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Supplemental Information

RB Maintains Quiescence and Prevents Premature Senescence through Upregulation of DNMT1 in Mesenchymal Stromal Cells

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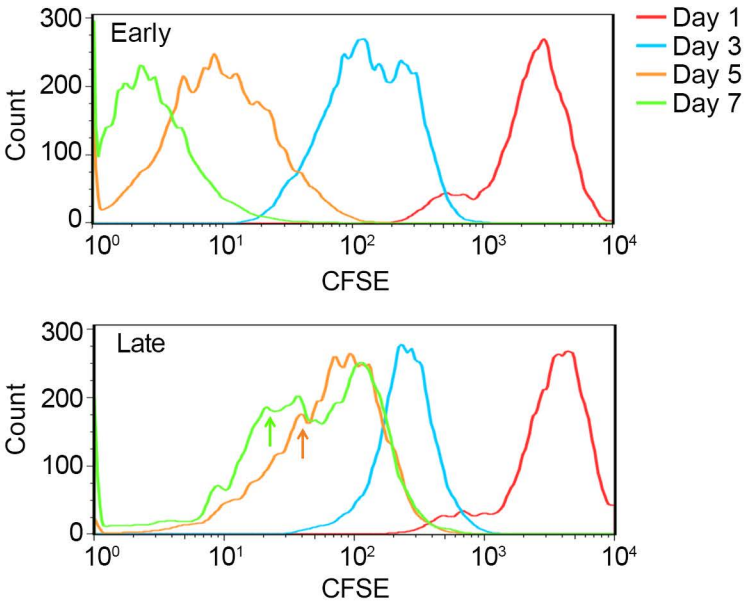
Early
Late
Count
CFSE
Day 1
Day 3
Day 5
Day 7
300
200
100
0
10^0
10^1
10^2
10^3
10^4

Figure S1

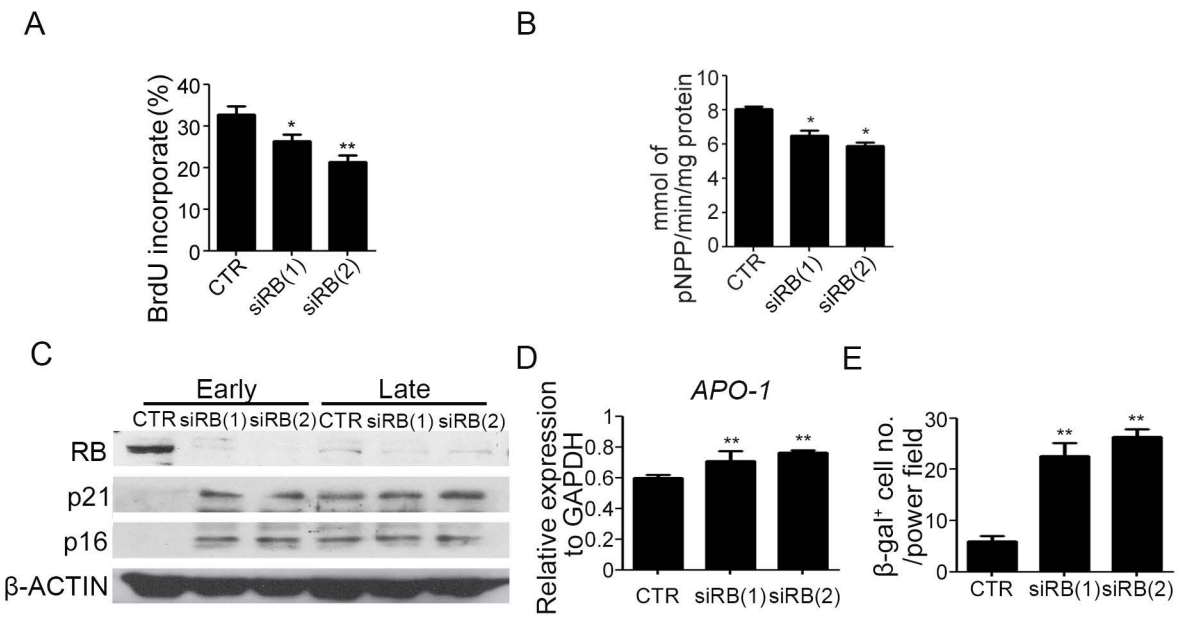
A
BrdU incorporate (%)
40
30
20
10
0
*
**
CTR
siRB(1)
siRB(2)
B
mmol of pNPP/min/mg protein
10
8
6
4
2
0
*
*
CTR
siRB(1)
siRB(2)
C
Early
Late
CTR siRB(1) siRB(2) CTR siRB(1) siRB(2)
RB
p21
p16
β-ACTIN
D
APO-1
Relative expression to GAPDH
1
0.8
0.6
0.4
0.2
0
**
**
CTR
siRB(1)
siRB(2)
E
β-gal+ cell no. /power field
30
20
10
0
**
**
CTR
siRB(1)
siRB(2)

