

REPROGRAMMING HUMAN FETAL LUNG FIBROBLASTS TO PLURIPOTENCY VIA LENTIVIRAL AND SENDAI VIRUS SYSTEMS

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Abstract

Introduction. Induced pluripotent stem cells (iPSCs) hold promises for disease modelling and regenerative medicine, but reprogramming efficiency and safety depend on the method used to deliver Yamanaka factors (OCT4, SOX2, KLF4, c-MYC). Lentiviral vectors offer high efficiency yet risk insertional mutagenesis, while non-integrating Sendai virus systems provide transient transgene expression.

Aim. This study aimed to compare these two commonly used delivery strategies by reprogramming primary human lung fibroblasts using both doxycycline-inducible lentiviral vectors and a non-integrating Sendai virus system.

Materials and Methods. Primary human fetal lung fibroblasts (20–21 weeks of gestation) were isolated via explant culture and reprogrammed using either doxycycline-inducible lentiviral vectors encoding OKSM factors or a Sendai virus-based kit (CytoTune 2.0). Emerging iPSC colonies were expanded and characterized by immunofluorescence for pluripotency markers (SSEA4, OCT4, TRA-1-60) and alkaline phosphatase (ALP) activity.

Results. Both methods generated iPSC colonies with typical morphology (compact structure, high nucleus-to-cytoplasm ratio). Colony emergence occurred on days 15-20 in both lentiviral and Sendai virus reprogramming systems. Immunofluorescence confirmed expression of SSEA4, OCT4, and TRA-1-60 in colonies from both approaches, and ALP staining was positive.

Conclusion. Primary human fetal lung fibroblasts can be reprogrammed into iPSCs using both integrating lentiviral and non-integrating Sendai virus systems. Both methods yield stable iPSC lines with proper characteristics. The choice of method depends on the required balance between efficiency and genomic safety.

Key words: lung fibroblasts, induced pluripotent stem cells, lentivirus, Sendai virus, reprogramming.

Introduction. The generation of Induced Pluripotent Stem Cells (iPSCs) represents a major advancement in regenerative medicine, providing a powerful approach to reprogram somatic cells into a pluripotent state with the capacity for self-renewal and dedifferentiation [1]. This technology enables not only the study of human development and disease modelling but also offers opportunities for patient-specific cell therapies and cellular rejuvenation. The concept of induced pluripotency was first established by Shinya Yamanaka in 2006 through the introduction of defined transcription factors (OCT4, SOX2, KLF4, and c-MYC), commonly referred to as Yamanaka factors, into somatic cells [2]. Since this discovery, numerous studies have explored alternative strategies to improve reprogramming efficiency, safety, and reproducibility.

Despite these advances, iPSC reprogramming remains a complex and highly variable process, influenced by multiple factors including the method of factor delivery, cell type, and

culture conditions [3-5]. Among these variables, the delivery system used to introduce reprogramming factors is particularly critical, as it directly affects both reprogramming efficiency and genomic integrity. Most studies have reported viral-based systems remain the most widely utilized due to their high efficiency. Current approaches broadly fall into two categories: integrating systems, such as lentiviral vectors, and non-integrating systems, including Sendai virus-based methods. Each approach presents distinct advantages and limitations, necessitating careful evaluation of how they affect the quality of iPSCs in terms of transcriptional signatures, epigenetic status, genomic integrity, stability, differentiation and tumorigenic potential [3,6-8].

Lentiviral vectors are integrating into genome and have been reported to have high efficiency among all methods. Due to its integrating feature, it gives a stable expression of Yamanaka factors. However, genomic integration is associated with several risks, including insertional mutagenesis, disruption of endogenous gene function following vector integration, clonal expansion resulting from proto-oncogene activation, and the potential reactivation of viral transgenes [9]. Also, studies report that this method can generate heterogenous iPSC lines which obscures the comparative analysis between lines [3,5,10,11].

