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Significant improvement of reprogramming efficiency by transient overexpression of ZGA inducer Dux but not Dppa2/4

Lei Yang[1], Xuefei Liu[1], Lishuang Song[1, 2], Anqi Di[1, 2], Guanghua Su[1, 2], Chunling Bai[1, 2], Zhuying Wei[1, 2], and Guangpeng Li[1, 2]

1 State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock (R_2BGL), Inner Mongolia University, Hohhot 010070, China.

2 College of Life Sciences, Inner Mongolia University, Hohhot 010070, China.

Correspondence: Guangpeng Li (gpengli@imu.edu.cn)

Running Title: Dux promotes somatic cell reprogramming

Abstract

Cloned animal have been reported by somatic cell nuclear transfer (SCNT) for many years. However, the SCNT biotechnology is extremely inefficient, and zygotic genome activation (ZGA) is required for SCNT and chemical mediated somatic cell reprogramming. To identify candidate factors that facilitate ZGA in SCNT reprogramming, we carried out a siRNA-repressor and mRNA-inducer screening in SCNT to identify epigenetic and chromatin regulators of the ZGA transcriptional program. Through a series of experiments, we demonstrate that transient overexpression of Dux not only improved SCNT efficiency, but also increased the efficiency of chemical reprogramming. Furthermore, transient overexpression of Dux combined with inactivation of DNA methyltransferases (Dnmts) further promote the overall development of SCNT-derived animals. These findings enhance our understanding of ZGA-regulators in SCNT and chemical mediated somatic cell reprogramming, and suggest that transient overexpression of Dux may serve as a useful tool to enhances the somatic reprogramming efficiency.

Introduction

The scientists at the Institute of Neuroscience (Chinese Academy of Sciences), recently cloned the world's first monkeys Zhong Zhong and Hua Hua (the Zhong Hua is a mandarin term for china) [1]. After the first mammal Dolly sheep to be cloned [2], the created non-human primate has once again attracted worldwide attention. Indeed, successful cloning of the sheep and monkey not only made reproductive cloning possible, but also raised the possibility of therapeutic cloning. Somatic cell nuclear transfer (SCNT) is the technical term for cloning in which terminal differentiated somatic cell is transferred into enucleated oocyte, in which the cytoplasmic factors reprogram the nucleus to become a zygote-like state [3]. Both SCNT derived and Yamanaka factors induced pluripotent stem (iPS) cells have the potential to generate patient-specific pluripotent stem cells for replacement therapy. Compared with iPS, the SCNT reprogramming is transgene-free and oncogene-free methodologies. Thus, SCNT derived pluripotent stem cells would be more suitable for human therapeutic applications.

Following fertilization, the newly formed zygotic genome is activated through a process known as the zygotic genome activation (ZGA), which enables zygotes can subsequently drive the differentiation of all the diverse cell types of the adult animal [4]. A similar mechanism is likely used in ZGA of SCNT embryos [3,5]. The 'erase-and-rebuild' strategy is used to establish the zygotic transcription program during SCNT reprogramming [5]. Consistently, our and others recent results have revealed that incomplete ZGA is a major SCNT reprogramming barrier [6-8]. Although many advances have been made in SCNT technology via different epigenetic regulators, the SCNT for producing cloned animals still inefficient.

Materials and Methods

Animals and Chemicals

Specific pathogen-free (SPF)-grade BDF1 (C57BL/6N × DBA/2), CD1, and Kun-Ming (KM) mice mice were purchased from Laboratory Animal Research Center (Inner Mongolia University) or Vital River Laboratories (Beijing, China). All animal experiments were approved by the Animal Care and Use Committee of Inner Mongolia University. All procedures were carried out in strict accordance with the recommendations made in the Guide for the Care and Use of Laboratory Animals of the National Veterinary and Quarantine Service. MERLV:: tdTomato transgenic mice, which carry a transgenic MERLV promoter LTR driven tdTomato reporter [8], were mated with the same positive mice. dDux-MEFs cells were isolated from E12.5 fetus. The MERLV:: tdTomato embryos used in the experiment were produced by MERVL::tdTomato sperm and MII oocytes from the littermates of transgenic mice. All chemicals used in this study were purchased from Sigma (USA), unless otherwise indicated.

