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Supplemental Information

Clonal Analysis Delineates Transcriptional Programs of Osteogenic and Adipogenic Lineages of Adult Mouse Skeletal Progenitors

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Figure S1

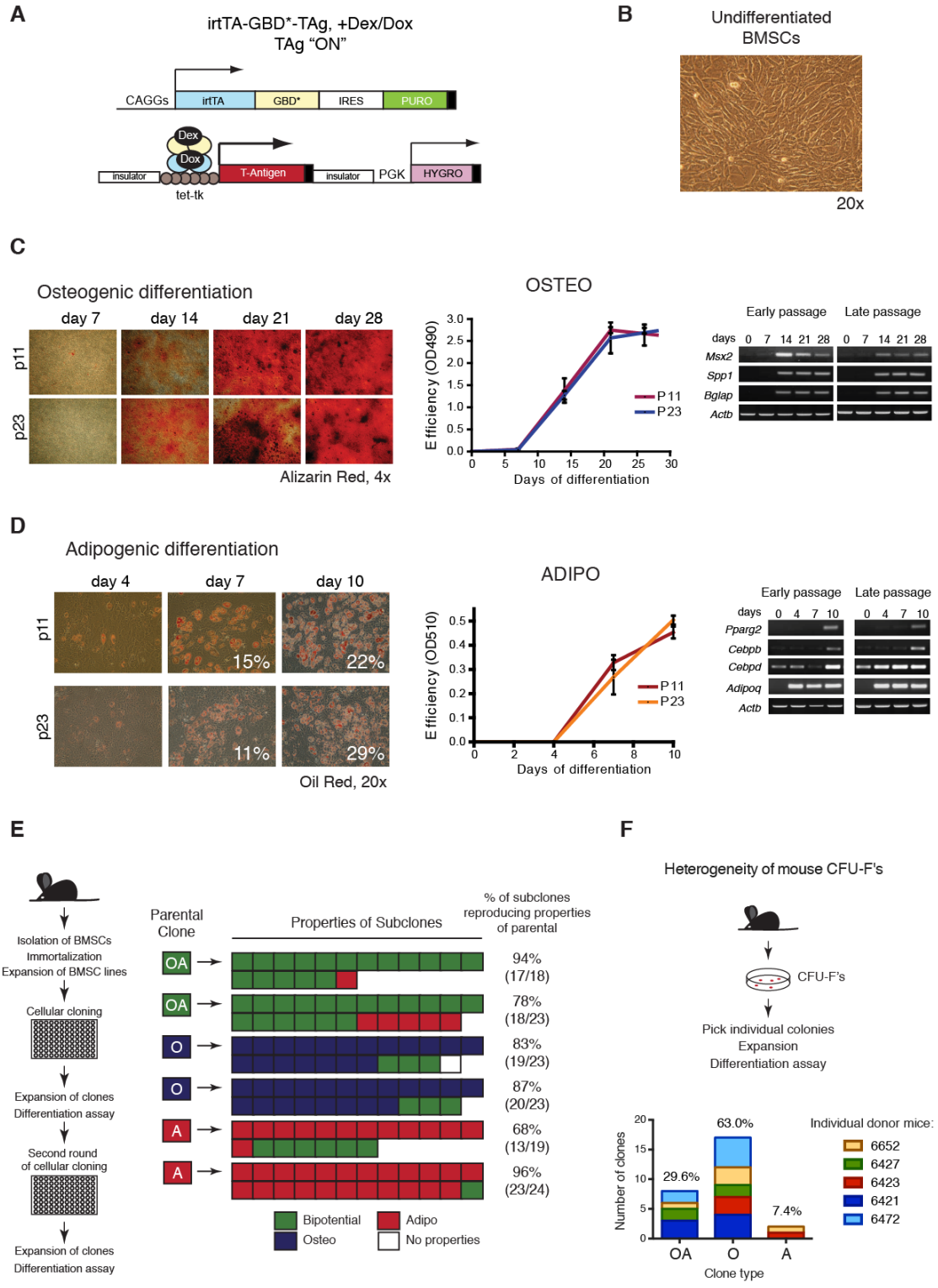
A
irtTA-GBD*-TAg, +Dex/Dox
TAg "ON"
CAGGs
irtTA
GBD*
IRES
PURO
Dex
Dox
insulator
T-Antigen
insulator
PGK
HYGRO
tet-tk
B
Undifferentiated
BMSCs
20x
C
Osteogenic differentiation
day 7
day 14
day 21
day 28
p11
p23
Alizarin Red, 4x
OSTEO
Efficiency (OD490)
P11
P23
Days of differentiation
Early passage
Late passage
days 0 7 14 21 28 0 7 14 21 28
Msx2
Spp1
Bglap
Actb
D
Adipogenic differentiation
day 4
day 7
day 10
p11
p23
15%
22%
11%
29%
Oil Red, 20x
ADIPO
Efficiency (OD510)
P11
P23
Days of differentiation
Early passage
Late passage
days 0 4 7 10 0 4 7 10
Pparg2
Cebpb
Cebpd
Adipoq
Actb
E
Isolation of BMSCs
Immortalization
Expansion of BMSC lines
Cellular cloning
Expansion of clones
Differentiation assay
Second round
of cellular cloning
Expansion of clones
Differentiation assay
Parental
Clone
Properties of Subclones
% of subclones
reproducing properties
of parental
OA
OA
O
O
A
A
94%
(17/18)
78%
(18/23)
83%
(19/23)
87%
(20/23)
68%
(13/19)
96%
(23/24)
Bipotential
Adipo
Osteo
No properties
F
Heterogeneity of mouse CFU-F's
CFU-F's
Pick individual colonies
Expansion
Differentiation assay
Number of clones
29.6%
63.0%
7.4%
OA
O
A
Clone type
Individual donor mice:
6652
6427
6423
6421
6472

Figure S1. Characterization of conditionally immortalized BMSCs lines, and clonal populations derived from established BMSC lines and from primary bone marrow. Related to Figure 1.
(**A**) Diagram showing the modified tetracycline-regulated system for conditional immortalization (Anastassiadis et al., 2010; Rostovskaya and Anastassiadis, 2012). Improved reverse tetracycline transactivator fused to a mutated glucocorticoid-binding domain (irtTA-GBD*) is constitutively expressed under control of the CAGGs promoter and induces transcription of SV40 Large T-Antigen in the presence of two ligands, dexamethasone and doxycycline (Dex/Dox).
(**B**) Morphology of BMSCs isolated form transgenic irtTA-GBD*-TAg mice expanded in the presence of Dex/Dox. The micrograph is reused from Figure 1A upper left panel.
(**C**) Conditionally immortalized BMSCs were assessed for osteogenic differentiation capacity at early (P11) and late passages (P23). Alizarin Red staining, 4x. Quantification was done by extraction of bound Alizarin Red and measurement of optical density of the eluate at 490 nm. Expression of differentiation markers was checked by RT-PCR, using β-actin as a loading control. Error bars indicate SD of technical replicates (measurements from independent wells).
(**D**) Adipogenic differentiation of conditionally immortalized BMSCs at early (P11) and late passages (P23). Oil Red staining, 20x. Percentages of adipocytes are indicated on the images. Accumulation of fat was evaluated by elution of Oil Red and measurement of OD510. Expression of differentiation markers was checked by RT-PCR, using β-actin as a loading control.
(**E**) Clonally derived BMSC progenitors with "OA", "O" and "A" properties were obtained from established BMSC lines by limiting dilutions, and then subjected to the second round of cloning and the resulting subclones were checked for differentiation properties. The properties of the cells are indicated as colors, the percentage and total numbers are shown. A majority of subclones reproduced the properties of parental populations indicating their homogeneity and stable inheritance of properties.
(**F**) Heterogeneity of bone marrow CFU-F's. Individual colonies (initiated by single CFU-F's) were established from five irtTA-GBD*-TAg transgenic mice, then immortalized, expanded and tested for osteogenic and adipogenic potential. Bi- ("OA") and unipotent ("O" and "A") populations were identified among clones. Clones established from each individual mouse are indicated with different color.

