Supplemental Data

Cyclin-dependent kinase inhibitor, $p21^{WAF1/CIP1}$, is involved in adipocyte differentiation and hypertrophy, linking apoptosis to obesity and insulin resistance

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Supplemental Method

Cell culture and differentiation

Primary MEF cells were harvested from 13.5-day post-coitus embryos of p21(+/+) and p21(-/-) mice. MEFs were cultured in high-glucose Dulbecco's modified Eagle's medium (GIBCO/BRL) with 10% FCS in 5% CO_2. MEFs were split into 60 mm dishes and cultured in α-modified Eagle's medium (GIBCO/BRL) with 10% FCS to confluence (Day -2). At Day 0, medium was replaced with the differentiation induction medium A (5 μg/ml insulin, 1 μM dexamethasone and 0.5 mM IBMX). This medium was renewed every other day (19).

Real-Time Quantity PCR

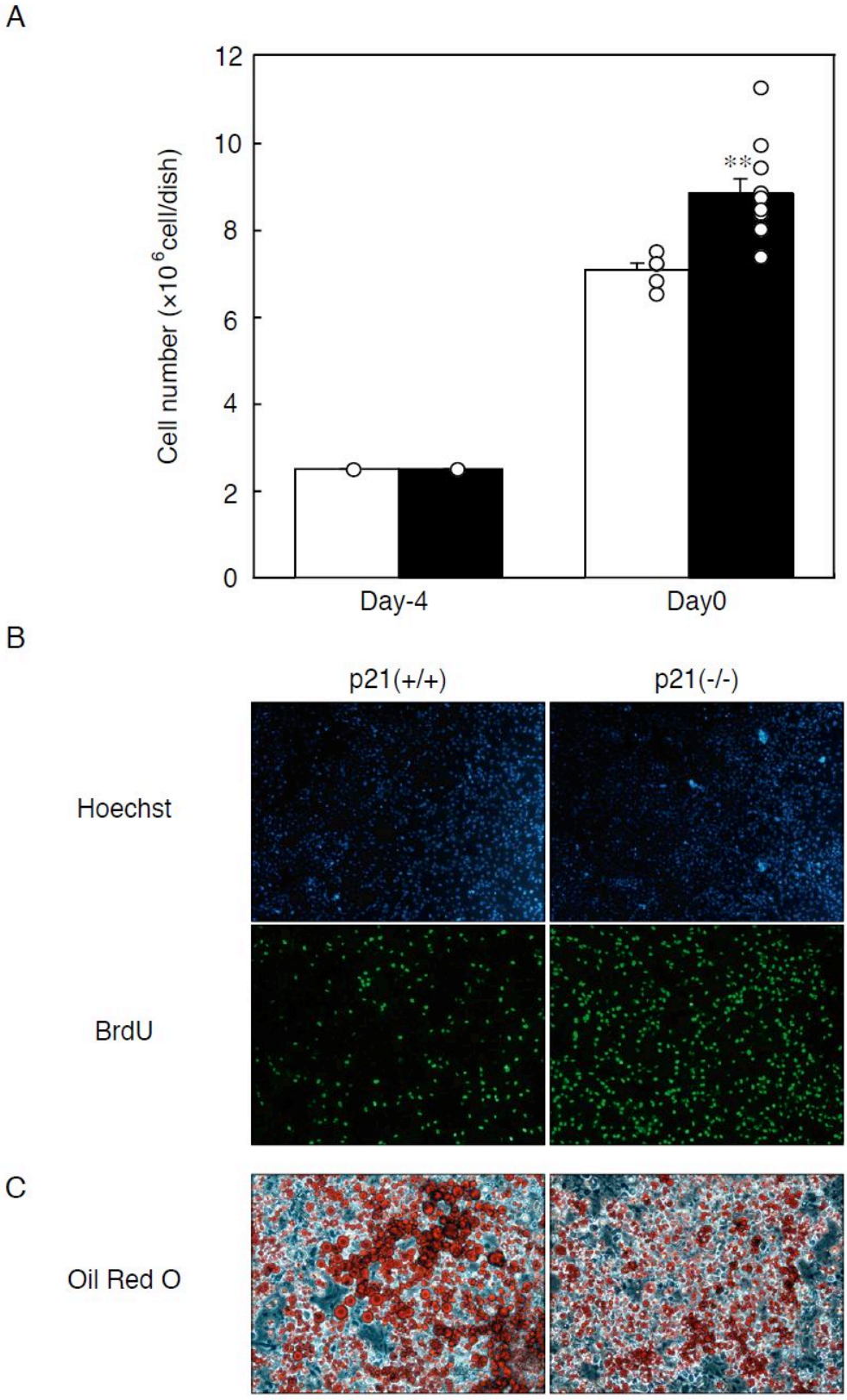
2.5μg of total RNA sample was reverse transcribed using High Capacity cDNA Archive Kit (Applied Biosystems). Expression of each gene was determined by real-time PCR on an ABI 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR reagents are from Applied Biosystems.