SCNT and embryo culture

The SCNT processes followed previously published studies [8,9]. Briefly, groups of ~50 MII oocytes were transferred to a chamber containing oil-covered M2 supplemented with 5 μg/ml cytochalasin B (CB). The spindle-chromosome complex (SCC) was removed by a blunt Piezo-

68 driven pipette (~10 μm internal diameter) on a 37°C heating stage of an inverted microscope
69 (Nikon, Japan). The nuclei of donor cells were drawn in and out of the injection pipette until its
70 plasma membrane was broken and was then injected into enucleated oocytes. The
71 reconstructed embryos were cultured in aMEM medium (Thermo, USA) containing 10% fetal
72 calf serum (FCS; Hyclone, USA) for 1 h before activation treatment. The reconstructed
73 embryos were activated in Ca^{2+} free KSOM medium containing 10 mM strontium and 5 μg/ml
74 CB for 6 h. Activated embryos were thoroughly washed and cultured in G1/G2 medium (1:1,
75 vol / vol; Vitrolife, Sweden). Induction of Dux was performed by administration of doxycycline
76 (2 μg/ml) in the G1/G2 medium.
77
78 **Immunofluorescence staining**
79 Embryos and CiPS cells were rinsed three times in phosphate-buffered saline (PBS) with 0.3%
80 BSA, fixed with 4% paraformaldehyde (PFA) overnight at 4°C, and then permeabilized with
81 0.2% (vol / vol) Triton X-100 for 15 min at room temperature, followed by washing thoroughly
82 in PBS containing 0.3% BSA. Fixed samples were blocked in 0.05% Tween 20 in PBS
83 containing 3% BSA (PBST) at 37°C for 1 h and then incubated with the primary antibodies
84 overnight at 4°C. Samples were incubated with primary antibodies against HA (Santa Cruz,
85 sc-7392, USA), Oct4 (Santa Cruz, sc-8629, USA), and Ssea1 (Santa Cruz, sc-21702, USA).
86 After incubating, the samples were needed to wash several times in PBST and then incubated
87 with appropriate secondary antibodies conjugated with Alexa Fluor 594 and Alexa Fluor 488
88 (Thermo, USA) for 1 h at 37°C. For imaging, the embryos were mounted in 10 μl anti-fade
89 solution with DAPI (Thermo, USA) and compressed with a cover-slip. All samples were
90 observed by laser scanning microscope (Nikon, Japan).
91
92 ***In vitro* mRNA synthesis and microinjection in oocytes**
93 The *Dux*, *Dppa2*, and *Dppa4* mRNA were cloned into T7-driven vectors, and mRNAs were
94 synthetized *in vitro* using mMESSAGE mMACHINE T7 Ultra Kit (Thermo, USA) following the
95 manufacturer's instructions. The integrity of manufactured mRNA was confirmed by

electrophoresis with formaldehyde gels. The final concentration of mRNA was diluted to 900 ng/ml before injection. As previously described [8], 8 pl of mRNA was microinjected into the cytoplasm of denuded oocytes. These oocytes were obtained by superovulating BDF1 female mice by intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG; Sansheng, China) and 10 IU human chorionic gonadotropin (hCG; Sansheng, China) 48 h apart. The cumulus-oocyte complexes (COCs) were collected from oviducts 14 h post-hCG, and the cumulus cells were dispersed by EmbryoMax FHM Mouse Embryo Media (Millipore, USA). Oocytes were injected using Piezo-operated blunt-end micropipette (3~5 μm internal diameter). After injection, oocytes were kept at room-temperature for 30 min and then moved into the incubator. The siRNA information was presented in Table S4.

