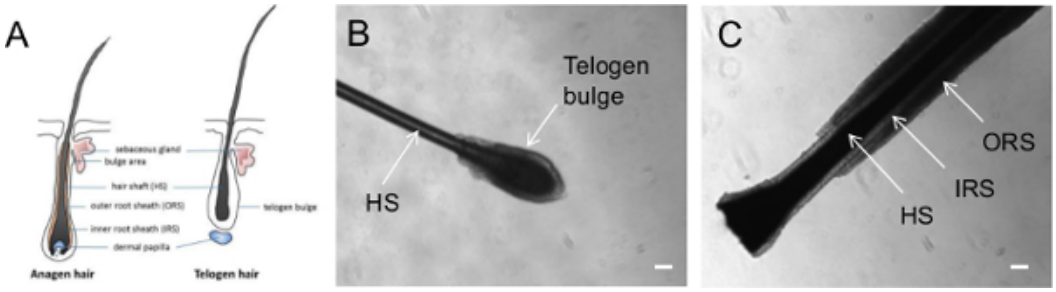

Figure 1. Representative images of plucked hairs at different growth phases. (**A**) Diagram illustrating hairs in the anagen or telogen phase. Phase contrast images showing a plucked hair in (**B**) telogen phase and (**C**) anagen phase. HS = hair shaft; IRS = Inner Root Sheath; ORS = Outer Root Sheath. Scale bar = 100 µm. Please click here to view a larger version of this figure.

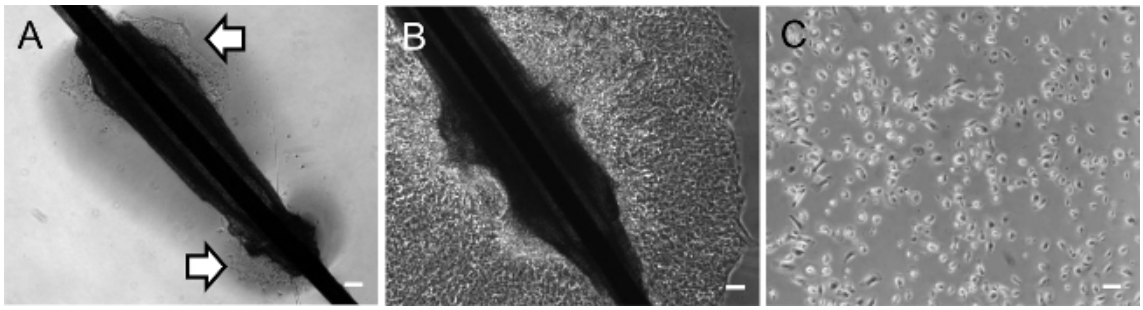

Figure 2. Representative images of hair-derived keratinocytes. Phase contrast images of a plucked hair plated down for (**A**) 3 days and (**B**) 10 days. White arrows mark the outgrowth of keratinocyte from a plucked hair. (**C**) Phase contrast image of Day 3 keratinocyte culture with a typical cobblestone morphology after passaging. Scale bar = 100 µm. Please click here to view a larger version of this figure.