Real-Time Quantity PCR Taq Man probe list

TaqMan Gene Expression Assays (Applied Biosystems)

Gene name	No.
p21(CDKN1a)	Mm00432448_m1
Leptin	Mm00434759_m1
Adiponectin	Mm00456425_m1
Resistin	Mm00445641_m1
Adipsin	Mm00442664_m1
TNFα	Mm00443258_m1
MCP-1	Mm00441242_m1
36B4(acidic ribosomal phosphoprotein P0)	Mm00725448_s1

Figure. A1 Proliferation and adipocyte differentiation of p21(+/+) and p21(-/-) MEFs.



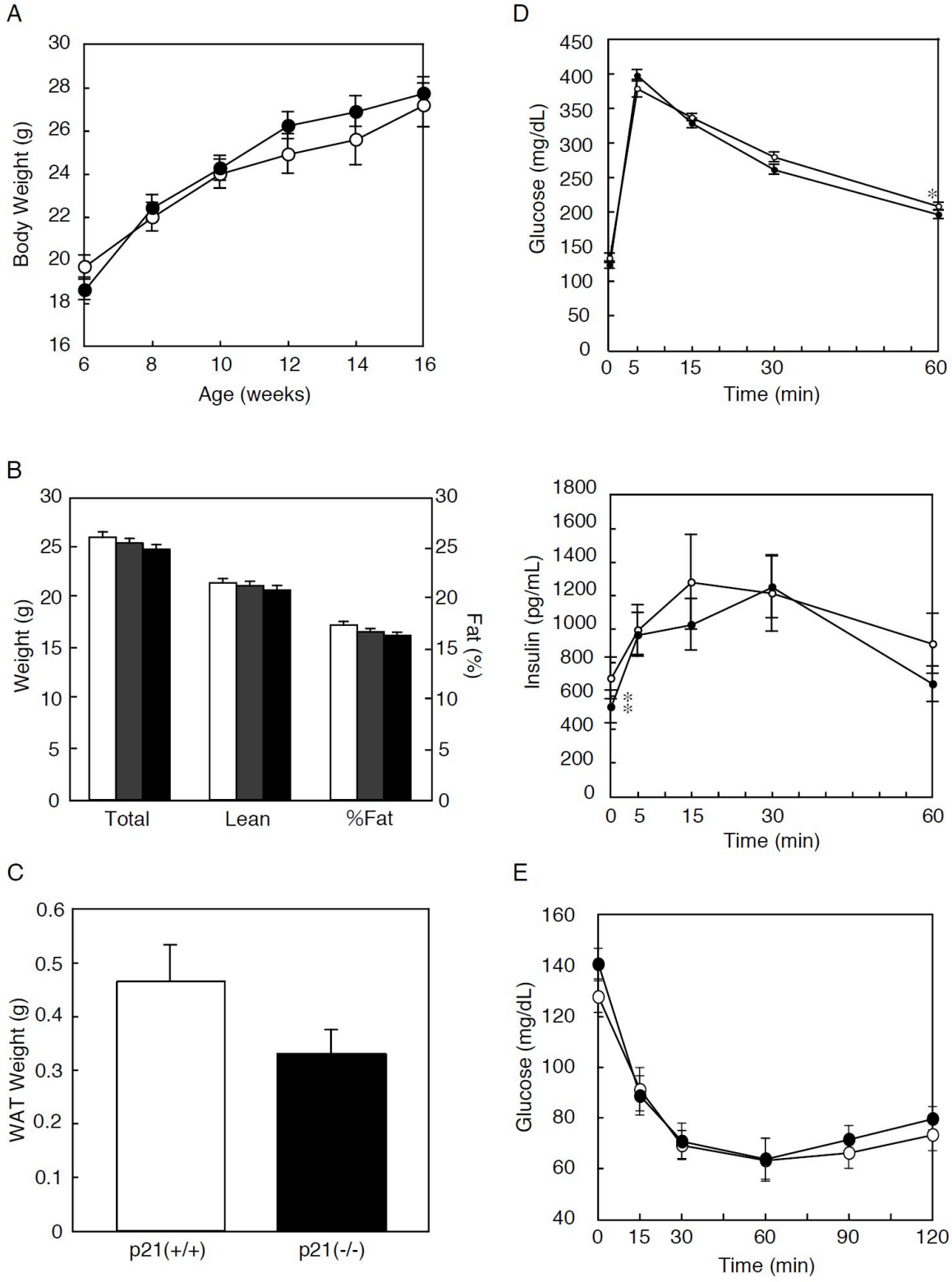
MEF cells were prepared from p21(+/+) and p21(-/-) mice, incubated for 4 days in regular media, followed by further culture in the adipocyte induction medium for 14 days.