Sendai virus systems are, on the contrary, non-integrating RNA virus, therefore the expression of OSKM factors is transient [12]. Sendai virus is an RNA virus that replicates in the cytoplasm and is eventually diluted out during cell division, making it particularly attractive for applications requiring genomic safety [13-16].

In this study, we aimed to compare these two commonly used delivery strategies by reprogramming primary human lung fibroblasts using both doxycycline-inducible lentiviral vectors and a non-integrating Sendai virus system. We further characterized the resulting iPSC line to assess their morphological and molecular properties, providing insight into the applicability of these methods for generating pluripotent cells from this specific primary cell type.

Materials and Methods

Ethical Issues

The protocol of the experimental study was approved by the Institutional Research Ethics Committee of the Medical School of Nazarbayev University (approval No. 74 – January 25, 2019; IREC No. 100/19112018).

Primary Human Fetal Lung Fibroblast Isolation and Culture

Human fetal lung tissue (male fetus, 20–21 weeks of gestation) was handled under sterile conditions in a tissue culture hood. Lung tissue was dissected using sterile scalpels and forceps and transferred to a sterile 10 cm Petri dish. The tissue was washed three times with Ham's F10 medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. The lung tissue was cut into approximately 1 cm³ (~1 g) explants and transferred to a fresh sterile Petri dish, followed by three additional washes with the same antibiotic-containing medium. The tissue was then finely minced into >20 small fragments using sterile scalpel blades. Tissue fragments were evenly distributed into 25 cm² tissue culture flasks using a sterile Pasteur pipette. Excess medium was carefully removed to allow the explants to adhere to the culture surface. Flasks were then inverted, and a growth medium consisting of Ham's F10 supplemented with 15% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin was added. Flasks were maintained in an inverted position for at least 1 hour at room temperature (~20°C) to facilitate tissue attachment, ensuring that sufficient medium was present to prevent desiccation of the explants. Following attachment, flasks were carefully returned to an upright position, allowing the medium to cover the tissue fragments, and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cells migrating from the explants were cultured under these conditions, and primary cultures were designated as passage 0. Upon reaching ~80%

confluence, cells were passaged at a 1:4 ratio using 0.25% trypsin (without EDTA) with minimal mechanical disruption. Culture medium (Ham's F10 supplemented with 15% FBS and antibiotics) was freshly prepared prior to use [17, 18].

Induced Pluripotent Stem Cell generation via lentiviral reprogramming and characterization

Primary human lung fibroblasts were expanded up to passage 4 prior to reprogramming. Cells were plated onto gelatin-coated culture vessels with a density of 0.5mln cells per 1 well of 6-well-plate and transduced with doxycycline-inducible lentiviral vectors encoding OKSM factors along with the TetR transactivator. The MOI was 3 and the virus titer was 50 CIU/ul. Vector constructs were obtained from Addgene as described by Gill.et.al [19]. A doxycycline-inducible reprogramming vector was created by inserting a GFP-IRES sequence into the FUW-tetO-hOKMS backbone (Addgene 51543) downstream of the tetracycline response element. This vector was used alongside FUW-M2rtTA (Addgene 20342). To produce viral particles, HEK293T cells were transfected with two packaging plasmids – pMD2.G (Addgene 12259) and psPAX2 (Addgene 12260), together with either FUW-tetO-GFP-hOKMS or FUW-M2rtTA. Integration of the virus cassette was assessed by expression of GFP upon Tet-activation by doxycycline. After 24 h, 2ug/ml doxycycline was added to the culture medium to induce transgene expression and initiate reprogramming. Cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, non-essential amino acids (NEAA), GlutaMAX, 20 ng/mL basic fibroblast growth factor (bFGF), and 50 μ M β -mercaptoethanol. On day 7 of reprogramming, cells were dissociated with Accutase and replated onto Matrigel-coated 6-well plates. After 24 h, the medium was replaced with mTeSR1, and cells were subsequently maintained in mTeSR1 with daily medium changes. By day 15-19, around 10 induced pluripotent stem cell (iPSC) colonies were observed. Individual colonies were manually picked and transferred to Matrigel-coated 24-well plates in mTeSR1 supplemented with 10 μ M ROCK inhibitor. Total duration of doxycycline induction was 29 days. Assessment of silencing of exogenous transgenes was done by evaluation of GFP expression. A colony that has lost the exogenous expression of lentiviral GFP was counted as a successfully reprogrammed IPS. This one colony was further selected and expanded until stable iPSC line was established. Pluripotency of established iPSC line was validated by immunofluorescence staining and alkaline phosphatase activity assays. Primary antibodies used for immunofluorescence included SSEA4-eFluor 660 (1:250, Invitrogen), OCT4 (1:250, Santa Cruz Biotechnology), and TRA-1-60 (1:200, Abcam). Alkaline phosphatase activity was assessed using the Vector Red Substrate Kit (SK-5100) according to the manufacturer's instructions. Stability of the expanded colony was checked by stable long-term passaging up to 12 times with consistent IPS morphology.