Generation of CiPS cells

This section was adapted from Deng's lab [10]. Reagents setup: Small molecules: VPA, CHIR99021, 616452, Tranylcypromine, Forskolin, Ch55, EPZ004777, DZNep, Decitabine, SGC0946, and PD0325901. Stage I medium: FBS/KSR-based medium supplemented with the small-molecule cocktail VC6TF5E (100 μM VPA, 40 μM CHIR99021, 10 μM 616452, 5 μM Tranylcypromine, 10 μM Forskolin, 1 μM Ch55, and 5 μM EPZ004777). N2B27-SII medium: N2B27-based medium supplemented with 10 ng/ml LIF, 50 μg/ml vitamin C, 25 ng/ml bFGF, 2 mg/ml Albumax-II, and the small-molecule cocktail VC6TF5ZDS (VPA at 1 mM). Stage III medium: N2B27-based medium with 3 μM CHIR99021, 1 μM PD0325901, 10 ng/ml LIF, and 50 μg/ml vitamin C. On day –1, MEFs were seeded at 50,000 cells per well of 6-well plate with MEF culture medium. The next day (day 0), change the medium into optimized stage I medium. From day 6~12, cells were cultured in optimized N2B27-SII medium. During day 12~16, cells were cultured in stage III medium with 500 μM VPA. On day 16, VPA was removed and cells were cultured in stage III medium. After another 4~10 days, CiPS colonies emerged. Induction of Dux was performed by administration of doxycycline (2 μg/ml) in the culture medium.

Transgenic mice generation

124 The pCW57.1-mDux-CA vector was a gift from Stephen Tapscott (Addgene 99284). The
125 pronuclear microinjection for the production of transgenic mice followed previously published
126 studies [11]. Briefly, the vector was injected into the well-recognized pronuclei. Injected zygotes
127 were transferred into pseudopregnant female mice (~ 30 zygotes per mouse) after 4 h
128 recovery culture in KSOM-AA medium (Millipore, USA). For founder identification, tail tips were
129 subjected to standard DNA extraction procedures. The amplified DNA fragments were
130 subjected to TA cloning and sequencing. The founder mice were crossed to the littermates of
131 founder mice for four generations to produce homozygous Dux mice.
132
133 **Alkaline phosphatase staining**
134 For alkaline phosphatase staining, CiPS cells were fixed with 4% PFA in PBS for 2 min, rinsed
135 once with PBS and detection was performed using a Alkaline Phosphatase Assay Kit
136 (VECTOR, USA) according to the manufacturer's protocol.
137
138 **Real-time qPCR analysis**
139 Total RNA was isolated using TRIzol reagent (Thermo, USA) and was immediately reverse-
140 transcribed using a Prime Script RT reagent kit (Takara, Japan). The reverse transcription
141 PCR was were amplified using Ex Taq (Takara, Japan). The qPCR was performed using a
142 SYBR Premix Ex Taq (Takara, Japan) and signals were detected with Applied Biosystems
143 7500 real-time PCR System (Thermo, USA). Relative mRNA expression was calculated use
144 the $2^{(-\Delta\Delta Ct)}$ method. The primer information was presented in Table S5.
145
146 **Chimeric mice and embryo transfer**
147 For chimeric experiments, CiPS cells were used one day before passaging, which showed an
148 optimal undifferentiated morphology. The CiPS cells were microinjected into CD1/KM
149 blastocysts using a Piezo (Primetech, Japan) microinjection pipette. After culturing for 3 h, the
150 embryos were transplanted into the uterus of pseudopregnant mice (~15 embryos per mouse).
151 The 2-cell stage SCNT embryos were transferred to the oviducts of E0.5 pseudopregnant (~20