A) Cell numbers of p21(+/+) MEF (white column) and p21(-/-) MEF(black column) at Day -4 (harvest) and Day 0 in a confluent condition just before addition of induction medium.
Dots in the graph are individual cell counts. Values are means ± SEM. [p21(+/+) MEFs : n=5 and p21(-/-) MEFs : n=9]
**; signicance versus p21 (+/+) MEFs at $p<0.01$

B) Proliferation of MEFs determined with Hoechst stain and BrdU uptake at Day 2 of adipocyte differentiation.

C) Oil Red O staining of cells at Day 14 of adipocyte differentiation.

Figure. A2 Metabolic parameters in p21 (+/+) and p21 (-/-) mice on a normal chow diet.



p21(+/+) and p21(-/-) mice were maintained on chow diet until 18 weeks of age.

A) Body weight change in p21 (+/+) and p21 (-/-) mice (n=17).

B) Total body mass and lean mass, % fat in p21 (+/+), p21(+/-) and p21 (-/-) mice at 10 weeks of age as estimated by DEXA (n =18).

C) Weights of epididymal fat pads from p21 (+/+) and p21 (-/-) mice. (n = 14).

D) Glucose tolerance in p21 (+/+) and p21 (-/-) mice. Mice were intraperitoneally injected with 2 mg/kg D-glucose. Blood glucose (upper panel) and plasma insulin (lower panel) concentrations were measured at the indicated times (n = 11).