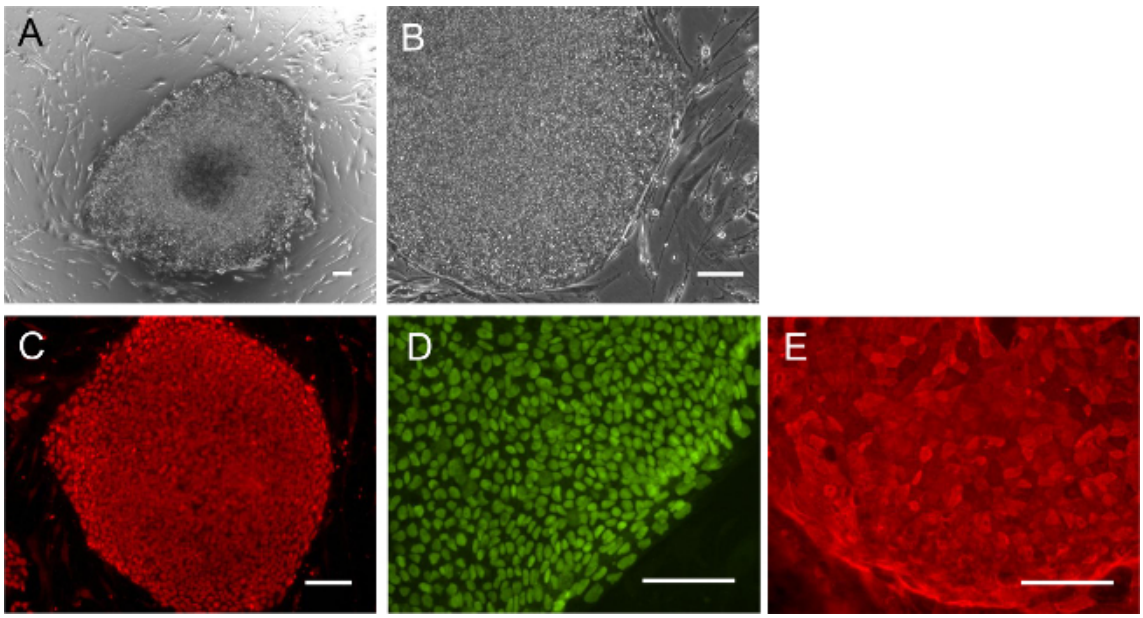

Figure 3. Representative images of keratinocyte-derived hiPSCs. (**A**) Phase contrast image of a Day 32 reprogramming culture showing a keratinocyte-derived hiPSC colony. (**B**) Manually selected keratinocyte-derived hiPSCs with morphology similar to hESCs. Immunostaining of hiPSCs with pluripotent markers (**C**) NANOG and (**D**) OCT4 and (**E**) TRA-160 in keratinocyte-derived hiPSCs. Scale bar = 100 µm. Please click here to view a larger version of this figure.

Discussion

Generation of patient-specific hiPSCs offers a unique approach for studying pathogenesis in the diseased cell types *in vitro*, and also provides a platform for drug screening to identify novel molecules that can rescue the disease phenotypes. This disease modeling approach using hiPSCs has yielded promising results for a variety of diseases, including Long QT syndrome, Huntington's disease, Parkinson's disease and amyotrophic lateral sclerosis [22]. Several initiatives are already underway to establish large-scale libraries of patient-specific hiPSCs, including consortiums in USA, Europe, Australia, China, South Korea and Japan [23,24].

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Here a protocol to establish hiPSCs from hair-derived keratinocytes is described, which has the potential to facilitate and fast-track the establishment of large-scale libraries of disease-specific hiPSCs. This protocol offers two advantages: Firstly, the episomal system utilized in this protocol generates integration-free hiPSCs using a cocktail of six reprogramming factors (OCT4, SOX2, KLF4, L-MYC, LIN28, shRNA for p53) at relatively high efficiency [4]. Compared to retroviral or lentiviral-mediated reprogramming methods, the use of episomal vector avoids undesirable genomic integration of foreign transgenes during reprogramming., Therefore, many initiatives favor the use of integration-free reprogramming methods for establishment of large-scale hiPSC libraries, such as episomal vectors, Sendai virus or mRNA, over integrative reprogramming methods [23].

Secondly, hair is easily accessible and can be harvested by the patients themselves without the use of invasive procedures or the attendance of medical staffs. This provides a unique opportunity for patients from different regions to collect and mail in their own hair samples to the laboratory for keratinocyte derivation and subsequent reprogramming. In support of the feasibility of this strategy, our unpublished data indicate that keratinocytes can be successfully isolated from plucked hair that was stored at 4 °C for up to 10 days.

The methodology described here will allow the derivation of keratinocyte from plucked hair. While keratinocytes can be derived from just a single hair, our recommendation is to collect at least 5 anagen hairs for keratinocyte derivation. One limitation of this protocol is that some hairs may not attach well and the keratinocytes may fail to grow. Thicker hair tends to attach better, while fine hair requires reduced amount of culture medium during plating to avoid floating and enhance attachment. Once keratinocytes are derived, it is advisable to expand and freeze down stocks using standard slow-freezing cryopreservation methods [16]. Subsequent reprogramming of keratinocytes can be performed following steps described in this protocol. It is important to note that the proliferation rate of the starting cells may affect the reprogramming efficiency, with decreased reprogramming efficiency observed in late passages as cells reach cellular senescence [25-27]. In this regard, use keratinocytes no later than passage 6 for reprogramming experiments. In addition, reprogramming efficiencies may vary between different individual's keratinocytes.

Recent studies suggest widespread genetic mosaicism in multiple types of somatic tissues [28], with ~30% of skin fibroblasts reported to have somatic CNVs in the genome [29]. These CNVs may be caused by errors in DNA replication, DNA repair or transposon mobilization. It is also possible that prolonged UV exposure to the skin can cause additional CNVs in epidermal cells including fibroblasts and keratinocytes, but the extent of this is not well understood. As CNVs in keratinocytes will be carried over during the reprogramming process, it is important to perform routine karyotyping or CNV analysis to ensure that the established hiPSCs maintain genomic stability. In summary, here we described procedures for generation of hiPSCs from hair-derived keratinocytes, which could be used for disease modeling and regenerative medicine.

Disclosures

The authors have no conflict of interest.

Acknowledgements

The authors wish to thank Harene Ranjithakumaran and Stacey Jackson for technical support. This work was supported in part by grants from the National Health and Medical Research Council (R.C.B. Wong, A. Pébay), the University of Melbourne (R.C.B. Wong), Retina Australia (R.C.B. Wong, S.S.C. Hung, A. Pébay) and the Ophthalmic Research Institute of Australia (R.C.B. Wong, S.S.C. Hung, A. Pébay); Australian Research Council Future Fellowship (A. Pébay, FT140100047), Cranbourne Foundation Fellowship (R.C.B. Wong); intramural funding from the National Institutes for Health (R.C.B. Wong, S.S.C. Hung) and operational infrastructure support from the Victorian Government.

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