Visualization of DNA Methylation in Cell Cycle Genes during iPSC Cardiac Differentiation

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ABSTRACT

Epigenetic DNA methylation is an essential mechanism controlling gene expression and cellular function. Endpoint analyses with conventional assays have generated significant insights into static states of DNA methylation, but were short of visualizing the dynamics of epigenetic regulation. Here, we adopted a genomic DNA methylation reporter (GMR) system to track the changes of DNA methylation during cardiac differentiation. The promoter region of *Cdk1* (Cyclin-dependent kinase 1) or *Sox2* (SRY-Box Transcription Factor 2) gene was cloned upstream of the small nuclear ribonucleoprotein polypeptide N (Snrpn) minimal promoter followed by a fluorescent reporter gene. Mouse induced pluripotent stem cells (iPSCs) carrying Sox2 GMR rapidly lost fluorescent reporter signal upon the induction of differentiation. Cdk1 GMR reporter signal was strong in undifferentiated iPSCs, and gradually decreased during directed cardiomyocyte (CM) differentiation. The present study demonstrated the dynamic DNA methylation along the course of cell cycle withdrawal during CM differentiation. The GMR reporter system can be instrumental to monitor real-time epigenetic DNA modification at single-cell resolution.

KEYWORDS: Epigenetics; DNA Methylation; Cardiac Differentiation; Cell Cycle; Genomics Methylation Reporter; Pyrosequencing; single-cell imaging; flow cytometry

INTRODUCTION

Epigenetic DNA methylation is highly associated with gene expressions in development and cellular functions such as cell cycle progression and differentiation. Methylation status of gDNA can be determined by a number of available methods, such as gene-specific analysis using Pyrosequencing and PCR quantification after bisulfite conversion, or digestion-based assay followed by PCR or qPCR, and whole-genome sequencing on similarly processed samples.[1] The mounting data in DNA methylation studies have generated significant insights into static states of DNA methylation but were short of depicting epigenetic perturbations in a real-time manner. A live-cell gDNA methylation reporter system is essential in visualizing dynamic genomic methylation states, such as in cardiac differentiation of induced pluripotent stem cells (iPSCs).

Mouse iPSCs have been widely used to study lineage differentiation, which is often associated with a perturbation in the cell cycle activity, but the exact temporal dynamics remained unclear for various lineages. Cardiomyocytes (CMs) withdraw from activity cell cycling shortly after birth,[2] but dynamic epigenetic DNA methylation during cardiac differentiation remained to be visualized.

The small nuclear ribonucleoprotein polypeptide N (*Snrpn*) gene is a maternally imprinted gene that is sensitive to DNA methylation of adjacent genomic regions.[3] *Snrpn* mini promoter region contains the conserved elements between human and mouse, with the endogenous imprinted differentially-methylated region (DMR). The *Snrpn* mini promoter can be used as a methylation sensor for real-time reporting of the DNA methylation state of adjacent sequences such as CpG regions of a particular gene.[4] In this study, we adopted this Snrpn mini-promoter-based

54 Genomics DNA methylation reporter (GMR) to track DNA methylation states of stemness and
55 cell cycle genes during CM differentiation from mouse iPSCs.

56

57 **MATERIALS AND METHODS**

58 **Mouse iPSCs cell culture –** Mouse iPSCs were maintained in ESC medium with 2iL [MEK
59 inhibitor (MEKi), GSK3 inhibitor (CHIR) and leukemia inhibitory factor (LIF)]. Stem Cell
60 Medium was made of DMEM (Gibco; # 11995065) supplemented with 20% FBS (Fetal Bovine
61 Serum; Gibco; #10082147), 1% penicillin/streptomycin (Gibco; #15070063), 1% NEAA (Non-
62 Essential Amino Acid; Gibco; #11140050) and 0.1 mM beta-mercaptoethanol (Sigma-Aldrich).
63 The medium was filtered through a Millipore Stericup 0.22 mm filtration system, and then added
64 with 2iL [10^3 Units/mL LIF (Millipore; #ESG1107), 1.0 μM MEKi (Stemgent; #PD0325901)
65 and 1.5 μM CHIR (Selleck Chemicals; #CHIR-99021)] before use in mouse iPSCs. All medium
66 was prepared under sterile conditions and cells were cultured in a incubator at 37°C temperature
67 and 5% CO_2.

