T2.1. Literature review on reprogramming methodologies for hiPSC generation from haplo-selected cord blood samples

Kuebler B, Alvarez-Palomo B, Aran B, Castaño J, Rodriguez L, Raya A, Querol Giner S, Veiga A. **Generation of a bank of clinical-grade, HLA-homozygous iPSC lines with high coverage of the Spanish population.** Stem Cell Res Ther. 2023 Dec 13;14(1):366. https://doi.org/10.1186/s13287-023-03576-1. PMID: 38093328
The paper describes the generation of the first clinical-grade, iPSC haplobank in Spain made from CD34+ cells from seven cord blood units homozygous for most common HLA-A, HLA-B and HLA-DRB1 haplotypes within the Spanish population by transduction with Sendai viral based vectors (Cyto Tune iPS 2.1 Sendai Reprogramming Kit) and their GMP-compliant expansion and banking.

Park S, Gwon Y, Khan SA, Jang KJ, Kim J. **Engineering considerations of iPSC-based personalized medicine**. *Biomater Res*. 2023;27(1):67. https://doi.org.10.1186/s40824-023-00382-x . Review article.
In this review, the authors summarize how engineering strategies have been applied to advance iPSC-based personalized medicine by categorizing the process into three distinctive steps: 1) production of therapeutic iPSCs; 2) engineering of therapeutic iPSCs; and 3) application of engineered iPSCs. For each step, the authors discuss the various engineering approaches and their implications.

Bohrer LR, Stone NE, Mullin NK, et al. **Automating iPSC generation to enable autologous photoreceptor cell replacement therapy.** *J Transl Med*. 2023;21(1):161. https://doi.org.10.1186/s12967-023-03966-2 .
The authors describe the use of the Cell X precision robotic cell culture platform to enable parallel production of clinical grade patient specific iPSCs. The Cell X is housed within an ISO Class 5 cGMP compliant closed aseptic isolator (Biospherix XVivo X2), where all procedures from fibroblast culture to iPSC generation, clonal expansion and retinal differentiation were performed. iPSCs were generated from skin derived human fibroblasts from patients with inherited retinal degenerative blindness by Sendai virus transduction (Cyto Tune iPS 2.1 Sendai Reprogramming Kit, Laminin 521, Essential E8 medium).

Yoshida S, Kato TM, Sato Y, et al. **A clinical-grade HLA haplobank of human induced pluripotent stem cells matching approximately 40% of the Japanese population**. *Med*. 2023;4(1):51-66.e10. https://doi.org/10.1016/j.medj.2022.10.003
The authors recruited donors whose human leukocyte antigens (HLAs) were homozygous. The peripheral or umbilical cord blood collected from the donors was used for iPSC production by electroporation of episomal vectors. They constructed a clinical-grade haplobank of 27 iPSC lines from 7 donors according to good manufacturing practice regulations. This haplobank provides HLA-matched iPSC lines for approximately 40% of the Japanese population.

Bohrer LR, Stone NE, Mullin NK, Voigt AP, Anfnson KR, Fick JL, Luangphakdy V, Hittle B, Powell K, Muschler GF, Mullins RF, Stone EM, Tucker BA. **Automating iPSC generation to enable autologous photoreceptor cell replacement therapy.** *Journal of Translational Medicine.* 2023;21:161; https://doi.org/10.1186/s12967-023-03966-2
The paper describes the use of the Cell X precision robotic cell culture platform to enable parallel production of clinical grade patient specifc iPSCs. The Cell X is housed within an ISO Class 5 cGMP compliant closed aseptic isolator (Biospherix XVivo X2), where all procedures from fibroblast culture to iPSC generation, clonal expansion and retinal diferentiation were performed. The iPSCs were generated by transduction of fibroblasts with CytoTune™-iPS 2 Sendai Reprogramming Kit 2.0 on Laminin 521 in E8 medium.

Powell KA, Bohrer LR, Stone NE, Hittle B, Anfinson KR, Luangphakdy V, Muschler G, Mullins RF, Stone EM, Tucker BA**. Automated human induced pluripotent stem cell colony segmentation for use in cell culture automation applications.** *SLAS Technol*. 2023;28(6):416-422. https://doi.org/10.1016/j.slast.2023.07.004.
The paper describes the development of a deep learning segmentation approach based on the U-Net architecture to automatically segment hiPSC colonies in high resolution large FOV phase contrast images of hiPSC cultures, as well as an algorithm for placement of automated pick locations within these segmented colonies. The iPSCs were generated by transduction of fibroblasts with CytoTune™-iPS 2.0 Sendai Reprogramming Kit on Laminin 521 in E8 medium.