Figure S2

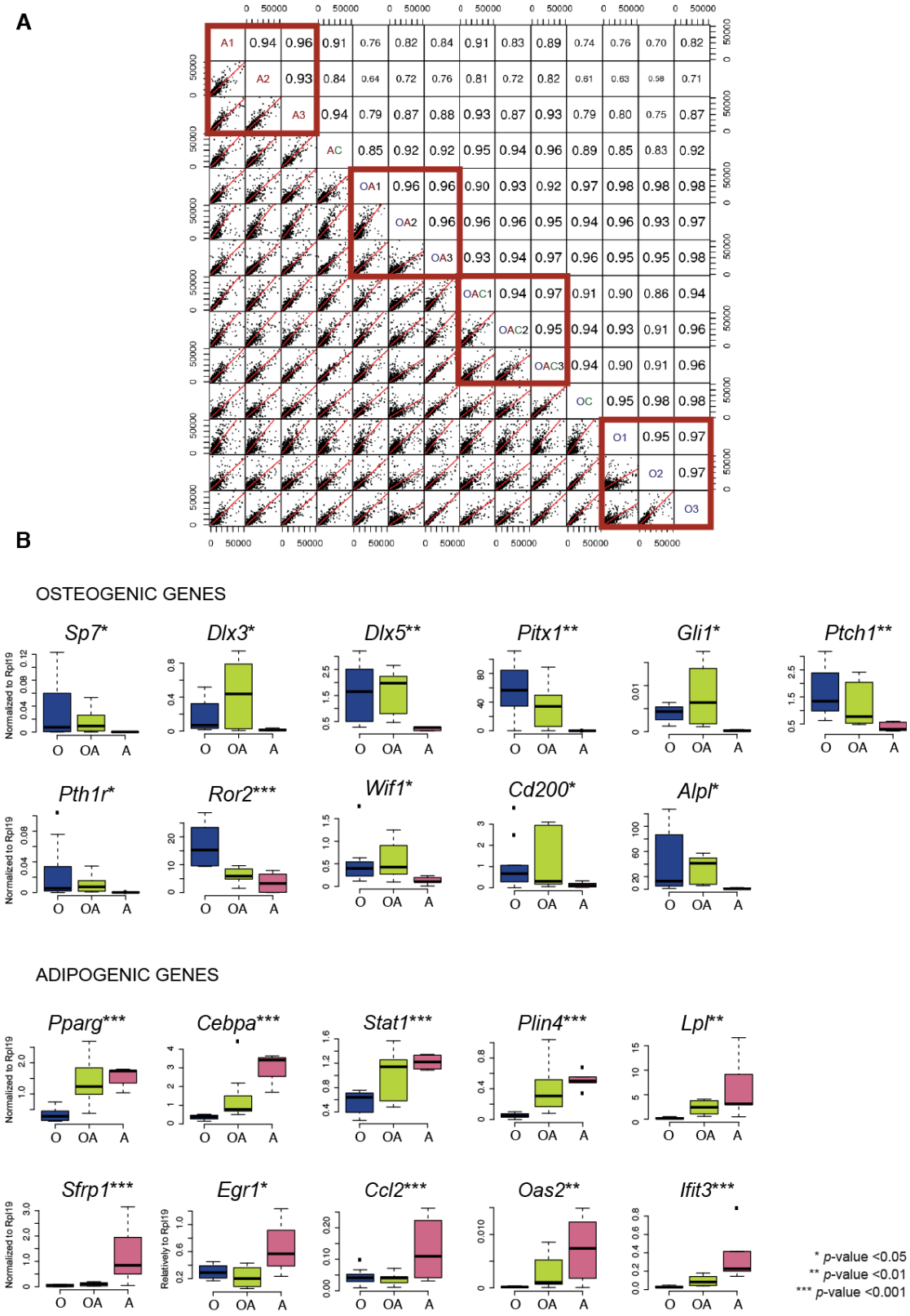
A
B
OSTEOGENIC GENES
Sp7*
Dlx3*
Dlx5**
Pitx1**
Gli1*
Ptch1**
Pth1r*
Ror2***
Wif1*
Cd200*
Alpl*
ADIPOGENIC GENES
Pparg***
Cebpa***
Stat1***
Plin4***
Lpl**
Sfrp1***
Egr1*
Ccl2***
Oas2**
Ifit3***
Normalized to Rpl19
Relatively to Rpl19
O
OA
A
* p-value <0.05
** p-value <0.01
*** p-value <0.001

Figure S2. RNA profiling of clonally derived skeletal progenitors with distinct differentiation potential. Related to Figure 2.

(**A**) Correlation between skeletal progenitors analyzed by RNA sequencing. The samples are sorted according to their properties and replicates are outlined with red boxes. Correlation coefficients and plots are shown for each pairwise comparison. Progenitor types "OAC", "OA", "O", "A" were analyzed in triplicates, whereas "OC" and "AC" were single samples. The clones for expression analysis were obtained from two mice (samples with numerical code "1" and "2"), and the third replicate (samples labeled with "3") always represented a pool of several clones with the same *in vitro* differentiation properties (2-6 clones) isolated from both mice. Pooling the clones presumably diminishes variability of the samples, therefore rare clone types ("OC" and "AC") were taken only as pools without replicates.

(**B**) Validation of differentially expressed genes resulted from RNA-seq. Expression of selected genes characteristic of osteogenic or adipogenic lineage was assayed by qRT-PCR in independent clones with "O", "OA" and "A" properties, which were not used for RNA-seq, thus representing biological replicates (6-10 clones with each property were tested). In the box plots the horizontal line indicates the median, the box shows 25^{th} and 75^{th} percentiles. Asterisks indicate statistical significance for the differential expression (*p*-value).