Sendai Virus-Mediated Reprogramming of Lung Fibroblasts

Primary human lung fibroblasts were expanded to approximately 50-60% confluence before reprogramming. Cells were seeded onto gelatin-coated culture vessels one day prior to transduction to ensure optimal cell density at the time of infection which was 0.5mln cells per one well of a 6-well-plate. Reprogramming was performed using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (catalog number: A16517) according to the manufacturer's recommendations. Briefly, fibroblasts were transduced with Sendai viral vectors encoding OCT4, SOX2, KLF4, and c-MYC at the recommended multiplicity of infection (MOI) for each vector component which was 5:5:3 (KOS: cMyc:KLF4). Viral particles were added directly to the culture medium, and cells were incubated overnight under standard culture conditions. Following transduction, the medium was replaced with fresh fibroblast growth medium, and cells were maintained for 5–7 days with regular medium changes every other day. During this period, cells were monitored for morphological changes associated with early reprogramming.

On day 7 post-transduction, cells were dissociated and replated onto Matrigel-coated plates at an appropriate density. After 24 h, the medium was transitioned to mTeSR1 medium, which was subsequently refreshed daily. 30 emerging iPSC colonies with human embryonic stem cell-like morphology were observed between days 14–21 post-transduction. 14 individual colonies were manually picked and transferred to Matrigel-coated plates in mTeSR1 supplemented with 10 μ M ROCK inhibitor to promote survival. 1 colony was expanded under standard pluripotent stem cell culture conditions until stable iPSC lines were established. Pluripotency of established iPSC lines was validated by immunofluorescence staining and alkaline phosphatase activity assays. Primary antibodies used for immunofluorescence included SSEA4-eFluor 660 (1:250, Invitrogen), OCT4 (1:250, Santa Cruz Biotechnology), and TRA-1-60 (1:200, Abcam). Alkaline phosphatase activity was assessed using the Vector Red Substrate Kit (SK-5100) according to the manufacturer's instructions. Sendai virus clearance was confirmed by performing thermal inactivation at 38C for one week as described by the manual.

Statistical Analysis

Reprogramming efficiency was determined by the following formula:

$$\text{Reprogramming Efficiency} = \frac{\# \text{ Emerging colonies}}{\# \text{ somatic cell seeded}} \times 100 \quad (1)$$

For lentiviral reprogramming system, 10 emerged colony from 0.5mln cells accounted for 0.002% reprogramming efficiency of lentiviral system. For Sendai Virus-Mediated reprogramming, reprogramming efficiency was 0.06%. The experiments were repeated two times independent from each other, and there was to variability between the repeats.

Results. Primary lung fibroblasts were successfully isolated using an explant-based method and expanded to passage 5. Cells exhibited typical fibroblast morphology and were used for subsequent reprogramming (Figure 1).