Buckberry S, Liu X, Poppe D, Ping Tan J, Sun G, Chen J, Viet Nguyen T, de Mendoza A, Pflueger J, Frazer T, Vargas-Landín DLB, Paynter JM, Smits N, Liu N, Ouyang JF, Rossello FJ, Chy HS, Rackham OJL, Laslett AL, Breen J, Faulkner GJ, Nefzger CM, Polo JM & Lister R. **Transient naïve reprogramming corrects hiPS cells funcionally and epigenetically.** Nature. 2023 Aug;620(7975):863-072. https://doi.org/10.1038/s41586-023-064234-7.
The paper describes a new reprogramming strategy used to produce human induced pluripotent stem cells from somatic cells results in epigenetic and functional profiles that are highly similar to those of human embryonic stem cells.

Lawrence M. **Human iPS cells for clinical applications and cellular products.** Handb Exp Pharmacol. 2023 Mar 25. https://doi.org/10.1007/164_2023_643
In this book chapter the author discusses the process whereby iPSCs are generated, key quality control steps which should be considered during manufacturing, the application of good manufacturing practice to production processes and iPSC-derived cellular products.

Stacey GN. Spotlight: **An HLA-homozygous haplobank resource to promote safer cell therapies**. Cell Stem Cell. 2023, 30, https://doi.org/10.1016/j.stem.2023.01.003
The author reviews and discuss the paper published by Yoshida et al. who reports on the establishment of an HLA-homozygous haplobank of iPSCs that covers approximately 40%

of the Japanese population and describe quality and safety considerations for manufacturing.

Guo T, Wei Q. **Cell reprogramming Techniques: Contribution to Cancer Therapy.** Cell Reprogram. 2023 Aug;25(4):142-153. https://doi.org/10.1089/cell.2023.0011 Review article.
This article reviews the recent progress of cell reprogramming technology in human cancer research, focuses on the application of reprogramming technology in cancer immunotherapy and the problems solved, and summarizes the malignant phenotype changes of cancer cells in the process of reprogramming and subsequent differentiation.

Poster presentation of ARVO Annual Meeting June 2023:
Maddileti S, Agrawal T, Mahato S, Pulimamidi VK, Mariappan I; **Generation and Characterization of a Clinical Grade Human iPSC line and its Differentiation into Retinal Organoids and Retinal Pigmented Epithelial Cells.** *Invest. Ophthalmol. Vis. Sci.* 2023;64(8):4627. ARVO Annual Meeting Abstract
The dermal fibroblast cells derived from skin biopsy of healthy volunteers were reprogrammed into hiPSCs using CTS™ CytoTune 2.1 Sendai reprogramming kit to obtain a clinical-grade hiPSC line which meets the minimal criterion for the stemness, pluripotency, safety and applicability for pre-clinical and clinical trial evaluations and for their ability to differentiate into neuro-retinal organoids and mature RPE cells.

Poster presentation of ARVO Annual Meeting June 2023:
Marmorstein AD, Knudsen T, Hill M, Atherton E, Trncic E, Kirkeby L, Resch Z, Batson H, Finnemann SC, Winters J, Wigle D. **Generation of clinical grade iPSCs for use in ocular cell therapy**. *Invest. Ophthalmol. Vis. Sci.* 2023;64(8):3855. ARVO Annual Meeting Abstract
The authors describe the production of regulatory compliant iPSC lines from 3 adult human donors by transduction of fibroblasts with Sendai viral based vectors (Cyto Tune iPS 2.1 Sendai Reprogramming Kit) to be used for the commercial production of RPE cells for clinical human allogeneic cell therapy.

Kunitomi A, Hirohata R, Arreola V, Osawa M, Kato TM, Nomura M, Kawaguchi J, Hara H, Kusano K, Takashima K, Fukuda K, Takasu N, Yamanaka S. **Improved Sendai viral system for reprogramming to naïve pluripotency**. Cell Reports Methods 2, 2022, 100317, November 21, https://doi.org/10.1016/j.crmeth.2022.100317
Kunitomi et al. develop an improved SeV vector system to generate naive human iPSCs from various somatic cells by changing the structure and combination of SeV vectors. This method allows rapid removal of the SeV vectors, resulting in transgene-free naive iPSCs with superior differentiation potential.