68 **Construction of cell cycle GMR, and generation of reporter iPSC cell lines –** Sox2se-CPG-
69 TV-dTomatto and Gapdh-CpG-TV-GFP plasmids (Addgene plasmid # 70155, and 70148) were
70 a gift from Dr. Rudolf Jaenisch.[4] The promoter of mouse *Cdk1* gene that includes 29 CpG was
71 cloned from wildtype C56/Bl6j mouse gDNA, using a primer pair: Cdk1-pro-F (5'-
72 ACG*GGTACC*AGCAATAGCCAGGTGGTGGTG-3') and Cdk1-pro-R (5'-
73 GAC*CAATTG*CATAAGGGATCCCGGACTTAG-3'). Then the PCR amplicon of *Cdk1* CpG
74 was digested by KpnI and MfeI (New England Biolabs) to remove the overhangs. The resultant
75 fragment was ligated to the Snrpn-dTomato backbone from Sox2se-CPG-TV, to generate the

76 Cdk1-Snrpn-dTomato plasmid, which was then amplified in MAX Efficiency DH5α Competent
77 Cells (Invitrogen).

78 To generate *Sox2* and *Cdk1* GMR reporter cell lines, we transfected Sox2se-CPG-TV-dTomato
79 and Cdk1-Snrpn-dTomato plasmids into mouse iPSCs using Lipofectamine 2000 Transfection
80 Reagent (Invitrogen) according to the provider's protocol. Twenty-four hours following
81 transfection, cells were treated with puromycin (0.75 μg/ml) for 3 days. Then the puromycin-
82 resistant cells carrying GMR genes were harvested and sorted with a BD Aria II cell sorter for
83 single-cell tdTomato expression. Cells were cultured in 96-well plates containing irradiated
84 mouse embryonic fibroblasts (iMEFs) feeder layer. Single colonies were passaged and used in
85 subsequent analyses.

86 **Directed cardiomyocyte differentiation from mouse iPSCs –** For differentiation assay, we
87 adopted Yashiro's method with some modifications.[5] Mouse Sox2 and Cdk1 GMR iPSC lines
88 were maintained in Stem Cell Medium with 2iL for three days, then harvested by trypsin
89 dissociation, and suspended in 2iL Stem Cell Medium medium, followed by incubation in a
90 incubator (5% CO_2, 37 °C) for 30 minutes to remove iMEFs. Cells were washed twice with
91 DPBS (Dulbecco's phosphate buffered saline, Gibco) before medium replacement. The iPSCs
92 were re-seeded into gelatin-coated 24-well plates at a density of 120,000 cells/well in 2iL ESC
93 medium. After 8 hours, medium was changed to Serum-free (SF) differentiation medium
94 containing the followings: 75% Iscove's modified Dulbecco's medium (IMDM, Gibco), 25%
95 Ham's F12 Nutrient Mix (Gibco), supplemented with 0.5% N-2 supplement (Gibco), 0.5% B-27
96 supplement (Gibco), 0.05% bovine serum albumin (BSA, Fisher scientific), 1% Pen-Strep
97 (Gibco), 2 mM L-glutamine (Gibco), 0.5 mM L-ascorbic acid (Sigma), and 0.45 mM 1-
98 Thioglycerol (Sigma); and cells were cultured for 2 days. It was counted as Day 0 when medium

