

Multigene Assembly for Construction of Synthetic Operons: Creation and Delivery of an Optimized All-IN-One Expression Construct for Generating Mouse iPS Cells

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2.1 INTRODUCTION

As our understanding of cell biology increases, the experiments needed to gain further knowledge become more complex. While overexpression (or knockdown or knockout) of a single gene has led to breakthroughs in our understanding of cellular signaling, perturbation of multiple genes (say in a signaling pathway) is now often necessary in order to gain a clear picture of how that pathway effects cellular function. An important consideration in the design of multigene delivery technology is the availability of a suitable vector to stably and stoichiometrically introduce multiple genes into living cells and coexpress these genes efficiently. Here, we describe use of Gateway technology to easily assemble multiple genes for controllable expression in cells. The mechanism for creating a Multisite construct is the same as that with a single fragment Gateway recombination. The difference is the att site

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specificity and the configuration (and orientation) of the att sites. This concept is key to the utility of the Multisite Gateway system.

With the appropriately configured vectors, the combination of multiple fragments is achieved by assembling the entry vectors with an appropriate destination vector containing the correct flanking att sites to capture the inserts. Using the same enzyme combination (LR Clonase) and selection criteria as with a single fragment recombination, essentially, the only way to get a colony is if the proper att sites line up and recombine with each other and the destination vector. Using the six currently available att site specificities, assemblies of more than 10 fragments are possible by creating intermediate destination vectors (1).

Using the Multisite Gateway system, modular assembly of expression constructs including promoters, ORFs, epitope and purification tags, can be achieved without the use of restriction enzymes and ligase. Once a set of entry vectors has been created, the elements can be mixed and matched in an LR reaction. Elements in a multifragment construct can be selectively removed via a specific BP recombination reaction creating an intermediate destination vector.

2.2 BACKGROUND

As an example of the utility of this system, we have developed all-in-one expression system for creation of induced pluripotent stem cells (iPSC) from differentiated tissue. The constructs described here harbor tandemly situated multiple cDNAs and their expression control signals in a single vector. Construction of multiple functional DNA elements in tandem on a single plasmid has been performed by stepwise Gateway recombination reactions using Multisite Gateway technology (Life Technologies) (1–5). The utility of this vector is amplified by combining it with a highly efficient cell transduction system. iPSC can be generated from somatic cells by transduction of four transcription factors, Oct4, Klf4, Sox2, and c-Myc. The intracellular delivery of these transcription factors has mostly entailed risks of tumorigenesis due to insertional mutagenesis and oncogenesis by the use of integrating retroviral or lentiviral vectors. Nonviral methods for generating iPSC using plasmid vectors and so on (6) have generally suffered from low reprogramming efficiencies mostly due to low transduction rates in primary somatic cells.

In an attempt to mitigate the risk of inspectional mutagenesis while maintaining the efficient delivery of viral particles, we chose to work with recombinant baculoviruses engineered to contain a mammalian expression cassette (7,8). These viral vectors containing mammalian cell-active promoters

upstream of the target genes were termed BacMam viruses. This system has evolved rapidly over the last several years (9), for example, for rapid and high-level protein production in mammalian cells for the purpose of drug screening (10), multiple transporters studies (11), and protein structural studies (12). Applications of the baculovirus system have been expanded to eukaryotic gene display, cancer therapy, tissue engineering and a potential vaccine delivery, and so on (13). Since the BacMam transduction system has a large payload capacity and does not lead to insertion of genetic material into the cellular genome, it provides a platform for footprint-free and effective stoichiometric expression of four factors that is assumed to important to generate fully reprogrammed iPS cells (14).

Generation of iPS cells from mouse embryonic fibroblast (MEF) was achieved successfully by BacMam-transduction of a polycistronic plasmid expression vector for coincident and optimized expression of the four reprogramming transcription factors, Oct4, Klf4, Sox2, and c-Myc in this order on a single plasmid. A four-in-one expression plasmid for construction of a bacmid, OKSM-2A2, outlined in Figure 2.1e was constructed by joining

