**T.C.**
**AKDENIZ UNIVERSITY**
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**DEPARTMENT OF GENE AND CELL THERAPY**

# GENERATION OF INDUCED PLURIPOTENT STEM CELLS FROM HUMAN FIBROBLAST CELL LINES

Muhammet Fatih AYDIN

MASTER OF SCIENCE THESIS

2025-ANTALYA

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**ADVISOR**
**Prof. Dr. Ahter Dilşad ŞANLIOĞLU**

,



2025-ANTALYA

**To the Institute of Health Sciences;**

This study has been approved by our jury as a Master of Science Thesis in the Department of Gene and Cell Therapy, Gene and Cell Therapy Master of Science program. ...../…...../….

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## DECLARATION

I hereby declare that this thesis is my own work; that it does not include any unethical act from the planning to the writing of the thesis; that I have acquired all the information included in this thesis following the ethical principles; that I have made due acknowledgements to all the information and comments that were not acquired by this thesis work; and that I have included all these references in the references list.

Muhammet Fatih AYDIN

Thesis Advisor
Prof. Dr. Ahter Dilşad ŞANLIOĞLU

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## ABSTRACT

**Objective:** Induced pluripotent stem cells (iPSCs) possess the remarkable abilities to differentiate into cell types from all three germ layers and to self-renew indefinitely. These features make them highly valuable for *in vitro* modeling of human biology and gene and cell therapies. However, current methods for iPSC generation are often inefficient and require prolonged culture periods, necessitating improved protocols with enhanced safety and efficacy. In this study, we aimed to reprogram HFF-1 human fibroblasts into iPSCs using integration-deficient lentiviral vectors (IDLV-OSKs) encoding the reprogramming factors Oct4, Sox2, and Klf4, along with sodium butyrate as a supporting small molecule to increase efficiency.

**Methods:** In our study, IDLV-OSK vectors encoding the transcriptional regulators Oct4, Sox2, and Klf4 were used to reprogram HFF-1 fibroblast cells into pluripotency. In addition to the viral vector, the epigenetic modulator sodium butyrate, which is a histone deacetylase inhibitor, was used as a supportive molecule. With our applied reprogramming protocol, colonies suitable for passaging were obtained within 9–12 days. The resulting iPSCs were characterized morphologically and also by the expression of pluripotency markers expressed on the cell surface (alkaline phosphatase, Tra-1-81, Tra-1-60) and those located in the nucleus (Oct4, Klf4, c-Myc, Nanog).

**Results:** We generated iPSCs using IDLV-OSKs with the support of a histone deacetylase inhibitor. The resulting colonies exhibited the expected morphological characteristics. The expression of surface and nuclear pluripotency markers was demonstrated in the colonies.

**Conclusion:** Our findings demonstrate that IDLV-OSK vectors, along with sodium butyrate, constitute an efficient and potentially safe approach for reprogramming human fibroblasts into iPSCs with our protocol. This method holds promise for efficient and safer applications in disease modeling and the development of experimental therapeutic strategies.



**Keywords:** Induced pluripotent stem cells, Sodium butyrate, Lentiviral vectors

## ÖZET

**Amaç:** İndüklenmiş pluripotent kök hücreler (iPKH'ler), her üç germ tabakasına ait hücrelere farklılaşabilme ve kendini sürekli yenileyebilme yetenekleri ile dikkat çeken, insan biyolojisinin *in vitro* modellenmesinde ve gen ve hücre tedavilerinde çok önemli potansiyel atfedilen hücrelerdir. Ancak iPKH oluşturma süreçleri, etkinliği düşük ve uzun süreli kültür gerektiren metodolojilerdir ve etkinlik ve güvenlilik açısından geliştirilmeleri gerekmektedir. Çalışmamızda, HFF-1 insan fibroblast hücrelerinin yeniden programlama faktörlerini kodlayan entegre olmayan lentiviral vektörler (integrase-deficient lentiviral vectors; IDLV-OSK'lar) aracılığıyla ve etkinliği artırıcı küçük molekül sodyum butirat desteği ile iPKH'lere yeniden programlanmasını hedefledik.

**Yöntem:** Çalışmamızda, HFF-1 fibroblast hücrelerinin pluripotensiye yeniden programlanması amacıyla, Oct4, Sox2 ve Klf4 transkripsiyonel regülatörlerini kodlayan IDLV-OSK vektörler kullanıldı. Viral vektörün yanı sıra, epigenetik modülatör destek molekül olarak histon deasetilaz inhibitörü olan sodyum butirat protokole dahil edildi. Uygulanan yeniden programlama protokolümüz ile 9-12 gün içinde pasajlanabilir koloniler elde edildi. Elde edilen iPKHler, morfolojik karakterizasyonun yanı sıra hücre yüzeyinde ifade edilen (alkalin fosfataz, Tra-1-81, Tra-1-60) ve nüklear konumlu (Oct4, Klf4, c-myc, Nanog) pluripotensi belirteçlerinin ekspresyonları ile karakterize edildi.

**Bulgular:** Tez çalışmamızda, IDLV-OSK'lar kullanılarak ve histon deasetilaz desteği ile iPKH'ler elde edildi. Elde edilen koloniler, beklenen morfolojik özellikleri sergiledi. Kolonilerde yüzey ve nüklear pluripotensi belirteçlerinin ekspresyonları gösterildi.

**Sonuç:** Çalışmamızda IDLV-OSK vektör sistemi ve sodyum butiratın birlikte kullanıldığı geriye programlama yaklaşımı, farklı hücrelerin etkin ve güvenlilik potansiyeli yüksek bir metodoloji aracılığıyla geriye programlanarak *in vitro* modelleme ve deneysel terapötik çalışmalarda kullanılma potansiyeli açısından önemlidir.



**Anahtar Kelimeler:** İndüklenmiş pluripotent kök hücre, Sodyum bütirat, Lentiviral vektör

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## ICONS AND ABBREVIATIONS

**AP** : Alkaline Phosphatase

**ASC** : Adipose-derived Stem Cell

**BMSC** : Bone Marrow Stem Cell

**BSA** : Bovine Serum Albumin

**c-Myc** : Cellular Myelocytomatosis Oncogene

**DMEM** : Dulbecco's Modified Eagle Medium

**DMSO** : Dimethyl Sulfoxide

**ESC** : Embryonic Stem Cell

**FBS** : Fetal Bovine Serum

**HFF-1** : Human Foreskin Fibroblast-1

**HSC** : Hematopoietic Stem Cell

**iPSC** : Induced Pluripotent Stem Cell

**IVF** : In Vitro Fertilization

**Klf4** : Kruppel-Like Factor 4

**mESC** : Mouse Embryonic Stem Cell

**Oct4** : Octamer-Binding Transcription Factor 4

**PBS** : Phosphate-Buffered Saline

**PFA** : Paraformaldehyde

**PSC** : Pluripotent Stem Cell

**SCNT** : Somatic Cell Nuclear Transfer

**Sox2** : SRY-box 2

**SRY** : Sex-Determining Region Y Protein

**TRA-1-60 :** Tumor Rejection Antigen 1-60

**TRA-1-81 :** Tumor Rejection Antigen 1-81

**VSEL :** Very Small Embryonic-Like Stem Cell

# 1. INTRODUCTION

The discovery that differentiated cells could be converted into pluripotency via the introduction of key transcriptional regulators marked a transformative milestone in stem cell studies. Techniques such as somatic cell nuclear transfer (SCNT) and cell fusion with embryonic stem cells (ESCs) initially demonstrated the feasibility of reversing cellular differentiation. Building on these foundational insights, a breakthrough occurred with a brilliant discovery: forced expression of the transcriptional factors Oct4, Sox2, Klf4, and c-Myc in differentiated cells initiated reversal of differentiation to produce induced pluripotent stem cells (iPSCs). This advancement provided a robust, embryo-free approach to generating ESC-like cells and opened up new avenues in regenerative medicine and cell-based therapies.

iPSC technology offers immense potential across a range of biomedical fields, enabling the study of developmental and disease-related mechanisms of human development, supporting the generation of patient-specific cell types for tissue repair, and providing valuable cell-based models for drug screening and toxicology testing. The ethical advantage of avoiding embryo use further enhances the appeal of iPSCs as a platform for translational research.

Despite these advantages, iPSC generation processes have relatively low efficiency and are associated with extended culture durations. Various gene delivery systems have been developed to support reprogramming, broadly categorized into viral and non-viral approaches. Among these, viral vectors, including but not limited to retroviral, lentiviral, adenoviral, and Sendai virus-based systems, tend to achieve higher efficiency due to their capacity to maintain high-level transgene expression during the critical early steps of reprogramming.