152 embryos per mouse). The cloned pups nursed by lactating CD1/KM females. SSLP analysis
153 was performed for D6Mit15 and D2Mit102. The primer information is presented in Table S5.
154
155 **Teratoma formation**
156 Teratoma formation analysis was carried out to evaluate the pluripotency of CiPS cells.
157 Approximately 10_6 CiPS cells were injected subcutaneously into the hind limbs of a 8-week-
158 old nude mice. After 6 weeks, fully formed teratomas were dissected and fixed with PBS
159 containing 4% PFA, then embedded in paraffin, sectioned and stained with haematoxylin and
160 eosin for histological analysis.
161
162 **Bisulfite genomic sequencing**
163 Genomic DNA was extracted by the tissue & blood DNA extraction kit (Qiagen, Germany) and
164 treated with the Methylamp DNA modification kit (Epigentek, USA). The bisulphite conversion
165 was performed as previously published studies [12].
166
167 **RNA-sequencing**
168 The single-cell RNA-seq method followed previously published studies [7,13]. The reverse
169 transcription was performed directly on the cytoplasmic lysate of individual embryos. The total
170 cDNA library was then amplified by 18~20 cycles for library construction, which was performed
171 following manufacturer's instructions (Illumina, USA). Paired-end sequencing was further
172 performed at Annoroad (Beijing, China). Three biological replicates were analyzed for each
173 treatment condition. After removing low-quality reads and adapters, the raw reads were
174 mapped to the mm9 genome using Tophat (v1.3.3) with default parameters [14]. Expression
175 levels for all RefSeq transcripts were quantified to fragments per kilobase of exon model per
176 million mapped reads (FPKM) using Cufflinks (v1.2.0) [15]. Gene Ontology (GO) analyses for
177 differentially expressed genes were performed by using the R package GO.db and Database
178 for Annotation, Visualization and Integrated Discovery (DAVID) [16].
179

180 **Statistics analysis and data availability**

181 Statistical analyses were done using the univariate analysis of variance (ANOVA) followed by
182 the Student t-test with SPSS 21.0 statistical software (Armonk, USA). $P < 0.05$ was considered
183 significant. Sequencing data have been deposited in the NCBI sequence read archive (SRA)
184 under accession codes: SCNT-1 (SAMN12871322), SCNT-2 (SAMN12871323), SCNT-3
185 (SAMN12871324); D-SCNT-1 (SAMN12871325), D-SCNT-2 (SAMN12871326), D-SCNT-3
186 (SAMN12871327); ICSI-1 (SAMN12871328), ICSI-2 (SAMN12871329), ICSI-3
187 (SAMN12871330).

188

189 **Results and Discussion**

190 We have recently established a MERVL::tdTomato based ZGA real-time monitor system
191 [8]. To identify candidate factors that facilitate ZGA in SCNT reprogramming, we carried out a
192 siRNA-repressor and mRNA-inducer screening in SCNT (Fig.1A). From the screen, we
193 identified a number of factors that when over-expression or knock-down resulted in reporter
194 activation (Supplementary information, Fig.S1A). Among the identified factors, Dux [17-19],
195 Dppa2 and Dppa4 [20-22] showed the strongest phenotype (Fig.1B, 1C). Intriguingly, we found
196 that most SCNT embryos injected with Dppa2 or Dppa4 mRNA arrested at the 1-cell stage
197 (called '1-cell block' for simplicity; Fig.1D, 1E; Supplementary information, Fig.S1B, Movie S1,
198 S2). Direct injection of Dux mRNAs had no significant impact on the blastocyst formation rate
199 (*vs.* canonical SCNT; 21.2% *vs.* 23.6%; Fig.1D; Supplementary information, Table S1). Indeed,
200 direct injection of Dux will increase the rate of SCNT embryo fragmentation (Fig.1D, 1E;
201 Supplementary information, Movie S2). Nevertheless, when Dux was over-expressed in SCNT
202 embryos, the rate of 2-cell block was significantly reduced (Fig.1D). These results suggest
203 that Dux (but not Dppa2/4) is a key factor whose overexpression can rescue 2-cell arrest in
204 SCNT embryos.

205

206 It is known that ZGA is governed by a time-dependent 'zygotic clock' (i.e. 24h post-
207 fertilization), and Dux is expressed as a brief pulse in early 2-cell stage [5, 20,21,23]. Therefore, we