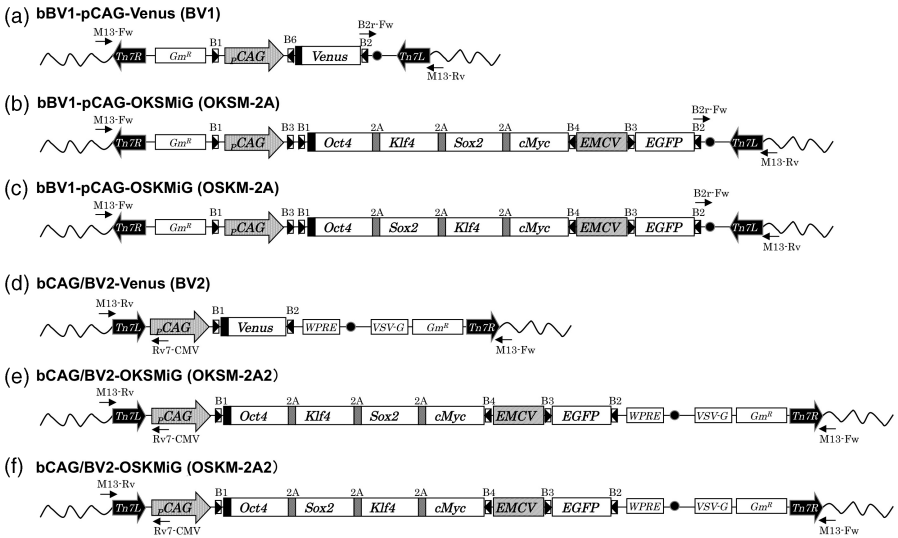


FIGURE 2.1 Diagrammatic maps of recombinant bacmids used. Schematic maps of (a) bBV1-pCAG-Venus (BV1), (b) bBV1-pCAG-OKSMiG (OKSM-2A), (c) bBV1-pCAG-OSKMiG (OSKM-2A), (d) bCAG/BV2-Venus (BV2), (e) bCAG/BV2-OKSMiG (OKSM-2A2), (f) bCAG/BV2-OSKMiG (OSKMiG). Closed circles represent transcriptional terminator signals (SV40 poly-adenylation signal). Wavy lines represent the bacmid backbone of bMON14272. Short arrows indicate placement of the PCR primers for verification of insertion of the expression cassette into the bacmid backbone by Tn7 transposase. 2A indicates EMDV 2A peptide sequences. B1–B6 indicate attB signals, remnants of Multisite Gateway cloning. *Gm^R* indicates a gentamycin resistance gene for selection of recombinant bacmid in DH10Bac.

three DNA fragments using Multisite Gateway technology. The plasmid backbone of this construct was version 2 that includes the VSV-G and WPRE elements. These cDNAs for four transcription factors were fused as a fusion gene in a single ORF via self-cleaving 2A peptides (15), in order to minimize the molecular size of the expression DNA cassette. The expression of these ORFs was directed by the CAG promoter.

In this chapter, we describe general methods of stepwise construction of an optimized multigene expression vector cassette for the BacMam system, and for transduction of MEF cells for reprogramming into iPS cells in a few weeks.

2.3 PROTOCOL FOR CLONING MULTIPLE GENES FOR SOMATIC REPROGRAMMING

2.3.1 Materials

2.3.1.1 Cloning of Reprogramming Factors as Gateway Entry Clones

1. Template DNA (cDNA, plasmid clones, etc., see Table 2.1a).
2. PCR primers for cloning (see Table 2.1a and b).
3. Platinum[®] Taq DNA Polymerase High Fidelity (Life Technologies) or equivalent and buffers.
4. Cloning vectors (pDONR-P1P4 (2), pCR[®]2.1-TOPO[®] (Life Technologies) (see Table 2.1a).
5. BP Clonase[®] Enzyme Mix (Life Technologies).
6. MAX Efficiency[®] DH10B[™] Competent Cells (Life Technologies).
7. Luria-Bertani (LB) agar plates containing the appropriate drug.
8. LB medium containing the appropriate drug.
9. PureLink[™] Quick Plasmid Miniprep kit (Life Technologies) or equivalent.
10. BigDye[®] Terminator v3.1 Cycle Sequencing kit (Life Technologies).
11. Sequence primers (see Table 2.1b, M13-Fw, M13-Rv, SeqL-A, SeqL-B).
12. Appropriate restriction enzymes (Sall, SpeI, XbaI) and buffers.
13. Agarose gel and loading buffers.
14. DNA fragment purification kits or following chemicals: phenol saturated with TE (pH 8.0), chloroform, isopropanol, 70% ethanol.
15. T4 DNA ligase and buffers.