In this study, we aimed to utilize and improve these foundational methods via the use of integrase-deficient lentiviral viruses as vectors (IDLV-OSKs) with a protocol supported by the small molecule sodium butyrate, which acts as a histone deacetylase inhibitor.

The fact that IDLV-OSK vectors do not express *c-myc*, which is commonly associated with increased tumorigenic risk in target cells, also addresses and eliminates one of the key safety concerns in iPSC generation.

# 2. LITERATURE REVIEW

## 2.1 General Features and Classification of Stem Cells

Stem cells represent a distinct cell population in the human body. They exist in all stages of human life, from embryonic development to adulthood. They are distinguished by their self-renewal and wide differentiating abilities, supporting continuing human development while also pursuing regenerative and restorative functions (Evans and Kaufman 1981, Menon, Shailendra et al. 2016). Self-renewal defines the ability to go through multiple cell divisions while preserving an undifferentiated state, whereas the capacity to differentiate into different cell types is defined as potency (Figure 1).

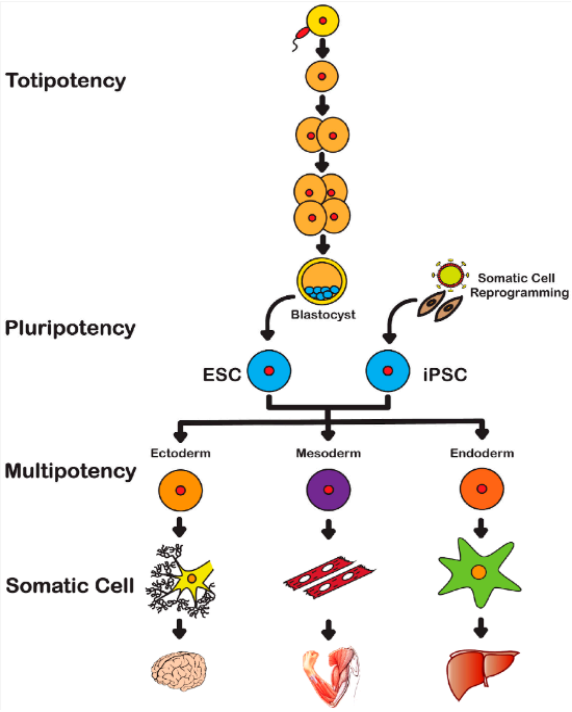


**Figure 2.1 Stem cell potencies.** The figure illustrates different levels of stem cell potencies. The developmental potency diminishes with each successive step (Menon, Shailendra et al. 2016).

Of the different potency types, totipotent cells have the highest differentiation capacity and can form all components of the embryo, as well as the extra-embryonic structures. As in a zygote, totipotent cells can divide and differentiate to establish the whole organism (Zakrzewski, Dobrzynski et al. 2019). Pluripotent stem cells, on the other

hand, cannot form the extraembryonic tissues, but have the potential to differentiate into cells of all germ layers. Examples are embryonic stem cells (ESCs), which originate from the inner cell mass of the blastocyst stage of the embryo, as well as the relatively recently defined induced pluripotent stem cells (iPSCs). Multipotent stem cells possess a more limited differentiation capacity compared to pluripotent stem cells (PSCs), but they can still give rise to specific cell types within a particular lineage, as the haematopoietic stem cells do. Interestingly, some multipotent cells have been claimed to differentiate into unrelated cell types, prompting debate about whether they should be classified as pluripotent (Ogawa, LaRue et al. 2013). One step further in specialization comes the oligopotent cells, whose differentiation potential is confined to a narrower range within their lineage. Oligopotent stem cells can differentiate into a few closely related cell types. For instance, a myeloid stem cell can produce certain white blood cells but not red blood cells. Unipotent stem cells, on the other hand, have the most limited differentiation potential, as they can produce only a single cell type, such as dermatocytes.

In terms of their origin, stem cells have been recently classified into five different groups (Liu, David et al. 2020):

(1) Embryonic Stem Cells (ESCs): ESCs are typically derived from early blastocyst masses, occurring around 4 to 7 days post-fertilization. In the normal process, they do not exist after 7 days, when the three embryonic tissue layers begin to form. Human ESCs (hESCs) represent a pioneering stem cell type used extensively in research, including ongoing clinical trials, yet are associated with ethical issues due to the destruction of human embryos.

(2) Very Small Embryonic-Like Stem Cells (VSELs): VSELs have been recently defined and included in the pluripotent stem cell types (Ratajczak, Ratajczak et al. 2019). The existence of VSELs has been corroborated by over 20 independent laboratories following initial identification in 2006 by Ratajczak et al., although some skepticism exists (Ratajczak, Zuba-Surma et al. 2006, Kucia, Halasa et al. 2007, Miyanishi, Mori et al. 2013, Suszynska, Zuba-Surma et al. 2014, Bhartiya, Shaikh et al. 2016, Ratajczak, Ratajczak et al. 2019). These cells, claimed to be found in adult tissues, exhibit characteristics of early developmental stem cells. They are termed VSELs based on their

small size, resembling cells in the inner cell mass (ICM) (Ratajczak, Ratajczak et al. 2017). In humans, they measure 5 to 7 μm, somewhat smaller than erythrocytes. These cells are described to express markers typical of ESCs, such as Oct-4A, SSEA, Rex1, and Nanog, as well as those related to the migrating primordial germ cells (PGCs), like Fragilis and Stella. Ratajczak and colleagues proposed a functional and developmental pattern for VSELs (Ratajczak, Ratajczak et al. 2019). This claimed pluripotent nature positions VSELs as potential alternatives to monopotent tissue-committed stem cells (TCSCs) in adult organisms.

(3) Nuclear Transfer Stem Cells (NTSCs): Originating from the somatic cell nuclear transfer (SCNT) technique pioneered in 1996, NTSCs represent another facet of stem cells that display a pluripotent nature. SCNT involves transferring a donor nucleus taken out of a differentiated somatic cell to an oocyte that has been enucleated, leading to genetic reprogramming and subsequent development into a blastocyst through mitotic divisions. This process results in a clone with nearly identical DNA to the nuclear donor, a technique applicable to therapeutic and reproductive cloning. Notably, the birth of Dolly the Sheep in July 1996 marked the first successful mammalian reproductive clone, showcasing the potential of SCNT (Campbell, McWhir et al. 1996, Garcia-Sancho 2015). Subsequent advancements have extended cloning techniques to various species, including the recent cloning of female macaque monkeys using fetal fibroblasts via SCNT, highlighting ongoing developments in this field (Liu, Cai et al. 2018).

(4) Adult Stem Cells (ASCs): Found in small numbers in most adult tissues, ASCs typically possess the ability to detect tissue damage and regenerate only the necessary cell types. Research has shown that the survival, dormancy, and activation of these cells depend on specific signals from their surrounding microenvironment (Rezza, Sennett et al. 2014). The initial discovery of these distinct cells generated considerable enthusiasm about their potential for clinical applications, though questions about their therapeutic effectiveness remain. ASCs are considered promising candidates for cell-based therapies, as the adult stem or progenitor cells residing within tissues offer a readily accessible source for many different therapies. It is known that externally applied small biological molecules may stimulate human ASCs to multiply and specialize into target

cell types in place. ASCs extracted from mature tissues can also be converted into induced pluripotent stem cells (iPSCs) (Kim, Zaehres et al. 2008).

(5) Reprogrammed Stem Cells: Since the first report by the Yamanaka team in 2006, direct reprogramming methods have developed significantly via the introduction of key transcriptional regulators as well as supporting molecules such as epigenetic regulators (Takahashi and Yamanaka 2006).

### 2.1.1 Background Information on Pluripotent Stem Cells

Pluripotent stem cells are divided into two main groups: embryonic stem cells (ESCs), whose primary source is the inner cell mass (ICM) of the blastocyst stage during embryonic development, and induced pluripotent stem cells (iPSCs), which are obtained by reprogramming of differentiated cells (Romito and Cobellis 2016). Following the fertilization process in human development, a blastocyst forms, which consists of the inner cell mass (ICM) and the trophectoderm (TE). As development proceeds, the TE differentiates to form essential support structures such as the placenta. While the TE specializes in providing structural and nutritional support, the ICM retains its undifferentiated and fully pluripotent state for a certain period, which has the potential to proliferate and develop into any human cell. After being derived in 1998 by James Thomson and his colleagues, human embryonic stem cells (hESCs) generated great excitement due to their ability for continuous growth and their potential to differentiate into cells of all three germ layers (Thomson, Itskovitz-Eldor et al. 1998). Induced pluripotent stem cells (iPSCs), on the other hand, are ESC-like cells that exhibit morphology similar to hESCs, express key ESC markers, and can form teratomas in immunosuppressed mice.