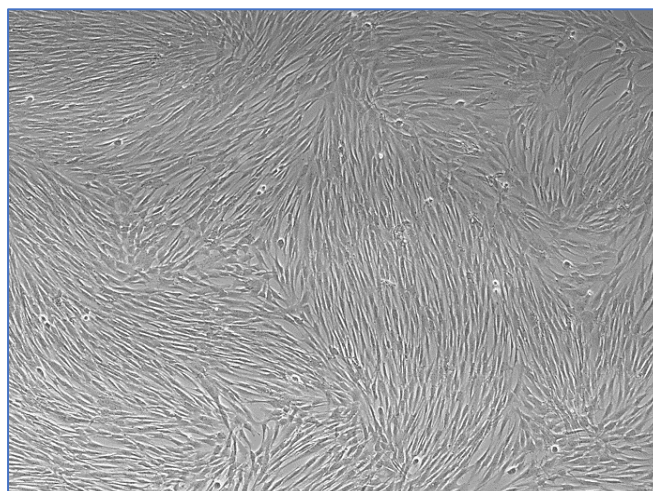


Figure 1. Expanded primary Lung Fibroblast cells in culture, passage 5.

Primary human lung fibroblasts were reprogrammed into induced pluripotent stem cells using both doxycycline-inducible lentiviral delivery of OKSM factors and a Sendai virus-based approach. In both systems, morphological changes characteristic of early reprogramming were observed within the first week, including a transition from elongated fibroblast-like cells to compact epithelial-like clusters. In lentiviral reprogramming conditions, colonies with human embryonic stem cell-like morphology emerged between days 16-19 post-induction (Figure 2-

A), whereas in Sendai virus–mediated reprogramming, colony formation was observed slightly earlier, between days 15–18 (Figure 3-A). This time difference was regarded as negligible since it does not show significant difference between the methods. In both cases, colonies displayed well-defined borders and a high nucleus-to-cytoplasm ratio (Figure 2-B, Figure 3-B). Individual colonies derived from both reprogramming approaches were manually picked and expanded under feeder-free conditions, resulting in stable iPSC lines. Immunofluorescence analysis confirmed robust expression of key pluripotency markers, including OCT4, SSEA4, and TRA-1-60, in iPSC colonies generated by both methods (Figure 2-D, Figure 3-D). Consistent with these findings, alkaline phosphatase staining demonstrated enzymatic activity in reprogrammed colonies, while no staining was detected in parental fibroblasts (Figure 2-C, Figure 3-C). Collectively, these results demonstrate the possibility of generation of pluripotent stem cells from primary human lung fibroblasts using both integrating lentiviral and non-integrating Sendai virus reprogramming approaches, with similar morphological and molecular characteristics.