TABLE 2.1 Cloning Strategies of Reprogramming Factors and Primer Sequences

(a) Cloning Strategies for Reprogramming Factors						
Clone Name	Sense Primer	Antisense Primer	Antisense Primer2	Template	Method	Vector
pENTR-L1-k-cMyc-*L4	B1-Sall-SpeI-hcMyc-Fw	B4*-hMYC-Rv		FLJ93327AAAN	BP reaction	pDONR-P1P4
pCR2.1-Oct4-2A	Sall-SpeI-hOct4-Fw	2A-hOct4-Rv	XbaI-2Apeptide-Rv	FLJ85586WAAN	TOPO cloning	pCR2.1-TOPO
pCR2.1-Sox2-2A	Sall-SpeI-hSox2-Fw	2A-hSox2-Rv	XbaI-2Apeptide-Rv	FLJ85594AAAN	TOPO cloning	pCR2.1-TOPO
pCR2.1-Klf4-2A	Sall-SpeI-hKlf4-Fw	2A-hKlf4-Rv	XbaI-2Apeptide-Rv	FLJ94042AAAN	TOPO cloning	pCR2.1-TOPO
(b) Primers						
Primer Name	Sequence					
B1-Sall-SpeI-hcMyc-Fw	GGGGCAAAGTTTGTACAAAAAAGCAGGAACCATGGTCGACACTAGTCCCTCAACGTTAGCTTC					
B4*-hMYC-Rv	GGGGCAACTTTGTATAGAAAAGTTGTACGCACAAGAGTTCGGTAG					
Sall-SpeI-hOct4-Fw	GTCGACACTAGTGGCGGGACACCTGGCTTCAGATT					
2A-hOct4-Rv	ATCAAAGTTAAGAGTTGTTTGTGACAGGAGCGACAATTTTGTGTAATGCATGGGAGA					
Sall-SpeI-hSox2-Fw	GTCGACACTAGTTACAACATGATGGAGACG					
2A-hSox2-Rv	ATCAAAGTTAAGAGTTGTTTGTGACAGGAGCGACAATTTTCATGTGTGAGAGGGGCAG					
Sall-SpeI-hKlf4-Fw	GTCGACACTAGTGCTGCAGCGACGCGCTG					
2A-hKlf4-Rv	ATCAAAGTTAAGAGTTGTTTGTGACAGGAGCGACAATTTTAAAAATGCCTCTTCATGTG					
XbaI-2Apeptide-Rv	TCTAGA TGGACCTGGATTGCTTTCTACATCCCCAGCCAGTTTGAGTAAATCAAAGTTAAGAGTTTG					
M13-Fw	CCCAAGTCACGACGTTGTAAACG					
M13-Rv	ACGGGATAACAATTTACACACAGG					
SeqL-A	TCGCGTTAACCGTAGCATGGATCTC					
SeqL-B	GTAACATCAGAGATTTTGAGACAC					
B2r-Fw	GGGGCAGCTTCTCTGTACAAAGTGG					
Rv7-CMV	CTATTGGCGTTACTATGG					

(a) Cloning strategies for reprogramming factors. The strategies for human Oct4, Sox2, Klf4, and c-Myc are summarized in this list. (b) Primers. Sequence of primers for PCR and sequencing are summarized in this list. Asterisks (*) in the names of clones and primers indicates the insertion of a stop codon. Bold sequences in the primer are parts of 2A peptides. Bold and underlined sequences are attB signals. Italic and underlined sequences represent restriction enzyme recognition signals of used in the cloning.

2.3.1.2 Construction of Recombinant BacMam Expression Clones by Multisite Gateway Method

1. Destination vectors (pBV1-DEST, pBV2-DEST (R&D of Life Technologies), pUC-DEST-R3R4 (4), -L4R2 (1)).
2. Entry clones (pENTR-R4-EMCV-R3 (16), -L3-EGFP-*L2 (16), -L1-P_{CAG}-R3 (5), -L3-HindIII-R1 (5), -L1-P_{CAG}-L6 (4), -R6-sdk-Venus-*L2 (4), -L1-sdk-Venus-*L2 (1)).
3. Donor vectors (pDONR-P3P4 (4), -P4rP2 (2)).
4. Appropriate restriction enzymes (HindIII and, etc.) and buffers.
5. Agarose gel and loading buffers.
6. DNA fragment purification kits or following chemicals: phenol saturated with TE (pH 8.0), chloroform, isopropanol, 70% ethanol.
7. T4 DNA ligase and buffers.
8. LR Clonase[®] II Plus Enzyme Mix and BP Clonase Enzyme Mix (Life Technologies).
9. One Shot[®] Mach1[™] T1 Phage-Resistant Chemically Competent *Escherichia coli* and Library Efficiency[®] DB3.1 Competent Cells (Life Technologies).
10. LB agar plates containing the appropriate drug.
11. LB medium containing the appropriate drug.
12. PureLink Quick Plasmid Miniprep kit (Life Technologies) or equivalent.

2.3.1.3 Conversion of BacMam Expression Plasmids into Bacmids by Transposition in Bacteria and Generation of Baculoviral Particles in Insect Cells

1. BacMam expression clones constructed as described in Section 2.3.2.
2. MAX Efficiency DH10Bac Competent *E. coli* (Life Technologies).
3. LB agar plates containing the appropriate drug.
4. LB medium containing the appropriate drug.
5. X-gal solution and isopropylthio- β -galactoside (IPTG) solution.
6. PureLink Quick Plasmid Miniprep kit (Life Technologies) or equivalent.
7. PCR primers for bacmid confirmation (see Table 2.1b, M13-Fw, M13-Rv, B2r-Fw, Rv7-CMV).
8. EX Taq DNA Polymerase (Takara Bio) or equivalent and buffers.
9. Agarose gel and loading buffers.