Generation of iPSCs was first reported while studies on embryonic stem cells (ESCs) continued. It was demonstrated in 2006 by Shinya Yamanaka and colleagues, through their work on mouse fibroblasts and later on human fibroblasts, that a differentiated cell could be reprogrammed into pluripotency (Takahashi and Yamanaka 2006, Takahashi, Tanabe et al. 2007). This groundbreaking work of Yamanaka and Sir John Gurdon, which earned them the 2012 Nobel Prize in Physiology or Medicine, was significant for

directly demonstrating the potential for induction of pluripotency in differentiated cells. In their study, Yamanaka and colleagues introduced 24 different factors believed to potentially induce reprogramming into fibroblasts using retroviral vectors in various combinations and quantities. From these, they identified four key transcription factors essential for inducing pluripotency: Oct4, Sox2, Klf4, and c-Myc. These factors, collectively known as the OSKM factors, play central roles in reprogramming, although c-Myc in particular has been raising concerns due to its very high carcinogenic potential.

iPSCs, which can be generated by reprogramming a patient's own differentiated cells, are considered more promising than ESCs in fields such as regenerative medicine, transplantation, disease modeling, and drug screening, particularly because they avoid many of the drawbacks associated with ESC-based approaches, such as the need for immunosuppression, difficulties in obtaining starting materials, and ethical concerns. Multiple studies have highlighted similarities between iPSCs and hESCs, including self-renewal and differentiation abilities. Thus, iPSC technologies provide a potentially limitless cell source for therapeutic and research purposes, offering promising solutions to ethical concerns related to hESCs.

## 2.2 Transcriptional Regulators Used in Original iPS Studies

### 2.2.1 Oct4 (Octamer-binding transcription factor 3/4)

The transcriptional regulator Oct4 (Pou5f1) is a POU family member. It is expressed in pluripotent stem cells (PSCs), as a widely recognized key genetic regulator, often referred to as the "master switch", responsible for initiating and maintaining both totipotency and pluripotency throughout mammalian development (Pesce, Marin Gomez et al. 1999). Oct4 is believed to occupy a central, upstream position in the regulatory network that controls pluripotency. Notably, the introduction of Oct4 in combination with specific chemical compounds has been shown to successfully reprogram somatic cells into induced pluripotency (Zhu, Li et al. 2010). Within Yamanaka's four-factor reprogramming model, Oct4 was considered indispensable, despite the existence of structurally similar homologs such as Oct1 and Oct6, which were unable to substitute its function (Jerabek, Merino et al. 2014).

### 2.2.2 Sox2 (SRY-related HMG box-2)

Sox2 is a member of the sex-determining region Y (SRY)-related high-mobility group (HMG) box (Sox) family, expressed during preimplantation in mammals in a tightly regulated spatiotemporal manner, contributing to key developmental processes (Singh, Marisetty et al. 2015). Sox2 serves as a reliable marker of pluripotency and maintenance of the self-renewal and undifferentiated state of ESCs. In fact, a reduction in Sox2 expression has been shown to disrupt pluripotency in human ESCs, as well as to impair self-renewal in mouse ESCs (Fong, Hohenstein et al. 2008, Singh, Marisetty et al. 2015, Wu, Zhang et al. 2015).

### 2.2.3 Klf-4 (Kruppel-like factor 4)

Klf4 has pivotal parts in governing apoptosis, proliferation and differentiation, preservation of tissue homeostasis, somatic cell reprogramming, and embryonic development (Shields, Christy et al. 1996). Functionally, it is a dual-purpose transcription factor, capable of either activating or repressing transcription (Evans and Liu 2008). Its association spans both tumor suppression and oncogenesis, contingent upon the specific cancer type under consideration. The contentious nature of its oncogenicity has rendered Klf4 a topic of debate in iPSC generation, with several studies suggesting that Klf4 may not be indispensable for the reprogramming process. Consequently, the substitution of Klf4 emerges as a pivotal stride toward the utilization of iPSCs as a potential therapeutic approach.

### 2.2.4 c-Myc (cellular Myc)

c-Myc protein arises from the proto-oncogene *c-Myc*, and is a significant actor in cellular processes such as growth, differentiation, proliferation, adhesion, senescence, apoptosis, as well as reprogramming (Boxer and Dang 2001). Notably, mutations within the *c-Myc* gene have been implicated as significant across several cancer types, such as colon, lung, prostate, and pancreatic cancers (Miller, Thomas et al. 2012). The potential oncogenic properties of this protein also make it a contentious regulator in iPSC generation (Guo, Li et al. 2009). Use of *c-myc* in reprogramming is one of the most

controversial issues, thus new methodologies that do not involve this gene in particular have gained importance.

### 2.3 Techniques Used in Reprogramming

Various viral and nonviral methods are utilized in the generation of iPSCs, each with distinct advantages and drawbacks concerning efficiency and safety (Figure 2.2) (Izady, Saltanatpour et al. 2022). Viral methodologies, in general, excel in their ability to express encoded genes at high rates over extended periods, a notable advantage compared to nonviral methods. Use of nonviral vectors, on the other hand, has its advantages, such as low toxicity, potential to transfer large cargo, ease of production, and regulatory simplicity. A major downside of some viral approaches is their integration into the host cell genome. Although this feature has the potential to provide long-term gene expression, it also raises safety concerns.

Integrating vectors have historically been favored for their efficacy in introducing reprogramming factors into cells and are still commonly used. In fact, integrating retroviruses have been used in the first study where fibroblasts were reprogrammed to pluripotency (Takahashi, Ichisaka et al. 2006). Nevertheless, the permanent and random integration of exogenous genes by integrating vectors carries inherent oncogenic risks, limiting their suitability for therapeutic applications. In response, non-integrating reprogramming strategies have emerged as alternatives to mitigate these concerns. These strategies include the utilization of Sendai viruses, plasmids, modified RNAs, or small molecules etc., all designed to induce reprogramming without genomic integration (Okita, Nakagawa et al. 2008, Stadtfeld, Nagaya et al. 2008, Kim, Kim et al. 2009, Jia, Wilson et al. 2010, Montserrat, Garreta et al. 2011). Of the viral vectors used in reprogramming studies, lentiviral vectors are highly effective gene delivery tools that are widely used today in both basic and clinical research (Escors and Breckpot 2010).

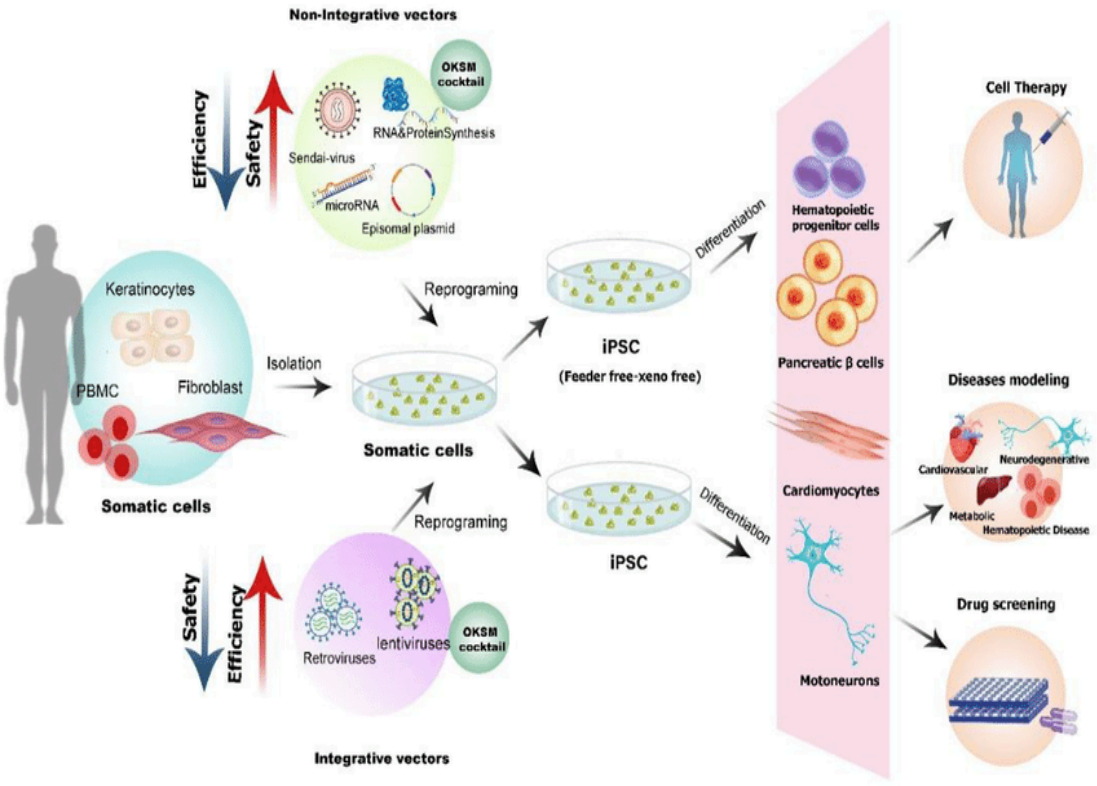


**Figure 2.2 iPSC generation and related applications.** Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) using a variety of methods, which may then be used for differentiation into various human cells (Izady, Saltanatpour et al. 2022).

