HAPLO-iPS

D4.1: Regulatory road map from donor samples to *iPSC production* for manufacture of cell *therapy products*

A regulatory roadmap from donor samples to human induced pluripotent stem cell (hiPSC) production requires progression from well-documented source materials to cGMP-compliant manufacturing by implementing rigorous quality control (QC), strict adherence to cGMP, defining critical quality attributes (CQAs), and maintaining strict donor traceability, and strict adherence to applicable guidelines (e.g., ISO standards, ICH guidelines), and regulatory agency (FDA/EMA) guidance, focusing on traceability of the cell line origins and final product safety, and potency. Key steps include risk assessment, validated donor screening/consent, xeno-free reprogramming methods (preferably non-integrating), master cell bank (MCB) establishment and characterization, and adherence to ICH guidelines for sterility, and safety, leading to IND/BLA submissions. Translation from research-grade to clinical-grade manufacturing in accordance with Good Manufacturing Practices (cGMP) needs a thorough product development process in order to maintain the same product characteristics and activity of the therapeutic product after full-scale clinical GMP production as originally developed within a research setting. Compliance requires comprehensive documentation, validated processes, and adherence to regulations like 21 CFR Part 312 for IND submissions.

1. Donor Qualification & Consent (Starting Material): Must adhere to stringent ethical and regulatory guidelines. Starting material is defined as material forming an integral part of the active substance (e.g. iPSCs that are differentiated into the final therapeutic DP; viral vector used to genetically modify the genome of a cell in order to generate a therapeutic cell product). Materials used during the manufacture of the active substance (e.g., culture media, growth factors, etc) that are not intended to form part of the active substance shall be considered as raw materials. Blood cells and skin fibroblasts are common starting material for manufacturing iPSC.

- **Donor selection record:** covering donor health, medical and social history obtained by a questionnaire and a confidential personal interview; Donors must be screened for high-risk behaviors and infectious diseases (e.g. HIV1/2, HBV, HCV, HTLV-I/II, Treponema pallidium, Trypanosoma cruzi, and West Nile Virus) following FDA/EMA guidelines,bacteremia, candidemia, and multidrug-resitant bacteria and fungi. If applicable HLA-typing for allogeneic banks should be performed. Tissue Procurement must follow cGMP regulations for starting materials to ensure traceability and safety.• **Starting cell (sample) procurement:** in Europe donated tissue from which the starting cells will be obtained (blood, skin…) must procured following the new European legislation for Substances of Human Origin (SoHO) (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ:L_202401938). According to this regulation the donor biological material must be procured in and through an accredited SoHO establishment.

- **Informed consent:** Must indicate that the donor understood the wide range of possibilities and yet to be discovered applications to which their cells might be used for including the generation of cell lines. Consent should include that cells would likely be subjected to reprogramming, genetic analysis including infectious agents, genetic disorders and whole genome sequencing, and commercial use.. Before signing the IC, the donor must be informed in detail about the nature of the donation and iPSC, including the potential applications of derived therapeutic cells. A plain language document describing this should be produced in advance and a personal interview scheduled with the investigator to allow the donor clarify any possible questions and concerns.

- **Traceability**: Strict coding systems (e.g., pseudonymization) are required to provide for robust traceability of cells from donor to final product.

- **HLA typing**: For the manufacturing of HLA homozygous iPSC (haplolines), intended for donor-recipient HLA matching, the donor HLA typing should be confirmed by high level resolution re-typing of the starting material (blood cells, fibroblasts, etc.).

2. Reprogramming & Line Derivation (GMP Compliance): Transition from laboratory methods to GMP-compliant, methods and reagents are generally preferred to minimize tumorigenic risk.

- **GMP-compliant reprogramming and culture conditions:**

Reprogramming technique: In order to ease the path to an acceptable iPSC-derived product it is helpful to avoid the use of vectors or other systems which disturb or replace genomic sequences. Furthermore, use of any reprogramming technology, including non-integrating vectors, could result in incomplete or variable reprogramming and requires careful validation. Investigation of cell function at cell line and product levels is therefore vital.