208 wonder whether Dux is time-depend on improving the efficiency of SCNT embryo development.
209 To answer this question, we generated transgenic mouse lines containing doxycycline (dox)
210 inducible Dux gene (Supplementary information, Fig.S2A-B). By switching the culture medium
211 between dox-containing and -lacking at various time points, the critical period for the dox-Dux
212 requirement was mapped to a 24-h window (Fig.1F). When the dox was supplied in this
213 window, the SCNT blastocyst formation rate was significantly increased (Fig.1G;
214 Supplementary information, Table S2). Subsequently, we performed RNA sequencing (RNA-
215 seq) to examine the possible changes of the ZGA related gene expressions in Dux-
216 overexpressed 2-cell SCNT embryos (D-SCNT). Principal component analysis (PCA)
217 revealed that the ICSI and D-SCNT generated embryos in the same cluster, and ZGA-genes
218 were upregulated in D-SCNT embryos (Fig.1H, 1I; Supplementary information, Fig.S3A-D;
219 Dataset S1, S2). These results indicating the improvement of Dux on SCNT embryonic
220 development depends on its treatment time.
221
222 The SCNT blastocyst rate was improved by injecting Kdm4d mRNA (K-SCNT) [6]. We next
223 compared the developmental potential of SCNT embryos derived by K-SCNT and D-SCNT.
224 We found that the blastocyst formation rate of D-SCNT was similar to K-SCNT (84.72% *vs.*
225 86.79%; Supplementary information, Fig.S4A, Table S2). Nonetheless, the cloned pup birth
226 rate of D-SCNT was higher than K-SCNT (10.71% *vs.* 7.81%; Fig.1J; Supplementary
227 information, Table S3). Despite D-SCNT or K-SCNT, the abnormally large placentas are still
228 observed in those cloned pups (Supplementary information, Fig.S4B, Table S3). Previous
229 studies have indicated that knock-down DNA-methyltransferases Dnmt3a/b led to less
230 placental abnormalities (Si-SCNT) [24]. We next investigated whether combined D-SCNT and
231 Si-SCNT could further improve SCNT embryonic development. Compared with canonical
232 SCNT, combined K-SCNT and Si-SCNT improved the pup birth-rate from 1.01% to 12.24%,
233 but less than D-SCNT combined with Si-SCNT (18.60%; Fig.1J). Genotyping analysis
234 confirmed that all the cloned pups were generated from donor cells (Supplementary
235 information, Fig.S4C). The large placental phenotype was rescued by the D-Si-SCNT

236 combined approach (Supplementary information, Fig.S4B).

237

238 Deng et al. identified a unique embryonic 2-cell like status as the key molecular event that
239 led to successful chemical reprogramming (CiPS) [10]. Thus, we tried to improve the CiPS by
240 overexpression of ZGA inducer Dux (D-CiPS). The dox-inducible mouse embryonic fibroblasts
241 (dDux-MEFs) cells were isolated from the transgenic mice. As shown in Fig.1K, we treated the
242 dDux-MEFs with dox for various durations, and observed that Dux displayed remarkable effect
243 at the stage-2 of CiPS. About 36 alkaline phosphatase-positive (AP_+) colonies could be
244 observed in D-CiPS wells (starting from 50,000 MEFs per well of 6-well plate), while only 26
245 AP_+ colonies could be observed in the canonical CiPS well (Fig.1K; Supplementary
246 information, Fig.S5A, S5B). Consistently, the ZGA related genes were significantly
247 upregulated after transient expression of Dux (Supplementary information, Fig.S5C). These
248 D-CiPS induced cells were pluripotent as demonstrated by standard characterization
249 procedures (Supplementary information, Fig.S5D-F). We found, based on bisulfite sequencing,
250 that successful demethylation occurred in the promoters of Oct4 and Nanog in the D-CiPS
251 induced cell lines (Fig.1L).

252

253 In summary, we demonstrate here that transient overexpression of Dux not only improved
254 SCNT efficiency, but also increased the efficiency of chemical reprogramming. The Dux factor
255 was recently identified as a inducer of ZGA in mouse, human, and likely all mammalian
256 animals [17,25]. Thus, we reasoned that transient overexpression of Dux could also improves
257 reprogramming efficiency of all mammals.

258

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Author contributions

L.Y. and G.L. conceived and designed the study. L.Y., X.L., L.S., A.D., G.S., C.B., and Z.W. performed the experiments. L.Y., X.L., L.S., A.D., and G.S. analyzed the data. G.L. supervised the project. L.Y. and G.L. wrote the paper.

Competing interests

The authors declare no competing interests.