Figure S3

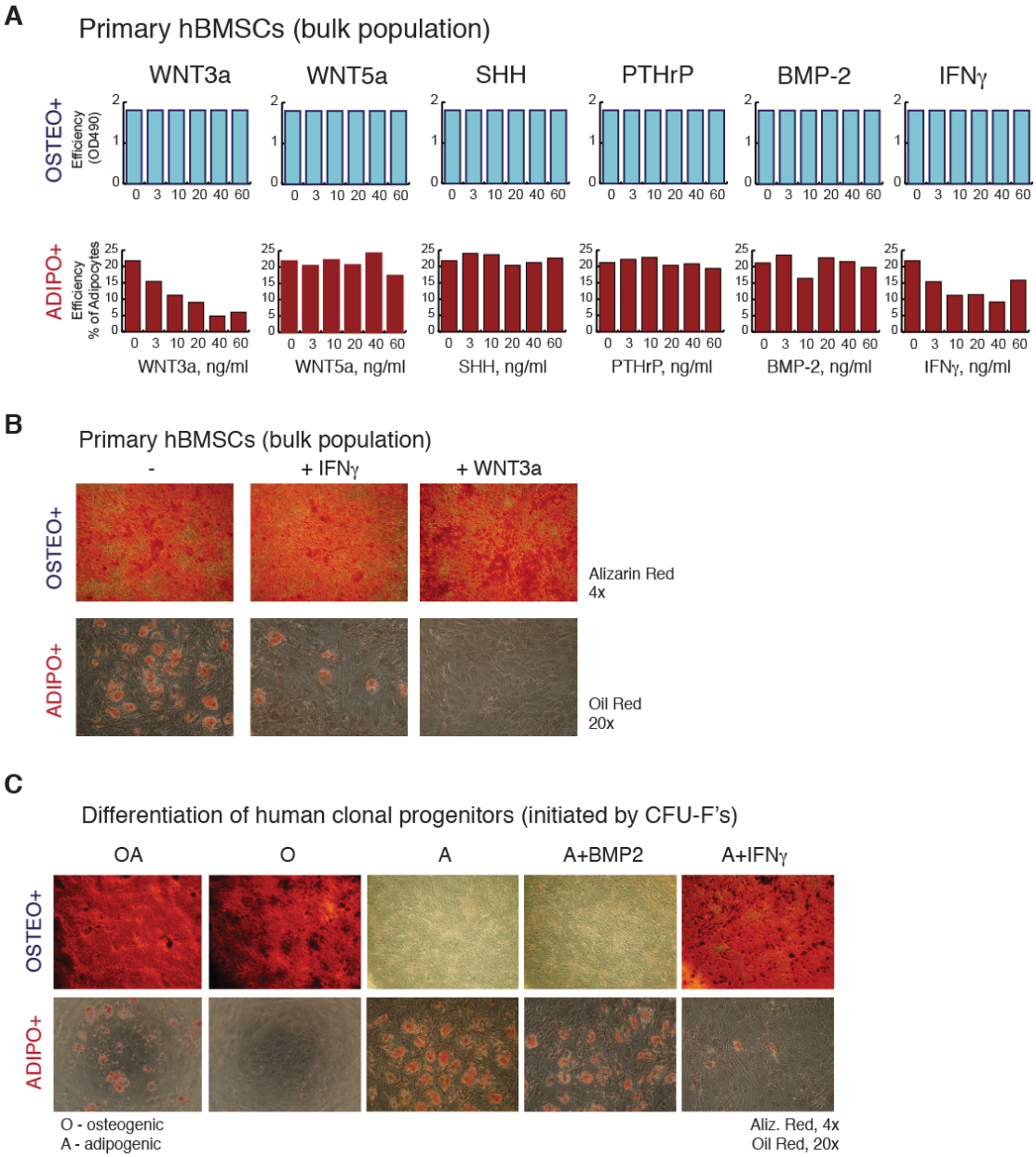

Figure S3. Signaling pathways in lineage commitment of human skeletal progenitors. Related to Figure 4.

(**A**) A set of signaling molecules (WNT3a, WNT5a, SHH, PTHrP, BMP-2, IFNγ) was tested for their effect on differentiation of primary human BMSC line into osteogenic and adipogenic lineages. Osteogenesis was confirmed in the presence of each of the tested compounds at concentrations ranging from 3 to 60 ng/ml. IFNγ and WNT3a inhibited adipogenesis in a dose-dependent manner. Osteogenic differentiation was assessed by extraction of Alizarin Red dye and measurement of OD490 of the eluate. Efficiency of adipogenesis was evaluated by counting proportion of adipocytes.
(B) Representative images of hBMSCs differentiated into osteogenic lineage (upper panel, Alizarin Red, 4x) or adipocytes (lower panel, Oil Red, 20x), in the absence or presence of IFNγ or WNT3a.
(**C**) Human bone marrow stromal cell clones initiated by single CFU-F's were individually picked and screened for osteo- and adipogenic capacity. "OA", "O" and "A" types of clones were identified. "A" clones were capable of osteogenic differentiation in the presence of IFNγ, whereas adipogenesis was inhibited. BMP-2 had no effect on the lineage commitment of the cells.

Figure S4

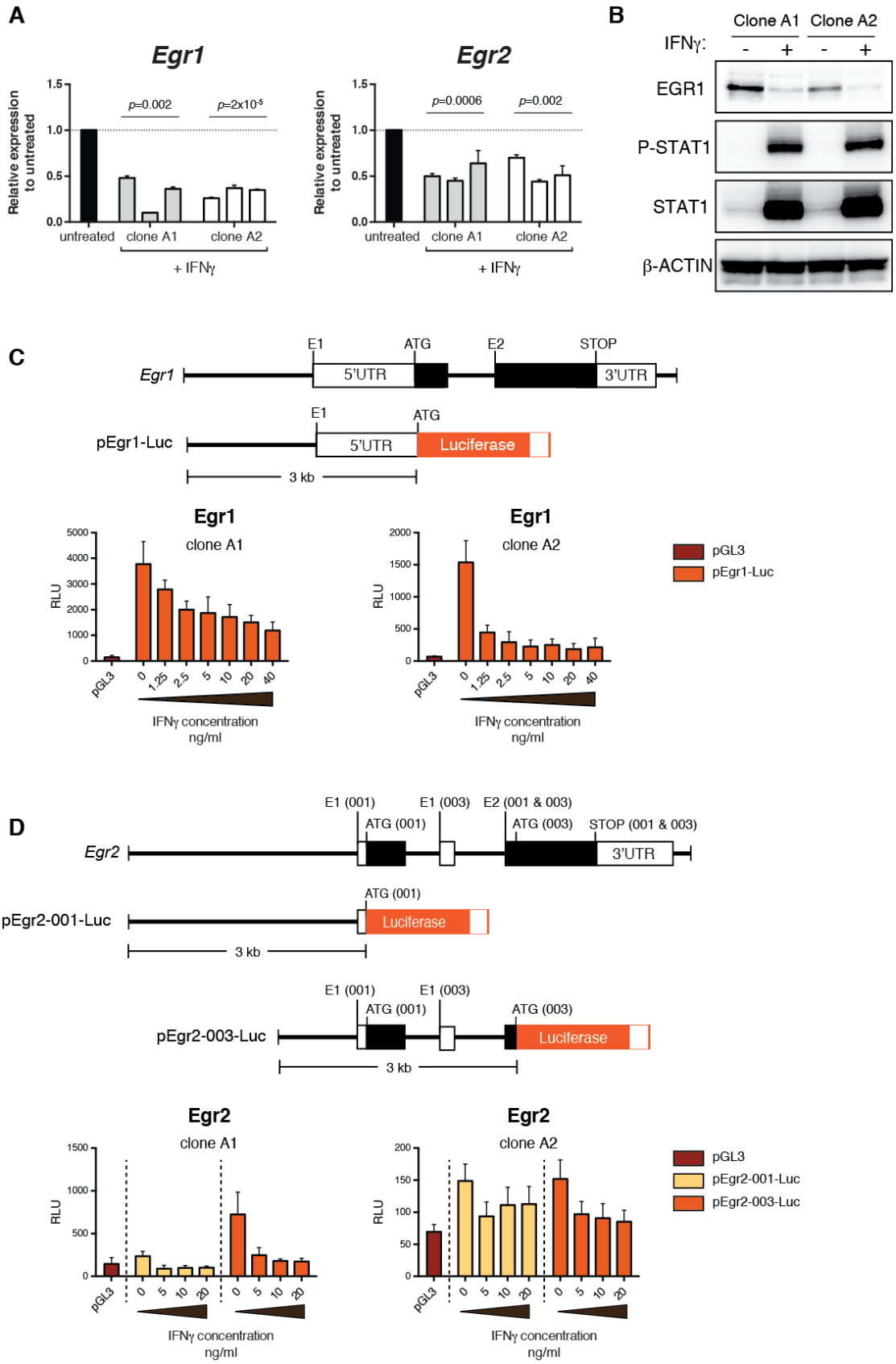
A
Egr1
Egr2
Relative expression to untreated
p=0.002
p=2x10-5
p=0.0006
p=0.002
untreated
clone A1
clone A2
+ IFNγ
B
Clone A1
Clone A2
IFNγ:
- + - +
EGR1
P-STAT1
STAT1
β-ACTIN
C
Egr1
E1
ATG
E2
STOP
5'UTR
3'UTR
pEgr1-Luc
Luciferase
3 kb
Egr1
clone A1
clone A2
RLU
pGL3
0 1.25 2.5 5 10 20 40
IFNγ concentration ng/ml
pGL3
pEgr1-Luc
D
Egr2
E1 (001)
E1 (003)
E2 (001 & 003)
ATG (001)
ATG (003)
STOP (001 & 003)
3'UTR
pEgr2-001-Luc
Luciferase
3 kb
pEgr2-003-Luc
Egr2
clone A1
clone A2
RLU
pGL3
0 5 10 20
IFNγ concentration ng/ml
pGL3
pEgr2-001-Luc
pEgr2-003-Luc

