Journal Club: iPSCs

Jesse Levine
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Gholson Lyon
Pluripotent Stem Cells Induced from Mouse Somatic Cells by Small-Molecule Compounds

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Goals

• Develop a combination of small molecule compounds capable of reprogramming mouse somatic cells into pluripotent stem cells in the absence of exogenous “master genes”
<table>
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<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Function</th>
<th>Structure</th>
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<tr>
<td>Valproic acid sodium salt</td>
<td>VPA, V</td>
<td>Histone deacetylase inhibitor</td>
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<tr>
<td>CHIR99021</td>
<td>CHIR, C</td>
<td>GSK3-β inhibitor</td>
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<tr>
<td>616452</td>
<td>6</td>
<td>TGF-β inhibitor</td>
<td><img src="image" alt="616452" /></td>
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<tr>
<td>Tranylcypromine</td>
<td>Tranyl, T</td>
<td>H3K4 demethylation inhibitor</td>
<td><img src="image" alt="Tranylcypromine" /></td>
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</tbody>
</table>

Table S1 (B)
• **Part 1: Find Oct4 substitute**
• Part 2: Test small molecule cocktail
• Part 3: Screen for late reprogramming molecule
• Part 4: Resolve incomplete reprogramming
• Part 5: Optimize cocktail
• Part 6: Screen for reprogramming booster
• Part 7: Additional cells of origin
• Part 8: Characterize CiPSC lines
• Part 9: Determine essential small molecules
• Part 10: Investigate role of small molecules
Part 1: Find Oct4 Substitute

Mice:

• OG - Oct4/EGFP transgenic C57BL/6J mice carrying EGFP (enhanced green fluorescence protein) under control of an Oct4 18-kb genomic fragment containing the minimal promoter and proximal and distal enhancers; can come close to mimicking the endogenous embryonic expression pattern of Oct-4 in transgenic mice
Part 1: Find Oct4 Substitute

Methods:

- Lentiviral infection: Sox2, Klf4 and c-Myc (SKM)

- Small molecule screen
  - 20k OG MEFs/well; 12 well plate
  - Infect with lentivirus encoding SKM
  - Replace with LIF-free ESC culture medium
  - Add individual chemicals from small-molecule libraries to each well
  - Change medium and chemicals every 4 days
  - 14-20 days or until GFP+ colonies
  - Primary hits confirmed and optimized

### Part 1: Find Oct4 Substitute
Small molecule libraries

<table>
<thead>
<tr>
<th>Library</th>
<th>Source</th>
<th>Number of small-molecule compounds</th>
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<tbody>
<tr>
<td>BBP-2080NPs library</td>
<td>BioBioPha</td>
<td>2,080</td>
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<tr>
<td>The Spectrum Collection</td>
<td>MicroSource Discovery Systems</td>
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<tr>
<td>Sigma LOPAC®(^{,1280})</td>
<td>Sigma</td>
<td>1,280</td>
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<tr>
<td>Prestwick Chemical Library(^{®})</td>
<td>Prestwick Chemical</td>
<td>1,200</td>
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<td>Tocriscreen(^{TM}) Total</td>
<td>Tocris</td>
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<td>ICCB Known Bioactives Library</td>
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<td>Protein Kinase Inhibitor Library I, II, III</td>
<td>Millipore</td>
<td>324</td>
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<tr>
<td>StemSelect Small Molecule Regulators</td>
<td>Calbiochem</td>
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<td>Nuclear Receptor Ligand Library</td>
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<td>76</td>
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<tr>
<td>Selected Small Molecules*</td>
<td>Our lab</td>
<td>88</td>
</tr>
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</table>

*This library was generated in-house, including 88 selected small molecules related to pluripotency, reprogramming or epigenetic modification.