## 2.4 Lentiviral Vectors as Reprogramming Tools

Lentiviral vectors (LVs) are efficient gene transfer vehicles with the ability to transduce both dividing and non-dividing cells, coupled with a highly efficient gene transfer capability, which underpins their widespread preference in scientific investigations (Escors and Breckpot 2010). Lentiviruses harbor a genomic composition comprising two RNA fragments and exhibit an enveloped structure. Present-day production methodologies leverage advanced and secure production kits, aiming to yield LVs devoid of replicative capacity while retaining robust gene delivery potential to target cells. Production typically involves the co-transfection of a transfer plasmid containing the gene of interest, packaging plasmids, and a plasmid encoding the envelope protein into 293T cells derived from embryonic kidney cells (Figure 2.3) (Olgun, Tasyurek et al. 2018).

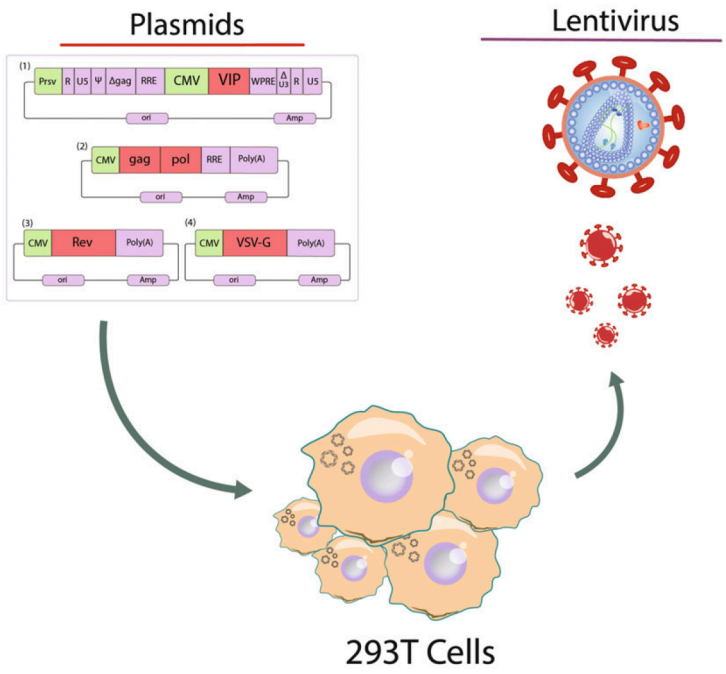


**Figure 2.3** Lentiviral vector production. The procedure includes the introduction of the transfer vector along with the packaging and envelope-coding components into 293T cells. Over the next few days, the cells begin producing the vectors. Once the process is complete, the vectors are collected from the cell culture supernatant (Olgun, Tasyurek et al. 2018).

LVs are categorized into different "generations" based on the packaging plasmid structure employed during production. Notably, the "third generation" LVs, with which the HFF-1 cells were transduced in our study, involve the provision of viral regulatory genes *rev* and *tat* via a separate fourth plasmid, distinct from the packaging plasmid. Furthermore, pseudotyping, achieved by substituting the *env* gene responsible for the viral envelope in HIV with the gene coding for the Vesicular Stomatitis Virus Envelope Protein (VSV-G), enhances viral tropism while reducing the risk of replication-competent viruses (RCL) due to the distinct envelope protein origin. Additionally, these vectors are crafted as self-inactivating (SIN) transfer vectors, eliminating the enhancer/promoter region in the 3'LTR region of the vector genome to preclude RCL formation and promoter interference. Consequently, third-generation LVs emerge as the highly recommended and favored choice in contemporary gene transfer studies, distinguished by their distinct envelope protein, utilization of at least four distinct plasmids segregating vector and packaging functionalities, elimination of select structural genes, and being produced as SIN vectors (Olgun, Tasyurek et al. 2018).

A noteworthy advancement in non-integrating viral vectors is the development of integrase-deficient lentiviruses (IDLVs) (Yew, Gurumoorthy et al. 2022). Unlike traditional lentiviruses, IDLVs are engineered to disable their integrase enzyme by use of a gag/pol plasmid in the production process that bears a mutation in the integrase gene. Thus, permanent integration into the host cell's genome is prevented. This strategic modification significantly enhances safety by minimizing genetic alterations, making IDLVs an attractive option for iPSC generation. IDLVs have become pivotal tools in modern molecular and cell biology research, offering a controlled and safe means of introducing and studying genes within target cells, thereby contributing to advancements in regenerative medicine and therapeutic development.

**2.5 Factors Increasing Reprogramming Efficiency**

Despite the numerous reprogramming strategies that have been devised, the process of reprogramming still remains inefficient. In response to this inefficiency, several research groups have effectively employed small molecules such as valproic acid, sodium butyrate, PD0325901, and others to increase the efficiency of reprogramming (Table 2.1). The small molecules known to enhance somatic cell reprogramming have a variety of different mechanisms of action, including histone deacetylase (HDAC) inhibitors, lysine methyltransferase inhibitors, cell signaling modulators, TGFβ inhibitors, GSK3 inhibitors, and MEK inhibitors (Federation, Bradner et al. 2014). Sodium butyrate, which we used in our study as a supporting small molecule, is a non-specific HDAC inhibitor.

Besides these molecules being used as supporting molecules in reprogramming, successful reprogramming has also been achieved using only small-molecule compounds (Hou, Li et al. 2013). Most small molecules known to enhance somatic cell reprogramming can effectively replace three of the four core transcription factors: Sox2, Klf4, and c-Myc. However, identifying compounds that can substitute directly for Oct4 has proven particularly challenging. Uncovering the mechanisms by which these molecules function is now a major area of interest. Further investigation into the molecular pathways involved in reprogramming, along with the development of

pharmacological strategies using current chemical biology tools, will likely reveal new targets and roles for small molecules.

**Table 2.1** A variety of compounds used to increase reprogramming efficiency (Federation, Bradner et al. 2014).

| Compound Name | Mechanism of Action |
|---|---|
| **Valproic Acid** | Histone deacetylase inhibition |
| **Sodium Butyrate** | Histone deacetylase inhibition |
| **PD0325901** | MEK inhibition |
| **A-83-01** | TGFβ-inhibition |
| **SB43152** | TGFβ-inhibition |
| **Vitamin C** | Enhances epigenetic modifiers, decreases p53 levels during reprogramming |
| **Thiazovivin** | ROCK inhibitor, promotes cell survival |

The rapidly evolving subject of cellular reprogramming has recently introduced non-integrative techniques for the generation of iPSCs, and the use of supporting small molecules for enhanced efficiency. In our study, we used IDLV-OSK vectors and sodium butyrate as a supporting molecule to effectively and safely reprogram HFF-1 cells into pluripotency, with an optimised protocol defined in the methods section (Figure 3.1).

# 3. MATERIALS AND METHODS

## 3.1 HFF-1 and BJ Fibroblast Cultures

HFF-1 (ATCC SCRC-1041) is a fibroblast cell line that was isolated from normal human foreskin pooled from two individuals. BJ cells also originate from human foreskin fibroblasts (CRL-2522).

**Reagents Used:**

- **Trypsin-EDTA**
- **Phosphate-Buffered Saline (PBS):**
  - 8 g NaCl
  - 0.2 g KCl
  - 1.44 g $Na_2HPO_4$
  - 0.24 g $KH_2PO_4$

All chemicals were weighed and dissolved in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl, and then the total volume was brought up to 1 L.