Figure S4. Regulation of *Egr1* and *Egr2* expression by IFNγ. Related to Figure 6.

(**A**) Expression of *Egr1* and *Egr2* in "A" clones treated with IFNγ (10 ng/ml) by qRT-PCR relative to untreated cells. Results of three independent experiments for 2 clones derived from different mice are shown. Both *Egr1* and *Egr2* showed significant downregulation in the presence of IFNγ. Error bars indicate SD of technical replicates of qPCR reactions.

(**B**) EGR1 was downregulated at the protein level in the presence of IFNγ as shown by Western blot. Phospho-STAT1 and total STAT1 were upregulated upon treatment. β-ACTIN was used as loading control.

(**C**) *Egr1* gene promoter activity in response to IFNγ. Schematic representation of mouse *Egr1* locus (top) and reporter construct containing luciferase gene inserted after the initiating Methionine (ATG) and 3 kb upstream regulatory sequence. The reporter construct (pEgr1-Luc) was transiently co-transfected with beta-actin-LacZ expression plasmid into two independent "A" clones established from different mice. Luciferase expression values were normalized to beta-galactosidase measurements for transfection efficiency. Luciferase expression was downregulated in the presence of IFNγ in a dose-dependent manner, indicating transcriptional regulation of *Egr1* expression by IFNγ. Error bars indicate SD of technical triplicates.

(**D**) *Egr2* gene promoter activity in response to IFNγ. Diagram of mouse *Egr2* locus (top) and two reporter constructs (middle and bottom), in which luciferase was inserted after the alternative ATG start codons either in exon 1 (pEgr2-001-Luc) or exon 2 (pEgr2-003-Luc) and included 3 kb of upstream sequences. The reporter constructs were transiently co-transfected with beta-actin-LacZ expression vector into 2 independent "A" clones derived from different mice. Luciferase quantification results were normalized to beta-galactosidase values. Luciferase expression was downregulated in the presence of IFNγ, showing transcriptional regulation of *Egr2* expression by IFNγ. pGL3 is a Luciferase promoterless vector. Error bars indicate SD of technical triplicates. RLU = Relative Luciferase Units.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

BMSC isolation and culture

BMSCs were isolated by flushing the femora and tibiae of irtTA-GBD*-TAg transgenic mice and cultured as described previously (Rostovskaya and Anastassiadis, 2012). In details, cells were cultured in Dulbecco's Modified Eagle's Medium, DMEM, with 1 g/L D-glucose (Gibco Life Technologies), supplemented with 10% Fetal Calf Serum (FSC, PAA) and 1x Penicillin/Streptomycin. At the first passage 10^{-7} M Dexamethasone (Dex) and 1 μg/ml Doxycycline (Dox, both from Sigma) were added to BMSC growth medium to induce expression of Large T-antigen. Conditionally immortalized BMSCs grew rapidly in the presence of Dex/Dox and were routinely passaged every 3-5 days at a dilution 1:5-1:10. To generate clones initiated by CFU-F's, $5x10^5$ mouse bone marrow cells were plated onto 10 cm tissue culture dishes, and grown until distinct single-cell derived clones appeared. The colonies were picked using cloning cylinders, and further conditionally immortalized in the presence of Dex/Dox. Cellular cloning was done by limiting dilutions or by single-cell deposition into 96-well plates using FACSAriaII cell sorter (BD Biosciences). The clones were passaged and assayed for differentiation in a 96-well plate format. Primary human BMSCs were isolated from healthy donors after informed consent and approval by the local ethics committee. Mononuclear cells were collected after centrifugation in Ficoll gradient and plated with a concentration of $5x10^5$ cells/cm^2 in BMSC growth medium. At confluency the cells were passaged at 1:4 dilution. To generate clones initiated by single CFU-F's, mononuclear cells were plated at 10^6 cells per 10 cm dish in alpha Minimum Essential Medium (aMEM, Gibco Life Technologies) supplemented with 20% FCS (PAA) and Penicillin/Streptomycin. Individual clones were transferred to a 96-well plate using cloning cylinders, and after reaching confluency split into differentiation conditions. For RNA sequencing the cells were cultured in the medium without Dex/Dox for 3 days, 10 ng/ml IFNγ or WNT3a were added where stated.