Table S1 (A)
Part 1: Find Oct4 Substitute
SKM/SK: Primary hits

Fig. 1
Part 1: Find Oct4 Substitute
SKM/SK: Primary hits

<table>
<thead>
<tr>
<th>Name</th>
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<tbody>
<tr>
<td>Forskolin</td>
<td>FSK, F</td>
<td>Activates adenylate cyclase</td>
<td><img src="image1.png" alt="" /></td>
</tr>
<tr>
<td>2-Methyl-5-hydroxytryptamine</td>
<td>2-Me-5HT</td>
<td>5-HT3 agonist</td>
<td><img src="image2.png" alt="" /></td>
</tr>
<tr>
<td>D4476</td>
<td></td>
<td>CK1 inhibitor</td>
<td><img src="image3.png" alt="" /></td>
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</table>
Part 1: Find Oct4 Substitute

Characterization of iPSC colonies induced from SKM or SK-infected MEFs with FSK treatment

Fig. S1
Part 1: Find Oct4 Substitute

Characterization of iPSC colonies induced from SKM or SK-infected MEFs with 2-Me-5HT or D4476 treatment

Fig. S2
• Part 1: Find Oct4 substitute  
• **Part 2: Test small molecule cocktail**  
• Part 3: Screen for late reprogramming molecule  
• Part 4: Resolve incomplete reprogramming  
• Part 5: Optimize cocktail  
• Part 6: Screen for reprogramming booster  
• Part 7: Additional cells of origin  
• Part 8: Characterize CiPSC lines  
• Part 9: Determine essential small molecules  
• Part 10: Investigate role of small molecules
### Part 2: Test small molecule cocktail

**VC6TF**

<table>
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Table S1 (B)
Part 2: Test small molecule cocktail

VC6TF

Mice: OG-MEFs

Methods:

• Plate cells: 50k/well; 6 well plate
• Replace medium with chemical reprogramming medium containing small molecule cocktail
• Change medium every 4 days
Part 2: Test small molecule cocktail

VC6TF: Characterization of GFP+ clusters; day 24

Fig. S3
• Part 1: Find Oct4 substitute
• Part 2: Test small molecule cocktail
• **Part 3: Screen for late reprogramming molecule**
• Part 4: Resolve incomplete reprogramming
• Part 5: Optimize cocktail
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• Part 8: Characterize CiPSC lines
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• Part 10: Investigate role of small molecules
Part 3: Screen for late reprogramming molecule

Mice:
• Infected OG MEFs
• MEFs with DOX-inducible Oct4 from Tet-On POU5F1 strain B6;129-Gt(ROSA)26Sortm1(rtTA*M2)Jae Col1a1tm2(tetO-Pou5f1)Jae/J

Methods:
• Infect OG MEFs with Fu-tet-hOct4 and FUdeltaGW-reTA lentiviruses
• Culture medium containing VC6T and DOX (DOX first 4-8 days)
• Individual chemicals from small-molecule library in each well
• Change medium and chemicals every 4 days
• Continue 16-24 days or until GFP+ colonies appear
• Confirm and optimize primary hits
Part 3: Screen for late reprogramming molecule VC6T + DOX: Primary hits

**Table S1 (B)**

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<td>3-deazaneplanocin</td>
<td>DZNep, Z</td>
<td>S-Adenosylhomocysteine Hydrolase inhibitor and histone methyltransferase EZH2 inhibitor</td>
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Part 3: Screen for late reprogramming molecule

VC6TFZ

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<td><img src="image" alt="3-deazaneplanocin" /></td>
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Table S1 (B)
Part 3: Screen for late reprogramming molecule

VC6TFZ: GFP positive cells induced

Fig. 1

Fig. S5
• Part 1: Find Oct4 substitute
• Part 2: Test small molecule cocktail
• Part 3: Screen for late reprogramming molecule
• **Part 4: Resolve incomplete reprogramming**
• Part 5: Optimize cocktail
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• Part 7: Additional cells of origin
• Part 8: Characterize CiPSC lines
• Part 9: Determine essential small molecules
• Part 10: Investigate role of small molecules
Part 4: Resolve incomplete reprogramming

VC6TFZ: timeline of CiPSC generation

Fig. 1
Part 4: Resolve incomplete reprogramming

VC6TFZ: RNA-seq analysis of GFP positive colonies (without 2i medium/incomplete) and CiPSCs