Luni C, Gagliano O, Elvassore N. **Derivation and differentiation of human pluripotent Stem cells in microfluidic devices**. Annu Rev Biomed Eng. 2022 Jun 6;24:231-248. doi: 10.1146/annurev-bioeng-092021-042744. Epub 2022 Apr 4.

Soltani, S., Eivazi, Z., Harvey, A. R., Voelcker, N. H., Parish, C. L., Williams, R. J., Elnathan, R., Nisbet, D. R., **Changing Fate: Reprogramming Cells via Engineered Nanoscale Delivery Materials**. *Adv. Mater.* 2022, 34, 2108757. https://doi.org/10.1002/adma.202108757 Review article.
This review presents the state-of-the-art research in cell reprogramming, focused on recent breakthroughs in the deployment of nanomaterials as cell reprogramming delivery tools.

Tan LS, Chen JT, Lim LY, Teo AKK. **Manufacturing clinical-grade human induced pluripotent stem cell-derived beta cells for diabetes treatment**. *Cell Prolif*. 2022;55(8):e13232. https://doi.org./10.1111/cpr.13232 . Review article.
This review focuses on the key processes and guidelines for clinical translation of human induced pluripotent stem cell (hiPSC)- derived β cells for diabetes cell therapy. The authors discuss the (1) key considerations of manufacturing clinical-grade hiPSCs, (2) scale-up and differentiation of clinical-grade hiPSCs into β cells in clinically compliant conditions and (3) mandatory quality control and product release criteria necessitated by various regulatory bodies to approve the use of the cell-based products.

Tian P, Elefanty A, Stanley EG, Durnall JC, Thompson H Elwood NJ. **Creation of GMP-compliant iPSCs from banked umbilical cord blood.** Front. Cell Dev. Biol. 2022 Mar 16; 10:835321. https://doi.org/10.3389/fcell.2022.835321
The paper describes a protocol to create clinical-grade iPSC from banked CB. This protocol uses a small volume of thawed CB buffy to first undergo ex-vivo expansion towards erythroid progenitor cells, which are then used for reprogramming using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit. Cells are maintained in a feeder-free, xeno-free environment, using fully defined, commercially available reagents. The authors state the efficient and robust creation of clinical-grade iPSC cell lines from small volumes of cryopreserved CB.

Kim JY, Nam Y, Rim YA, Ju JH. **Review of the Current Trends in Clinical Trials Involving Induced Pluripotent Stem Cells.** *Stem Cell Rev Rep*. 2022;18(1):142-154. https://doi.org/10.1007/s12015-021-10262-3. Review article.
In this review, ClinicalTrials.gov, the WHO ICTRP, and several country-specific clinical trial databases were consulted to investigate clinical studies involving iPSCs.

Lam ATL, Ho V, Vassilev S, Reuveny S, Oh SKW. **An allied reprogramming, selection, expansion and differentiation platform for creating hiPSC on microcarriers.** *Cell Prolif*. 2022;55(8):e13256. https://doi.org/10.1111/cpr.13256
The paper describes reprogramming of human somatic cells (2 fibroblast lines, PBMCs, CD3+ T-cells and CD34+ cells) using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit in microcarrier cultures for large-scale generation.

Becker-Kojić ZA, García-Verdugo JM, Schott AK, Herranz-Pérez V, Zipančić I, and Hernández-Rabaza V. **Membrane-to-Nucleus Signaling in Human Blood Progenitor Cells**

Reveals an Efficient GM-Free Reprogramming to Pluripotency. *Possibilities and Limitations in Current Translational Stem Cell Research*, IntechOpen, Rijeka, 2022, Diana Kitala. Book chapter. https://doi.org/10.5772/intechopen.108950
The authors describe de-differentiation of PBMCs from healthy donors w/o genetic manipulation, based on the activation of the GPI-linked protein ACA through antibodies.