10. Sf9 Cells, SFM Adapted (Life Technologies).
11. Sf900 SFM (Life Technologies).
12. Grace's Insect Cell Culture Medium, Unsupplemented (Life Technologies).
13. Cellfectin® II Reagent (Life Technologies).
14. Sf900 II (x1.3) (Life Technologies).
15. 4% Agarose solution.
16. 1% Neutral Red solution.

2.3.1.4 Transduction of Mammalian Cells with BacMam Baculoviral Particles

1. BacMam baculoviral particles prepared as described in Section 2.3.3.
2. Mouse embryonic fibroblast cells.
3. HEK293T cells.
4. Embryonic fibroblast medium (EFM): 10% fetal bovine serum (FBS), 0.1 mM (1×) MEM nonessential amino acids solution (NEAA), 1× penicillin–streptomycin–glutamine (PSQ), 100 μM 2-mercaptoethanol (2-ME) in IMDM (Iscove's Modified Dulbecco's Medium). Store at 4°C.
5. DMEM (10% FBS): 10% FBS, 1× PSQ in DMEM (Dulbecco's Modified Eagle Medium), low glucose. Store at 4°C.
6. 1× PBS(–).
7. 0.05% trypsin–EDTA.
8. Premo™ enhancer (Life Technologies) or 1 mM trichostatin A (TSA).
9. Embryonic stem cell medium (ESM): 20% FBS, 0.1 mM (1×) NEAA, 1× PSQ, 100 μM 2-ME, 1× human Leukemia Inhibitory Factor (hLIF) in IMDM. Store at 4°C.
10. RIPA buffer: 150 mM NaCl, 10 mM Tris-HCl (pH 7.2), 5 mM EDTA–NaOH, 0.1% SDS, 1% Triton X-100, 1% deoxycholate in sterile distilled water. Store at 4°C.
11. Complete protease inhibitor, EDTA-free (Roche).
12. Appropriate protein electrophoresis equipments, buffers, and gels.
13. Polyvinylidene fluoride (PVDF) membrane.
14. 1× PBST: 0.05% Tween 20 in 1× PBS.
15. Nonfat milk (NFM).
16. Primary rabbit antibodies (Oct3/4 (Santa Cruz), Sox2 (Novus Biologicals), Klf4 (Santa Cruz), and c-Myc (Santa Cruz)).

17. Secondary antibody (Anti-Rabbit IgG-HRP (GE Healthcare)).
18. Tetramethylbenzidine (TMB) membrane peroxidase substrate (KPL).

2.3.1.5 Isolation of Reprogrammed iPSC Colonies, Maintenance, and Characterization

1. ESM and EFM.
2. $1 \times$ PBS(-).
3. 0.25% trypsin-EDTA.
4. Feeder cells (MEF cells treated with $15 \mu\text{g/mL}$ of mitomycin C for 3 h).
5. BamBanker™ (Lymphotec) or equivalent.
6. Alkaline phosphatase kit (Sigma-Aldrich) or equivalent.
7. 0.01% poly-L-lysine solution.
8. 4% paraformaldehyde in $1 \times$ PBS.
9. 0.2% Triton X-100 in $1 \times$ PBS.
10. 5% NFM in $1 \times$ PBS.
11. Primary antibodies (SSEA-1, rabbit (Millipore), Nanog, mouse (Reprocell)).
12. Secondary antibodies (Anti-rabbit IgG, Alexa Fluor® 568 Conjugate (Life Technologies), Anti-mouse IgM, Alexa Fluor® 488 Conjugate (Life Technologies)).

2.3.2 Methods

2.3.2.1 Cloning of Reprogramming Factors as Gateway Entry Clones

1. cDNAs of human reprogramming factors (c-Myc, Oct4, Sox2, and Klf4) (17) were amplified by either one-step adaptor PCR (18) using primers and templates listed in Table 2.1a. Template clones with ID (e.g., FLJ93327AAAN) are Gateway entry clones that were derived from the Human Genome and Protein Database (HGPD) (19,20).
2. Then the PCR products were cloned into the vectors by the methods listed in Table 2.1a and confirmed by sequencing. Sequences of the PCR and sequencing primers are also shown (Table 2.1b).
3. pCR2.1-Oct4-2A, pCR2.1-Sox2-2A, or pCR2.1-Klf4-2A, was digested with SalI and XbaI and the cDNA fragment with C-terminal foot-and-mouth disease virus (FMDV) 2A peptide was excised. Then each fragment was inserted between neighboring SalI and SpeI sites at N-terminal end of pENTR-L1-k-cMyc-*L4. Resulting entry clones

with tandemly linked cDNA by FMDV 2A peptide sequence also have neighboring SalI and SpeI sites at the N-terminus. Subsequently, another cDNA fragment with 2A peptide sequence digested with SalI and XbaI was inserted between the SalI and SpeI sites and this step was repeated until all four factors are linked with 2A peptide.

4. Two entry clones were constructed in this manner (*attL1-k-Oct4-2A-Sox2-2A-Klf4-2A-cMyc-attL4* and *attL1-k-Oct4-2A-Klf4-2A-Sox2-2A-cMyc-attL4*) and used in further experiments.