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335

Figure legends

Figure 1 Dux act as promote factors for SCNT- and chemical-mediated somatic cell reprogramming.

(A) Schematic illustration of the screen strategy.

(B) Quantification of embryos that expression ZGA reporter (MERVL::tdTomato) after injection with siRNA-repressor or mRNA- inducer. N, total number of embryos analyzed for each condition. Error bars, mean ± S.D.; three independent experiment replicates were performed.

(C) Representative fluorescence image of SCNT embryos derived by different methods. The SCNT embryos produced by transfer of MERVL::tdTomato cumulus cell into WT enucleated oocytes. Scale bar, 100 μm.

(D) Stacked bar plots show fraction of embryos at blastocyst stages after injection with different mRNA as indicated. At least three independent experiment replicates were performed. N, total number of embryos analyzed for each condition.

(E) Representative live-cell images of dynamics ZGA reporter (MERVL::tdTomato) expression during SCNT embryos preimplantation development. The selected images from a series acquired every 15 min. The time after starting observation is shown on the bottom of image. Scale bar, 50 μm.

(F) Schematic transient induction of Dux. All timings reported in this paper is hour post-activation.

(G) The preimplantation development of SCNT embryos derived from D-SCNT or SCNT. Shown is the percentage of embryos that reaches each indicated stage. Scale bar, 100 μm.

(H) Unsupervised hierarchical clustering. The D-SCNT embryos mostly resembled the ICSI derived embryos.

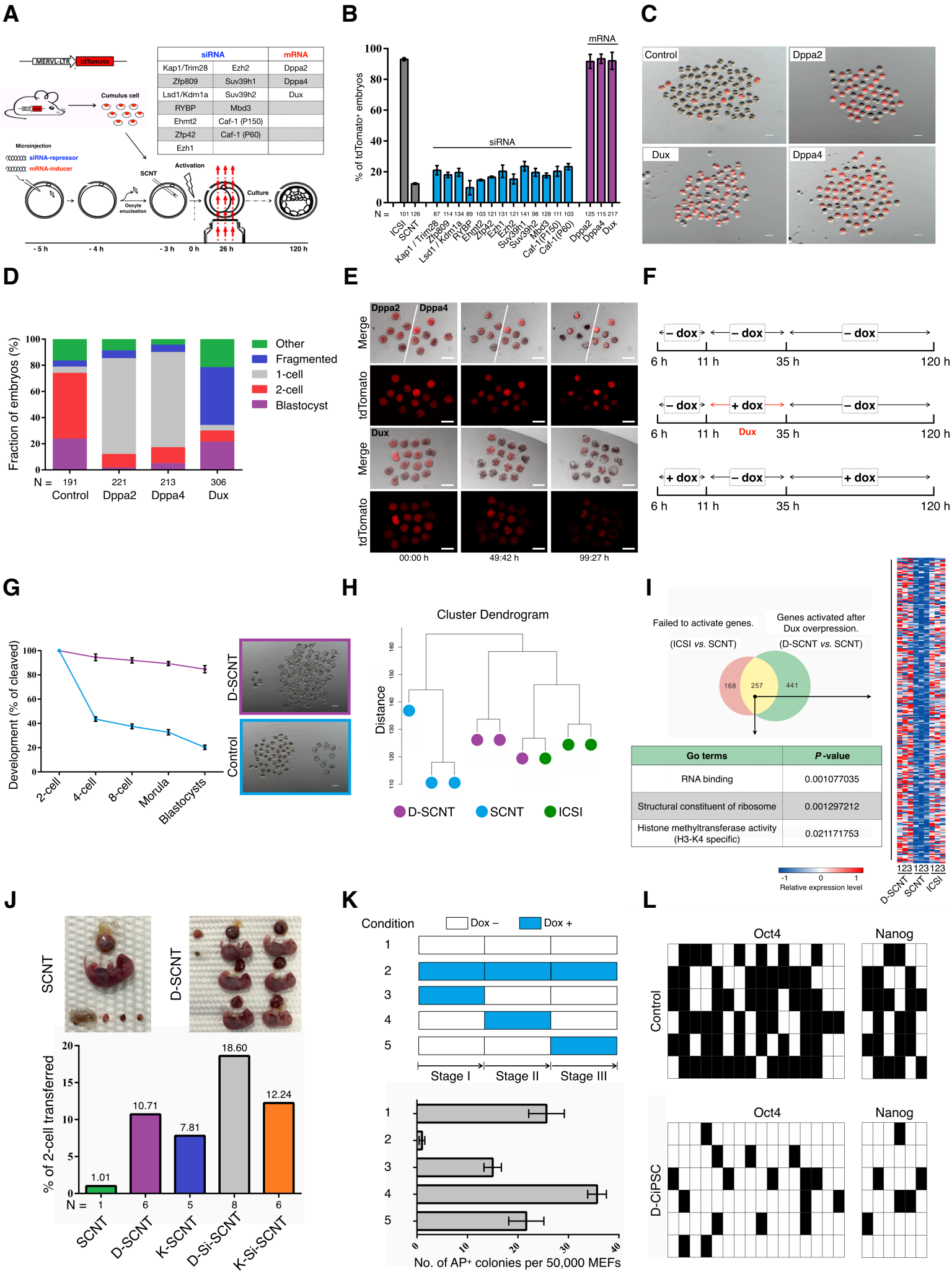
(I) Venn diagram showing the overlap between the genes that failed to be activated in SCNT 2-cell embryos and derepressed in D-SCNT. Heatmap, GO terms showing the expression pattern of 257 overlap genes.