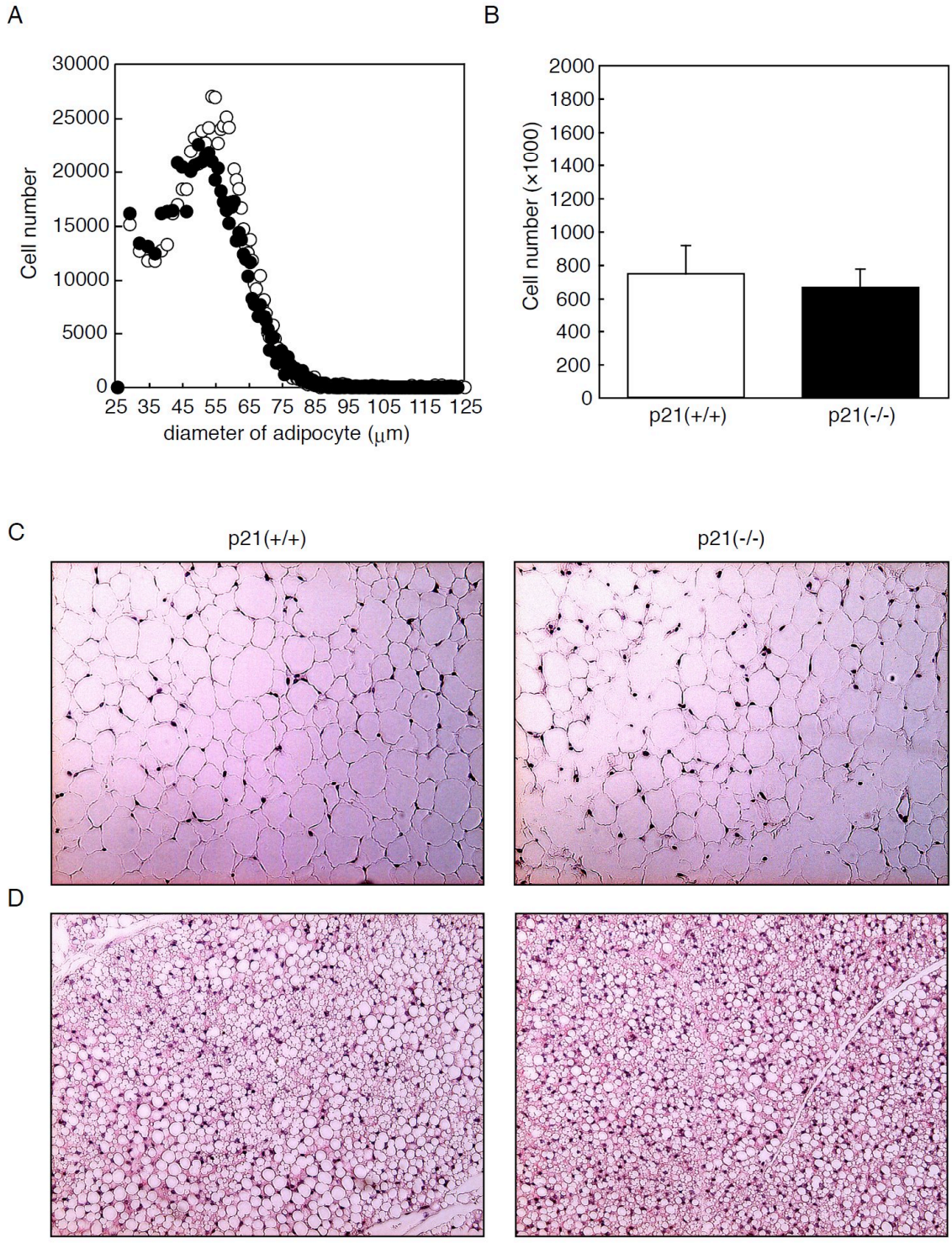
E) Insulin tolerance in p21 (+/+) and p21 (-/-) mice. Mice were injected with 0.5 U/kg insulin. Blood glucose levels were measured at the indicated times.

Columns and circles; white: p21(+/+), gray: p21(+/-), black: p21(-/-); n=11.