Kitano, Y., Nishimura, S., Kato, T.M., Ueda, A., Takigawa, K., Umekage, M., Nomura, M., Kawakami, A., Ogawa, H., Xu, H., Hotta A., Takasu N, Tsukahara M. **Generation of hypoimmunogenic induced pluripotent stem cells by CRISPR-Cas9 system and detailed evaluation for clinical application**. Mol. Ther. Methods Clin. Dev. 2022. 26 15–25. https://doi.org/10.1016/J.OMTM.2022.05.010

Abberton KM, McDonald TL, Diviney M, Holdsworth R, Leslie S, Delatycki MB, Liu L, Klamer G, Johnson P, Elwood NJ. **Identification and re-consent of existing cord blood donors for creation of induced Pluripotent Stem Cell lines for potential clinical applications.** Stem Cells Translational Medicine. 2022, 11, 1052-1060. https://doi.org/10.1093/stcltm/szac060
The authors present a pathway toward the creation of a clinical grade cord blood-derived induced pluripotent stem cell lines, within a strong quality framework, and with the appropriate regulatory, government and ethics approvals, along with a dynamic follow-up and re-consent process of cord blood donors from the public BMDI Cord Blood Bank.

Yoshida S, Kato TM, Sato Y, Umekage M, Ichisaka T, Tsukahara M, Takasu N, Yamanaka S. **A clinical-grade HLA haplobank of human induced pluripotent stem cells matching approximately 40% of the Japanese population.** Med, 2023, 4:1-6, https://doi.org/10.1016/j.medj.2022.10.003
The authors describe the construction of a clinical-grade haplobank of 27 iPSC lines from 7 donors according to good manufacturing practice regulations. The lines were generated by electroporation of blood cells with episomal plasmids. This haplobank provides HLA-matched iPSC lines for approximately 40% of the Japanese population.

Kawase, E., Takada, K., Nakatani, R., Yamazaki, S., and Suemori, H. **Generation of clinical-grade human embryonic stem cell line KthES11 according to Japanese regulations.** Stem Cell Res. 2021. 54, 102383. https://doi.org/10. 1016/J.SCR.2021.102383.
The paper describes the derivation of the human embryonic stem cell line, KthES11, from a normal healthy blastocyst donated for clinical research. Cell line derivation, its propagation and storage were performed without feeders in an animal product-free environment according to current Good Manufacturing Practice (cGMP) standards.

Scesa G, Adami R, Bottai D. **iPSC Preparation and Epigenetic Memory: Does the Tissue Origin Matter?** *Cells*. 2021; 10(6):1470. https://doi.org/10.3390/cells10061470 Review article.
In this article, the authors review the impact of reprogramming methods and the choice of the tissue of origin on the epigenetic memory of the iPSCs or their differentiated cells. Next, they describe the importance of induction methods to determine the reprogramming efficiency and avoid integration in the host genome that could alter gene

expression. Finally, they compare the significance of the tissue of origin and the inter-individual genetic variation modification that has been lightly evaluated so far, but which significantly impacts reprogramming.

Sullivan S, Fairchild PJ, Marsh SGE, Müller CR, Turner ML, Song J, Turner D. **Haplobanking induced pluripotent stem cells for clinical use.** Stem Cell Research. 2020;49 102035. https://doi.org/10.1016/j.scr.2020.102035
In this article, the authors discuss what is practically involved in developing and executing an iPSC haplobanking strategy.

Huang CY, Liu CL, Ting CY, Chiu YT, Cheng YC, Nicholson MW, Hsieh PCH. **Human iPSC banking: barriers and opportunities**. *J Biomed Sci*. 2019;26(1):87. Published 2019 Oct 28. Review article. https://doi.org/10.1186/s12929-019-0578-x
In this review, the authors compare the construction and operation strategy of some iPSC banks as well as their ongoing development. They also introduce the technical challenges and offer future perspectives pertaining to the establishment and management of iPSC banks.

Wang Q, Vossen A, Ikeda Y, Devaux P. **Measles vector as a multigene delivery platform facilitating iPSC reprogramming**. *Gene Ther*. 2019;26(5):151-164. https://doi.org/10.1038/s41434-019-0058-7
The authors produced a one-cycle measles virus (MV) vector as a highly efficient multi-transgene delivery system based on a vaccine strain of MV, a non-integrating RNA virus that has a long-standing safety record in humans. Introduction of the four reprogramming factors OCT4, SOX2, KLF4, and cMYC via a single, “one-cycle” MV vector efficiently reprogrammed human somatic cells into iPSCs, whereas MV vector genomes are rapidly eliminated in derived iPSCs. The authors present a MV vector system as a new reprogramming platform for genomic modification-free iPSCs amenable for clinical translation.