Heat map:
- Value in the color key indicates $\log_2$ changes
- Generated using R

Fig. S6
Part 4: Resolve incomplete reprogramming

2i medium:
- Small molecule inhibition of MAP kinase (MEK) and glycogen synthase kinase 3 (GSK3)
- MEK inhibition is the main reprogramming cue in 2i and also exerts selection against pre-iPS cells
  - Phospho-Erk (p-Erk) signal extinguished
  - Upregulation of Nanog expression
- GSK inhibition generates intracellular β-catenin, which interacts with Tcf3 and abolishes its repressor effect on multiple genes in the pluripotent network
- GSK inhibition also supports embryonic stem cell propagation through stimulatory effects on metabolic and biosynthetic processes
- 2i treatment does not select for expansion of an already resident pluripotent subpopulation, but actively induces conversion to pluripotency in pre-iPS cell

Hamazaki et al, 2006
Wray et al, 2011
• Part 1: Find Oct4 substitute
• Part 2: Test small molecule cocktail
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• Part 4: Resolve incomplete reprogramming
• **Part 5: Optimize cocktail**
• Part 6: Screen for reprogramming booster
• Part 7: Additional cells of origin
• Part 8: Characterize CiPSC lines
• Part 9: Determine essential small molecules
• Part 10: Investigate role of small molecules
Part 5: Optimize cocktail
VC6TFZ: concentrations and treatment durations of individual chemicals
• Part 1: Find Oct4 substitute
• Part 2: Test small molecule cocktail
• Part 3: Screen for late reprogramming molecule
• Part 4: Resolve incomplete reprogramming
• Part 5: Optimize cocktail
• **Part 6: Screen for reprogramming booster**
• Part 7: Additional cells of origin
• Part 8: Characterize CiPSC lines
• Part 9: Determine essential small molecules
• Part 10: Investigate role of small molecules
Part 6: Screen for reprogramming booster

VC6TFZ + TTNPB: effect of TTNPB and characterization of generated CiPSCs
### Part 6: Screen for reprogramming booster

VC6TFZ + TTNPB: effect of TTNPB and characterization of generated CiPSCs

<table>
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<tr>
<th>Name</th>
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<th>Function</th>
<th>Structure</th>
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<td>TTNPB</td>
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<td>Selective and highly potent retinoic acid analog with affinity for retinoic acid receptors (RAR) α, β, and γ, which are nuclear transcription factors. Produces ligand-activated transcription of genes that possess retinoic acid responsive elements.</td>
<td>![Structure Image]</td>
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</table>

Table S1 (B)
• Part 1: Find Oct4 substitute
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• Part 8: Characterize CiPSC lines
• Part 9: Determine essential small molecules
• Part 10: Investigate role of small molecules
Part 7: Additional cells of origin

Methods:
- Plate cells: 50k/well; 6 well plate
- Replace medium with chemical reprogramming medium containing small molecule cocktail
- Change medium every 4 days
- DZNep added day 16 or day 20
- Small molecule cocktail (including DZNep) removed day 20; replace with 2i medium

Fig. 1
Part 7: Additional cells of origin

VC6TFZ: morphology of CiPSC colonies generated from MNFs, MAFs, ADSCs and WT MEFs; genomic PCR analyzing pOct4-GFP cassettes in the CiPSCs derived from WT MEFs
• Part 1: Find Oct4 substitute
• Part 2: Test small molecule cocktail
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• Part 4: Resolve incomplete reprogramming
• Part 5: Optimize cocktail
• Part 6: Screen for reprogramming booster
• Part 7: Additional cells of origin
• Part 8: Characterize CiPSC lines
• Part 9: Determine essential small molecules
• Part 10: Investigate role of small molecules
Part 8: Characterize CiPSC lines
VC6TFZ: CiPSCs free of transgene contamination
Part 8: Characterize CiPSC lines

VC6TFZ: MEF-derived
Part 8: Characterize CiPSC lines

VC6TFZ

MNF

ADSC

MAF

WT MEF

Fig. S11
Part 8: Characterize CiPSC lines

VC6TFZ

Fig. S12
Part 8: Characterize CiPSC lines
VC6TFZ: Histone H3 modifications at Oct4, Sox2 and Nanog promoter regions