- **Dulbecco's Modified Eagle's Medium (DMEM):**
  - DMEM powder medium (Sigma-Aldrich, D5648)
  - 10% Fetal Bovine Serum (FBS) (Biochrom, 50115)
  - 3.7 g/L Sodium Bicarbonate (Sigma, S5761)
  - 1% Sodium Pyruvate
  - 1% Penicillin-Streptomycin (Biological Industries)

To prepare the medium, DMEM powder was solubilized in 800 ml of distilled water, and sodium bicarbonate (3.7 g) was added. The pH of the medium was adjusted to 7.4 with HCl. Final amount was then made 1 L. In a laminar flow cabinet, sodium pyruvate (10 ml), penicillin-streptomycin (10 ml), and FBS (100 ml) were added to the medium. It was then filtered via a 0.22 μm filter and stored at +4°C.

- **Dimethyl Sulfoxide (DMSO) (Sigma, D2650)**

Cells were thawed in DMEM medium (with 10% FBS, 1% penicillin-streptomycin, 4.5 g/L D-glucose, 4 mM L-glutamine, sodium pyruvate). Culturing was performed in 10 cm dishes (Sarstedt 83.3902) in DMEM medium at 37°C in 5% $CO_2$. The cultured cells, passaged using Trypsin-EDTA, were frozen in medium with 10% DMSO and stored at -80°C followed by transfer to a liquid nitrogen tank.

### 3.2 Production of the Integrase-Deficient Lentiviral Vectors

Integrase-deficient lentiviral vectors (LVs) encoding the Oct4, Sox2, and Klf4 reprogramming factors (IDLV-OSK) were previously produced in our lab. Human embryonic kidney (HEK) 293 cells and their derivatives are commonly used in the production of third-generation LVs through the co-transfection of four separate plasmids. The most widely used variant for this purpose is the simian virus 40 (SV40) large T antigen-expressing HEK293T cell line, which is known to be more efficient for vector production due to enhanced cell growth and transfection efficiency, allowing for high-titer production.

To generate the IDLV-OSK vectors, the pKP332 Lenti-OSK1 transgene plasmid (Addgene) was used. This plasmid contains a 9692 bp pDL171 viral backbone and a 3589 bp hOct4-2A-hSox2-2A-hKlf4 insert, and was propagated in Stbl3 chemically competent *E. coli*. Additionally, to produce the intact virus, the following plasmids were used: the envelope plasmid pMD2.G (Addgene), which encodes the VSV-G viral envelope protein; the integrase-deficient packaging plasmid psPAX2-D64V (Addgene), which contains *gag* and *pol* but includes a point mutation in the integrase gene; and the pRSV-Rev (Addgene) plasmid, which encodes the rev protein. Briefly, the plasmids were transfected into the HEK293T producer cells using chloroquine, followed by incubation in roller bottles and ultracentrifugation (Olgun, Tasyurek et al. 2018).

### 3.3 iPSC Derivation with Integrase-Deficient Lentiviral Vectors

**Reagents Used:**

- **Polybrene**
- **TeSR™-E7 medium (E7) (Stem Cell Technologies, 5914)**
- **TeSR™-E8 medium (E8) (Stem Cell Technologies, 5990)**
- **mTeSR™Plus medium (Stem Cell Technologies, 05825)**
- **Sodium butyrate**
- **Vitronectin XF (Stem Cell Technologies, 07180)**
- **ROCK inhibitor / Y-27632 (Sigma, SCM075)**
- **ReLeSR (Stem Cell Technologies, 5873)**

For the IDLV-OSK-mediated iPSC generation protocol, HFF-1 cells were transduced at day 0 with the IDLV-OSK vectors (Figure 3.1). For this, each well was seeded with $3x10^5$ cells and maintained under standard conditions (37°C, 5% $CO_2$) for 24 hours to allow the cells to settle. The next day, when cells had approximately 70% cell confluency, transduction with IDLV-OSK vectors was performed in DMEM with 1 µg/mL polybrene. Polybrene increases transduction efficiency by increasing the adsorption of the virus on target cell membranes in a receptor-independent manner, when used in non-toxic concentrations for the corresponding cell (Davis, Morgan et al. 2002). The following day, medium was replaced with fresh DMEM containing 0.2 mM sodium butyrate.

The cells were trypsinized on day 9 after transduction and put into non-tissue culture-treated 6-well culture plates laminated with vitronectin. Vitronectin is a defined, xeno-free cell culture matrix that supports the growth of human pluripotent stem cells. TeSR-E7 culture medium was used at this point, renewed with fresh media every day for 3 days, after which it was changed to TeSR-E8 medium, also with daily refreshment for 3 days. On day 16, the culture was maintained in mTeSR medium, which is suitable for renewal every 2 days. These media are specifically formulated to meet the requirements of the reprogrammed cells. TeSR-E7 promotes the continued development of the primary colonies, while TeSR-E8 facilitates their progression into secondary or mature colonies. Finally, mTeSR-Plus, in combination with appropriate inhibitors, maintains

and supports the stability of these mature colonies for the subsequent stages of the experimental process. Colonies were passaged using the non-enzymatic ReLeSR agent, which allows for the selective removal of undifferentiated aggregates from the culture medium by taking advantage of the principle that differentiated cells adhere more firmly to the culture surface.

Rock Inhibitor (Y-27632) was also used in our protocol following the growth of colonies. Y-27632 is a selective inhibitor against the Rho-associated coiled-coil containing protein kinase (ROCK). It performs this action via competing with ATP for the catalytic region (Davies, Reddy et al. 2000). It is included in pluripotent stem cell cultures primarily because it helps protect pluripotent stem cells from dissociation-induced apoptosis (anoikis) when in a single-cell state, and because it preserves the viability of frozen human pluripotent stem cells after thawing (Watanabe, Ueno et al. 2007, Krawetz, Li et al. 2009).

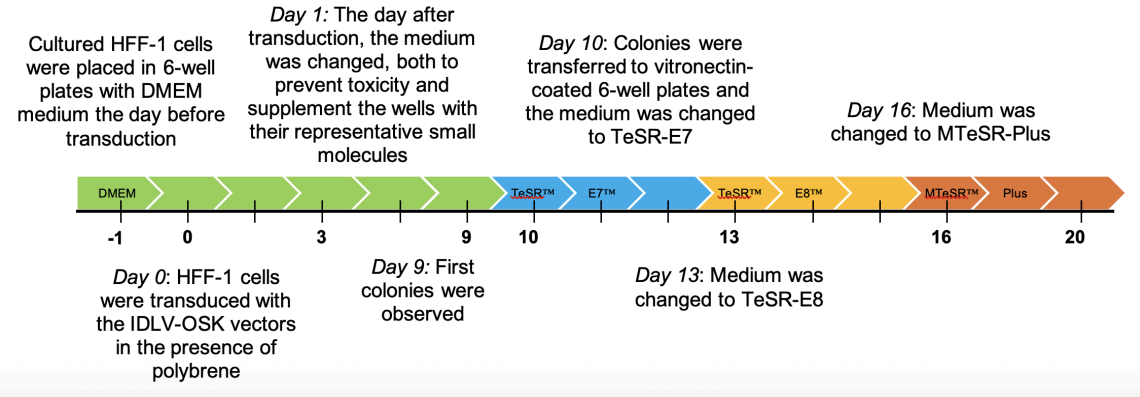


**Figure 3.1** Chronological representation of the reprogramming timeline. Events are ordered chronologically from left to right.

### 3.4 Characterization of the Obtained iPSCs

#### 3.4.1 Morphological Characterization

The obtained iPSCs were evaluated for their morphology, according to the typical morphological characteristics of pluripotent stem cells. A good example of an iPSC colony cultured on V-XF was defined as having round, defined edges, being compact, composed of small round cells, and having a semi-transparent appearance.

#### 3.4.2 Characterization by Surface Pluripotency Marker Expressions

**Live Staining for Alkaline Phosphatase (AP)**

**Reagents:**

- Alkaline Phosphatase Live Staining Kit (Thermo Fisher A14353)
- DMEM/F12 Medium (Stem Cell Technologies)

In live-cell staining, the molecule used diffuses out of the cells after fluorescence emission, so no toxic effects are observed in the cells, and the culture can be continued with the same cells.

For the procedure, the culture medium was aspirated, and 2-3 min rinsing was performed twice with DMEM/F12. The medium was removed, and 1XAP live staining solution (prepared by mixing 3 µL of 500X AP live stain stock solution with 1.5 µL of DMEM/F12) was added. The wells were incubated for 20-30 min at RT. The 1XAP live stain solution was aspirated, and the wells were gently rinsed with DMEM/F12 for 5 minutes, taking care not to disturb the cells. Following visualization, the DMEM/F12 medium was replaced with mTeSRTM Plus medium, and the culture was maintained.