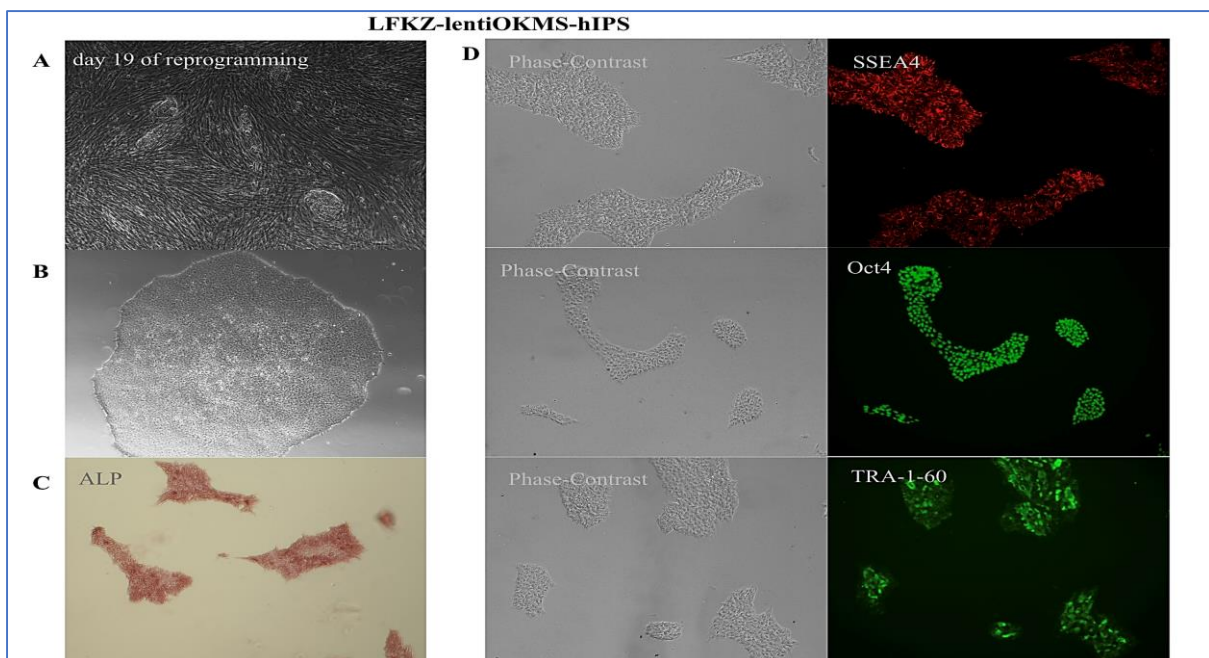


Figure 2. IPS cells derived from lung fibroblasts via lentiviral reprogramming. A) day 19 of reprogramming, B) morphology of expanded iPSC on passage 12, C) ALP staining, D) Immunofluorescence staining for pluripotency markers: SSEA4 expression, OCT4 expression, TRA-1-60 expression.