Differentiation assays

Prior to all differentiation assays conditionally immortalized mouse BMSCs were cultured for 3 days without Dex/Dox to deinduce Large T-antigen. For osteogenic differentiation, BMSCs were plated at a concentration of $2x10^4$ cells/cm^2 in growth medium supplemented with 10^{-8} M Dex, 10 mM β-glycerophosphate, 300 μM ascorbic acid. After 4 weeks the cells were fixed with methanol for 10 min at -20°C, stained with 2% Alizarin Red at pH: 4.3 and dried. To quantify the efficiency, the dye was extracted from the stained samples with 4 M guanidine chloride at 37°C overnight, and the optical density of the eluate was measured at 490 nm. Alkaline phosphatase staining was done on cells fixed with ice-cold methanol for 2 min, using 0.4 mg/ml Nitro-Blue-Tetrazolium Chloride (NBT) and 0.19 mg/ml 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) in buffer containing 100 mM Tris-HCl, pH: 9.5, 100 mM NaCl and 10 mM $MgCl_2$. Adipogenic differentiation was induced in BMSCs at 75-90% confluency with 10^{-7} Dex, 5 μg/ml Troglitazone and 5 μg/ml insulin added to the growth medium for 7 days. The cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 30 min and stained with Oil Red (0.3% solution in isopropanol mixed 3:2 with water and filtered) for 5 min. Quantification was done by counting adipocytes using phase contrast microscopy in at least 3 fields of view, or by extraction of bound Oil Red with isopropanol and assessing optical density at 510 nm. For chondrogenic differentiation, pellets prepared from 10^6 cells by centrifugation were cultured in DMEM with 4.5 g/L glucose (Cat. 61965, Gibco Life Technologies) supplemented with 1% FCS, 10 ng/ml TGF-β1 (Peprotech), 10^{-7} M Dex, 6.25 μg/ml apo-transferrin, 6.25 μg/ml insulin and 50 μg/ml ascorbate-2-phosphate. After three weeks the pellets were fixed with 4% formaldehyde in PBS for 10 min at room temperature, embedded in paraffin and sectioned. Immunostaining on sections was done with anti-collagen II antibody (1:200, MAB8887, Chemicon) and visualized with Vectastain ABC kit (Vector Labs) according to the manufacturer's recommendations. All chemicals were purchased from Sigma, unless otherwise stated. Growth factors and cytokines, such as WNT3a, SHH, PTHrP, BMP-2, IFNγ (all from Peprotech) or WNT5a (R&D Systems) were added to the cells differentiating to osteocytes or adipocytes in the range of concentrations 3, 10, 20, 40 and 60 ng/ml.

Transplantation assays

In order to assess the osteogenic potential *in vivo*, $2x10^6$ cells were loaded onto hydroxyapatite/tricalcium phosphate particles (HA/TCP, 40 mg, 100–200 μm; Zimmer, Warsaw IN) and embedded in a fibrin gel to generate carrier-cell constructs. The constructs were subcutaneously transplanted in the backs of 6-15 weeks-old female SCID/beige mice (CB17.Cg-*Prkdc*^{scid}*Lyst*$^{bg\text{-}J}$/Crl; Charles River Laboratories International, Inc., Wilmington, MA). Heterotopic transplants were harvested after 8 weeks, fixed in 4% buffered formaldehyde, decalcified by a neutral solution of EDTA

and embedded in paraffin. 5 μm thick paraffin sections were stained with hematoxylin and eosin or Sirius red and analyzed by transmitted light microscopy or polarized microscopy. A nested PCR was used for identifying the transgenes (irtTA and T-Ag) in the transplants. Genomic DNA was isolated from the paraffin embedded transplant using DNA lysis buffer (10 mM Tris pH: 8.0, 150 mM NaCl, 10 mM EDTA, 0.1% SDS) containing 0.1 mg/ml proteinase K. After overnight digestion at 55°C, genomic DNA was extracted with Phenol/Chloroform and Glycogen was added to visualize the pellet after Isopropanol precipitation and centrifugation. The first PCR was performed on 1/10 v/v of the DNA solution using published irtTA and T-Ag genotyping primers (Anastassiadis et al., 2010). For the nested PCR 1/20 v/v of the first PCR was used with the following primers (5' to 3'): irtTA-3: CAAGAGCTTCAGATGTGCCTTG and irtTA-4: GCATACAAGGCATTTTCCAGGG or T-Ag-3: CAATGTACTGCAAACAATGGCC and T-Ag-4: ACACCACAGAAGTAAGGTTCCT. Nested product size: irtTA = 150 bp, T-Ag = 203 bp.

qRT-PCR

1 μg of total RNA was treated with DNase I (Invitrogen) and used for cDNA synthesis (Affinity Script Multiple Temperature cDNA Synthesis kit, Agilent). Quantitative PCR was done with GoTaq qPCR Master Mix (Promega) in 384-well plates using LightCycler480 (Roche).

List of qRT-PCR primers

Gene	Forward 5' - 3'	Reverse 5' - 3'	Product size
Alpl	GGGACGAATCTCAGGGTACA	TTCAAGGTCTCTTGGGCTTG	135
Sp7	TCCTCGGTTCTCTCCATCTG	GGACTGGAGCCATAGTGAGC	101
Dlx3	AGCCCAGTATCTGGCCTTG	GGCACCTCCCCATTCTTATAG	132
Dlx5	AGCCCCTACCACCAGTACG	CCATTCACCATCCTCACCTC	101
Pitx1	CTGATCTGCCAGACAAGGAG	CTTAGCTGGGTCCTCTGCAC	101
Gli1	CTGGAGAACCTTAGGCTGGA	CGGCTGACTGTGTAAGCAGA	179
Ptch1	TGATTGTGGAAGCCACAGAA	GACAAGGAGCCAGAGTCCAG	121
Pth1r	CTGTTTCTGCAATGGTGAGG	ACCATTGGGCCATAGCTGTA	123
Ror2	CATGGAGATGCCACTCATCA	GTGGCCTTTGTAGACCTTGC	161
Wif1	CAGGAAAGCCCAACAAAGAA	GAGCGCAGAGACAGGAACTC	114
Cd200	CTGGAAACGTCACCGAAATC	TCCCTCCTGCTTTTCTTTCA	145
Msx2	AATTCCGAAGACGGAGCAC	CGGTTGGTCTTGTGTTTCCT	107
Spp1	TGTGATGAGACCGTCACTGC	AGGTCCTCATCTGTGGCATC	170
Bglap	TGACAAAGCCTTCATGTCCA	TTTGTAGGCGGTCTTCAAGC	175
Cebpb	CAAGCTGAGCGACGAGTACA	AGCTGCTCCACCTTCTTCTG	156
Cebpd	ATCGCTGCAGCTTCCTATGT	AGTCATGCTTTCCCGTGTTC	177
Adipoq	GGAACTTGTGCAGGTTGGAT	CCTTCAGCTCCTGTCATTCC	170
Actb	GGCCCAGAGCAAGAGAGGTATCC	ACGCACGATTTCCCTCTCAGC	440
Pparg1	TGAAAGAAGCGGTGAACCACTG	TGGCATCTCTGTGTCAACCATG	91
Pparg2	CTCCTGTTGACCCAGAGCAT	AATGCGAGTGGTCTTCCATC	153
Cebpa	GCAGTGTGCACGTCTATGCT	AGCCCACTTCATTTCATTGG	198
Plin4	GCCACATACAGCACAACCAG	CTACCAACAGCCTCCACCAT	148
Fabp4	GATGGTGACAAGCTGGTGGT	AATTTCCATCCAGGCCTCTT	100
Egr1	GAGCGAACAACCCTATGAGC	GAGTCGTTTGGCTGGGATAA	104
Egr2	CTCTACCCGGTGGAAGACCT	GGAGATCCAGGGGTCTCTTC	148
Stat1	AAGATTTTGGAAAATGCCCA	TGCACATGACTTGATCCTTCA	127
Lpl	CGAGAGGATCCGAGTGAAAG	TGGCATTTCACAAACACTGC	114
Sfrp1	GTACAACCGTGTGTCCTCCA	CCCAGCTTCAAGGGTTTCTT	163
Ccl2	AGGTCCCTGTCATGCTTCTG	TCATTGGGATCATCTTGCTG	132
Oas2	CAAGTGTCTGTACCAGGGCA	AGACACAGGAGCTGGGATTC	91
Ifit3	TTCCCAGCAGCACAGAAAC	TGCACACCCTGTCTTCCATA	148
Rpl19	CTGATCAAGGATGGGCTGAT	GGCAGTACCCTTCCTCTTCC	126