**Immunocytochemical staining for Tra-1-60 and Tra-1-81**

**Solutions and Antibodies Used:**

- **Fixative Solution:**

4% Paraformaldehyde was made ready by dissolving 2 g PFA in 50 ml PBS, and adding 200 µl NaOH.

- **Permeabilization Solution (PBS-T):**

Prepared by 20 µl Tween 20 added to 20 ml PBS.

- **Blocking Solution (1% BSA):**

500 mg BSA was dissolved in 50 ml PBS-T.

- **Primary Antibodies:**
    - Tra-1-60: AB-16288-100, 1/300 dilution
    - Tra-1-81: AB-16289-100, 1/300 dilution
- **Secondary Antibody:**
    - ThermoFisher A32727, Goat-Anti Rabbit, 1/500 dilution

**Procedure:** Dissociated cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature (RT). Following fixation, the cells were permeabilized using PBS containing 0.1% Triton X-100 (PBS-T) for 15 minutes at RT. After permeabilization, the solution was removed, and the wells were washed with PBS supplemented with 0.05% Tween-20 (PBS-T:20). To block non-specific binding, a 1% bovine serum albumin (BSA) solution was applied, and the samples were incubated for 1 hour at RT. Meanwhile, primary antibodies were diluted at 1:300 in 1% BSA. A volume of 500 µL of the diluted antibody solution was added to each well, which were then covered with aluminum foil and incubated overnight (O/N) at 4°C. The next day, primary antibodies were removed, and the wells were washed three times with PBS-T:20 for 5 minutes each. Secondary antibodies were diluted at 1:500, and 500 µL of the diluted solution was added to each well, followed by incubation at RT for 1 hour in the dark. Subsequently, the wells were washed three times with PBS for 10 min each. Nuclear staining was performed using 2 drops of DAPI in 500 µL PBS per well, incubated for 15 minutes at RT. Finally, the wells were washed with 1 mL PBS, and 500 µL of fresh PBS was added to each well for imaging of the stained cells.

### 3.4.3 Characterization by Nuclear Pluripotency Marker Expressions

**Solutions and Antibodies Used:**

- **Fixative Solution:**

4% Paraformaldehyde was made ready by dissolving 2 g PFA in 50 ml PBS, and adding 200 µl NaOH.

- **Permeabilization Solution (PBS-T):**

Prepared by 20 µl Tween 20 added to 20 ml PBS.

- **Blocking Solution (1% BSA):**

500 mg BSA was dissolved in 50 ml PBS-T.

- **Primary Antibodies:**
    - Oct4: Bioss bs-1111R, 1/300 dilution
    - Klf4: Bioss BSM-52850R, 1/300 dilution
    - Nanog: Bioss bs-0829R, 1/300 dilution
    - c-Myc: Bioss bs-4963R, 1/300 dilution
- **Secondary Antibody:**
    - Invitrogen A32731, Goat-Anti Rabbit, 1/500 dilution

The culture medium was removed, and a PBS wash was performed. Following this step, 1 ml of fixative solution was added to each well, and cells were kept at room temperature (RT) for 20 min. 3xPBS wash was performed following the removal of the fixative solution, and the permeabilization solution was added. Following 15 minutes of incubation at RT, the permeabilization solution was removed, and a 3xPBS wash was performed. The cells were incubated at RT for 1 h following the addition of the blocking solution. After washing the cells with PBS-T, primary antibodies were added to each well at the appropriate dilution in 500 µl aliquots. The culture dish was then wrapped in aluminum foil and incubated O/N at 4°C. The primary antibody solution was removed the next day, and a 3xPBS wash for 5 min was performed. Subsequently, the cells were kept in RT for 1 h with the diluted secondary antibody. This was continued by 3xPBS washes, each lasting 10 minutes. Two drops of DAPI and 500 µl of PBS/well were added, and incubation at RT was performed for 15 min. A final 1 ml PBS wash was done, and 500 µl of PBS was put into each well, and imaging was performed.

# 4. RESULTS

## 4.1 Functional Testing of the IDLV-OSK Vectors was Performed

Integrase-deficient lentiviral vectors encoding the Yamanaka factors (IDLV-OSK) were previously produced in our lab. The protocol briefly involved transfection of the transgene plasmid and the psPAX2-D64V (gag and pol), pRSV/Rev (rev), and pMD2.G (VSV-G envelope protein) plasmids into 293T producer cell lines with chloroquine, followed by incubation in roller bottles and ultracentrifugation (Olgun, Tasyurek et al. 2018). Functional testing was performed in BJ fibroblast cell lines, which are also human foreskin fibroblasts like HFF-1 cells (Figure 4.1). Cells were efficiently transduced with the vector, as evidenced by the efficient expression of one of the transgenes encoded by the vector, *Klf4*, in nearly 100% of the cultured cells.

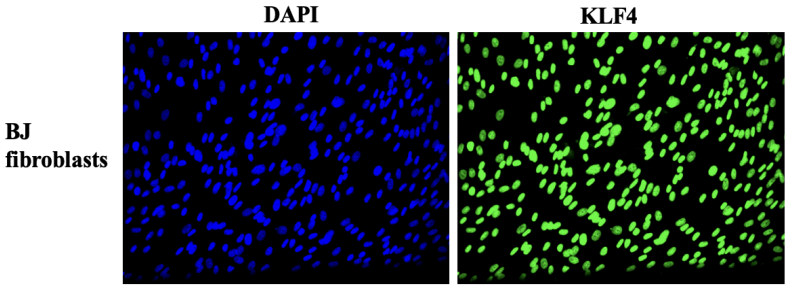


**Figure 4.1** Confirmation of IDLV-OSK vector functionality. BJ fibroblast cells were transduced with IDLV-OSK vectors and immunocytochemically stained for Klf4 to test the transduction efficiency.

## 4.2 Induced Pluripotent Stem Cells were Generated using IDLV-OSK Vectors

We used the integrase-deficient IDLV-OSK vectors along with sodium butyrate as a supporting small molecule to produce induced pluripotent stem cells (iPSCs) from HFF-1 human fibroblasts, maintained the colonies in culture for growth via passages, and characterized the colonies in terms of pluripotency. As outlined in our methodology (Figure 3.1), the process began with the transfer of the HFF-1 cells cultured in petri dishes to 6-well plates. Each well was seeded with $3x10^5$ cells and maintained under

standard conditions (37°C, 5% $CO_2$) for 24 hours to allow the cells to settle prior to the transduction procedure. The next day, cells were transduced with IDLV-OSK vectors in DMEM with 1 µg/mL polybrene, and the medium was replaced the following day with fresh DMEM containing sodium butyrate. Transfer of the cells to vitronectin-coated plates was performed at day 9 of culturing, when the first colonies started to appear (Figure 4.2). Colonies were sequentially cultured in TeSR-E7, TeSR-E8, and mTeSR-Plus media, which are specifically formulated to support the distinct developmental phases of the colonies during maturation.

Morphological changes throughout the reprogramming process were prominent. The starting HFF-1 fibroblast cells displayed a typical fibroblastic appearance, as long, thin, spindle-shaped cells spreading out across the surface and growing in a monolayer (Figure 4.2). At the early reprogramming stage (days 2-5), cells started to lose their fibroblastic shapes and became shorter, and started to gather up at specific loci where soon the colonies were to appear. Between days 5-10, usually defined as the mesenchymal-to-epithelial transition (MET) stage, cells at certain loci started to gain a more epithelial morphology with rounded/cuboidal shapes and tighter packing.