Values are means ± SEM

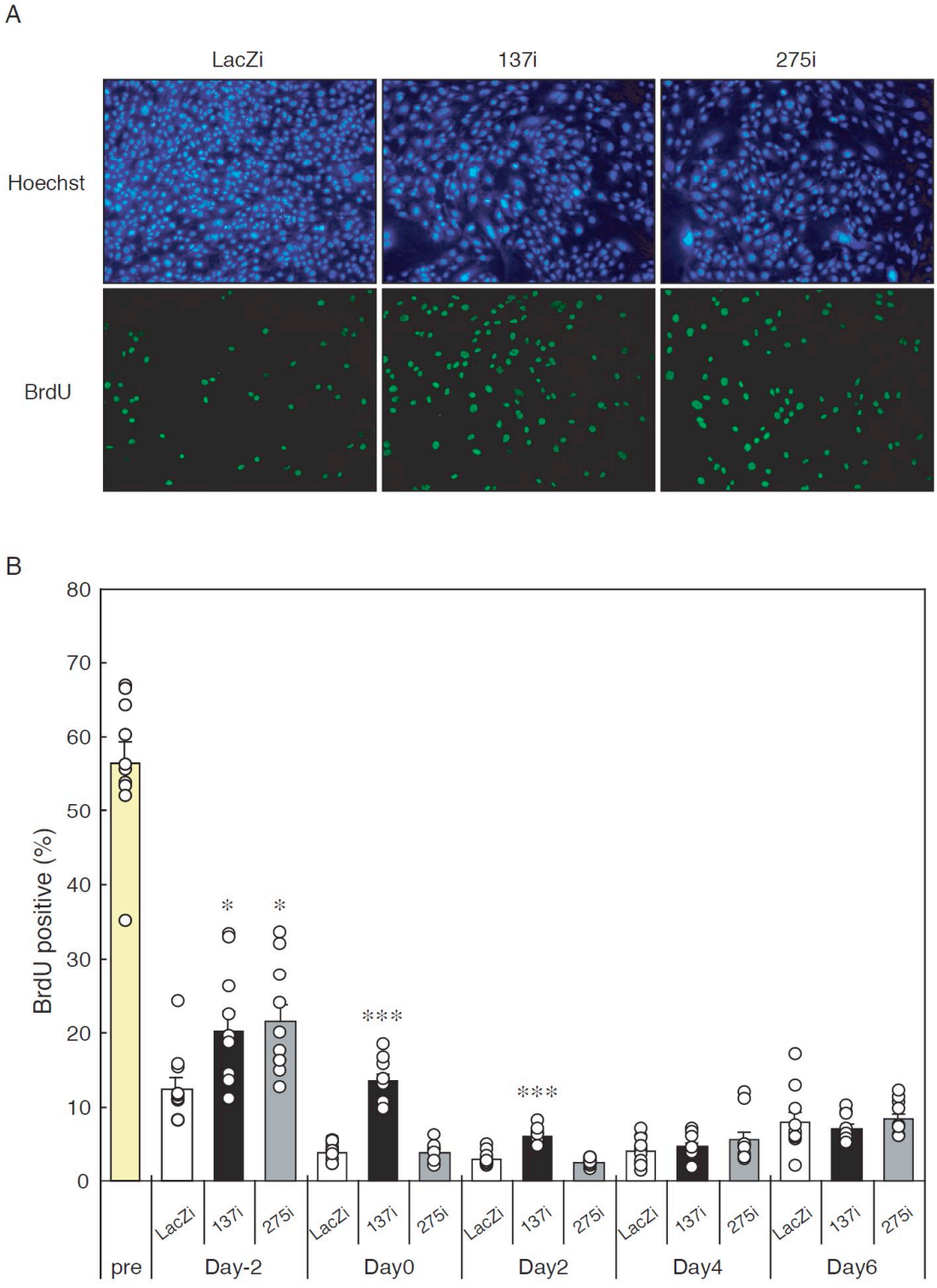
** at $p<0.01$ for p21(-/-) compared to p21(+/+) groups.

Figure. A3 Analysis of white adipose tissue in p21 (+/+) and p21 (-/-) mice on a normal chow diet.



p21(+/+) and p21(-/-) mice were maintained on chow diet until 18 weeks of age.
Size distribution (A) and total cell number (B) of adipocytes from epididymal fat pads. Cell size distribution was as quantified by Coulter counter. Microscopic examination of epididymal white fat pad (C) and brown adipose tissue (D) (HE staining).
Columns and circles; white: p21(+/+), black: p21(-/-). Values are means ± SEM of 6 mice per group.