Fig. S13
Part 8: Characterize CiPSC lines
VC6TFZ: genetic integrity of CiPSCs
Part 8: Characterize CiPSC lines

VC6TFZ: pluripotency of CiPSCs

Fig. 3
Part 8: Characterize CiPSC lines

VC6TFZ: pluripotency of CiPSCs

Fig. S15
Part 8: Characterize CiPSC lines
VC6TFZ: in vivo developmental potential of CiPSCs
• Part 1: Find Oct4 substitute
• Part 2: Test small molecule cocktail
• Part 3: Screen for late reprogramming molecule
• Part 4: Resolve incomplete reprogramming
• Part 5: Optimize cocktail
• Part 6: Screen for reprogramming booster
• Part 7: Additional cells of origin
• Part 8: Characterize CiPSC lines
• **Part 9: Determine essential small molecules**
• Part 10: Investigate role of small molecules
Part 9: Determine essential small molecules

Fig. 4
Part 9: Determine essential small molecules

Characterization of CiPSCs induced by C6FZ
• Part 1: Find Oct4 substitute
• Part 2: Test small molecule cocktail
• Part 3: Screen for late reprogramming molecule
• Part 4: Resolve incomplete reprogramming
• Part 5: Optimize cocktail
• Part 6: Screen for reprogramming booster
• Part 7: Additional cells of origin
• Part 8: Characterize CiPSC lines
• Part 9: Determine essential small molecules
• **Part 10: Investigate role of small molecules**
Part 10: Investigate role of small molecules

Biological activity of FSK during chemical reprogramming

Fig. S17
Part 10: Investigate role of small molecules

Function of DZNep in chemical reprogramming

Fig. 4
Part 10: Investigate role of small molecules

Function of DZNep in chemical reprogramming

[Graph showing relative ratio of SAH/SAM and number of CIPS colonies per 300,000 replated cells]
Part 10: Investigate role of small molecules

Function of DZNep in chemical reprogramming

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<th>32D</th>
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**Fig. S19**
Part 10: Investigate role of small molecules

VC6TFZ: Gene expression during chemical reprogramming

Fig. S21
Part 10: Investigate role of small molecules

VC6TFZ: Gene expression during chemical reprogramming
Part 10: Investigate role of small molecules
VC6TFZ: Gene expression during chemical reprogramming

Fig. S22 & S23
Part 10: Investigate role of small molecules
VC6TFZ: effect of specific chemicals on gene expression
Part 10: Investigate role of small molecules

Overexpression of Sall4 and Sox2: Oct-4 promoter-driven luciferase reporter

Fig. S25
Part 10: Investigate role of small molecules

VTZ: overexpression of Sall4 and Sox2

Fig. 4

Fig. S26
Part 10: Investigate role of small molecules

VC6TFZ: Effects of knockdown on gene expression

Fig. S27
Part 10: Investigate role of small molecules

VC6TFZ: Effects of knockdown on expression of Oct4 and iPSC formation
Part 10: Investigate role of small molecules
Summary

Fig. S30
What’s next

• Human somatic cells
• Improve efficiency
• Differentiation of CiPSCs
• Direct reprogramming
Additional information
Part 1: Find Oct4 Substitute

Characterization of iPSC colonies induced from SKM or SK-infected MEFs with FSK treatment

Methods:
• Immunofluorescence
  - Primary antibodies: SSEA-1, OCT4, NANOG, UTF1
  - Secondary antibodies: Rhodamine-conjugated
• Chimera:
  - Blastocyst injection:
    • Injection needle
    • 10-15 CiPSCs into embryo cavity of F2 or CD-1 female mice at 3.5 days post coitum
    • Transferred into 2.5 day pseudopregnant females
  - Eight cell embryo injection:
    • XYClone laser system
    • Collected from female mice at 2.5 days; 7-10 CiPSCs injected into each embryo
    • Transferred into .5 day pseudopregnant females
  - Chimeric mice identified by coat color
  - Assessed for germline transmission by mating with ICR mice