99 was changed to SF medium with 8 ng/mL recombinant Activin A (R&D Systems), 5 ng/mL
100 recombinant human VEGF 165 Protein (R&D Systems) and 0.25 ng/mL recombinant human
101 BMP-4 Protein (R&D Systems).Cells were cultured for 28 hours; and then the medium was
102 changed to StemPro-34 Serum-free Medium (SFM, Gibco) supplemented with 2 mM L-
103 glutamine, 0.5 mM L-ascorbic acid (Sigma), 5 ng/ml recombinant human VEGF 165 protein
104 (R&D Systems), 10 ng/ml human bFGF (Invitrogen), and 50 ng/ml recombinant human FGF-10
105 protein, and cells were cultured for another 3.5 days. Subsequently, cells were harvested, and
106 passaged into gelatin-coated 24-well plates in 500k cells/well density in StemPro-34 SF
107 supplemented medium. After 2 days, RPMI 1640 medium (with L-glutamine, Gibco) with B-27
108 supplement (minus vitamin A, Gibco) was used for further cell culture of the differentiated
109 myocytes.

110 **Flow cytometry –** Mouse iPSCs and differentiated cells in tissue cultures were dissociated by
111 incubation with Trypsin-EDTA (0.25%, Gibco) and then fixed with 4% paraformaldehyde and
112 0.2% saponin in PBS (Gibco). The fixed cells could be stored in 10% DMSO in FBS at -80°C
113 for future use. Mouse iPSCs were stained with Oct4-AF488 (Millipore MAB4419A4) to verify
114 the stemness gene. The cells harvested on different days of cardiac differentiation were stained
115 with cTnT antibody (Troponin T, mouse monoclonal antibody, Thermo Fisher Scientific, MS-
116 295-P, 1:200) and then with donkey anti-mouse IgG (H+L) secondary antibody (Alexa Fluor
117 647; Thermo Fisher; A31571; 1:1000). Immuno stained cells were analyzed on an FACScan
118 Canto RUO flow cytometer (BD) and the result data were analyzed with FlowJo software
119 (FlowJo; Ashland, OR).[6-8]

120 **Statistics –** After the derivation of GMR reporter clones, experiments were carried out with at
121 least biologically replicated, unless otherwise specified. Group data are expressed as Mean±

122 SEM unless otherwise specified. Difference between groups was assessed by t test, with a P
123 value <0.05 consider significant different.

124

125 **RESULTS**

126 **Mouse iPSC with GMR for Sox2 and Cdk1 –** To test the utility of Snrpn mini promoter's
127 DNA methylation sensing function, we generated mouse iPSC lines carrying Sox2-GMR
128 construct that composites of Sox2 super enhancer CpG island region fused with the minimal
129 promoter of Snrpn, followed by dTomato reporter. iPSCs transfected with Sox2-GMR were
130 subjected to puromycin-resistant selection. Cells with high expression of dTomato reporter
131 signals were collected by fluorescence-activated cell sorting (FACS), and single clones were
132 expanded, among which clone C7 was used for subsequent experiments (**Figure 1**). Similar
133 approaches were used to generate mouse Cdk1-GMR iPSC lines, among which clone B9 was
134 used for subsequent analyses. The expression of stemness protein such as Oct4 or Nano, Sox2 in
135 GMR lines remained at comparable to wildtype mouse iPSC as assessed by immunostaining and
136 flow cytometry. Fluorescent reporter signals in both Sox2 and Cdk1 GMR lines were readily
137 detected with epifluorescent microscopy imaging.

138 **Diminished reporter signals in cells differentiated from Cdk1 and Sox2 GMR iPSCs –** Cell
139 differentiation is accompanied with epigenetic reprogramming that sets the landscape for
140 transcriptional activity for lineage specification, cell identity and functions. As a proof of
141 concept to the utility of GMR, we examined the reporter signal and cardiac gene expression in
142 cells differentiated from the GMR iPSCs lines. By day 2.5 of directed cardiac differentiation,
143 dTomato reporter signals in both Sox2 GMR and Cdk1 GMR cells reduced remarkably (**Figure**