Lee M, Ha J, Son YS, Ahn H, Jung KB, Son MY, Kim J. **Efficient exogenous DNA-free reprogramming with suicide gene vectors.** *Exp Mol Med*. 2019;51(7):1-12. Published 2019 Jul 19. https://doi.org/10.1038/s12276-019-0282-7
The paper describes a new episomal vector-based reprogramming method employing the CD/5-FC combination for the easy and rapid isolation of EF-iPSCs and EF-induced neural stem cells (iNSCs) from human fibroblasts. The authors could negatively select cells with an integrated copy of the CD gene and promptly isolate EF-reprogrammed cells within seven days. They propose that this CD episomal vector system offers the easiest and cheapest method for producing safe reprogrammed cells.

Haase A, Glienke W, Engels L, Goehring G, Esser R, Arseniev L, Martin U. **GMP-compatible manufacturing of three iPS cell lines from human peripheral blood.** Stem Cell Res. 2019. https://doi.org/10.1016/j.scr.2019.101394

The paper describes the development of a GMP-compatible protocol for the reprogramming of CD34+ hematopoietic stem cells from peripheral blood (CD34+ PBHSC) into hiPSCs using Sendai virus-based reprogramming vectors. Three GMP-compatible hiPSC (GMP-hiPSC) lines were manufactured and characterized under these conditions. The cells were transduced using the CTS Cyto Tune iPS 2.1 Sendai Reprogramming Kit and seeded on CTS ™ Recombinant Human Vitronectin (Thermo Fisher Scientific)-coated plates in iPS-Brew GMP Medium (Miltenyi Biotec).

Wiley LA, Anfinson KR, Cranston CM, Kaalberg EE, Collins MM, Mullins RF, Stone EM, Tucker BA. **Generation of Xeno-Free, cGMP-compliant patient-specifc iPSCs from skin biopsy.** Curr Protoc Stem Cell Biol. 2018, 42: 4A.12.1-4A.12.14. https://doi.org/10.1002/cpsc.30.
The paper describes how using xeno-free reagents in an ISO class 5 environment, isolation and culturing of dermal fibroblasts, the generation of clinical-grade iPSCs and derivation of autologous retinal cells via 3D differentiation. The iPSCs were generated by transduction of fibroblasts with CytoTune™-iPS 2.0 Sendai Reprogramming Kit on Laminin 521 in E8 medium.

Rim YA, Park N, Nam Y, et al. **Recent progress of national banking project on homozygous HLA-typed induced pluripotent stem cells in South Korea**. *J Tissue Eng Regen Med*. 2018;12(3):e1531-e1536. https://doi.org/10.1002/term.2578
The paper describes the screening of the Catholic Hematopoietic Stem Cell Bank of Korea for the most frequent homozygous HLA types of the South Korean population. Blood cells with the selected homozygous HLA types were obtained and transferred to the GMP facility in the Catholic Institute of Cell Therapy. Cells were reprogrammed to iPSCs inside the facility and went through several quality controls (they don't mention how the iPSCs were generated).

Haake K, Ackermann M, Lachmann N. **Concise Review: Towards the Clinical Translation of Induced Pluripotent Stem Cell-Derived Blood Cells—Ready for Take-Off.** Stem Cells TM, 2018.
https://doi.org/10.1002/sctm.18-0134 Review article.
In this review article, the authors provide an overview of the current advances and challenges of the clinical translation of iPSC-derived blood cells and highlight the most pressing problems that have to be overcome.