Figure S2

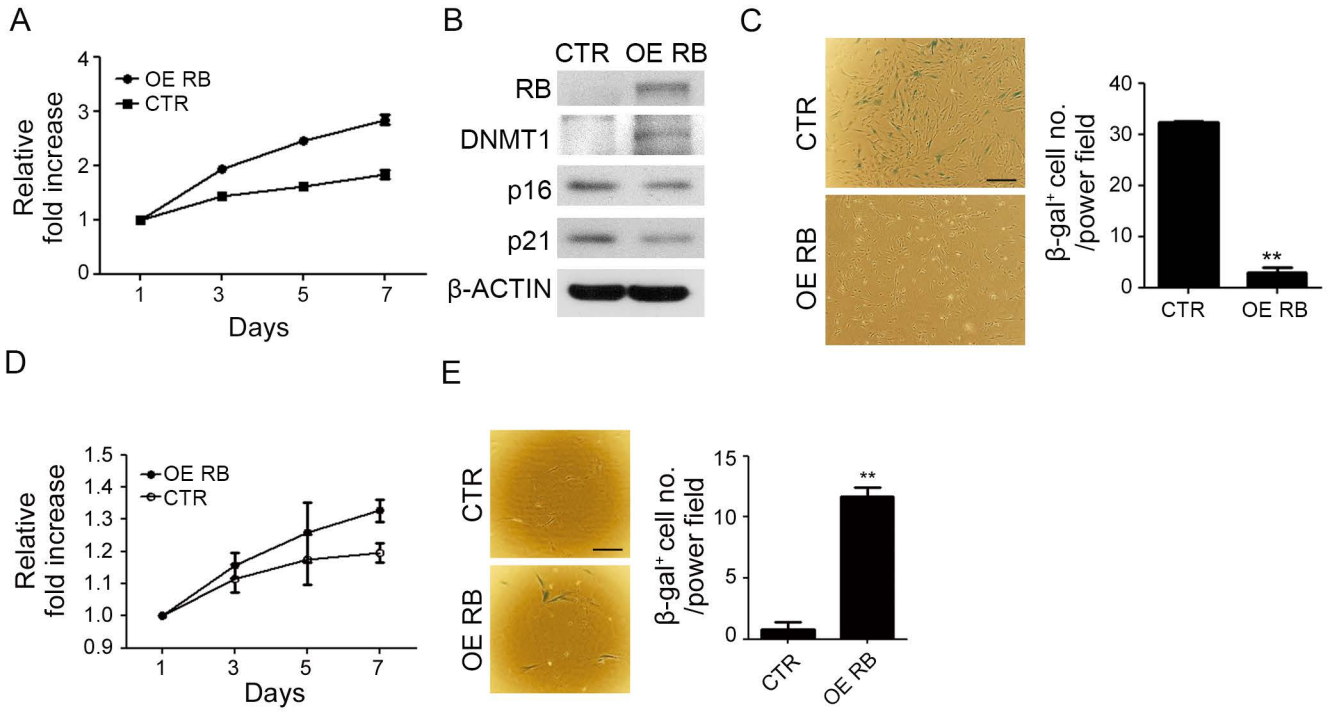
A
Relative fold increase
OE RB
CTR
Days
B
CTR OE RB
RB
DNMT1
p16
p21
β-ACTIN
C
CTR
OE RB
β-gal+ cell no. /power field
**
D
Relative fold increase
OE RB
CTR
Days
E
CTR
OE RB
β-gal+ cell no. /power field
**