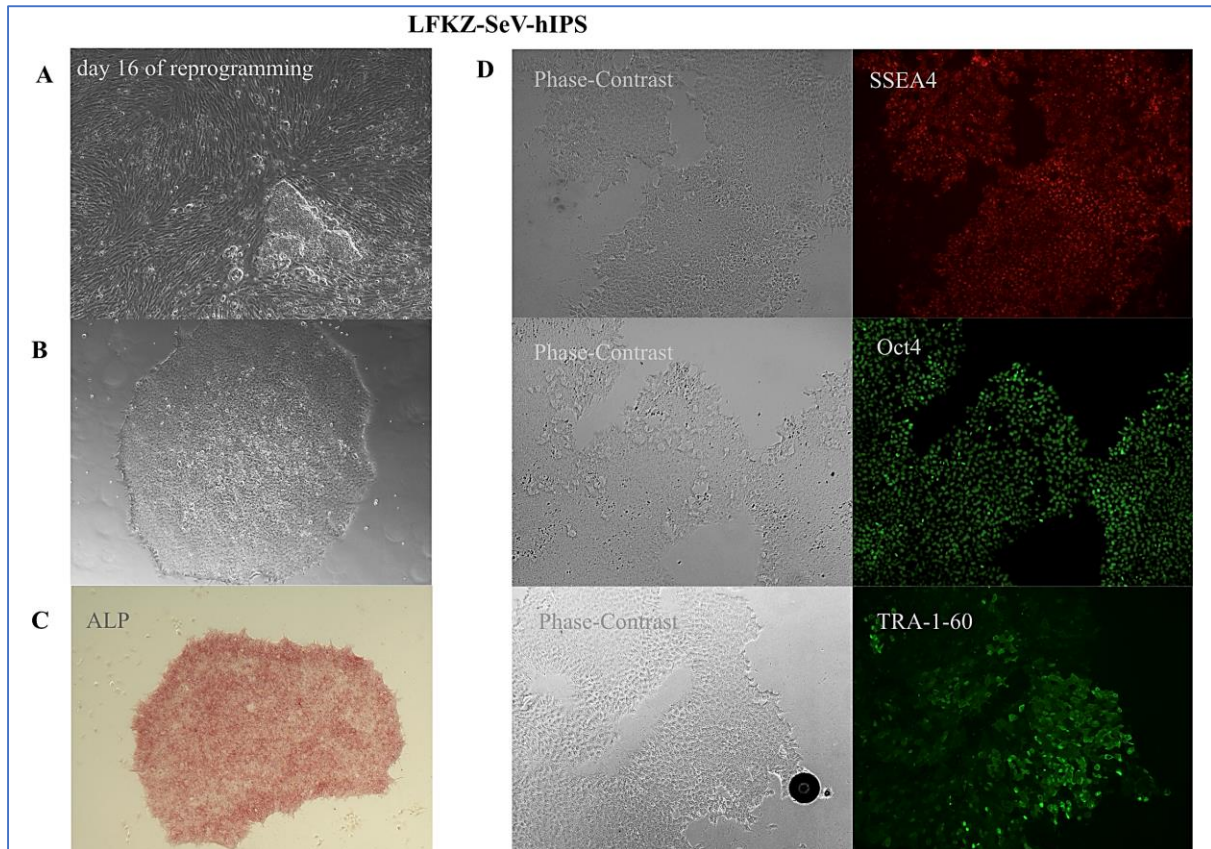


Figure 3. IPS cells derived from lung fibroblasts via Sendai Viral reprogramming. A) day 16 of reprogramming, B) morphology of expanded iPSC on passage 12, C) ALP staining, D) Immunofluorescence staining for pluripotency markers: SSEA4 expression, OCT4 expression, TRA-1-60 expression.

Discussion. The generation of iPSC by the introduction of Yamanaka factors has developed many methods for their delivery. Lentiviral vectors are regarded as the gold standard in laboratory settings [9, 20]. In our experiments, the lentiviral reprogramming system has raised iPSC colonies on day 19. This timeframe is consistent with previously reported reprogramming kinetics for integrating viral systems, where colony formation typically occurs between days 15 and 25 post-transduction [21]. The observed timing suggests efficient delivery and sustained expression of reprogramming factors, which are critical for the successful induction of pluripotency. Furthermore, the ability to generate colonies within this timeframe indicates that primary human lung fibroblasts are amenable to lentiviral-mediated reprogramming, despite known variability associated with primary cell sources. Pluripotency of the obtained iPSC lines were also validated with the expression of SSEA4, OCT4, and TRA-1-60 markers and positive Alkaline Phosphatase activity [22].

The Sendai virus-based system resulted in the emergence of iPSC-like colonies approximately on day 16 post-transduction. This onset of colony formation in the Sendai system may be attributed to its non-integrating nature and efficient cytoplasmic replication, which enables rapid expression of reprogramming factors without the need for genomic integration. In contrast, lentiviral systems rely on integration into the host genome, which may introduce a delay in the establishment of stable transgene expression. These findings are consistent with previous reports indicating that non-integrating viral systems can facilitate more rapid induction of pluripotency while avoiding risks associated with genomic integration [23]. However, further

quantitative analysis would be required to determine whether these differences in kinetics also translate into variations in overall reprogramming efficiency.

Fetal fibroblasts are mitotically active, have longer telomeres, and carry fewer age-associated epigenetic marks compared to adult or postnatal fibroblasts. This is relevant because residual DNA methylation and histone modification patterns from donor cells can restrict full reprogramming and impair lineage-specific differentiation, a burden that is substantially reduced in fetal-origin cells [24].