364 (J) The birth rate of SCNT embryos derived by different methods.

365 (K) Durations of dox addition at different time points during chemical induction (upper) and
366 assessment of AP_+ colonies (bottom). Error bars, mean ± S.D.; the Dox inducible Dux-
367 MEFs were used for chemical reprogramming.

368 (L) Bisulfite sequencing analysis of Oct4 and Nanog demethylation in D-CiPS or canonical
369 CiPS cells (filled squares are methylated CpGs and empty ones indicate unmethylated
370 sites).

Fig.1


A
MERVL-LTR
tdTomato
Cumulus cell
Microinjection
siRNA-repressor
mRNA-inducer
SCNT
Activation
Oocyte enucleation
Culture
- 5 h
- 4 h
- 3 h
0 h
26 h
120 h
siRNA
mRNA
Kap1/Trim28
Zfp809
Lsd1/Kdm1a
RYBP
Ehmt2
Zfp42
Ezh1
Ezh2
Suv39h1
Suv39h2
Mbd3
Caf-1 (P150)
Caf-1 (P60)
Dppa2
Dppa4
Dux
B
% of tdTomato+ embryos
siRNA
mRNA
N =
ICSI
SCNT
C
Control
Dppa2
Dux
Dppa4
D
Fraction of embryos (%)
Other
Fragmented
1-cell
2-cell
Blastocyst
N =
191
221
213
306
Control
Dppa2
Dppa4
Dux
E
Merge
tdTomato
Dppa2
Dppa4
Dux
00:00 h
49:42 h
99:27 h
F
– dox
+ dox
6 h
11 h
35 h
120 h
Dux
G
Development (% of cleaved)
2-cell
4-cell
8-cell
Morula
Blastocysts
D-SCNT
Control
H
Cluster Dendrogram
Distance
D-SCNT
SCNT
ICSI
I
Failed to activate genes.
(ICSI vs. SCNT)
Genes activated after Dux overexpression.
(D-SCNT vs. SCNT)
168
257
441
Go terms
P-value
RNA binding
0.001077035
Structural constituent of ribosome
0.001297212
Histone methyltransferase activity (H3-K4 specific)
0.021171753
Relative expression level
D-SCNT
SCNT
ICSI
J
SCNT
D-SCNT
% of 2-cell transferred
1.01
10.71
7.81
18.60
12.24
N =
1
6
5
8
6
SCNT
D-SCNT
K-SCNT
D-Si-SCNT
K-Si-SCNT
K
Condition
Dox –
Dox +
Stage I
Stage II
Stage III
No. of AP+ colonies per 50,000 MEFs
L
Oct4
Nanog
Control
D-CiPSC