Figure S3

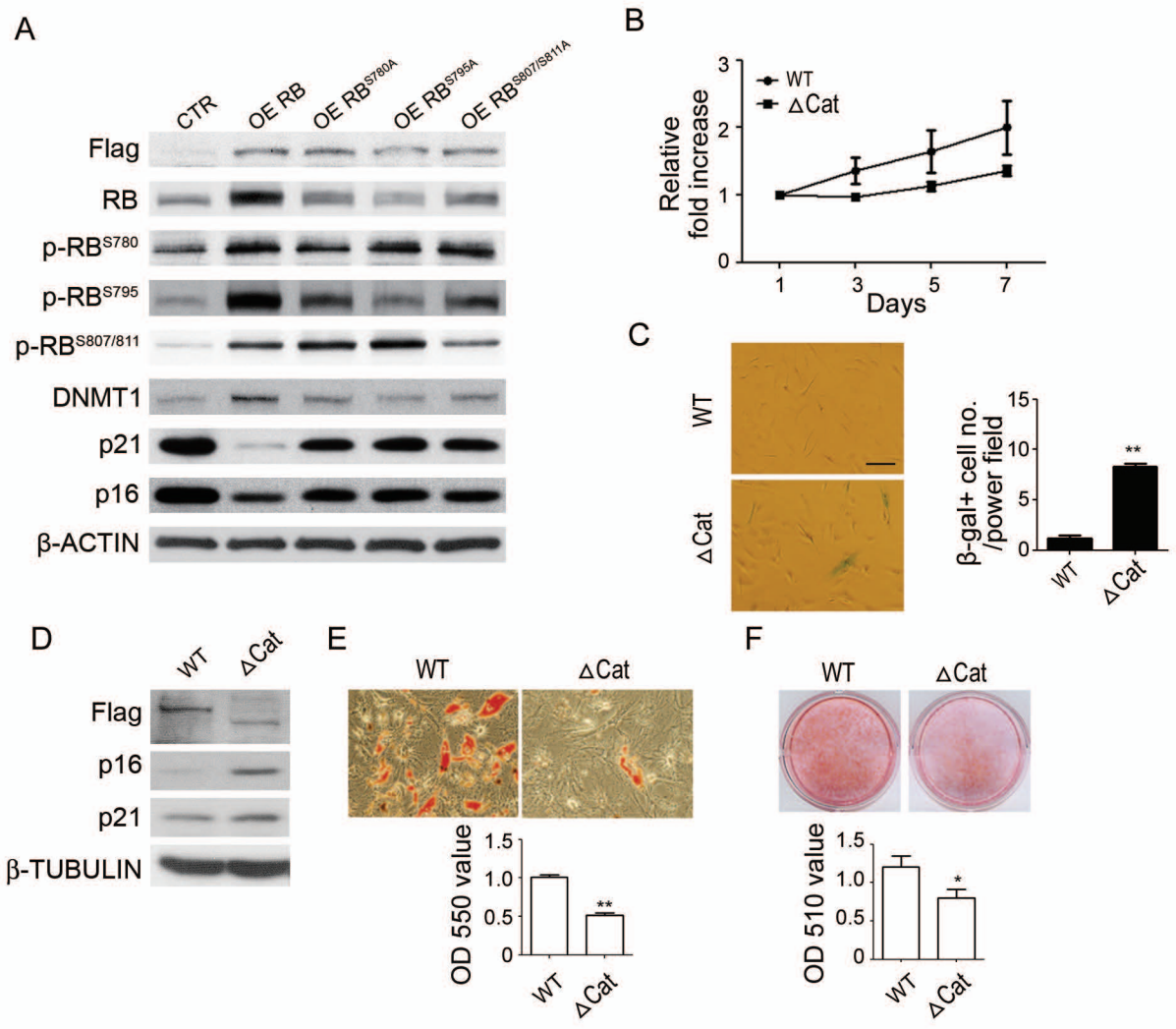
A
CTR
OE RB
OE RB S780A
OE RB S795A
OE RB S807/S811A
Flag
RB
p-RB S780
p-RB S795
p-RB S807/811
DNMT1
p21
p16
β-ACTIN
B
WT
ΔCat
Relative fold increase
Days
C
WT
ΔCat
β-gal+ cell no. /power field
D
WT
ΔCat
Flag
p16
p21
β-TUBULIN
E
WT
ΔCat
OD 550 value
F
WT
ΔCat
OD 510 value