Despite the generation of iPSCs from lentiviral and sendai-virus mediated systems and validation of obtained colonies quality, the study has several limitations. The donor samples which are primary fibroblasts were obtained from single fetal lung specimen, therefore the results cannot be extrapolated to a broader population of fetal lung fibroblasts. Another limitation and an important direction for future work is the differentiation potential of established iPSCs. Within the scope of the present study, pluripotency and genomic stability were assessed through morphological analysis, and immunostaining for standard pluripotency markers, which collectively provided sufficient evidence for the pluripotent state of the generated colonies. Nevertheless, definitive confirmation of iPSC quality requires demonstration of trilineage differentiation potential – that is, the capacity to generate cells representative of all three embryonic germ layers (endoderm, mesoderm, and ectoderm). This constitutes a critical next step and will be the focus of subsequent investigations.

Conclusion. Our data provides several conclusions. First, primary lung fibroblasts can be reprogrammed into iPSC lines using the OSKM transcription factors. These iPSCs expressed pluripotency markers and ALP activity. Second, both approaches resulted in the formation of colonies exhibiting characteristic iPSC-like morphology, confirming the effectiveness of these methods in this cell type. These findings highlight the impact of delivery strategy on the dynamics of reprogramming while supporting both integrating and non-integrating systems.

Conflicts of Interest. The authors declare no conflict of interest.

Authors' contribution. Experimental work, review and editing - Sholpan Nurzhan; writing, review, and editing, supervision, and project administration – Dulat Azhibek. All authors have read and agreed to the published version of the manuscript. The authors declare that this material has not been previously published and is not under consideration by other publishers.

Funding. The study was supported by program-targeted funding from the Ministry of Science and Higher Education of the Republic of Kazakhstan “Prolonging healthy longevity: using new technologies and machine learning to control reversal of aging in old cells” (Grant No. BR24992900).

Data availability statement. All available data was indicated within manuscript text.

Acknowledgements. The authors are thankful to the School of Medicine, Nazarbayev University (Astana, Kazakhstan), for the administrative and technical support provided.

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АДАМ ҰРЫҒЫНЫҢ ӨКПЕ ФИБРОБЛАСТТАРЫН ЛЕНТИВИРУСТЫҚ ЖӘНЕ СЕНДАЙ ВИРУС ЖҮЙЕЛЕРІ АРҚЫЛЫ ПЛЮРИПОТЕНЦИЯҒА ДЕЙІН ҚАЙТА БАҒДАРЛАМАЛАУ

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Түйіндеме

Кіріспе. Индукцияланған плюрипотентті дің жасушалары (iPSCs) ауруларды модельдеу және регенеративті медицина үшін үлкен мүмкіндіктер береді, алайда репрограммалау тиімділігі мен қауіпсіздігі Яманака факторларын (OCT4, SOX2, KLF4, c-MYC) жеткізу үшін қолданылатын әдіске байланысты. Лентивирустық векторлар жоғары тиімділікті қамтамасыз етеді, бірақ инсерциялық мутагенез қаупін тудыруы мүмкін, ал интеграцияланбайтын Сендай вирусы жүйелері трансгендердің уақытша экспрессиясын қамтамасыз етеді.

Мақсаты. Бұл зерттеудің мақсаты бастапқы адам өкпе фибробластарын доксициклин-индукцияланатын лентивирустық векторлар және интеграцияланбайтын Сендай вирусы жүйесі арқылы репрограммалау негізінде екі кеңінен қолданылатын жеткізу екі стратегиясын салыстыру.

Материалдар мен әдістер. Бастапқы адам ұрығының өкпе фибробласттары (жүктіліктің 20–21 аптасы) экспланттық культура әдісі арқылы бөлініп алынды және OKSM факторларын кодтайтын доксициклин-индукцияланатын лентивирустық векторлар немесе Сендай вирусына негізделген жинақ (CytoTune 2.0) көмегімен репрограммаланды. Пайда болған iPSC колониялары кеңейтіліп өсірілді және плюрипотенттілік маркерлеріне (SSEA4, OCT4, TRA-1-60) арналған иммунофлуоресценция және сілтілік фосфатаза (ALP) белсенділігі бойынша сипатталды.