144 **2A**). By day 6.5 of differentiation, dTomato signal was minimally detected in ~2.08% in Sox2-
145 GMR cells, consistent with the loss of stemness in differentiated cells assessed by flow
146 cytometry on the gene expression of Sox2 and Oct4. dTomato signal in Cdk1 GMR cells also
147 reduced significantly in differentiated cells (6.5 days) compared to iPSCs (64.3%±7.1%
148 compared to 95.5%±1.5% (n=3, $p<0.01$). By 3 weeks, there is barely detectably dTomato signal
149 from Cdk1 GMR-derived cells that were mostly myocytes expressing cTnT as detected by
150 immunostaining and flow cytometry (**Figure 2B, C**).

151 **Correlations between GMR signals and gene expression –** To determine the correlation
152 between GMR signal in live cells and gene expression during cardiac differentiation, we
153 performed RT-qPCR on cells differentiated from Cdk1 GMR iPSCs. After 4.5 days of cardiac
154 differentiation, *Tnnt2* was expressed and remarkably increased in myocytes of later stage (13.5
155 days) as compared to earlier stages. On the other hand, the transcript *Cdk1* increased in 4.5 days
156 differentiated cells compared to Cdk1 GMR iPSC, then tapered: *Cdk1* in differentiated cells at
157 day 13.5 was reduced to only 25% level of that in undifferentiated iPSC (**Figure 3**).

158

159 **DISCUSSION**

160 Epigenetic DNA and histone modifications, and subsequently the changes in chromatin
161 accessibility, are the critical mechanisms controlling gene expression, thereby affecting cellular
162 phenotype and function, such as differentiation and proliferation.[6,9-15] Several approaches can be
163 used to reveal the status of DNA methylation; for example, fluorescent proteins (FPs) in
164 conjugation with methyl-CpG-binding domain (MBD) or Synthetic-molecule/protein hybrid
165 probes,[16] modular fluorescence complementation-based epigenetic biosensors that combine

166 engineered DNA-binding proteins with domains recognizing defined epigenetic marks, both
167 fused to non-fluorescent fragments of a fluorescent protein.[17] The live-cell reporter system
168 leveraging the mini-promoter of imprinted *Snrpn* gene to visualize the CpG island regions of a
169 target gene represents unique and highly specific system with detail temporal and spatial
170 resolutions critical in the study of DNA methylation dynamics.[4]

171 In this study, we confirmed the fidelity of CpG-*Snrpn* based GMR approach in mouse iPSC and
172 differentiated myocytes. It is important to note that methylation status of genes that have no or
173 short CpG regions may not be reflective in this GMR system. C2c12 cell lines carrying *Gapdh*
174 GMR (GFP) reporter and Sox2SE GMR (tdTomato) were also used to verify the expected GMR
175 signals. In addition, there was no reporter signals in cells transfected with pre-methylated Sox2
176 or Gapdh GMR gene constructs (Data not shown). Live cell imaging data showed that the
177 positive GMR reporter signal reflects well the transcript expression of corresponding gene.
178 Differentiated cells, like cardiomyocytes, have slower proliferation and the cell cycle can be
179 arrested shortly after birth.[2] Our data show that during early differentiation (4.5 days), there was
180 an increased expression of *Cdk1* then reduced significantly in more mature cardiomyocytes (e.g.,
181 13.5 days). It may be plausible that there was a fast proliferative phase right after transition to
182 cardiac mersoderm lineage, leading to the higher *Cdk1* transcript expression, and the changes in
183 transcription activity may be delayed behaind the GMR reporter signal for that short period.[18] It
184 is worth noting that Cdk1 itself can affect histone epigenetic landscape as shown in mouse
185 embryonic stem cells.[19] Combination with other approaches will likely further delineate the
186 epigenetic-transcriptional regulations in cell differentiation and cell cycle regulation. A caveat of
187 using this GMR is that it is a "negative-positive" system in which the reporter signal represents
188 hypomethylation rather than hypermethylation.