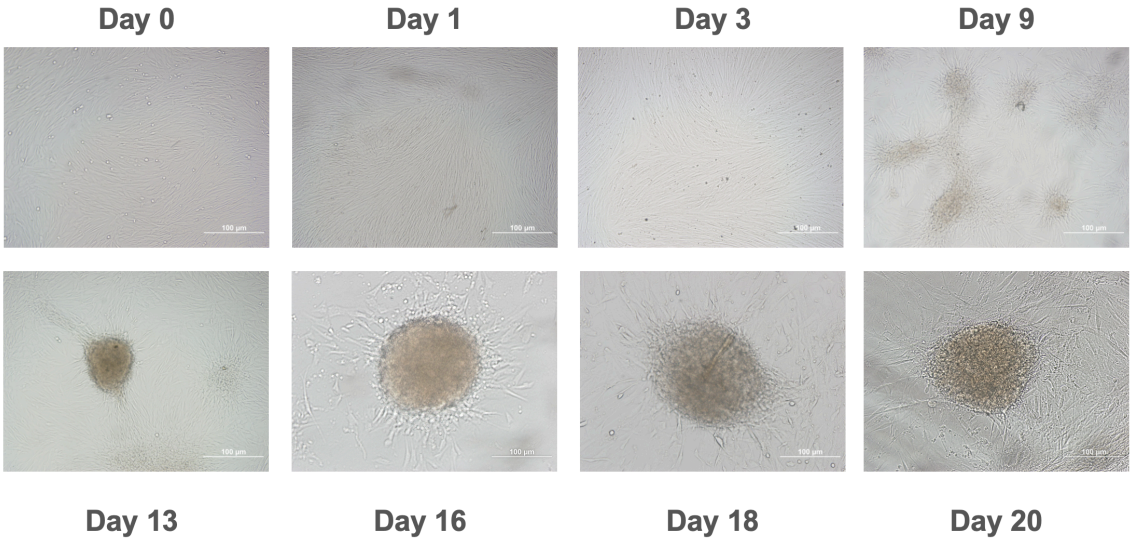


**Figure 4.2** Sequential images of HFF-1 cells undergoing reprogramming. This figure illustrates different stages of the HFF-1 cell reprogramming process. Day 0 shows the pre-transduction state of the fibroblasts cultured in 6-well plates. On day 1, the cells were monitored following medium renewal to remove excess vectors and polybrene. By day 3, subtle deviations from typical fibroblast morphology became observable. On day 9, newly formed primary colonies were passaged onto vitronectin-coated plates, and the medium

was changed to TeSR-E7, initiating the vitronectin stage. On day 13, a second passage onto fresh vitronectin-coated plates was performed, accompanied by a medium change to TeSR-E8. By day 20, colonies were passaged again and maintained in mTeSR-Plus to preserve their matured state. The pre-vitronectin stage spans the first 8 days, during which cells were treated with sodium butyrate.

We also wanted to compare the efficiency of reprogramming between protocols supported or unsupported with sodium butyrate. For this, cultures without any supporting small molecule use and those that were supported with sodium butyrate were established and followed until the appearance of the first colonies. With $3x10^5$ cells seeded in the beginning, reprogramming cultures with no supporting small molecules produced 102 colonies, whereas those supported with 0.2 mM sodium butyrate gave out 125 colonies. This proved the increase in the efficiency of reprogramming of HFF-1 cells via IDLV-OSK vectors upon use of the histone deacetylase inhibitor sodium butyrate.

## 4.3 Characterization of the Obtained iPSC Colonies

### 4.3.1 Morphological Characterization

The obtained iPSC colonies were morphologically characterized as round, compact masses with defined borders that looked flat and tightly packed (Figure 4.2). They displayed adherent growth on the vitronectin feeder-free matrix. Cells at the periphery of the growing colonies usually appeared more elongated, a usual sign of differentiation onset at the borders, as frequently reported in iPSC cultures (Castro-Vinuelas, Sanjurjo-Rodriguez et al. 2021, Cheng, Xu et al. 2023). iPSC colonies are passaged many times to eliminate these spontaneously differentiated cells to eventually obtain pure colonies, before progressing onto further experimental procedures. Growing iPSC colonies in culture presented a heterogeneous appearance, in different sizes and shapes (Figures 4.3 and 4.4).

### 4.3.2 Characterization with Cell Surface Pluripotency Markers

We characterized the obtained iPSCs via confirmation of human surface pluripotency marker expressions (Figure 4.3). For this, live immunocytochemical fluorescent staining was performed for Alkaline Phosphatase (AP), an early pluripotency marker, and the Tra-1-60 and Tra-1-81 markers, which are characteristic of the human iPSCs. The obtained colonies stained positive for all three markers.

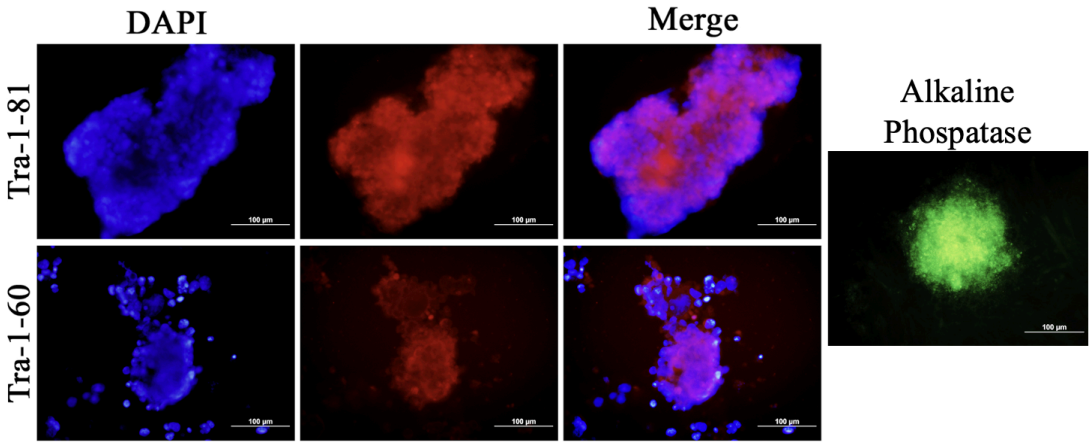


**Figure 4.3** Demonstration of surface pluripotency marker expressions in iPSCs. The figure depicts immunofluorescence images of iPSCs generated through reprogramming, showing expression of surface markers TRA-1-60, TRA-1-81, and alkaline phosphatase. DAPI: 4',6-diamidino-2-phenylindole, a nuclear stain used for labeling cell nuclei.

### 4.3.3 Characterization with Nuclear Pluripotency Markers

To characterize the pluripotency properties of the iPSCs obtained in our study, immunocytochemical/immunofluorescent staining was performed for the nuclear pluripotency markers Oct4, Klf4, c-Myc, and Nanog. iPSC colonies expressed all four markers (Figure 4.4).

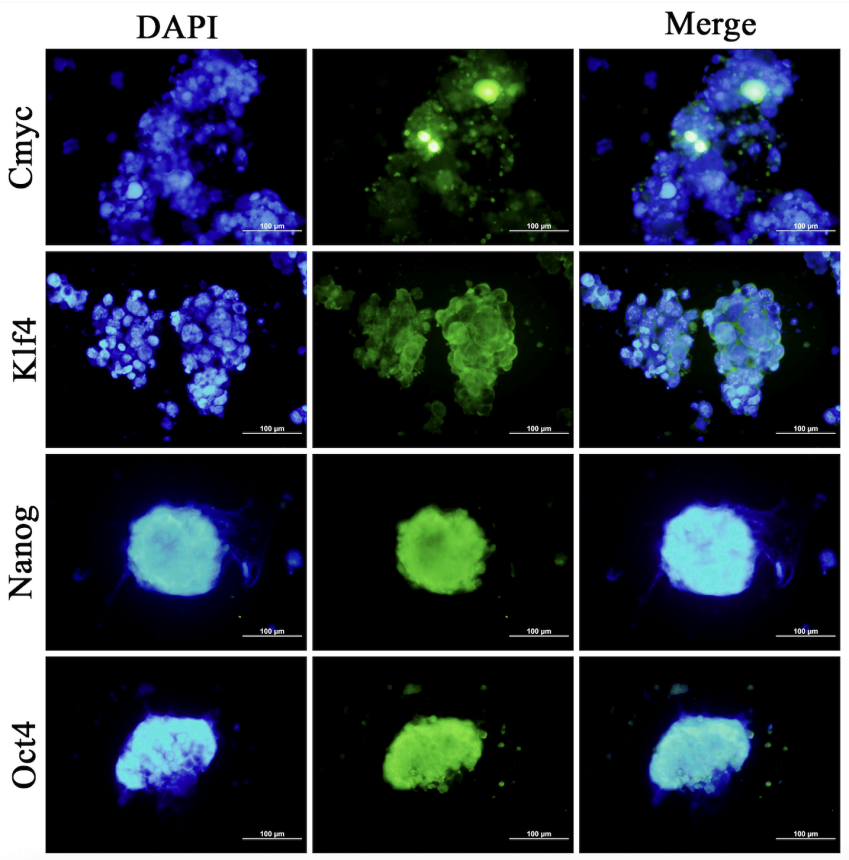


**Figure 4.4** Characterization images of iPSCs using nuclear pluripotency markers.
The figure depicts the immunocytochemical staining images of the nuclear pluripotency markers Oct4, Nanog, Klf4, and c-Myc, in iPSC colonies. DAPI: 4',6-diamidino-2-phenylindole, a nuclear stain used for labeling cell nuclei.