De Sousa PA, Downie JM, Tye BJ, Bruce K, Dand P, Dhanjal S, Serhal P, Harper J, Turner M, Bateman M. **Development and production of good manufacturing practice grade human embryonic stem cell lines as source material for clinical application.** Stem cell research. 2016;17(2):379-90. https://doi.org/10.1016/j.scr.2016.08.011
The paper describes the derivation of human embryonic stem cells (hESC) while developing and implementing quality assured standards of operation in a facility operating in compliance with European Union (EU) directives and United Kingdom (UK) licensure for procurement, processing and storage of human cells as source material for clinical application and targeted to comply with an EU Good Manufacturing Practice

specification. They describe the evolution and specification of the facility, its operation and outputs, complementing hESC resource details communicated in Stem Cell Research Lab Resources

Task2.2 Literature review and consultation with expert networks on hiPSC characterisation criteria and reprogramming experts meeting selection of the hiPSC characterisation criteria

Critical characteristics and suggested testing techniques

Data from a few of the several clinical trials that have been conducted utilizing iPSC-derived cells are already accessible (www.clinicaltrials.org). Although they have enormous therapeutic potential and have come a long way from the bench to the bedside since their first derivation, the use of iPSC should clear a few obstacles (Rehakova et al. 2020). The requirement for uniformity is one of them. Making a bank or several banks of clinical-grade iPSC lines available for additional processing in a particular application is the most practical way to take advantage of the cells. So, reaching a consensus regarding the banked cell lines' quality assessment is crucial since we need to be able to compare them. Additionally, characterization of iPSC is critical to ensure their quality, safety, and functionality before their use in clinical applications. Lack of proper characterization can lead to inconsistent and potentially dangerous results, such as tumor formation or failure to differentiate into desired cell types. Therefore, it is essential to establish clear and rigorous criteria for evaluating iPSCs.

Variability among different iPSC lines still raises serious questions when utilizing iPSCs and their derivatives for cell treatment and disease modelling. There is a vast variation in the potential for iPSC differentiation, tumorigenicity, instability of the genome and epigenetics. Identity verification, microbiological sterility, endotoxin, genetic fidelity and stability (karyotyping and residual vector testing), potency determination, pluripotency marker expression, and post-thawed viability are among the essential quality attributes for clinical-grade iPSC generation. Variations might still occur, nevertheless, in certain iPSC cell banks during the processes of iPSC differentiation, reprogramming, colony selection, culture system selection, and purification. To address such issues, regular and ongoing validation of the iPSCs is necessary (Huang et al. 2019).

In this sense, as the initial stage of creating a global network of clinical-grade iPSC haplobanks, consensus-building around shared standards should be defined. Every test should be taken into account for the information it offers when determining the justification behind the establishment of clinical-grade iPSC key quality features.

Morphology

"Normal" undifferentiated iPSC colonies exhibit clear borders with defined edges with small, rounded cells, large nucleus/cytoplasma ratio, and prominent nucleoli. This typical morphology can be easily assessed in a brightfield or phase contrast microscope and established as an initial attribute to be determined (Koh et al. 2022; Souralova et al. 2022). Some authors have proposed 90% as the threshold for considering appropriate colony's shape and morphology (Novoa et al. 2024a; Novoa et al. 2024b).

Viability is important since provides essential information about the state of the culture, although it is not an indicator of iPSC's quality or functionality. Additionally, it is recommended not to measure viability immediately after thawing (Rehacova) and 48h after is a preferred period (Sullivan et al. 2018). Among the different techniques proposed, flow cytometry of clinical grade iPSC or ESC with a release criterion above 50% (Shafa et al. 2018) or 70% (Novoa et al. 2024a; Novoa et al. 2024b)have been proposed. On the other hand, growth rate is also an important characteristic to monitor that can be recorded as population doublings when possible.

Self-Renewal Capacity

iPSCs should exhibit the ability to self-renew indefinitely while maintaining their pluripotent state. This can be assessed by performing colony-forming assays or by measuring the growth rate and population doubling time of the cells over extended culture periods. Additionally, and in parallel to colony formation, karyotyping after at least 20 passages (Novoa et al. 2024a; Novoa et al. 2024b)should be included to ensure genetic stability after several passages.

Pluripotency Assays

There are several indicators associated with the pluripotent state of iPSC that can be used to identify them. The transcription factors OCT3/4, SOX2, and NANOG, tumor rejection antigens TRA-1-60 and TRA-1-81, and stage-specific embryonic antigens SSEA3 and SSEA4 are among the most well-known pluripotency indicators (International Stem Cell et al. 2007). Besides, several techniques have been also suggested, being flow cytometry one of the most popular techniques. The combination of one surface marker (TRA-1-60, TRA-1-81, SSEA3, SSEA4) with an intracellular one (OCT3/4, SOX2, NANOG) (Andrews 2009; Andrews et al. 2015; Sullivan et al. 2020; Sullivan et al. 2018) could offer quantitative data that can be easily obtained and compared.

