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An optimized Sendai viral vector platform for reprogramming to naive pluripotency

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Technologies to reprogram somatic cells into iPSCs have advanced significantly, however challenges to the derivation of iPSCs remain. In this issue of *Cell Reports Methods*, Kunitomi et al. address some of these challenges by developing a straightforward protocol to derive naive human iPSCs using Sendai virus vectors.

Although protocols to generate human iPSCs have existed for over a decade, conventional reprogramming protocols lead to the generation of primed induced pluripotent stem cells (iPSCs), a cell type that closely resembles pluripotent stem cells (PSCs) from the post-implantation embryo. Human PSCs can also be found in pre-implantation embryos, and these PSCs that arise early in embryonic development (known as naive PSCs) are physiologically distinct from primed iPSCs.[1] Although both cell types are able to differentiate into any cell of the adult body, their morphology, transcription profile, and signaling requirements are distinct. Furthermore, compared with primed PSCs, naive PSCs have greater potential to differentiate into extra-embryonic cell types *in vitro* and can integrate far more readily into developing pre-implantation embryos than primed PSCs.[1,2] Thus, the development of optimized protocols to derive naive iPSCs is not only important for the study of early human embryonic development but may also have practical applications in biomedicine as well; such as in the derivation of extra-embryonic tissues, synthetic embryos, and in the generation of interspecies human-animal chimeras.[2–4] Technologies that can be used to model human disease and may one day be used to generate transplantable human organs.

Numerous different approaches have now been developed that allow for the generation of human naive PSCs *in vitro*, however there is still a need for the development of simple protocols that facilitate reprogramming robustly across different cell types. Sendai virus (SeV) represents an attractive vector for delivering the reprogramming factors *OCT4*, *SOX2*, *KLF4*, and *CMYC* (OKSM) into somatic cells to generate iPSCs. The virus is non-pathogenic to humans and has an RNA genome that persists cytosolically, thus eliminating the risk of integration of OKSM or viral genes into the genome. Furthermore, its use in reprogramming somatic cells into iPSCs is well established and commercial kits toward this purpose are readily available. Recently it has also been demonstrated that SeV can be used to reprogram human dermal fibroblasts (HDFs) into naive iPSCs.[5,6] However, current SeV can persist long term in iPSCs, leading to long term expression of OKSM genes in cells, which not only brings an increased risk of tumorigenicity but can also hinder the differentiation potential of iPSCs.[7] In addition, current SeV reprogramming approaches are only able to reprogram HDFs into naive iPSCs and are unable to generate naive iPSCs from more readily accessible cell types, such as peripheral blood mononuclear cells (PBMCs).

Kunitomi et al. set out to develop a more robust SeV protocol to generate naive iPSCs.[8] They first tested current SeV protocols to derive naive human iPSCs and confirmed previous reports demonstrating that SeV could be applied to generate naive iPSCs from HDFs but not from PBMCs.[6] Previous work from the Yamanaka lab has demonstrated that *LMYC* is more efficient than *CMYC* in generating iPSCs.[9] The authors thus tested if substitution of *CMYC* for *LMYC* would improve their ability to derive naive iPSCs from different cell types and found that this change in the SeV reprogramming platform allowed for the derivation of naive iPSCs from both HDFs and PBMCs. The authors then collected samples from reprogrammed cells over time and performed qPCR to determine to what extent Sendai virus persisted in iPSCs following reprogramming. Tracking iPSC colonies over the course of 94 days, the authors found that Sendai viral genomes persisted in all iPSC clones tested, a finding that was secondarily confirmed by immunohistochemistry.

The authors then further examined the makeup of the commercially available CytoTune-iPS 2.0 SeV reprogramming kit that was used to generate naive iPSCs. Upon examination, the authors noted that although two of the three SeV vectors used to generate iPSCs had multiple mutations in the viral polymerase P gene that conferred temperature sensitivity, one of the SeV vectors (which delivered *KLF4* to cells), had only a single mutation in the P gene.[10] The authors thus hypothesized that this single mutation in the P gene of the SeV-KLF4 vector (SeV-KLF4-TS) conferred only weak temperature sensitivity to the virus and was allowing SeV vectors to persist in iPSCs long term. To test this hypothesis and address the issue of SeV persistence in iPSCs, the authors then generated a series of three KLF4-SeV vectors with modifications to the vector genome. The authors generated KLF4-SeV vectors with: (1) a viral backbone that had multiple previously described mutations in the P gene, that conferred enhanced temperature sensitivity to the SeV vector (SeV-KLF4/TS12)[10]; (2) the incorporation of a

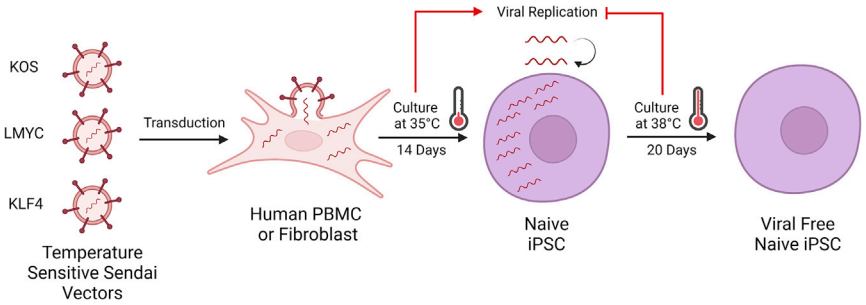

Figure 1. A modified Sendai virus reprogramming method facilitates efficient generation of human naive PSCs