2.3.2.2 Construction of BacMam Expression Clones by Multisite Gateway Method

1. Two BacMam destination vectors, pBV1-DEST and pBV2-DEST were kindly provided by R&D of Life Technologies.
2. A modified version of pBV2-DEST was constructed by inserting a CAG promoter (1747 bp), which was excised from HindIII-digested pENTR-L1-P_{CAG}-R3 (5), into unique HindIII site of pBV2-DEST.
3. Expression clones were constructed by Multisite Gateway LR reactions (2,21). The entry clones and destination vectors used to construct each expression clone are listed in Table 2.2. The expression cassettes, which are flanked by Tn7R and Tn7L, are common with that of the bacmids shown in Figure 2.1.

TABLE 2.2 Cloning Strategies for Reprogramming Factors and Primer Sequences

Expression Clone	Destination Vector	Entry Clones (‘pENTR-’ is abbreviated)
pUC-B3-B1-OSKM-B4	pUC-DEST-R3R4	L3-HindIII-R1, L1-k-OSKM- <i>*L4</i>
pUC-B3-B1-OKSM-B4	pUC-DEST-R3R4	L3-HindIII-R1, L1-k-OKSM- <i>*L4</i>
pUC-B4r-EMCV-B3-EGFP-B2	pUC-DEST-L4R2	R4-EMCV-R3, L3-EGFP- <i>*L2</i>
pBV1-pCAG-Venus	pBV1-DEST(R1R2)	L1-P _{CAG} -L6, R6-sdk-Venus- <i>*L2</i>
pBV1-pCAG-OSKMiG	pBV1-DEST(R1R2)	L1-P _{CAG} -R3, L3-B1-OSKM-L4, R4-EMCV-B3-EGFP-L2
pBV1-pCAG-OKSMiG	pBV1-DEST(R1R2)	L1-P _{CAG} -R3, L3-B1-OKSM-L4, R4-EMCV-B3-EGFP-L2
pCAG/BV2-Venus	pCAG/BV2-DEST (R1R2)	L1-sdk-Venus- <i>*L2</i>
pCAG/BV2-OSKMiG	pCAG/BV2-DEST (R1R2)	L1-k-OSKM- <i>*L4</i> , R4-EMCV-B3-EGFP-L2
pCAG/BV2-OKSMiG	pCAG/BV2-DEST (R1R2)	L1-k-OKSM- <i>*L4</i> , R4-EMCV-B3-EGFP-L2

Expression clones were constructed by the Multisite Gateway cloning method with the combination of a destination vector and multiple entry clones summarized in this list. For the names of entry clones, “pENTR” is abbreviated.

4. If more than two *attB* signals with the same number exist in the final construct (e.g., Both pBV1-pCAG-OKSMiG and pBV1-pCAG-OSK-MiG have two *attB*1 and two *attB*3 sites in their expression cassette (Figure 2.1b and c), a stepwise LR reaction method is required, essentially as described (1,4,5).
5. For example, in order to construct pBV1-pCAG-OKSMiG, the expression cassette of which is common with the bacmid bBV1-pCAG-OKSMiG (Figure 2.1b), pUC-B3-B1-OKSM-B4 and pUC-B4r-EMCV-B3-EGFP-B2 were first constructed by Multisite Gateway LR reactions (Table 2.2, line 3 and 4). Then the temporary expression clones were converted to modular entry clones, pENTR-L3-B1-OKSM-L4 and pENTR-R4-EMCV-B3-EGFP-L2, by BP reaction with pDONR-P3P4 and pDONR-P4rP2, respectively. Finally, the modular entry clones and another entry clone, pENTR-L1-P_{CAG}-R3 were cloned into pBV1-DEST (R1R2) by LR reaction (Table 2.2, line 7).

2.3.2.3 Conversion of the BacMam Expression Plasmids into Bacmids and Generation of Baculoviral Particles

1. Generation of recombinant BacMam bacmids and viral particles was performed essentially following the manual of the Bac-to-Bac Baculovirus Expression System (Life Technologies).
2. The purified BacMam expression clone plasmid DNA was used to transform DH10Bac chemical competent cells. The plasmid expression cassette, which is flanked by left and right arm of Tn7 sequences, were transposed into a mini-*att*Tn7 target site of the bacmid, bMON14272, contained in DH10Bac, catalyzed by Tn7 transposase encoded by a helper plasmid, pMON7124, also contained in DH10Bac (22).
3. The recombinant bacmid clones were identified as white colonies by blue–white selection because transposition into the mini-*att*Tn7 target site disrupts the LacZ α peptide in which the target site is situated.
4. After purification of the bacmid DNA, PCR analysis using the primers M13-Rv and one of M13-Fw, B2r-Fw, or Rv-7 CMV was performed to verify the presence of the expression cassette in the recombinant bacmid (see Figure 2.1).
5. Once the recombinant bacmids were obtained, they were transfected into SF9 cells by using Cellfectin Reagent to generate recombinant baculoviral particles (P1) that can be used for preliminary expression experiments.