189 In summary, the *Snrpn* mini-promoter-based GMR system,[4] such as for *Cdk1* and other cell cycle
190 genes, can be instrumental to report dynamic DNA methylation states during cell differentiation
191 and cell cycling.

192

193 **Declaration of Competing Interest**

194 The authors declare no conflicts of interest.

195

196 **AUTHOR CONTRIBUTIONS**

197 Y.Z. conceived the study. Y.Z. and N.L. designed and performed experiments, collected and
198 analyzed data. B.T.N. contributed to gene expression analysis. Z.Z. conducted experiments on
199 cell culture. N.L. and Y.Z. wrote the manuscript. All co-authors provided their edits to the
200 manuscript.

201

202 **Acknowledgments**

203 The authors thank Yun-Yu Wu and Lovina Abdi for technical supports, and Dr. W. Robb
204 MacLellan for assistantce in discussing the experiments. The work was in part supported by
205 research grants from American Heart Association (18IPA34110210 to Y.Z.) and the National
206 Institute of Health (1R56HL131898 to Y.Z.) and University of Washington Institute for Stem
207 Cells and Regenerative Medicine (Innovation Pilot Awards to Y.Z.).

208

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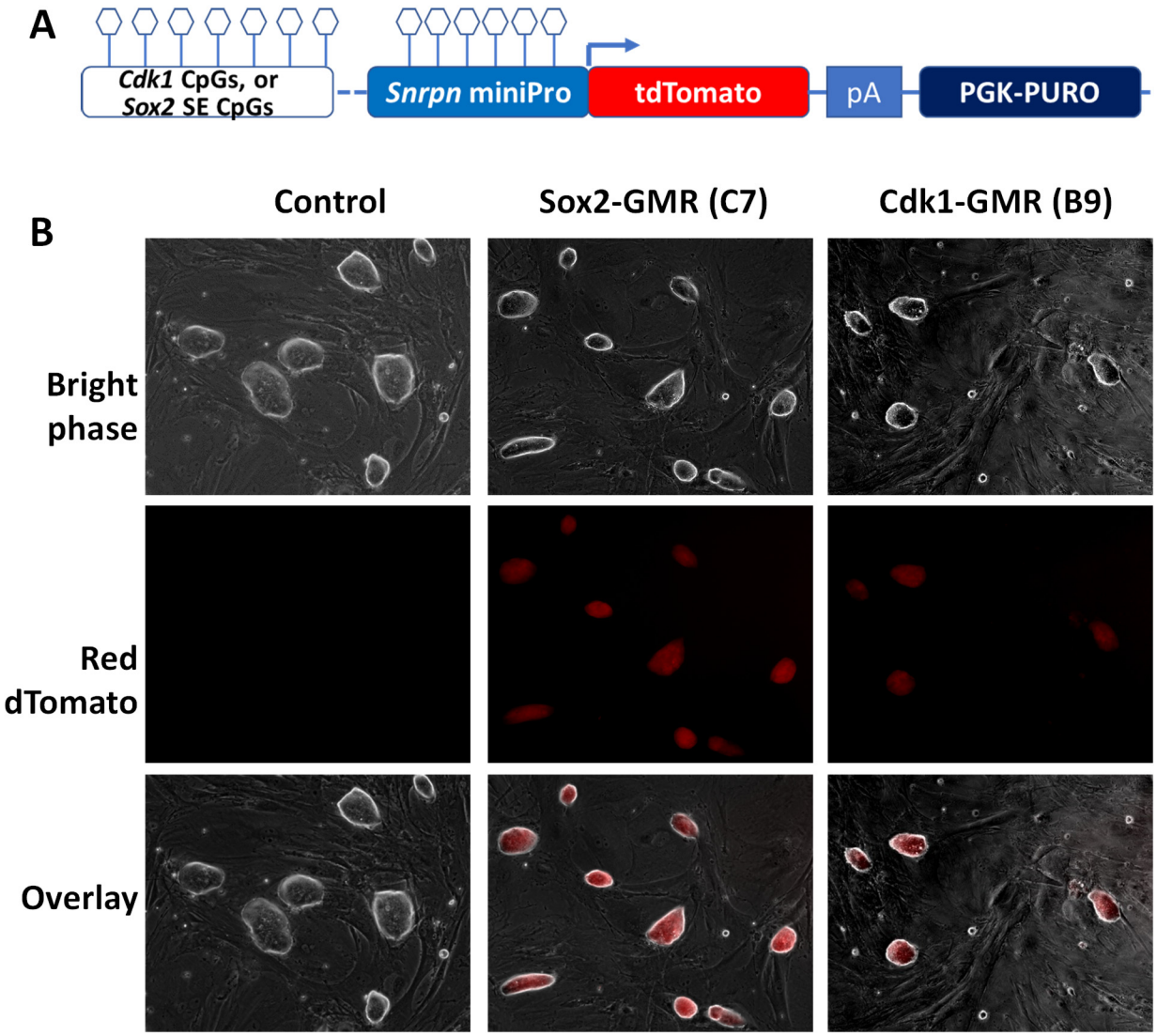
263 **Figures and Figure Legends**