• RT-PCR
  - Isolate RNA
  - Convert to cDNA
  - Carry out PCR
  - Analysis of data using delta-delta Ct method
• Scatter plot – DNA microarray
  - Total mRNA was labeled with Cy5, hybridized to a mouse Oligo Microarray
  - Red line = boundary for two-fold change
  - R = Pearson’s coefficient

Fig. S1
Cell culture

• Cells used in reprogramming were passage 1-5
• Cells cultured in DMEM/High glucose containing 10% fetal bovine serum

• ESCs, iPSCs and CiPSCs maintained on feeder layers of mitomycin C-treated (halts division) MEFs in ESC culture medium (KnockOut DMEM containing 10% knockout serum replacement, 10% FBS, 2mM GlutaMAX-I, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol, 1% penicillin-streptomycin and 1,000 U/ml leukemia inhibitory factor)

• For CiPSC induction, LIF-free ESC culture medium supplemented with 20-100ng/ml bFGF
Part 2: Test small molecule cocktail
VC6TF: Characterization of GFP+ clusters; day 24

Methods:
• Bisulfite genomic sequencing
  - Genomic DNA modified by bisulfite treatment and purified
  - Amplified fragments cloned into pEASY-blunt vector
  - Ten randomly picked clones from each sample were sequenced
• RNA-seq:
  - RNA sequencing libraries constructed
  - Fragmented and randomly primed 200bp paired-end libraries were sequenced using Illumina HiSeq 2000

Fig. S3
Part 3: Screen for late reprogramming molecule

VC6TFZ: GFP positive cells induced

Methods:
Flow cytometry analysis:
• Cultured cells trypsinized into single cells and resuspended in PBS containing 3% FBS
• Using endogenous Oct4-GFP, FACS analyses preformed with FACSCalibur instrument
Part 6: Screen for reprogramming booster

2-Me-5HT

IBMX

PGE2

SRT1720

TTNPB

UNC0638

RG108

SF1670

DY131

Table S1 (B)
Part 8: Characterize CiPSC lines
VC6TFZ: Histone H3 modifications at Oct4, Sox2 and Nanog promoter regions

Methods:
Chromatin immunoprecipitation (ChIP):
• EZ-Magna ChIP A/G kit
• Anti-H3K27me3, Anti-H3K9me2, Anti-H3K4me3, Anti-H3K9ac antibodies used
• Following immunoprecipitation, DNA analyzed by real-time PCR

Fig. S13
Part 8: Characterize CiPSC lines

VC6TFZ: genetic integrity of CiPSCs

Comparative genomic hybridization analysis:
• Genomic DNA extracted and hybridized to mouse whole-genome tiling arrays

Fig. S14
Part 8: Characterize CiPSC lines
VC6TFZ: pluripotency of CiPSCs

Methods:
Teratoma formation:
• $10^5$ iPSC cells were injected into the kidney capsule of one severe combined immunodeficient beige mouse
• Teratomas were recovered 4 weeks after grafting
• Control mice were injected with 1 million MEFs and failed to form teratoma
• Embedded in paraffin and processed with hematoxylin and eosin staining
Part 10: Investigate role of small molecules
Overexpression of Sall4 and Sox2: Oct-4 promoter-driven luciferase reporter

Methods:
• MEFs plated 40,000 cells/well; 24 well plate
• Transiently transfected with Oct4 promoter reporters using Lipofetamine LTX and Plus Reagent
• pRL-TK plasmids cotransfected in each well as internal references
• Total DNA concentrations for all transfections were equalized by adding empty pLL3.7-ΔU6 vector
• At 48 hours, cells washed and lysed
• Luciferase activity measured with Dual-luciferase Reporter Assay system and normalized to Renilla luciferase activity
• Empty expression vector plasmids used as negative control

Fig. S25
Part 10: Investigate role of small molecules

VC6TFZ: Effects of knockdown on gene expression

shRNA silencing

Fig. S27