6. Titer of the P1 viral stock was determined by plaque assay and single plaques were purified (if necessary).
7. The P1 stock or the plaque-purified stock was used for a second and third round of transduction into SF9 cells to generate P2 and P3 stocks. After the baculoviral stocks were amplified and the titer was determined, these high-titer stocks (about 5×10^7 pfu/mL) were stored at 4°C in the dark.

2.3.2.4 Transduction of Mammalian Cells with BacMam Baculoviral Particles

1. Transduction of MEF cells with BacMam particles was performed essentially following the Premo™ Cameleon Calcium Sensor manual (Life Technologies).
2. Mammalian cells were seeded in culture media (EFM or DMEM, 10% FBS) for MEF or HEK293T, respectively, at appropriate density (1.3×10^5 cells/well or 4.0×10^5 cells/well for MEF or HEK293T, respectively) in 6-well plate 1 day before transduction. The amount of the materials was reduced (or increased) according the scale of the experiment.
3. On the day of the first transduction, the medium was replaced with a mixture of 400 µL BacMam particles and 700 µL PBS medium then incubated for 4 h at room temperature. Then the medium was removed and 2 mL EFM containing 2 µL of Premo enhancer was added followed by incubation for 2 h at 37°C, 5% CO₂. Medium was changed to normal EM medium and incubated for 3 days at 37°C, 5% CO₂.
4. The second and the third transductions were performed on day 3 and day 6, if necessary. The protocols were almost identical with the first transduction unless incubation time with BacMam particles were shortened from 4 to 2 h and ESM were used instead of EFM at the step of final medium change.
5. The expression of fluorescent protein was confirmed by fluorescence microscopy (Figures 2.2 and 2.3) and the transduction efficiency was measured by FACS analysis (Figure 2.2). The expression of the reprogramming factors were confirmed by Western blotting (Figure 2.3).

2.3.2.5 Isolation of Reprogrammed iPSC Colonies, Maintenance, and Characterization

1. Transduction of MEF with BacMam particles expressing reprogramming factors was performed as described in Section 2.3.4, then 2 days

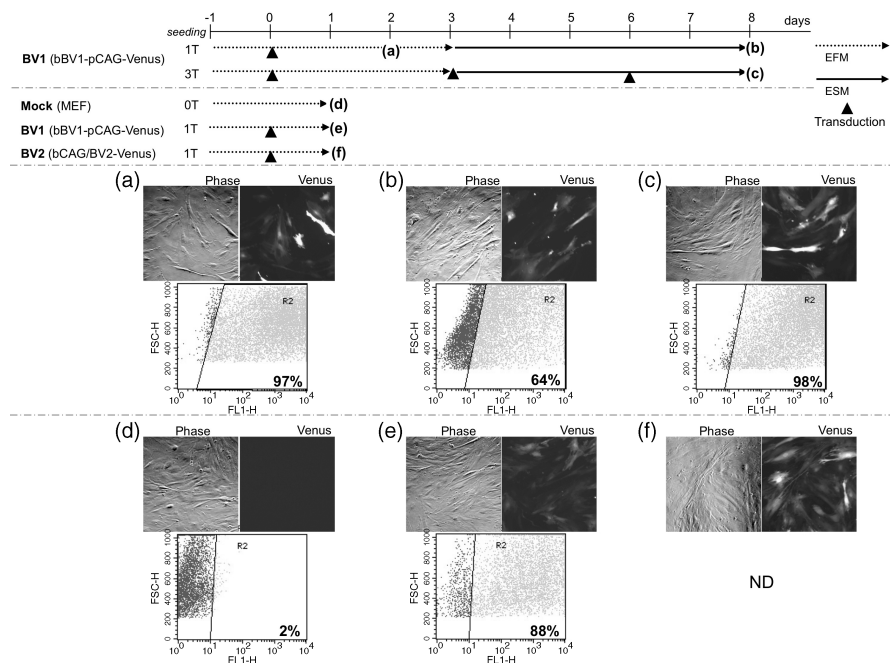


FIGURE 2.2 Transduction efficiency with a reporter Venus gene directed from CAG promoter. For investigating transduction efficiency of MEF cells, we used BacMam particle bBV1-pCAG-Venus (BV1, Figure 2.1a) as a reporter. The diagram at the top shows the timeline of baculoviral transduction and culturing conditions. Dotted arrows indicate the period of culturing in EFM. Solid arrows indicate the period of culturing in ESM. Closed triangles indicate baculoviral transductions. The middle panels (a–c) represent phase contrast and Venus fluorescence images shown together with a FACS scatter plots for each condition. In the first experiment, a single transduction and successive triple transduction are compared. On the 2nd day after the first transduction (a), 97% of MEF Venus fluorescence positive and 98% retained fluorescence on the 8th day after three transductions (c), while only 64% retained fluorescence for MEF with single transduction (b). In the bottom panels (d–f), two BacMam reporter with different backbones (BV1 and BV2, Figure 2.1d), are compared. On the day after transduction, BV2 with VSV-G and WPRE elements (f) was obviously more efficient in transducing MEF cells than BV1, which lacks VSV-G and WPRE elements (e). MEF with mock transduction (d) had barely detectable fluorescence. (See the color version of this figure in Color Plates section.)