264 **Figure 1.**

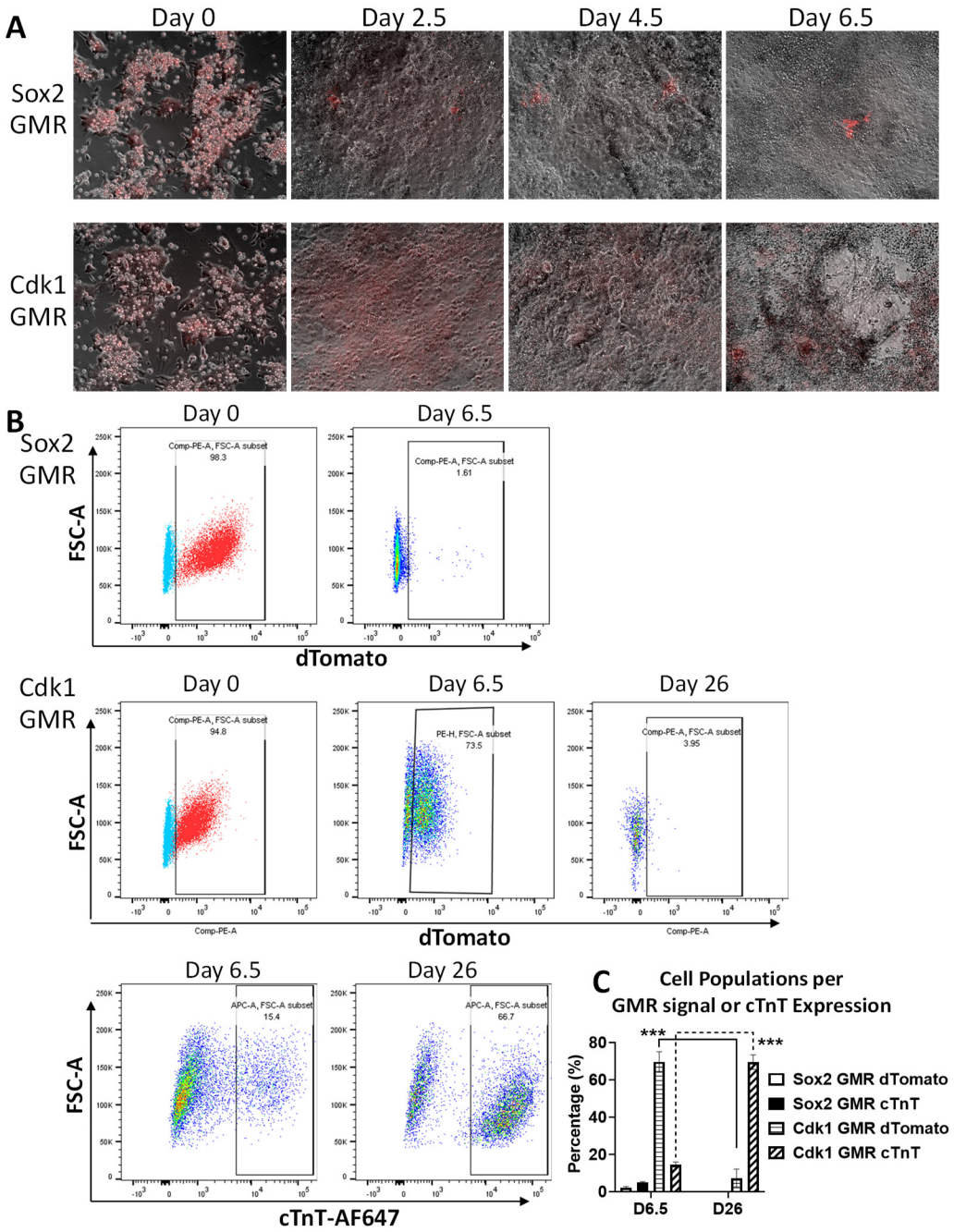


265

266 **Figure 1. Generation of mouse GMR iPSC lines. A**, Gene construction of Snrpn mini-
267 promoter based genomic DNA methylation reporter system. **B,** Microscopy images of wildtype
268 iPSC (control) and GMR iPSC lines for carrying GMR for Sox2 super enhancer or Cdk1
269 promoter's CpG region.

270

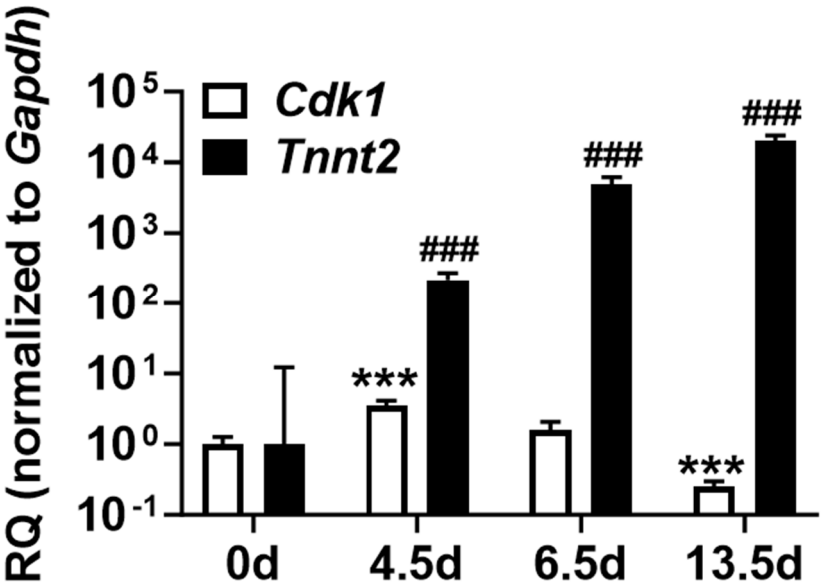
271 **Figure 2.**



272

273 **Figure 2. Dynamic reporter signal during cardiac differentiation of GMR iPSCs. A**,
274 Microscopy images of Sox2-GMR and Cdk1-GMR iPSCs (day 0) and differentiated cells. **B,**
275 Flow cytometry dot plots showing the signal of GMR reporter dTomato and cardiomyocyte
276 marker cTnT (cardiac troponin T). **C**, Cell population quantified by the expression of GMR
277 signal dTomato of cardiac myocyte marker cTnT.

278 **Figure 3.**



279

280 **Figure 3. Expression of Cdk1 and Tnnt2 (cTnT) gene during cardiac differentiation from**
281 **the Cdk1 GMR iPSCs.** Relative quantification of mRNA as normalized to *Gapdh.* *** and ###,
282 $p<0.05$ vs 0d; n=3.