Culture conditions: Culture media, cell attachment matrix and gaseous atmosphere will all impact on the properties of cells either due to biochemical effects on phenotype or adventitious agent contamination. **:** Utilization of well-defined, xeno-free, chemically defined media to minimize genomic disruption and adventitious agents is recommended. In addition, any changes in physical culture conditions, notably temperature and pressure, may also influence the performance of cultured iPSCs, their culture and differentiation, and final product function.

- **Monoclonality & Quality Control:** Single-cell cloning assures that only one cell clone is selected for manufacturing as a control point to reduce the risk of variant, and potentially aberrant, clones being present in the final product. Rigorous characterization for stem cell markers, functional assays for pluripotency (see “Identity and Purity” below)., identity (a precise match with the donor, see below), and genomic integrity (karyotyping or a technique demonstrated to provide be at least equivalent evaluation).

3. Characterization & Quality Control (QC): Defining meaningful critical quality attributes (CQAs) and developing specific, quantitative assays to measure the ability of the final product to produce the desired therapeutic effect is crucial for clinical approval. Stringent QC using robust (well validated) assays is essential to ensure safety and efficacy. The precise identification of

- **Identity & Purity:** Confirmation of identity of the manufacturing cell line enables the product developer (manufacturer) to confirm a match with the donor origin and absence of cell line switching at later stages of cell line use). Markers of self-renewal (e.g., OCT4, NANOG) and hPSC nature (e.g., TRA antigens, SSEA-3) are important to assure that the typical features of hPSCs are retained. It is also vital from a patient safety perspective that residual undifferentiated cells are excluded from the final product as these have the potential to cause benign teratomas in patients. Furthermore, the selected manufacturing cell line should not exhibit a transformed state that could generate teratinocarcinomas or other malignant phenotypes. These considerations are important to mitigate the risk of product cells giving rise to teratomas or other malignancy in patients.

- **Safety Testing:** Screening for adventitious agents (bacteria/sterility, mycoplasma, viruses, prions), karyotype abnormalities, and tumorigenicity (see “identity and purity” above)). Key objectives of these tests are to assure acceptable levels of risk of causing patient disease (infection and/or malignancy). Immunological issues and release of other bioactive molecules with potential for adverse effects in patients are also important considerations. Whilst “haplobanking” to better match patient HLA markers, as opposed to use of an allogenic and thus

donor-unmatched hPSC, offer a strategy to reduce acute rejection. However, many cell glycoprotein types are involved in immunological rejection and these would not be covered by HLA matching alone and this has been addressed in approaches using so-called autologous hPSCs (i.e. hPSCs derived from the patient). Whatever, patient matching strategy is used, this does not exclude the formal possibility of changes occurring in immunologically active phenotype of manufacturing cells during complex hPSC manufacturing processes and such changes may need to be born in mind.

- **Potency Assays:** Developing validated assays that demonstrate the functional capacity of the final cell product . For cell banks (see below), it is beneficial to demonstrate that a cell line can differentiate into at least the early stages of tri-lineage committment as this is a key characeristic of pluripotent cells[1,2] and furthermore, a single cell bank may be used as the starting material for multiple product cell types (see also “Characterisation & QC” above)

[1] In principle, hPSCs do not necessarily need to be fully pluripotent to meet regulatory requirements to support the manufacture of efficacious and suitably safe products.

[2] There are no truly accepted „markers“ of pluripotency. Numerous ISCI and ISCBI publications (see www.iscbi.org/publications) as well as recent ISSCR guidance for researchers have made this point for the characterisation of hPSC lines for clinical use.

- **Raw Material Sourcing:** Documentation and validation of all materials, particularly serum-free media and growth factors, ensuring xeno-free components where possible.