Immnohistochemistry of different surface and intracellular markers has been also used for the characterization of clinical grade ESC, although it has been recommended to be just an informative method according to different guidelines for PSC characterization.

Finally, PSC express significant amounts of alkaline phosphatase (AP), so, its staining is commonly used for the characterization of iPSC cells. This enzyme's ability to convert a

colorimetric reagent from a soluble to a precipitated state makes it simple to measure AP expression (Marti et al. 2013).

Differentiation potential

The differentiation capacity of iPSC is also essential to ensure that they can from a stem cell state to form different types of differentiated cells. iPSC should be able to differentiate into all three germ layers: ectoderm, mesoderm, and endoderm. This can be evaluated through in vitro differentiation assays, where iPSCs are induced to form embryoid bodies or specific cell types. The successful differentiation can be confirmed by the expression of lineage-specific markers.

Traditionally, teratoma formation has been considered the gold standard for demonstrating the differentiation potential of stem cells. The test lies in injecting undifferentiated PSC into an immunocompromised mouse., and after several weeks of growth, tumors which can be histologically analyzed for tissues of all 3 germ layers. However, from an ethical aspect, the usage of an experimental animal should be avoided and only used if there are not any alternative methods.

Embryoid bodies (EB) formation is the spontaneous differentiation of PSC when cultured in suspension in the absence of FGF (Itskovitz-Eldor et al. 2000). EB should spontaneously differentiate into the three germ layers (ectoderm, mesoderm, and endoderm) (Marti et al. 2013). EB are generated in ultra-low attachment plates and expression of at least one marker per germ layer is evaluated. SOX17 and FOXA2 expression are used for endoderm detection, whereas TBXT, and CD71 are used for mesoderm. Ectoderm is normally demonstrated by PAX6 and NESTIN expression (Koh et al. 2022).

Another option is the directed differentiation using commercial kits such as STEMDiff trilineage differentiation kit (StemCell Technologies) or already published methods. For instance, the differentiation towards neural cells (ectoderm), cardiomyocytes (mesoderm) and definitive endoderm In vitro with published methods which have been referenced and described in detail (Tian et al. 2022). After differentiation, expression analysis by immunofluorescence is usually performed.

Genetic testing

In order to provide information about chromosomal aberrations, different tests have been proposed. On the one hand, the standard technique used for genome stability if karyotyping. This assay is based on arresting the cells in the metaphase stage of cell division and it is followed by staining (Marti et al. 2013). Giemsa-banding (G-banding) is the commonest technique. For this, chromosome counts of 20-30 metaphases and additional banding patters analysis of a minimum of 8 metaphases is required (Andrews 2009).

Single nucleotide polymorphism (SNP) arrays can detect genome changes with higher resolution than G-banding. This method is based on the analysis of copy number variations (CVN) although do not allow to detect inversions, balanced chromosomal translocations and 20% of mosaicisms (Rehakova et al. 2020).

Identity

Single tandem repeat (STR) genotyping of iPSC using commercially available kits is recommended for ensuring cell identity. The STR profile should match the cell donor. For instance, 16 STR specific sites were used (Haase et al. 2019).

DNA methylation

It is known that an epigenomic transformation rather than a genetic one is what gives a somatic cell its pluripotent potential through reprogramming. Moreover, Lister and cols. demonstrated that whereas ES cells and iPSC have relatively comparable global methylomes, each iPSC line exhibits notable diversity in reprogramming when compared to ES cells and other iPSC (Lister et al. 2011). Additionally, they showed specific differential DNA methylation and that iPSC' differently methylated areas are frequently transferred to differentiated cells (Lister et al. 2011). The promoters of pluripotency genes are significantly demethylated and exhibit the characteristic activating histone H3K4me3 in PSC, whereas pluripotency genes stay muted in somatic cells due to DNA methylation and repressive histone modifications (Poetsch et al. 2022). Most iPSC lines have their characteristic DNA methylation and histone modification landscapes correctly reprogrammed, although in partially reprogrammed iPSCs, they can be partially reset (Mikkelsen et al. 2007).