Нәтижелер. Екі әдіс те типтік морфологиясы бар iPSC колонияларын қалыптастырды: ықшам құрылым және ядро-цитоплазмалық қатынастың жоғары болуы. Колониялардың пайда болуы лентивирустық және Сендай вирусы арқылы репрограммалау жүйелерінде 15–20-күндері байқалды. Иммунофлуоресценция екі тәсілмен алынған колонияларда SSEA4, OCT4 және TRA-1-60 экспрессиясын растады, сондай-ақ ALP боялуы оң нәтиже көрсетті.

Қорытынды. Бастапқы адам ұрығының өкпе фибробластарын интеграцияланатын лентивирустық және интеграцияланбайтын Сендай вирусы жүйелерін қолдану арқылы iPSC-ке қайта программалауға болады. Екі әдіс те тиісті сипаттамалары бар тұрақты iPSC желілерін алуға мүмкіндік береді. Әдісті таңдау тиімділік пен геномдық қауіпсіздік арасындағы қажетті теңгерімге байланысты.

Түйінді сөздер: өкпе фибробласттары, индукцияланған плюрипотентті дің жасушалары, лентивирус, Сендай вирусы, қайта программалау.

ПЕРЕПРОГРАММИРОВАНИЕ ФИБРОБЛАСТОВ ЛЕГКИХ ПЛОДА ЧЕЛОВЕКА ДО ПЛЮРИПОТЕНТНОСТИ С ПОМОЩЬЮ ЛЕНТИВИРУСНОЙ И СЕНДАЙСКОЙ ВИРУСНОЙ СИСТЕМ

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Аннотация

Введение. Индуцированные плюрипотентные стволовые клетки (иПСК) обладают значительным потенциалом для моделирования заболеваний и регенеративной медицины, однако эффективность и безопасность репрограммирования зависят от метода, используемого для доставки факторов Яманаки (OCT4, SOX2, KLF4, c-MYC). Lentiviral векторы обеспечивают высокую эффективность, однако несут риск инсерционного мутагенеза, тогда как неинтегрирующие системы на основе вируса Сендай обеспечивают транзиторную экспрессию трансгенов.

Цель. Целью данного исследования было сравнить две широко используемые стратегии доставки путём репрограммирования первичных фибробластов лёгких человека с использованием как доксициклин-индуцируемых лентивирусных векторов, так и неинтегрирующей системы на основе вируса Сендай.

Материалы и методы. Первичные фибробласты лёгких плода человека (20–21 неделя гестации) были выделены методом эксплантной культуры и репрограммированы с использованием либо доксициклин-индуцируемых лентивирусных векторов, кодирующих факторы OKSM, либо набора на основе вируса Сендай (CytoTune 2.0). Формирующиеся колонии иПСК были экспандированы и охарактеризованы методом иммунофлуоресценции на маркеры плюрипотентности (SSEA4, OCT4, TRA-1-60), а также по активности щелочной фосфатазы (ALP).

Результаты. Оба метода обеспечили получение колоний иПСК с типичной морфологией (компактная структура, высокое соотношение ядра к цитоплазме). Появление колоний наблюдалось на 15–20-е сутки как в лентивирусной системе репрограммирования, так и в системе на основе вируса Сендай. Иммунофлуоресценция подтвердила экспрессию SSEA4, OCT4 и TRA-1-60 в колониях, полученных обоими подходами, а окрашивание на ALP было положительным.

Заключение. Первичные фибробласты лёгких плода человека могут быть репрограммированы в иПСК с использованием как интегрирующих лентивирусных, так и неинтегрирующих систем на основе вируса Сендай. Оба метода позволяют получать стабильные линии иПСК с надлежащими характеристиками. Выбор метода зависит от необходимого баланса между эффективностью и геномной безопасностью.

Ключевые слова: фибробласты лёгких, индуцированные плюрипотентные стволовые клетки, лентивирус, вирус Сендай, репрограммирование.