Immunostaining

For immunostaining the cells were fixed with 4% formaldehyde for 10 min at room temperature, then permeabilized for 15 min with 0.5% Triton X-100 and blocked for 30 min in 3% bovine serum albumin (BSA, Sigma) and 0.1% Tween-20, all solutions were in PBS. Immunostaining was done using the

following primary antibodies for the following antigens: SP7 (1:200, clone A-13, sc-22536-R, Santa Cruz), PPARγ (1:250, MAB3872, Millipore), C/EBPα (1:100, 14-AA, sc-61, Santa Cruz), EGR2 (1:50, H-220, sc-20690, Santa Cruz), STAT1 (1:100, Cat. 610116, BD Biosciences). The secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC)-conjugated goat anti-mouse (both 1:500, Jackson Immunoresearch Laboratories). Nuclei were counterstained with DAPI, and the imaging was done using confocal laser scanning microscope Leica SP5.

Western Blot

Whole cell protein extracts were prepared by freezing and thawing of cells in extraction buffer (20 mM Hepes pH: 8.0, 350 mM NaCl, 10% glycerol, 0.1% Tween-20, 2mM EDTA, 1 mM DTT, 1 mM PMSF, 1% Protease Inhibitor Cocktail, all Sigma). 20 µg of proteins were resolved in 10% polyacrylamide gel and transferred to nitrocellulose membrane using semi-dry blotting. Blocking was done overnight in 5% milk in PBS with 0.1% Tween-20 at +4°C. The membranes were incubated with primary antibodies against PPARγ1/2 (1:250, E-8, sc-7273, Santa Cruz), C/EBPα (1:500, 14AA, sc-61, Santa Cruz), STAT1 (1:1000, Cat. 610116, BD Biosciences), phospho-STAT1 (1:1000, 58D6, #9167, Cell Signaling Technology), SP7 (1:1000, A-13, sc-22536-R, Santa Cruz), EGR1 (1:1000, T.126.1, MA5-15009, Thermo Fisher Scientific) and β-ACTIN (1:10000, mAbcam 8224, abcam) for 1 hour at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (1:2000, Pierce Thermo Scientific) in the same conditions. The detection was performed using SuperSignal West Femto Substrate kit (Pierce Thermo Scientific).

Library preparation for sequencing

Total RNA was isolated by chloroform extraction from cell lysates prepared with Trizol (Invitrogen) followed by isopropanol precipitation. Isolation of mRNA was done using bead based poly-dT selection followed by chemical fragmentation and further processing for Illumina short read sequencing (HiSeq 2000). For strand specific sequencing two different versions of the dUTP method (Parkhomchuk et al., 2009) were applied.

<u>Strand specific RNA sequencing protocol version 1 (used for library preparations from conditionally immortalized clonal skeletal progenitors)</u>

Library preparation was done as described elsewhere (Thieme et al., 2013). For strand specific RNA sequencing library preparation mRNA was enriched from total RNA using 15 µl Sera-Mag Oligo(dT) beads (Thermo Scientific) in 50 µl 10 mM Tris-HCl. Samples were treated with 1 U Turbo DNAse (Ambion) and purified with Agencourt RNAclean XP beads. Eluted mRNA (18 µl) was chemically fragmented with NEBNext Magnesium RNA Fragmentation Module (NEB; New England Biolabs), re-purified with RNAclean XP beads, and eluted in 13.5 µl nuclease-free water. First strand cDNA synthesis was performed using 0.15 µg/µl Random Primers (NEB), 1x First Strand Synthesis Reaction Buffer (NEB), 10 U/µl Superscript II (Invitrogen) with an initial hybridization for 5 min at 65°C with mRNA and primers followed by incubation at 25°C for 10 min, 42°C for 50 min and 70°C for 15 min. After purification with Agencourt Ampure XP - beads (Beckman Coulter), second strand synthesis was done using the Second Strand Synthesis module (NEB) replacing the 2nd strand synthesis buffer with a NTP-free buffer and adding 2.5 mM of each d(A/C/G/U)TP-nucleotides. Incubation for 2.5 h at 16°C was followed by Ampure XP beads purification as described above. End-Repair was done with the NEBnext End Repair Module (NEB) followed by XP beads purification and A-Tailing using the NEBnext dA-Tailing Module. Adaptors were ligated (Adaptor-Oligo 1: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3', Adaptor-Oligo 2: 5'-P-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3') using 1x NEBnext Quick Ligation Buffer (NEB), 0.3 µM DNA Adaptors, 1 µl Quick T4 DNA Ligase (NEB) in 50 µl. XP beads purification was followed by dUTP cleavage with 1U USER enzyme mix per sample and direct enrichment using the PCR Enrich Adaptor Ligated cDNA Library module (NEB) with indexed primers. After XP beads purification libraries were quantified using Qubit dsDNA HS Assay Kit (Invitrogen). For Illumina flowcell production, samples were equimolarly pooled and distributed on all used lanes for 75 bp single read sequencing on Illumina HiSeq 2000.

<u>Strand specific RNA sequencing protocol version 2 (used for library preparations from clonal skeletal progenitors of "O" and "A" types, and "A" cells treated with IFNγ)</u>

mRNA was enriched from total RNA by poly-dT enrichment using the NEBNext Poly(A) mRNA Magnetic Isolation Module according to the manufacturers instructions. Eluted mRNA (18 µl) was directly subjected to the workflow of the NEBNext Ultra Directional RNA Library Prep Kit for

Illumina. For ligation custom adaptors were used (Adaptor-Oligo 1: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3', Adaptor-Oligo 2: 5'-P-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'). Indexing was done during the PCR enrichment step using custom amplification primers carrying the index sequence indicated with 'NNNNNN'. (Primer1: Oligo_Seq AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT, primer2: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT, primer3: CAAGCAGAAGACGGCATACGAGAT NNNNNN GTGACTGGAGTT). After XP beads purification libraries were quantified using Qubit dsDNA HS Assay Kit (Invitrogen). For Illumina flowcell production, samples were equimolarly pooled and distributed on all lanes used for 75 bp single read sequencing on Illumina HiSeq 2000.

