Published online January 2, 2004

Nucleic Acids Research, 2004, Vol. 32, No. 1 ***e1***
DOI: 10.1093/nar/gnh001

Dual-regulated expression of C/EBP-α and BMP-2 enables differential differentiation of C2C12 cells into adipocytes and osteoblasts

Cornelia Fux, Barbara Mitta, Beat P. Kramer and Martin Fussenegger*

Institute of Biotechnology, Swiss Federal Institute of Technology, ETH Hoenggerberg, HPT D74, CH-8093 Zurich, Switzerland

Received July 9, 2003; Revised October 20, 2003; Accepted November 3, 2003

ABSTRACT

CCAAT/enhancer-binding proteins (C/EBPs) as well as bone morphogenic proteins (BMPs) play essential roles in mammalian cell differentiation in shaping adipogenic and osteoblastic lineages in particular. Recent evidence suggested that adipocytes and osteoblasts share a common mesenchymal precursor cell phenotype. Yet, the molecular details underlying the decision of adipocyte versus osteoblast differentiation as well as the involvement of C/EBPs and BMPs remains elusive. We have engineered C2C12 cells for dual-regulated expression of human C/EBP-α and BMP-2 to enable independent transcription control of both differentiation factors using clinically licensed antibiotics of the streptogramin (pristinamycin) and tetracycline (tetracycline) classes. Differential as well as coordinated expression of C/EBP-α and BMP-2 revealed that (i) C/EBP-α may differentiate C2C12 myoblasts into adipocytes as well as osteoblasts, (ii) BMP-2 prevents myotube differentiation, (iii) is incompetent in differentiating C2C12 into osteoblasts and (iv) even decreases C/EBP-α's osteoblast-specific differentiation potential but (v) cooperates with C/EBP-α on adipocyte differentiation, (vi) osteoblast formation occurs at low C/EBP-α levels while adipocyte-specific differentiation requires maximum C/EBP-α expression and that (vii) BMP-2 may bias the C/EBP-α-mediated adipocyte versus osteoblast differentiation switch towards fat cell formation. Dual-regulated expression technology enabled precise insight into combinatorial effects of two key differentiation factors involved in adipocyte/osteoblast lineage control which could be implemented in rational reprogramming of multipotent cells into desired cell phenotypes tailored for gene therapy and tissue engineering.

INTRODUCTION

Mesenchymal stem cells are multipotent cells which can differentiate into adipocyte, chondrocyte, myoblast and osteoblast lineages (1–4). Recent evidence suggested that adipocytes and osteoblasts originate from a common mesenchymal precursor cell (5), which raises particular interest on how molecular lineage control is implemented in those cells. CCAAT/enhancer-binding proteins (C/EBPs) comprise a family of transcription factors that have key metabolic and differentiation missions in many tissues (6–8). To date, six members of the C/EBP family (C/EBP-α, -β, -δ, -γ, -ε, -ζ) have been identified, which are expressed in various cell types including adipocytes and osteoblasts (9–11). All C/EBPs share a highly conserved DNA-binding domain as well as a bZIP dimerization domain (12,13), which enables homo- and heterodimeric binding to sequence-specific operators (14, 15). While ectopic expression of C/EBP-α was sufficient for differentiation of mouse fibroblasts into adipocytes (16), fat-cell-specific differentiation of G8 mouse myoblasts required coordinated expression of C/EBP-α and PPARγ (a member of the nuclear hormone receptor family) as well as addition of diverse lipids and/or lipid-like compounds including 5,8,11,14-eicosatetrayonic acid (ETYA) to the culture medium (17–19).

Previous studies have implied that the bone morphogenic protein 2 (BMP-2) promotes osteoblast-specific differentiation of C2C12 myoblasts via induction of several osteogenic master transcription factors including Runx2 and Osterix (20–23). BMP-2 belongs to the transforming growth factor β family of differentiation factors that play key roles in chondrogenic or osteogenic lineage control, bone remodeling as well as differentiation of mesenchymal cells (24,25). However, BMP function seems not to be restricted to bone or cartilage formation since some family members have been found (i) to prevent myotube differentiation of myoblasts (26), (ii) to commit pluripotent mouse fibroblast cells (C3H10T1/2) to osteoblasts, chondroblasts and adipocytes (27–29) and (iii) to stimulate adipocyte formation of mesenchymal precursor cells (30).

Based on recent advances in heterologous transcription control technology, we have designed dual-regulated expression configurations which enable independent regulation of two different transgenes (31–37). Pioneering dual-regulated

*To whom correspondence should be addressed. Tel: +41 1 633 34 48; Fax: +41 1 633 12 34; Email: fussenegger@biotech.biol.ethz.ch

expression technology combined two compatible gene regulation systems, the streptogramin-(PIP) and tetracycline-(TET) responsive transgene control configurations which follow a similar design concept (38): a *Streptomyces coelicolor/Escherichia coli* antibiotic resistance response regulator (Pip [pristinamycin-induced protein]/TetR) fused to a mammalian transactivation domain (VP16) (39) reconstitutes a streptogramin-/tetracycline-dependent transactivator (PIT/tTA) that binds and activates specific promoters [P_{PIR}/$P_{hCMV*-1}$; assembled from Pip-/TetR-specific tandem operator sites (ptr/tetO) and minimal eukaryotic promoters ($P_{hCMVmin}$)] in a pristinamycin-/tetracycline-adjustable manner (38). Whereas in the presence of pristinamycin/tetracycline, PIT/tTA fails to bind and transactivate P_{PIR}/$P_{hCMV*-1}$, desired transgene expression is fully induced in the absence of regulating antibiotics (34,38). Combination of PIP and TET systems enabled four different digital expression configurations: ON/ON, OFF/ON, ON/OFF and OFF/OFF (34,36, 37,40).