4. Bank Establishment (MCB/WCB): -
Development of a Master Cell Bank (MCB) and Working Cell Bank (WCB) under full GMP compliance.

- **Generating a well-characterized MCB:** serves as starting material for clinical-grade, off-the-shelf, cell therapy products; A stable hiPSC line must be fully characterized, including identity testing, sterility, mycoplasma testing, karyotype analysis, and pluripotency verification.

- **GMP Compliance:** The MCB must be produced in a certified, validated cleanroom environment under conditions that would meet the requirements of cGMP. Cell bank production may be included under a GMP manufacturing licence

- **Stability Studies:** Continuous testing of the MCB to confirm that storage does not affect viability or functionality. Product developers should establish a formal regime to assure stability.

- **Documentation:** Maintain a complete, traceable, and transparent "paper trail" for the entire process, supporting the Drug Master File (DMF) for regulatory submission. The following information may be required:

Depositor information: Name of owner of cell line; Address (registered company and manufacturing sites where applicable); Primary contact; Telephone number(s); Evidence of ownership*;

Shipping records: Signed records of inventory shipped and cross check of received goods, including ‘chain of custody’ documentation; Records of temperature monitoring data; Record of courier used; Record of arrival at repository including transport time/temperature and condition on receipt;

Provenance: Donor information related to the donation of primary tissue**; Original, anonymized donor consent and medical history (this may not always be available depending on national laws and regulations);

Culture/banking details: Description of the culture conditions related to (where applicable):

tissue or embryo culture; cell line derivation; cell line expansion; reagent documentation, traceability and cryopreservation. This should include, for example, passage number (or population doublings where possible) of seed lots and subsequent banks that were created up to the point of manufacture relevant to the material being received by the repository;

Quality control test results: Characterization and safety test results both provided by the depositor and generated by the repository and given with associated passage or population doubling levels;

Facility and equipment details: Qualification records: records of use, maintenance, calibration, validation, re-verification, repair;

Environmental monitoring records: Records of and trends in scores of contamination for testing applied to the environmental conditions, which may include: viable and non-viable particle counts; active air sampling, air pressures, temperature, relative humidity, operator finger dabs, ambient temperatures in critical storage areas;

Deviations from standard procedures (SOPs): Records of deviations from normal procedure, which may affect the specific cell line, for example failure of an incubator in which the line was processed;

Change controls: Records of change control investigations relevant to the cell line, for example impact of changes to QC test specifications or moving storage location of cryopreserved material; Records of staff training and illness of an infectious nature: Records of training and return to work procedures to ensure staff infectious status is not a risk to cell cultures;

5. Regulatory authorities engagement: Depending on the country in Europe, iPSC are considered ATMP or critical cellular intermediate. In the case that the iPSC MCB is considered an ATMP, the manufacturing process can get the GMP certification from a regulatory agency upon auditing and approval. If on the contrary it is considered a cellular intermediate, the GMP compliance can be achieved indirectly as part of the manufacturing process of a derived ATMP. It is advisable to engage in early conversations with the national regulatory agencies before starting the manufacturing process. And in any case, a GMP compliant process to produce the MCB is highly recommendable and most likely mandatory.

6. Process Development & Scale-Up: A robust, reproducible manufacturing process must be established, including in-process controls. Identification of critical process parameters (CPPs) to ensure reproducibility, focusing on automated systems to reduce operator-dependent variability. Many CPPs may be obvious but some are very subtle for cell culture. Any changes or modifications newly introduced in the manufacturing or analytical platform require careful consideration because the consequences of some specific changes might be unpredictable.

- Transition from research-scale to GMP-compliant, often closed-system, manufacturing entails the following key issues:
  - What is the impact of changing raw materials to GMP manufacturing quality which may alter them.
  - Are established analytical systems suitable for GMP use and data recording? They are if they are properly qualified, validated, and managed within a controlled quality system. In the pharmaceutical industry, these systems must comply with stringent regulations—such as FDA 21 CFR Part 11 and EU GMP Annex 11—to ensure data integrity,
  - Shift to GMP is costly;
  - Consider an overall manufacture validation matrix that is usually part of the Validation Master Plan (VMP) listing all processes, equipment, utilities, and computerized systems requiring validation and assigns appropriate validation approaches based on risk.