Analysis of RNA-seq data

Sequencing resulted in 35 million 75 bp single-end reads on average per sample. Alignment of the short reads to the mm9 transcriptome was performed with pBWA (Peters et al., 2012) and a table of readcounts per gene was created based on the overlap of the uniquely mapped reads (on average 73% of the total sequenced reads) with the Ensembl Genes annotation v. 61 for mm9, using BEDtools, v. 2.11 (Quinlan and Hall, 2010). Normalization of the raw read counts based on the library size and testing for differential expression between the different cell types/treatments was performed with the DESeq R package, v.1.8.3 (Anders and Huber, 2010). Sample to sample Euclidean distance as well as Pearson's correlation coefficient (r) were computed based on the normalized gene expression level in order to explore the correlation between biological replicates and different libraries. For testing for differential expression, the count data were fitted to the negative binomial distribution and a cut-off of 0.01 was selected for the resulting p-values to assess the statistical significance of the fold change. Functional annotation clustering using DAVID (Huang da et al., 2009) was performed on lists of differentially expressed genes to discover enrichment of specific molecular functions, biological processes and terms related to cellular components. A list of primers used for validation of RNA-seq data is available in Table: List of qRT-PCR primers.

shRNA knockdown

The gene knockdowns were done via retroviral transduction of small hairpin RNA (shRNA)-encoding vectors pLKO.1-puro listed Table: shRNA sequences, kindly provided by Mark Bickle (MPI-CBG, Dresden). The pLKO.1 plasmids in combination with packaging constructs pCMV-VSV-G and pCMV-dR8.91 were co-transfected to 293T cells using Lipofectamine LTX (Invitrogen). The viral supernatants were collected 48 and 72 hours after transfection and used for BMSC infection in the presence of 8 μg/ml polybrene. shRNA-infected BMSCs were checked for gene expression 72 hours after infection by qRT-PCR on total RNA preparations. The cells were expanded further and tested for their differentiation potential to osteocytes and adipocytes.

shRNA sequences

Gene		shRNA sequence (5' to 3')
Egr1	shRNA1	CACTCCACTATCCACTATTAACTCGAGTTAATAGTGGATAGTGGAGTG
	shRNA2	GCTCTTAATACCACCTACCAACTCGAGTTGGTAGGTGGTATTAAGAGC
Egr2	shRNA1	CCACTCTCTACCATCCGTAATCTCGAGATTACGGATGGTAGAGAGTGG
	shRNA2	CCAGAAGGTATCATCAATATTCTCGAGAATATTGATGATACCTTCTGG

Reporter constructs

The Egr1 and Egr2 Luciferase reporters were generated by conventional cloning. A genomic fragment of 3 kb upstream of the start codon of *Egr1* or *Egr2* was cloned directly before the Luciferase gene in pGL3-basic vector (Promega). The Egr1 reporter was cloned into two steps. Two genomic fragments (distal and proximal) upstream of the start codon (ATG) were amplified by PCR from a BAC vector carrying *Egr1* (RP23-108C3) using primers containing restriction sites. For the distal fragment the primers are: mEgr1-Luc-Xho-F: 5'-ATACATGCTCGAGAATACGACTTAGCCAGG-3' and mEgr1-Luc-HindIII-R: 5'-GACTTGGCTTTGGGCAGTCTGG-3'. The PCR product after XhoI/HindIII digestion is 1583 bp and is cloned into XhoI and HindIII sites of pGL3. The proximal fragment was amplified using the primers: mEgr1-Luc-HindIII-F: 5'-AGAGGGCAGCACCGAGCCGTAAA-3' and mEgr1-Luc-NcoI-R: 5'-GTCTTCCATGGCCCGGACCAGCGAGCTGGAGA-3' and cloned after HindIII/NcoI digestion (1434 bp) into HindIII and NcoI sites between distal fragment and Luciferase gene. There are at least 7 transcripts annotated for *Egr2*, utilizing different start codons (ENSEMBL GRCm38.p5). We made two reporter constructs: Egr2-N1-Luc and Egr2-N3-Luc. The first uses an

upstream ATG annotated in transcript Egr2-201= ENSMUST00000048289.13 and the second a downstream ATG (transcript Egr2-203 ENSMUST00000127820.1). As for Egr1 both Egr2 reporters were cloned in two steps. Two genomic fragments (distal and proximal) upstream of the start codon (ATG) were amplified by PCR from a BAC vector carrying *Egr2* (RP23-231M5) using primers containing restriction sites. For Egr2-N1-Luc the distal part was amplified with Egr2-N1-Luc-XhoI-F: 5'-ATACATGCTCGAGACAATTGCTTTGGCAGAGATGCC-3' and Egr2-N1-Luc-NcoI-R: 5'-CCCAACCCCATGGTATCCTTCC-3' and the product (1842 bp) was cloned into XhoI and NcoI sites of pGL3. The proximal part (1187 bp) was amplified with mEgr2-N1-Luc-NcoI-F: 5'-GGAAGGATACCATGGGGTTGGG-3'and mEgr2-N1-Luc-ATG-R: 5'-GTCTTCCATGGCTCCAGCCTGGGTAGGGAAGAAATGG-3' and cloned into NcoI site and checked for orientation. For Egr2-N3-Luc the proximal part was amplified with Egr2-N3-Luc-HindIII-F: 5'-ATAGGATCAAGCTTAGATGCACCTGGTCACCAAAAG-3' and Egr2-N3-Luc-NcoI-R: 5'-GTCTTCCATGGCTGGGGAGGGAAAAGTCAGGG-3' and cloned (1188 bp) into HindIII and NcoI of pGL3. Then the distal part (1916 bp) was amplified with Egr2-N3-Luc-XhoI-F: 5'-ATACATGCTCGAGGGTGTGTAGTGTGTAGCGTAAGG-3' and Egr2-N3-Luc-AscI-R: 5'-GATGGACCTGCTTCAAGGACCAGG-3' and cloned after digesting with AscI and XhoI into the intermediate vector digested with the same enzymes. Every cloning step was confirmed by sequencing.

Luciferase assay

The Luciferase reporter constructs were transfected together with beta-actin-LacZ vector into two adipogenic "A" clones (6472-B7 and 6652-F2) cultured in 24-well plates using Lipofectamine LTX (Invitrogen) and following the manufacturers instructions. The cells were incubated with different amounts of IFNγ (0, 1.25, 2.5, 5, 10, 20 and 40 ng/ml) for 48 h. Cell extracts were made in 24-well plates using extraction buffer (20 mM Hepes pH: 8.0, 350 mM NaCl, 10% Glycerol, 0.1% Tween-20, 2 mM EDTA) containing 1 mM PMSF by three cycles of freezing and thawing. Luciferase activity was measured with a GLOMAX 96 microplate Luminometer (Promega) using standard protocols. Beta-galactosidase assay was done using standard methods and measured with a TECAN Reader.