# 5. DISCUSSION

Stem cells are exclusive cells allowing human development as well as tissue regeneration and repair. In 1998, James Thomson derived the first human embryonic stem cells (hESCs) from the inner cell mass of the human blastocyst (Thomson, Itskovitz-Eldor et al. 1998). hESCs have mediated many critical discoveries, particularly in elucidating the mechanisms of human embryonic development. The groundbreaking discovery by Yamanaka and colleagues in 2006 followed this significant progression, reporting reprogramming of differentiated mammalian cells into pluripotency via forced expression of critical transcriptional regulators to obtain the induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006).

While hESCs have faced many challenges, especially in obtaining the starting material and the related ethical concerns, iPSCs attracted attention in terms of combining the benefits of adult stem cells with the unique features of embryonic stem cells, as well as the potential to be generated from a person's own cells (Martin 2017). These distinct cells are suitable for use in various fields, such as *in vitro* disease modeling for mechanism studies and drug screening, and treatment-based approaches for human diseases (Ovics, Regev et al. 2020).

Although various approaches involving iPSCs are currently being evaluated in clinical trials for many different human conditions, both *in vitro* and *in vivo* approaches are known to require refinement (Yamanaka 2020). The reprogramming potential of different starting cell types varies, as well as the efficiency and safety of the type of reprogramming methodology utilized. Potential risks, including tumorigenicity, possible immune rejection, and cellular heterogeneity, remain critical considerations, especially in therapeutic contexts. Thus, new detailed studies involving different cell types and novel methodological approaches are critical for the development of the field.

In our research, we reprogrammed HFF-1 human fibroblast cells into iPSCs via integrase-deficient lentiviral vectors (IDLV-OSKs) encoding the reprogramming factors Oct4, Sox2, and Klf4 (Figures 4.2, 4.3, and 4.4). Recent findings highlight that the

technique employed to generate iPSCs plays a more critical role than previously recognized (Kaneko and Yamanaka 2013). The immune tolerance or rejection observed with the use of iPSCs and their differentiated derivatives appears to be closely associated with the gene delivery method used during reprogramming. Therefore, investigation of the effect of different gene delivery approaches on various starting cell types and reprogramming processes remains a crucial focus of ongoing research. Retroviral integrase is an enzyme produced by retroviruses, such as HIV, that facilitates the integration of viral genetic material into the genome of the host cell. As is well known, this gives the viral vectors derived from retroviruses a unique characteristic: if the vector is constructed with a functional integrase, the transduced DNA is randomly integrated into the target genome. While this integration mechanism can be beneficial in some experimental and gene therapy contexts, especially in terms of long-term gene expression, it carries considerable risks due to the random nature of insertion, which may cause unwanted consequences such as disruption of vital genes or activation of oncogenes. Thereby, the use of these vectors in clinical settings may be limited for safety purposes. An effective solution to this problem appears to be the use of integrase-deficient lentiviral vectors as gene transfer tools in reprogramming studies, maintaining the effectiveness of transduction and gene expression while putting a much safer vector into the scene. We previously produced integrase-deficient lentiviral vectors encoding the OSK Yamanaka factors (IDLV-OSK) in our lab, and tested the transduction efficiency to see that these vectors very efficiently transduce the target BJ human fibroblast cells (Figure 4.1). This cell line originates from human foreskin fibroblast cells, just as the HFF-1 cells, and is routinely used in our lab in many processes, including testing lentiviral functionality. Testing of the IDLV-OSK vectors in new experimental settings and different starting cells will be important.

We added the histone deacetylase inhibitor sodium butyrate in our protocol as a supporting small molecule to increase reprogramming efficiency, which increased the number of colonies obtained from 102 to 125, compared to the protocol that did not involve sodium butyrate. Various options can be used in reprogramming studies to enhance efficiency, which are still associated with low efficiency. These include small molecules acting in epigenetic regulation, such as histone deacetylase inhibitors, DNA

methyltransferase inhibitors, and histone methyltransferase inhibitors (Sun, Fu et al. 2011, Malik and Rao 2013). We chose to apply the histone deacetylase (HDAC) inhibitor sodium butyrate in our protocol, and tried the 0.2 mM concentration to be applied in combination with the IDLV-OSK vectors for increased efficiency. Inhibitors of HDACs interact with intrinsic transcription factors and diverse signaling pathways to control stem cell pluripotency, self-renewal, and fate decisions. Sodium butyrate specifically facilitates the induction of the miR302/367 cluster, which increases the reprogramming efficiency by accelerating the mesenchymal-to-epithelial transition (MET), a crucial early step in reprogramming (Zhang and Wu 2013, Pei, Shu et al. 2019). We showed that together with the non-integrating IDLV-OSK vectors, the applied concentration of sodium butyrate was effective in increasing the number of colonies that emerged. It is very likely that the use of sodium butyrate partly compensated for the lack of c-Myc among the reprogramming factors we used. Different concentrations and different timing of use may be tested with new studies.

The original Yamanaka factors in reprogramming included c-Myc, as well as the Oct4, Sox2, and Klf4 transcriptional regulators (Takahashi and Yamanaka 2006, Karagiannis, Takahashi et al. 2019). Although providing increased efficiency in reprogramming, *c-Myc* is a proto-oncogene that produces a nuclear phosphoprotein playing a key role in regulating cell cycle progression, cellular transformation, and apoptosis. Amplification of the *c-Myc* gene is frequently observed in a wide range of cancers (Miller, Thomas et al. 2012). Moreover, chromosomal translocations involving this gene are strongly associated with malignancies. For these reasons, reprogramming protocols without c-Myc started to be tested, and different combinations of reprogramming factors such as the OSNL (Oct4, Sox2, Nanog, Lin-28) factors or less number of the original reprogramming factors (OSK; Oct4, Sox2, Klf4) proved to give efficient results (Yu, Vodyanik et al. 2007, Rizzi, Di Pasquale et al. 2012). The IDLV-OSK vectors we used in our study do not contain the *c-Myc* gene, supporting the ultimate goal in this field to activate intracellular reprogramming machinery without the necessity of using oncogenic genes (Ohnuki and Takahashi 2015). *Klf4*, which is in the set of genes we used for reprogramming, may also pose a problem in that regard, as it may play a role in tumorigenesis (He, He et al. 2023). However, removing *c-Myc* substantially reduces

tumorigenic risk during the process, as it is a well-known oncogene with significantly higher oncogenic potential than other factors used (Dhanasekaran, Deutzmann et al. 2022).

Following the generation and growth of the iPSC colonies, we applied methods for characterization, such as morphological characterization and characterization by detection of the common surface and nuclear pluripotency markers (Figures 4.3 and 4.4). These methodologies are usually considered the basic terms of characterization of iPSCs, and although effective in testing pluripotency, supporting techniques such as embryoid body formation to test differentiation potential of the colonies to cells from the three germ layers; mycoplasma testing for proving the absence of mycoplasma contamination; and chromosome analysis for proving an intact chromosomal pattern are also necessary for a more extensive characterization. We aim to complete these steps in our further studies.

Thus, we hereby display the effectiveness of a non-integrating viral vector-mediated reprogramming protocol combined with a histone deacetylase inhibitor small molecule.

## 6. CONCLUSION and SUGGESTIONS

Induced pluripotent stem cells (iPSCs) and related studies are currently under focus for their high potential of use in different fields, including toxicity testing, cancer vaccine research, drug screening, and generation of new cell sources. Morphologically, iPSCs closely resemble embryonic stem cells (ESCs), and in addition to sharing basic stem cell characteristics, they also show similar potential to differentiate into cells of the three germ layers. iPSCs can be generated directly from adult somatic cells through reprogramming, and are central to the development of new gene and cell therapy approaches for various diseases. Studies in this area provide support by testing various groups of transcriptional regulators, and also different vector systems and supporting molecules for improved protocols.

Testing the efficiency and safety of non-integrating but effective vectors and inducing systems in iPSC production, rather than the integrating approaches that carry insertional mutagenesis risk, is an important field of study focusing on different starting cells in the process. In our study, we used a reprogramming protocol in HFF-1 cells, involving integrase-deficient lentiviral vectors encoding OSK factors (Oct4, Sox2, Klf4), supported by a histone deacetylase inhibitor as a supporting small molecule to enhance efficiency and ensure greater safety of the targeted cells.

We demonstrated that our non-integrating protocol was efficient in the generation of iPSCs, and the inclusion of sodium butyrate significantly enhanced efficiency. Studies to test different starting cells, as well as different concentrations and timing of the small molecule used, will be important in evaluating the further improvement of the described approach to give an efficient and safe alternative to the existing iPSC technologies.

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# RESUME

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