Figure. A4 BrdU uptake in 3T3-L1 adipocytes infected with p21 RNAi adenoviruses



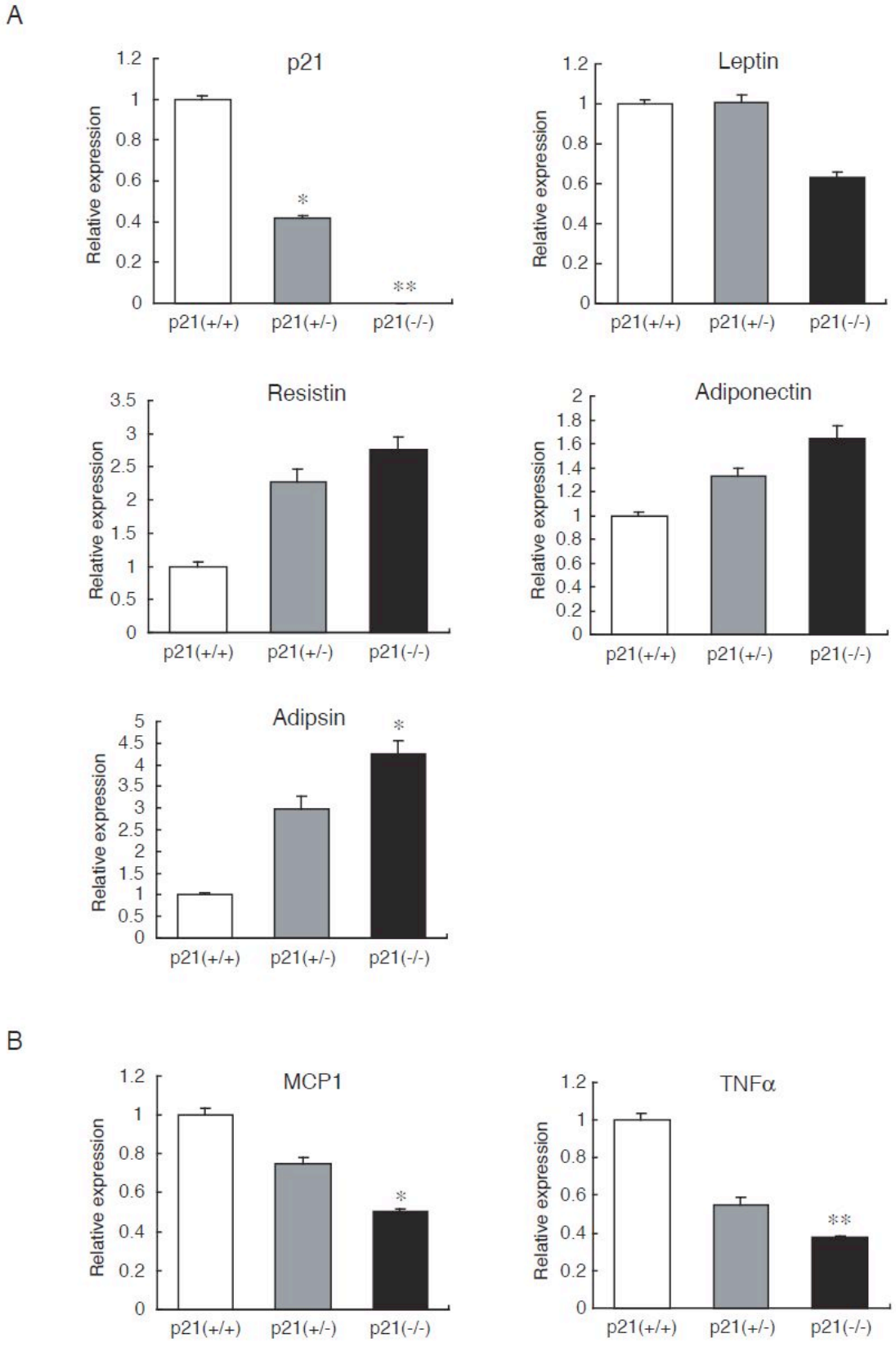
Under the same condition as Figure.1C-E, 3T3-L1 fibroblasts infected with p21 RNAi adenoviruses at Day -3. The cells started differentiation at Day 0 with differentiation induction medium A and changed to differentiation induction medium B at Day 2 (See the Experimental Procedures). Proliferation of the cells at the indicated days was determined as estimated by BrdU uptake and Hoechst stain.