Erosion of X-chromosome inactivation

While iPSC undergo global epigenetic remodelling during reprogramming, they maintain both the active and inactive copies of chromosome X of their somatic progenitor (Tchieu et al. 2010). Nevertheless, iPSC cultures are thoughtto be clonal, indicating that each line descends from a single somatic progenitor cell, rather than being mosaic for Xi (Tchieu et al. 2010). However, in extended cultures, hiPSCs exhibit epigenetic heterogeneity, including X-chromosome inactivation degradation (Anguera et al. 2012). The erosion is associated with the loss of expression of the long non-coding RNA XIST, which is required to achieve X-chromosome inactivation (Topa et al. 2024). Thus, for female iPSC X,-chromosome inactivation should be also included as a part of their characterization.

Sterility testing

Any product that is meant to be used in a clinical setting needs to be sterilised. Since there is no way to sterilize the product without killing it, testing the product is essential. A series of tests must be conducted on the product to guarantee its safety with regard

to contamination by bacteria, funghi, or viruses, even though local pharmacopoeias may differ.
Although PCR-based methods are also recognized by certain authorities (Andrews et al. 2015), culture-based approaches can be employed for mycoplasma testing. Moreover, direct staining with DAPI or Hoechst33258 have been also proposed (Andrews et al. 2015).

Anaerobic and aerobic bacteria should both be examined for bacterial contamination using broth-based or culture-based techniques, whereas limulous amoebocyte lysate, or LAL, assay is used to measure endotoxin levels.

Finally, in vitro and in vivo non-specific and specific virus screening (HIV, HBV, HCMV, HCV, etc) should be performed to ensure non detectable contamination.

CA21151 HAPLO-iPS; WG2 hiPSC characterization criteria (T2.2)

Characteristic	Method	Recommended Technique	Acceptance/Release criteria	Status	References
Morphology	Morphology	PC/BF microscopy Rounded cells, large nucleus/cytoplasma ratio, 2 nucleoli	≥90% of colonies with characteristic iPSC morphology and shape; lack of spontaneously differentiated cells	Mandatory	Baghbaderani et al. 2016; Baghbaderani et al. 2015
Self-renew Passage (stem cells)	Morphology, Genomic stability (Karyotype)	Colony formation and chromosome count of 20 metaphases and G-band	Normal (46XX or 46XY) after 20 passages	Mandatory	Baghbaderani et al. 2016; Baghbaderani et al. 2015; Rim et al. 201
Reprogramming vectors clearance	PCR	qPCR for SENDAI	No PCR signal obtained within 35 cycles on the target gene	Mandatory	Novoa et al 2024
Expression of pluripotency markers	Flow cytometry	Quantitative analysis of SSEA-3, SSEA-4, OCT3/4, TRA1-60, SOX-2, NANOG expression	≥ 70% of at least 2 markers	Mandatory	Novoa et al. 2024 Tan et al. 2022
	Immunofluorescence	Qualitative analysis of SSEA-3, SSEA-4, OCT3/4, TRA1-60, SOX-2, NANOG expression	Expression of at least 2 markers (qualitative)	Mandatory	Novoa et al. 2024
	qPCR	OCT3/4, NANOG, SOX-2, E-CAD, LIN28A expression	Expression of at least 2 markers (qualitative)	Informative	Sanjaya et al. 2022
Differentiation potential/Potency	EB formation	Detection of at least one marker per germ layer by immunofluorescence	Detection of at least one marker per each of the 3 germ layers	Mandatory	Baghbaderani et al. 2016; Baghbaderani et al. 2015
	Directed differentiation	Detection of at least one marker per germ layer by immunofluorescence	Detection of at least one marker per germ layer	Mandatory	Tian et al. 2022
Identity	Genetic fingerprinting	STR	All alleles match parent cell line		Rao et al. 2018
	DNA methylation	Human Methylation 450 BeadChip (Illumina) WGS			Araki et al. 2024 ; Lister et al. 2011

CA21151 HAPLO-iPS; WG2 hiPSC characterization criteria (T2.2)

For female hPSCs erosion of X-chromosome inactivation	Allele specific expression/XIST expression				Topa et al. 2024
Sterility	Endotoxin	Pharmacopeial methods (LAL assay)	Negative	Mandatory	Andrews et al. 2015; Baghbaderani et al. 2015; Haase et al. 2019; O'Shea et al. 2020; Shafa et al. 2018; Souralova et al. 2022; Sullivan et al. 2020

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