- Validation of expansion and differentiation protocols (EudraLex-Vol.4-Public Health-European Commission: *Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products*): involves establishing documented evidence that applied expansion and differentiation processes consistently produce cells with predefined identity, purity, viability, and potency. This process requires strict adherence to aseptic conditions and regulatory guidelines

to ensure safety and reproducibility, typically involving a three-stage validation lifecycle (Process Design, Process Qualification, and Continued Process Verification).

- **Small Scale Setup:** Initially define the Process Parameters (PPs) and Critical Material Attributes (CMAs) in small-scale,, GMP-compliant systems (e.g., shake flasks, small bioreactors). It is recommended to identify potential testing variations in key process parameters to assess their impact the Critical Quality Attributes (CQAs) of a product, such as its purity, strength, or stability. In a small-scale environment, these parameters are monitored intensely to define the "design space"—the range of conditions within which the product consistently meets quality standards.

- **Scale-Up Technology:** Transition to larger-scale platforms such as automated, closed-system stirred-tank bioreactors or automated adherent culture systems (e.g., bioreactor systems) to ensure scalability. **:** Validate that the scaled-up, closed-system process maintains product CQAs, specifically ensuring high cell viability and purity during expansion and differentiation. Key environmental factors driving this transition include:
  - **Stringent Sterility and Contamination Control:** The immediate manufacturing environment can pose risks to sensitive cell cultures (e.g., CAR T-cell therapy). Closed systems provide a physical barrier, ensuring sterility and enabling manufacturing in lower-classification cleanrooms, which reduces operational costs and risks.
  - **Need for Homogeneous Environmental Parameters:** As cell cultures grow, they require precise control over parameters like dissolved oxygen (DO), pH, and nutrient concentration. Large-scale bioreactors utilize automated systems to prevent gradients (e.g., oxygen depletion in the upper regions) that can decrease bioprocess efficiency and lead to high byproduct concentrations.
  - **Mitigation of Biological Inhomogeneities:** Large-scale systems face challenges with dead zones, nutrient starvation, and pH fluctuations, which can be overcome by automated, optimized mixing strategies.
  - **Shear Stress and Physical Sensitivity:** For adherent cells or shear-sensitive cells, automated adherent systems offer a "scalability-friendly" environment that mimics static culture while reducing mechanical stress, compared to traditional stirred-tank systems.
  - **Environmental Sustainability in Manufacturing:** The shift to closed-system, single-use technologies addresses environmental concerns by reducing the need for intensive, energy-demanding Cleaning-in-Place (CIP) and Sterilization-in-Place (SIP) procedures.
  - **Reducing Labor-Related Contamination Risks:** Manual handling of large numbers of vessels increases contamination risks. Automated systems automate steps like media exchange and passage, enhancing the consistency of the cell culture environment and increasing productivity per square meter of facility space.

- **Purification & Harvesting:** Implement robust methods (e.g., chromatography, centrifugal separation) to remove impurities and target cell types, ensuring removal of undifferentiated iPSCs.

- **Quality by Design (QbD):** Utilize QbD to define critical process parameters to ensure consistent, large-scale production of high-quality cell therapy product

Manufacturing of the iPSC-derived ATMP

7. Preclinical Safety & Efficacy of the iPSC-derived ATMP**:** Extensive *in vivo* and *in vitro* studies must demonstrate safety (e.g., no tumor formation) and efficacy of the differentiated product.