A) Time course analysis for BrdU uptake of 3T3-L1 adipocytes after p21 knockdown at Day -3 Hoechst 33342 for nuclear staining (upper) and BrdU staining (lower) are shown at magnification of x400.

B) Quantification of BrdU positive cells (green) ratio corrected to the Hoechst33342 (blue).

Dots in the graph are individual cell counts. Values represent mean ± SEM from 8-10 fields of 800-1000 cells per group. *** at $p<0.0001$ for 137i and 275i versus LacZi adenoviruses infected cells, * at $p<0.05$

Figure.A5 RT-PCR quantification of in white adipose tissue of p21(-/-) mice on a HFHS diet.



mRNA levels of indicated genes were measured by Real Time quantity PCR for RNA samples used for northern blot analysis in the Figure.4A. Expression of each gene was normalized to 36B4 (acidic ribosomal phosphoprotein P0).

** at $p<0.01$ for p21(+/-) and p21(-/-) versus p21(+/+), * at $p<0.05$

Columns and circles; white: p21(+/+), gray: p21(+/-), black: p21(-/-); n=6

Values are means ± SEM.

Table. A1 Metabolic parameters of p21 (+/+) and (-/-) mice on a normal diet (18 week old)

	Fasted		Fed	
	p21(+/+)	p21(-/-)	p21(+/+)	p21(-/-)
TG (mg/dL)	175 ± 9	183 ± 18	118 ± 25	107 ± 14
T-Cho (mg/dL)	103 ± 6	99 ± 4	94 ± 9	85 ± 4
NEFA (mEq/L)	1.43 ± 0.16	1.38 ± 0.08	0.83 ± 0.14	0.64 ± 0.08
Leptin (ng/mL)	0.14 ± 0.09	0.06 ± 0.02	1.05 ± 0.21	0.48 ± 0.07

Values are means ± SEM. (n=16) ∗; signicance versus p21(+/+) mice at $p<0.05$ and ∗∗ at $p<0.01$

Table. A2 Metabolic parameters of p21 (+/+) and (-/-) mice on a HFHS diet

p21(+/+) and p21(-/-) mice at 10 weeks of age were put fed on a HFHS diet for 6 weeks.

	Fasted		Fed	
	p21(+/+)	p21(-/-)	p21(+/+)	p21(-/-)
Glucose (mg/dL)	210 ± 9	193 ± 10	201 ± 9	204 ± 5
Insulin (pg/mL)	1727 ± 303	855 ± 185∗∗	5497 ± 994	2625 ± 619∗
TG (mg/dL)	93 ± 7	97 ± 11	112 ± 7	102 ± 10
T-Cho (mg/dL)	215 ± 6	183 ± 13∗	218 ± 10	185 ± 13∗
NEFA (mEq/L)	0.68 ± 0.05	0.81 ± 0.07	0.49 ± 0.04	0.56 ± 0.04
Liver TG (mg/g)	35 ± 5	38 ± 4	N/D	N/D
Liver T-Cho (mg/g)	3.8 ± 0.7	4.7 ± 0.7	N/D	N/D
Leptin (ng/mL)	3.2 ± 1.3	2.1 ± 1.4	8.8 ± 2.7	4.7 ± 1.8

Values are means ± SEM. (n=6-16)

∗; signicance versus p21 (+/+) mice at $p<0.05$ and ∗∗ at $p<0.01$

N/D = not determined

Table. A3 Insulin secretion of p21 knockout mice at normal diet

p21(+/+) and p21(-/-) mice were maintained on chow diet. Mice were either fasted for 24 h or refed at 12 h after a 24 h starvation.

	Fasted		Refed	
	p21(+/+)	p21(-/-)	p21(+/+)	p21(-/-)
Glucose (ng/dL)	81 ± 5.3	81 ± 4.8	201 ± 21	208 ± 14
Insulin (pg/mL)	420 ± 190	198 ± 35	3316 ± 551	5786 ± 2316

Values are means ± SEM. (n=6)