Chromatin Immunoprecipitation (ChIP)

One 15 cm plate with confluent cells was used per antibody. Cross-linking was done in two steps, at first 2 mM disuccinimidyl glutarate (DSG) in PBS was added to the cells for 45 min, followed by wash with PBS and the second incubation with 1% formaldehyde in DMEM for 20 min. Glycine was added at 125 mM concentration for 5 min, all at room temperature. Then the cells were scraped from the plates in cold PBS, pelleted and resuspended in lysis buffer (10 mM Tris HCl pH: 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) containing protease inhibitors (Roche). The lysate was centrifuged and the pellet was resuspended in 3 ml sonication buffer (10 mM Tris HCl pH: 8.0, 1 mM EDTA, 0.5 mM EGTA). Chromatin was sheared by sonication for 10 min, duty cycles 10%, intensity 8, cycles per burst 200 (Covaris) followed by centrifugation to remove debris, supernatant was further used for the ChIP. 1% of total amount of the chromatin was saved as input. For immunoprecipitation, the Protein G-Sepharose beads (100 µl per IP sample) were blocked with 1.5% gelatin and 0.02% salmon sperm DNA in PBS overnight at +4°C. The chromatin was pre-cleared by incubation with Sepharose beads (without protein G) in the ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris HCl pH: 8.1, 167 mM NaCl) for 1h at +4°C, the volume was 2.2 ml per IP. Then the Sepharose beads were removed by centrifugation and the chromatin was incubated with 2-5µg of respective antibody rotating overnight +4°C. Pre-blocked Protein G beads were added then to the sample and incubated for 3 h at +4°C. The beads were pelleted by centrifugation and sequentially washed: once in Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH: 8.1, 150 mM NaCl), 2 times in High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH: 8.1, 500 mM NaCl), once in LiCl Immune Complex Wash Buffer (0.25 M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH: 8.1), once in TE (10 mM Tris HCl pH: 8.0, 1 mM EDTA). Then the beads were resuspended in 250 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$), incubated for 15 min at room temperature and pelleted, this step was then repeated and two eluates were combined. Input was used from this step, elution buffer was added to 500 µl to equalize with the samples. For de-crosslinking NaCl was added to concentration 200 mM, the incubation was done for 4 h at 65°C. DNA was purified by phenol-chloroform and isopropanol precipitation, and dissolved in 300 µl water and subsequently used for qPCR. Positions of putative EGR1/2 binding sites and primer sequences are shown in the Tables below.

EGR1/2 binding sites and ChIP primers (5' to 3') for regulatory regions of adipogenic factors

Gene	Position of putative site	Sequence	Primer pair	Position of FWD	Product size	FWD	REV
Pparg	-332	GGCGCGGGAGG	PPARg_A	-422	130	GCCGCCTCAGGTCAGAGT	TCACCTTGTCGTCACACTCG
	-310	CTCGTGGGCGC					
	-288	TGCGGGCGCGG					
	-254	CCGCCCCCGGT					
	-140	TCACCGACGCA					
Cebpa		none (ctrl)	CEBPa_A	-2615	142	CCTGTCAAACACCCTTGTCC	GCAGGAAGTCTCTGGAAGGA
	-1187	ACTCCTACCCA	CEBPa_B	-1304	194	GTGCAAAAACGAGAGACGTG	CTACGAAGCCTTTCGAGCAC
	-1166	CCTGTAGGCGG					
	-717	CCGCCTCCGCT	CEBPa_C	-764	101	CCTAGGCGAGTGGACGAG	CCACGGGCTCTTCAGAGTAG
	-506	CCTCCCGCGCC	CEBPa_D	-536	140	GCTAGGTTGCTGGTCCAAAG	AATGTTGGTTTTGAGCTACCG
	-270	CGTGTGTGCGG	CEBPa_E	-324	210	GGCTGGAAGTGGGTGACTTA	CGCCTTCTCCTGTGACTTTC
	-264	TGCGGGGGCGA					
	-247	CGCCGGGGTGG					
	-195	TCAGTGGGCGT					
Fabp4	-2692	TGTGTGGCTGT	FABP4_A2	-2700	137	GCTGTGTCTGTGTGGCTGTT	AATCCCCTAAGTTCGCTGCT
	-1151	AATGTGGGTGT	FABP4_B	-1111	129	TAGGGACTTGGGAGCAGGTA	CACAGCAGAATGATGGGAGA
Plin4	-2187	GCACTCACGCA	Plin4_A	-2258	103	AGCACACAATGATGCCAGAG	AAGGGAACAGAAGCCCTAGC
	-1195	CCTCCCACCCA	Plin4_C	-915	158	GTTCAGGGGCTGTCTGAATC	TGTGAGAACCCAATCTGGTG
	-1191	CCACCCACCCC					
	-857	(AC)9					
	-340	AGAGTGGGAGG	Plin4_D	-420	149	GAAGGCTGTGGGTGAGAAAG	CCACCTGTGGGTCTCAGTTT
	-228	AGAGTGGGAGG	Plin4_E	-291	126	TGCCTGGTAAGAAAGGCATC	GCCAAGCAGCCTCTGTTTAT
Intergenic region		none (ctrl)	chr9			TGGCAGCTTGTGTTGTTAGA	CCACTATGGTGTCAGGAAGG

EGR1/2 binding sites and ChIP primers (5' to 3') for regulatory regions of osteogenic factors

Gene	Position of putative site	Sequence	Primer pair	Position of FWD	Product size	FWD	REV
Sp7	-2738	(GT)16	Sp7_A	-2770	177	TGAATGTTGGGCTCACAGAG	CCAAGAGTTCCAGGTTCCAA
	-1965	AGTGTGGGCAT	Sp7_B	-2122	195	ACCCAGGGAAGCAAGATGAT	AGGACTGAGGGACAGAGCAA
			Sp7_C	-1815	193	TCCCACTCTCTACCACTTTCT	GTGCATGCATGATCATGTATTT
	-1698…-1001	(AC)15…(AC-rich)…(AC)24 (34 predicted sites within the region)	Sp7_D2	-1001	200	TGCCACCCTGAACTAGTGGT	CATGAGCAAACACGGAGATG
	-771	TGTGTGAGTGT					
	-55	CCACCCCCACC	Sp7_F2	-45	111	CCCCCAGTCCCTTCTAACTT	GGAGTCAGGCAGATGGAGAG
Dlx3	-1863	CCGCCCCCGCT	Dlx3_A2	-2044	113	CTATTACCCCAAGGCTCCTG	CGGGAGTTCGTTCATTCATT
	-1658	CCGCCCCCGCC	Dlx3_B2	-1564	130	CTCTGCCCAGCCAACTCTAC	AGGTAAGCAGGCAGACAGGA
	-1652	CCGCCCCCACC					
	-247	ACCCCTCCGCA	Dlx3_C	-340	125	TGGGTAAATTCCCCTCCTTC	CTCCGATAAATCCCCCAAAT
	-178	(GT)22	Dlx3_D	-235	132	GGGGGATTTATCGGAGGTAG	CCAACCCTCTCAACCCTCTT
	-84	AGCGCGAGCGT	Dlx3_E	-119	170	AAGAGGGTTGAGAGGGTTGG	GTCCCAAGCCACAGTCAAAT
Dlx5	-1792	AAACCCACACA	Dlx5_A	-1834	121	CAAAGGCAAGGGATCAGTGT	CACCCCTTTCTTCATCTGGA
	-264	(TC)27(AC)17(TC)41	Dlx5_B	-340	244	CCCAACAGGGAACAACTCAT	GCAACACCCTAACTCGTCCA
	-51	TGCGTAAGCGC	Dlx5_C	-116	152	GGACGAGTTAGGGTGTTGCT	GAGTCGTGGAGGCTCTGTCT
Intergenic region		none (ctrl)	chr9		249	TGGCAGCTTGTGTTGTTAGA	CCACTATGGTGTCAGGAAGG

The Tables show positions relative to transcription start sites and sequences of putative EGR1/2 binding sites predicted using the Jaspar database (http://jaspar.genereg.net/). Primer pairs are designed in proximity to single binding sites or to a group of closely located binding sites, which cannot be resolved by ChIP-qPCR approach. Product size is calculated in bp. The intergenic region in Chromosome 9 serves as negative control.

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