Figure S4

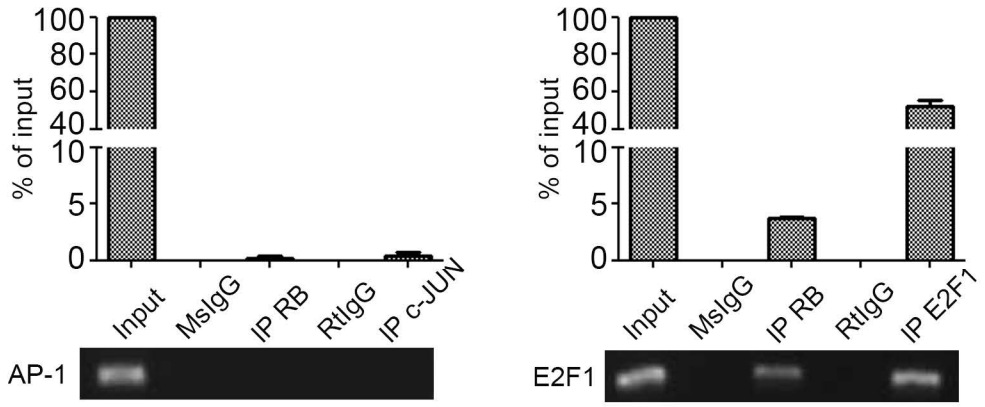
100
80
60
40
10
5
0
% of input
Input
MsIgG
IP RB
RtIgG
IP c-JUN
AP-1
100
80
60
40
10
5
0
% of input
Input
MsIgG
IP RB
RtIgG
IP E2F1
E2F1

Figure S5

Supplemental Figures Legends

FigureS1. Early-passage MSCs replicate as a more homogenous population than late-passage MSCs. Related to Figure 1. Carboxyfluorescein (CFSE) dye dilution assay for detecting cell proliferation rate. Aliquots of 10^5 CFSE labeled early- and late-passage MSCs were seeded in 10-cm dishes and incubated for 1, 3, 5, 7 days. Fluorescence of MSCs was quantified by flow cytometry. At day 5 and day 7, late-passage MSCs were separated into two groups. Only a portion of cells (arrow) replicated at a speed slower than early-passage MSCs, while the rest of the cells stopped replication**.**

Figure S2. Knockdown of RB in early-passage MSCs decreased BrdU incorporation rate, alkaline phosphatase activity and induced premature expression of senescence markers. Related to Figure 2. (A) MSCs without or with RB knockdown were seeded at 2×10^5 in 10-cm dishes for 48 h, bromodeoxyuridine (BrdU) were added into each dish and incubated for 18 h, and then detected by flow cytometry. (B) Alkaline phosphatase activity was analyzed at 1 week. (C) Western blot analysis, (D) quantitative RT-PCR, and (E) β-galactosidase staining of MSCs without or with RB knockdown. The results are expressed as mean ± SD of three independent experiments. Asterisks indicate significant differences (**P < 0.01).

Figure S3. Over-expression of RB affected proliferation rate and expression of senescence markers in late-passage MSCs and IMR90 cells. Related to Figure 3. (A-C) Over-expression of RB via retroviral transduction in late passage MSCs increased proliferation rate and reduced senescence markers. Late-passage MSCs were retroviral transducted with control (CTR) or RB overexpression vectors (OE RB). (A) MSCs without or with RB overexpression were seeded at 4.5×10^3 /cm^2 and cultured for 7 days. MTT assay was performed at indicated period, and data are shown as relative fold increase. (B) Western blot analysis. (C) β-galactosidase staining of MSCs without or with RB overexpression. Bar = 100 μm. (D-E) Over-expression of RB in IMR90 reduced proliferation rate and increased senescence marker. IMR90 cells transfected with control (CTR) or RB overexpression vectors (OE RB) were seeded at 4.5×10^3/cm^2 and cultured for 7 days. (D) MTT assay was performed at indicated period, and data are shown as relative fold increase. (E) β-galactosidase staining. Bar = 50 μm. The results are expressed as mean ± SD of three independent experiments. Asterisks indicate significant differences (**P < 0.01).