- **Tumorigenicity Testing:** Essential *in vivo* or other studies to assess the risk of tumor formation (teratoma) from residual undifferentiated hPSCs in the drug product, often requiring high-sensitivity assays (e.g., qPCR, imaging). Detection of transformed cells which might cause

malignancy is also important and this may require more complex in vivo/in vitro assays to assure product safety. A range of assays has been proposed based on animal models, molecular assays and other functional assays (e.g. Sato et al. (2019) Cytotherapy 21: 1095-1111) (See also above). A critical issue is whether the results can be directly related to patient risk and at this stage there is no definitive assay which could do this.

From a regulatory point of view, it is essential and sufficient to assess the risk of tumorigenicity of the DP administered to the patient. From a manufacturers/sponsors point of view, it is recommended and reasonable to test genomic integrity as measure for tumorigenicity risk also already at the cell line//MCB/WCB level and as part of in-process monitoring – although it is well established that not every mutation in a gene that is part of an oncogene panel can cause a tumor (

• **Proof of Concept (POC):** In vivo studies in relevant animal models to demonstrate the therapeutic mechanism, dosage, and efficacy.

The challenge is to model the modeling the physiological, biochemical and immunological disease background and not just a physical defect/lesion. The challenge of translating non-clinical animal data to human safety information must be taken on.

Recent initiatives from the FDA and EMA reflect a significant shift toward replacing in vivo animal testing for tumorigenicity with New Approach Methodologies (NAMs), driven by legislative changes like the FDA Modernization Act 2.0 (2022) and advancements in cell-based assays.

EMA and ICH Perspectives include:

- **ICH S12 Guideline (Adopted 2023):** ICH Guideline S12 on nonclinical biodistribution of gene therapy products, adopted by the EMA, provides recommendations to facilitate the development of gene therapy products while avoiding unnecessary use of animals.
- **EMA Roadmap (2026):** The European Commission is developing a roadmap (due 2026) to transition away from animal testing, emphasizing the validation of alternative methods for chemical and drug safety.
- **ATMP Guidelines:** EMA guidelines for advanced therapy medicinal products (ATMPs) allow for case-by-case evaluation of tumorigenicity, often accepting in vitro assessments of genetic stability rather than mandatory long-term animal studies, especially when utilizing non-pluripotent cells.

Humanized animal models, particularly mice engrafted with human immune systems (HIS) or human tissues, offer significant value in tumorigenicity testing by overcoming the limitations of conventional models that lack human-relevant immune responses. Their primary value lies in providing a **physiological bridge between in vitro testing and human clinical trials**, allowing for the assessment of tumor growth, immunotherapy efficacy, and the safety of advanced therapies in a human-relevant environment.

• **Biodistribution & Persistence:** Studies to track the migration, engraftment, and long-term survival of the transplanted cells. Key requirements for any ATMP biodistribution and persistence study are:

- **Timing of Studies:** Studies are generally required before first-in-human (FIH) testing, although they can be integrated into pivotal proof-of-principle studies.
- **Relevance of Models:** Animal species and disease models must be as relevant as possible to the expected situation in humans. The use of immunocompromised animals may be necessary to prevent rejection of human cells.
- **Target and Non-Target Assessment:** Studies must evaluate distribution to intended target organs as well as non-target, ectopic, or unintended tissues to identify potential safety risks.
- **Analytical Techniques:** Methods must be validated, sensitive, and capable of distinguishing between ATMP cells and host cells. Common techniques include polymerase chain reaction (PCR) for DNA/RNA detection, in situ hybridization (ISH), and immunohistochemistry (IHC).
- **Persistence and Clearance:** Studies must investigate the kinetics of persistence, including how long the product survives in the body and its clearance rate, which directly impacts safety and efficacy.