Sendai virus has been applied to generate human naive iPSCs, however previous methods were only able to generate naive iPSCs from HDFs and Sendai viral vectors were difficult to eliminate from reprogrammed cells. Through replacement of *CMYC* with *LMYC*, the use of temperature-sensitive SeV vectors and a two-step reprogramming protocol to first ensure retention of (culture at 35°C) followed by elimination (culture at 38°C) of Sendai vectors from cells; Kunitomi et al. have developed a straightforward method to derive human naive iPSCs from both HDFs and PBMCs.
KOS: *KLF4*, *OCT3/4*, *SOX2*.

microRNA binding site for the microRNA miR-367 (which is specifically expressed in PSCs) into the vector genome to reduce viral persistence after de-differentiation via RNA knockdown (SEV-KLF4/miR/TS)[11]; and (3) tested the synergistic combination of incorporating the miR-367 binding site into the SeV-KLF4/TS12 vector (SeV-KLF4/miR/TS12).

Somatic cells were reprogrammed into iPSCs with all three vectors, by first culturing cells at 35°C for 14 days to ensure persistence of the SeV vectors, cells were then moved to 38°C for 20 days to eliminate SeV vectors by temperature inhibition (Figure 1). The authors found that they could derive naive iPSCs with all vectors tested. They also found that all three modified SeV-KLF4 vectors lead to the rapid loss of SeV genomes in iPSCs over time, decreasing below the limit of detection 34 days after the onset of transduction, while the original SEV-KLF4/TS vector continued to persist in iPSCs even at 42 days post transduction. The authors also noted that their modified SEV-KLF4 vectors lead to the generation of more naive iPSC colonies and appeared to cause less cytotoxicity in cells over time, compared to the use of the original SeV-KLF4 vector. Having found that both the introduction of temperature-sensitive point mutations in the P gene of SeV and the incorporation of the miR-367 binding site into the SeV genome were effective for preventing viral persistence, the authors proceeded with the use of the synergistic SeV-KLF4/miR/TS12 vector in their reprogramming protocol.

The authors then validated the naive pluripotency of cells generated using their protocol by performing RNA-seq and comparing the transcriptome of their naive cells to primed cells. They found that their reprogrammed cells had a distinct expression pattern from primed cells, that matched the previously reported expression profiles of naive PSCs. Furthermore, iPSCs generated from their protocol clustered distinctly with other naive PSCs by principal component analysis and their reprogrammed iPSCs were demonstrated to have undergone X chromosome reactivation, a key developmental phenotype that differentiates naive iPSCs from primed iPSCs. To functionally validate their reprogrammed naive iPSCs, the authors then investigated their ability to generate embryoid bodies (EBs) and to differentiate into trophectoderm (an extra-embryonic tissue). The reprogrammed naive iPSCs were able to successfully generate EBs and notably, compared to cells reprogrammed using the original SeV protocol, these cells were significantly better able to differentiate into EBs. The authors based this finding on both the more regular morphology of EBs generated with their optimized SeV protocol and by gene expression analysis. Furthermore, these reprogrammed naive iPSCs were able to differentiate into trophectoderm *in vitro* at similar efficiencies as other naive PSC lines.

Altogether, Kunitomi et al. have refined the use of SeV vectors to generate naive iPSCs from different cell types and have addressed the issue of long-term persistence of SeV vectors in both naive and primed iPSCs. Furthermore, they have functionally validated the efficacy of their approach for generating naive iPSCs. Although alternative approaches for generating naive iPSCs exist, including the use of mRNA transfection of OKSM factors into somatic cells and chemical reprogramming, these approaches are often inefficient, and in the case of mRNA transfection require repeated application to reprogram cells. In developing a simple approach to reprogram somatic cells into naive iPSCs by building upon a commercially available platform, Kunitomi et al. have generated a protocol that is readily accessible to any group interested in studying or applying naive iPSCs as part of their research (Figure 1).

The development of this protocol comes at an opportune time in PSC research as naive iPSCs are not only a valuable resource for studying early human development but also have recently been demonstrated to have significant practical applications in biomedicine as well,[2–4]including as part of technologies that could one day be used to create transplantable human organs. Perhaps this reprogramming protocol will be used to generate naive iPSCs that will be used for this purpose. Important challenges to the application of naive iPSCs in biomedicine still exist, however. For instance, naive iPSCs have been found to have significantly greater genome instability than primed iPSCs and the authors found that this issue still persists even when naive iPSCs are generated using their protocol. However, while challenges to the application of naive iPSCs in biomedicine still exist, Kunitomi et al.'s straightforward reprogramming protocol marks a step forward in our ability to derive this cell type.

DECLARATION OF INTERESTS

H.N. is a co-founder and shareholder in ReproCELL, Megakaryon, and Century Therapeutics.

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