Figure S4. RB maintains proliferation rate, differentiation potential and reduces senescence markers through up-regulation of DNMT1 in MSCs. Related to Figure 4. (A) Loss-of-function mutations in each of the phosphorylation sites are associated with a decrease in the upregulation of DNMT1 by RB. Western blot analysis of late-passage MSCs overexpressed with wild-type RB or RB with indicated mutation (RB^{S780A}, RB^{S795A}, or $RB^{S807/811A}$). The MSCs overexpressed loss of function mutated RB decreased in the upregulation of DNMT1 and the downregulation of p21 and p16. (B-F) Over-expression of DNMT but not catalytic domain truncated DNMT1 in late passage MSCs increased proliferation rate, *in vitro* osteogenic and adipogenic differentiation potential, and reduced senescence markers. Late-passage MSCs transfected with full length (WT) or catalytic domain truncated (ΔCat) DNMT1 overexpression vectors were seeded at 4.5×10^3 /cm^2 and cultured for 7 days. (B) MTT assay was performed at indicated period, and data are shown as relative fold increase. (C) β-galactosidase staining. (D) Western blot analysis. (E) Cells were treated with adipogenic induction medium for 2 weeks followed by staining with Oil Red O. Upper panel shows representative pictures of Oil Red O staining at 2 weeks. Lower panel shows optical density measurement of extracted Oil Red O staining. (F) Cells were treated with osteogenic induction medium followed by staining with Alizarin Red S. Upper panel shows representative pictures of Alizarin Red S staining at 2 weeks. Lower panel shows optical density measurement of extracted Alizarin Red S staining. The results are expressed as mean ± SD of three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$).Bar = 50 μm.

Figure S5. RB does not bind to c-JUN in the DNMT1 promoter of non-MSC cells ChIP assay of H1299 cells. Related to Figure 5. The chromatin was incubated with antibodies against RB, c-JUN or E2F1 or their isotype IgG antibodies. Fragments of AP-1 (left panel) and E2F1 site (right panel) on DNMT1 promoter were amplified by PCR (lower panel) and quantitative PCR (upper panel). Input, 1% of total input lysate. The results are expressed as mean ± SD of three independent experiments. MsIgG, mouse IgG2a is the isotype of anti-RB antibody. RtIgG, rabbit IgG is the isotype of anti-c-JUN and anti-E2F1 antibody.

Table S1. Primer sequences for real-time PCR. Related to experimental procedures.

Primer name	Primer sequences
RB F	5′-GCTAGCCTATCTCCGGCTAAA-3′
RB R	5’-CTGGAAAAGGGTCCAGATGA-3′
LPL F	5’- CCCTAAGGACCCCTGAAGAC-3’
LPL R	5’- GGTTTTGCTGCTGTGATTGA-3’
PPAR-γ2 F	5’-CCTATTGACCCAGAAAGCGATTC-3’
PPAR-γ2 R	5’-GCATTATGAGACATCCCCACTGC-3’
RUNX2 F	5’-TTCAGGAGGGAGAAGAGCAA-3’
RUNX2 R	5’-TGGTTTTGTGAGCTGTCTGC-3’
BSP F	5’-AACCTACAACCCCACCACAA-3’
BSP R	5’-GTTCCCCGTTCTCACTTTCA-3’
APO-1 F	5’-CAAGGGATTGGAATTGAGGA-3’
APO-1 R	5’-ACCTGGAGGACAGGGCTTAT-3’
DNMT1 F	5’-ACCGCTTCTACTTCCTCGAGGCCTA-3’
DNMT1 R	5’-GTTGCAGTCCTCTGTGAACACTGTGG-3’
GAPDH F	5’-CTCTGCTCCTCCTGTTCGACA-3’
GAPDH R	5’-ACGACCAAATCCGTTGACTC-3’
p16 F	5’-GCCTTTTCACTGTGTTGGAGTTT-3’
p16 R	5’-CGCAAGAAATGCCCACATG-3’
p21 F	5’-GCCGAAGTCAGTTCCTT-3’
p21 R	5’-TCATGCTGGTCTGCCGC-3’
DNMT1-AP1-F	5’-CCCCGTTTTACAGATGAGGA-3’
DNMT1-AP1-R	5’- GGTTTGTGAGAGCCCTTGAG-3’
DNMT1-E2F1-F	5’-GCCTCTCTCCGTTTGGTACA-3’
DNMT1-E2F1-R	5’-TCGGAGGCTTCAGCAGAC-3’