- **Tumorigenicity Risk:** For cell-based products, investigation of ectopic engraftment, unintended differentiation, or uncontrolled proliferation (neoplastic lesions) is crucial.
- **Vector Shedding:** For gene therapies, shedding into body fluids (e.g., urine, saliva) and the environment must be assessed.
- **Route of Administration:** The studies should follow the intended clinical route of administration (e.g., systemic or in situ)

Specific Considerations include:;

- **Risk-Based Approach (RBA):** A risk-based approach is often used to guide the extent of studies, which may be reduced if abundant clinical experience exists with similar cell types.
- **Repeated Dosing:** If clinical development includes multiple doses, repeated dose biodistribution studies may be necessary.
- **Immune Reactions:** Monitoring for immune reactions (autoantibodies, cytokine release) is required because ATMPs can elicit significant, potentially life-threatening immune responses.

• **Toxicology Studies for hPSC-based products:** fn.Comprehensive preclinical safety evaluations designed to ensure the product is safe, stable, and non-tumorigenic before entering human clinical trials. These studies focus on the unique risks associated with stem cells, such as uncontrolled proliferation and unexpected differentiation, and must be conducted in compliance with Good Laboratory Practices (GLP) and regulatory guidelines (e.g., ICH S6).

Key Components of hPSC-Based Product Toxicology Studies:

- **Tumorigenicity Assessment:** This is the most critical safety study. It involves assessing the risk of teratoma or malignant tumor formation from residual, undifferentiated hPSCs in the final product.

  - Biodistribution and Persistence: Studies tracking where the cells go after administration, how long they stay in the target tissue, and whether they migrate to off-target organs.

  - Genomic Stability Analysis: Extensive testing to ensure the cells have not acquired dangerous genetic rearrangements or mutations during expansion and differentiation. This often includes karyotyping and whole-genome sequencing (WGS).

  - Cell Purity and Phenotype Assessment: Using techniques like single-cell RNA sequencing (scRNA-seq) or flow cytometry to quantify the exact composition of the product, ensuring the absence of residual undifferentiated pluripotent stem cells.

  - In Vivo Safety and Toxicology (Animal Models): Administration of the product to animal models to identify potential systemic toxicities, local reactions at the injection site, and acute or delayed adverse effects.

  - Immunogenicity: Assessing the immune response to allogeneic (donor) cells and evaluating the safety of any required immunosuppression protocols.

Common Methodologies in Toxicology Studies:

- **3D Organoids and Tissue Models:** Advanced in vitro 3D models are increasingly used to mimic human organ structures (liver, heart, neural tissue) for toxicity testing.
- **Functional Assays:** Validating that the differentiated cells function as intended (e.g., glucose-responsive insulin secretion for pancreatic cells or contractions for cardiomyocytes) and do not produce toxic metabolites.
- **Repeated-Dose Toxicity:** For chronic therapies, studies evaluating the effect of long-term exposure to the drug or cell product.

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APPENDIX - Formal Definitions:

Starting Material: the initial biological substances—specifically cells, tissues, or vector stocks of human or animal origin—from which the active substance or finished product is manufactured;

Raw material: any ingredient, substance, or biological component used during the manufacturing process—such as growth factors, culture media, or reagents—that does not become an integral part of the final drug substance

Identity: a fundamental quality parameter designed to confirm that the specific biological or genetic material present in the product matches its intended composition

Purity: critical quality attribute strictly defined by the absence or controlled limitation of impurities

Potency: a quality parameter for Advanced Therapy Medicinal Products (ATMPs) is defined as the measure of the biological activity using a suitably quantitative biological assay (also called potency assay or bioassay), based on the attribute of the product, which is linked to the relevant biological properties.

Comparability (in the context of ATMPs**):** the scientific exercise of demonstrating that a product has a comparable quality, safety, and efficacy profile following changes to its manufacturing process or a transfer to additional manufacturing sites;

Manufacturing Consistency: defined operationally as the reproducible performance of the manufacturing process to deliver a finished product that continuously conforms to established specifications;

Critical Quality Attributes (CQA): a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality, safety, and efficacy

Critical Process Parameter (CPP): A process parameter whose variability has an impact on a critical quality attribute (CQA) and therefore should be monitored or controlled to ensure the process produces the desired quality.

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