after third transduction (day 8), cells were passaged onto feeder cells plated at a density of 1×10^6 cells/10 cm dish 1 day before usage. Isolation of mouse iPSC colonies were essentially followed the protocol distributed by Center for iPS Cell Research and Application (CiRA), Kyoto University (23).

2. The medium was changed every 2 days until visible colonies were formed on MEF.

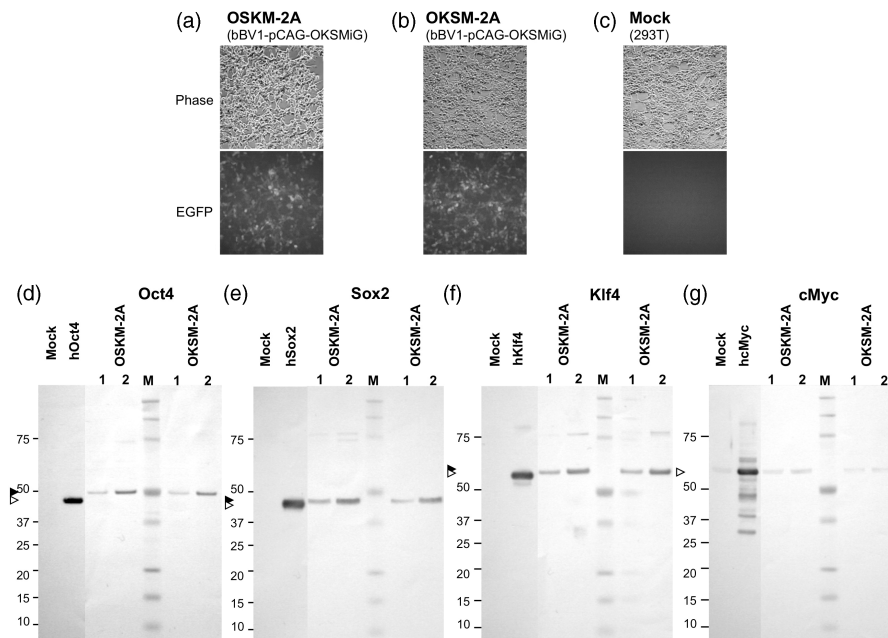


FIGURE 2.3 Western blot analyses to ascertain expression of reprogramming factors. BacMam particles were prepared for OSKM-2A (Figure 2.1c) and OKSM-2A (Figure 2.1b). Single transduction of HEK293T cells using these BacMam particles was carried out at a cell density of 1×10^5 cells/well in 24-well plate. Two days after transduction, fluorescence of EGFP was observed by microscopy for OSKM-2A (a), OKSM-2A (b), and mock (c). The cellular proteins were then harvested by lysing the cells in 50 μ L RIPA buffer. Protein electrophoresis was performed with appropriate equipment, buffers, and gels. Ten micrograms of total protein was loaded to each well. Lysates of 293T cell transfected with expression clones of single reprogramming factors (hOct4, hSox2, hKlf4, hCMyC) were used as positive controls. (d–g) After transferring the proteins to a PVDF membrane, the protein expression of reprogramming factors was detected by primary antibodies of Oct4 (d), Sox2 (e), Klf4 (f), and cMyc (g), followed by secondary antibody conjugated with HRP and TMB. The lanes marked by M are size markers. Open triangles indicate the sizes of native reprogramming factors and closed triangles indicate the sizes of recombinant reprogramming factors with 2A peptides. All of the reprogramming factors were expressed at detectable level, and lot 2 for both OSKM-2A and OKSM-2A had superior transduction efficiency than lot 1 in four panels shown in (d–g). Endogenous c-Myc is expressed in 293T cells of mock transduction (g). (See the color version of this figure in Color Plates section.)

3. When colonies were observed (days 20–35), they were each picked using a sterile micropipette tip and transferred separately to 10 μ L of 0.25% trypsin–EDTA in 96-well plate and incubated for 5 min at 37°C.
4. After 90 μ L of ESM was added to each well and the cells suspended by pipetting, they were transferred to 500 μ L of ESM in 48-well plate

(which were previously plated by feeder cells at a density of 2.5×10^4 cells/well 1 day before usage).

5. The clonal iPS cell lines were maintained by passaging every 2 days in ESM. Freeze stocks were made using Bambanker™ at early stages of maintenance.
6. The character of the iPS cell lines was initially validated by alkaline phosphatase staining and immunofluorescence analyses with Nanog and SSEA-1 as pluripotency markers (Figure 2.4). The conditions of the alkaline phosphatase assay and immunofluorescent analysis are as described in a previous report (24).

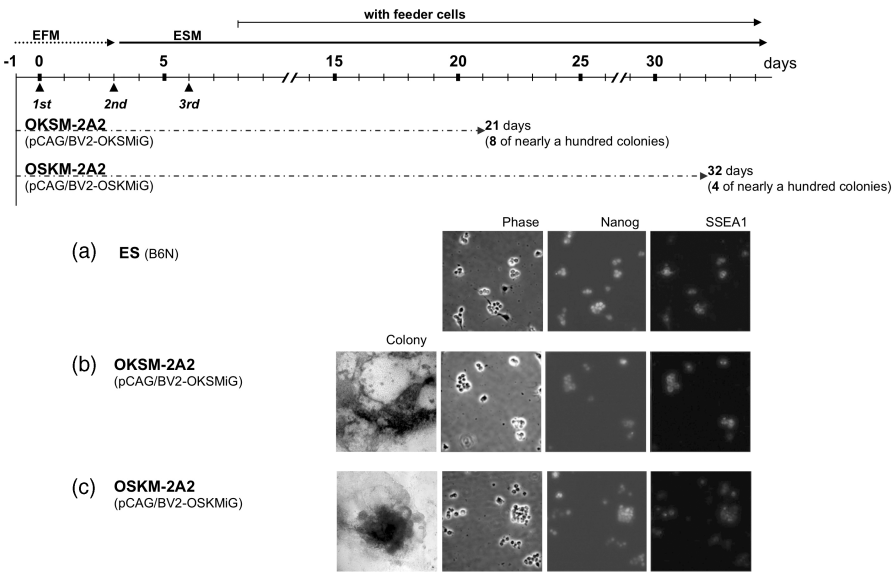


FIGURE 2.4 Generation of iPS cells using BacMam particles harboring OKSM and OSKM constructs. Successive triple transduction of MEF with OKSM-2A2 (Figure 2.1e) and OSKM-2A2 (Figure 2.1f) BacMam particles at a cell density of 1.3×10^5 cells/well in a 6-well plate was performed and colonies of iPS cells were isolated under timelines and conditions shown in the diagram at the top. In the end, 8 and 4 iPS cell lines were obtained from nearly a hundred colonies for each, OKSM-2A2 and OSKM-2A2 BacMam particles respectively. The efficiency and the timing of colony formation were significantly better for OKSM-2A2 than OSKM-2A2. Although the efficiency was lower, iPS colonies were also obtained by single transduction of the same particles and successive triple transduction of version 1 BacMam particles (OKSM-2A and OSKM-2A) (data not shown). Imaging profiles and immunofluorescent analyses for Nanog and SSEA-1 are shown for the iPS cell lines derived from OKSM-2A2 (b) and OSKM-2A2 (c) transductions. The control imaging panels with mouse ES cells (PRX-B6N) are also shown (a). (See the color version of this figure in Color Plates section.)

2.4 PERSPECTIVES

Creation of induced pluripotent stem cells is one example of a system where expression of multiple genes in proper stoichiometric amounts is necessary for a particular outcome. While the exact mechanism of cellular reprogramming is not clear at this time, it is evident that the process is most likely complex and involves multiple signaling pathways. The days of one gene, one effect and perhaps waning and a more systematic approach must be taken in the study of cellular function.

Expressing a collection of genes, reporters, and so on, from a single construct has several advantages. These include stoichiometric expression of the genes of interest relative to each other when the construct is delivered transiently (as in the example described above using baculovirus). Further, when a multigene construct is targeted to a specific locus, one can control the absolute expression of several genes since the resulting cell line will have a defined copy number. This can be a double edged sword in that a specific locus may be highly expressed in one circumstance (i.e., embryonic stem cells) and partially or completely repressed in another (i.e., the same cells differentiated down a lineage). There are techniques to mitigate this, such as use of genetic insulators but one is generally better served if a well behaved locus is fully validated prior to creation of a cell line.

There are several ways to assemble multiple DNA fragments in a single reaction and we feel that Multisite Gateway is the most efficient for many applications. However, demand for new and more robust gene assembly technology is coming from the field of synthetic biology so it may not be long before other methods gain acceptance and can be used either as a compliment or instead of what is currently available. Regardless of the evolution of technology, expression of multiple genes and other elements in a single, controllable operon will remain essential for future functional genomics applications.

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system. Gateway Platinum, pCR, TOPO, BigDye, One Shot, Max Efficiency, Library Efficiency, Cellfectin, and Alexa Fluor are registered trademarks of Life Technologies. Clonase, PureLink, pDONR, DH10B, DH10Bac, Mach1, Premo, Pentr, and pDEST are trademarks of Life Technologies.

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