In this study we used dual-regulated expression of C/EBP-α and BMP-2 for rational reprogramming of C2C12 to reveal mechanisms underlying C2C12 to adipocyte and C2C12 to osteoblast differentiation. Multiregulated multigene-based therapeutic interventions as presented here are expected to enable design of desired cell phenotypes for gene therapy and tissue engineering in the not-too-distant future.

MATERIALS AND METHODS

Vector constructions

pCF141 was constructed by excising human C/EBP-α [pCMVα (41)] from pCF50 (pA_I-C/EBPα←$P_{hCMV*-1}$-stuffer-P_{PIR8}→E7-HA-pA_{II}; C.Fux *et al.*, unpublished) using BamHI/ClaI and cloning it into the corresponding sites (BamHI/ClaI) of pCF93 ($P_{hCMV*-1}$-MCS-pA_I-P_{PGK}-puro-pA_{II}; C.Fux, unpublished) to result in pCF141 ($P_{hCMV*-1}$-C/EBPα-pA_I-P_{PGK}-puro-pA_{II}). pCF240 was constructed following a two step procedure: (i) C-terminally hemagglutinin A (HA)-tagged human BMP-2 (BMP-2-HA) was constructed by amplifying BMP-2 cDNA (W.Weber, unpublished) using oligonucleotides OWW15: 5′-ggaagcttgaattcCCACCATG-GTGGCCGGGACCCGC-3′ (annealing sequence upper case, HindIII and EcoRI sites underlined) and OWW16: 5′-ggagatctgaattcatttaaatTCAAGCGTAATCTGGAACA-TCGTATGGGTA*gcgacacccacaaccctccacaac*-3′ (annealing sequence upper case, BglII, EcoRI and SwaI sites underlined, HA-Tag in italic) followed by subsequent cloning in sense orientation into pEF6/V5-His TOPO (Invitrogen, Carlsbad, CA) to create pWW16. (ii) BMP-2-HA was excised from pWW16 using EcoRI and cloned into the corresponding site (EcoRI) of pCF217 (P_{PPI8}-MCS-pA_I-P_{PGK}-zeo-pA_{II}; C.Fux, unpublished) to give pCF240 (P_{PPIR8}-BMP2-HA-pA_I-P_{PGK}-zeo-pA_{II}). Lentivector pBP252 was constructed by excising C/EBP-α from pCF257 by EcoRI/StuI and ligating it into the EcoRI/SwaI of pMF359 (42). Likewise, BMP-2 was excised from pCF241 using EcoRI/MluI and cloned into EcoRI/MluI-restricted pMF359 to give pBP253. Details on pCF257 and pCF241 are available upon request.

Cell culture, transfection, lentiviral particle production and transduction

Wild-type C2C12 (C2C12, ATCC:CRL-1772) were cultivated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad CA) supplemented with 10% FCS (PAA Laboratories GmbH, Linz, Austria; catalog no. A15-022, lot no. A01129-242) (basic medium). The cell line $C2C12_{PIT/tTA}$ stably expressing the streptogramin-(PIT) and the tetracycline-(tTA) dependent transactivators was cultivated in basic medium that also contained 800 μg/ml G418. The cell line $C2C12_{PIT/tTA}$-C/EBP$α_{TET}$, a $C2C12_{PIT/tTA}$ derivative harboring a tetracycline-responsive CEBP-α expression unit (pCF141; $P_{hCMV*-1}$-CEBPα-pA_I-P_{PGK}-puro-pA_{II}) was cultivated in basic medium supplemented with 800 μg/ml G418 and 1 μg/ml puromycin. The cell line $C2C12_{PIT/tTA}$-CEBP$α_{TET}$-BMP_{PIP}, a $C2C12_{PIT/tTA}$-C/EBP$α_{TET}$ engineered for streptogramin-responsive BMP2 expression (pCF240; P_{PIR8}-BMP2-HA-pA_I-P_{PGK}-zeo-pA_{II}) was cultivated in medium supplemented with 800 μg/ml G418, 1 μg/ml puromycin and 40 μg/ml zeocin. Transfection of C2C12 derivates was performed using a modified $CaPO_4$-based protocol (32). 120 000 cells per 6 well were transfected with 1.2 μg DNA, which resulted in a typical transfection efficiency of 40 ± 5%. Production and transduction protocols using replication-incompetent self-inactivating lentiviral particles have been described before (42).

Construction of stable cell lines

The stable cell line $C2C12_{PIT/tTA}$-CEBP$α_{TET}$ was generated by transfection of pCF141 ($P_{hCMV*-1}$- C/EBPα-pA_I-P_{PGK}-puro-pA_{II}) into $C2C12_{PIT/tTA}$. The mixed stable population was selected for 3 weeks in basic medium supplemented with selective (800 μg/ml G418, 1 μg/ml puromycin) and regulating (5 μg/ml tetracycline) antibiotics prior to FACS-mediated single-cell sorting. Similarly, stable cell line $C2C12_{PIT/tTA}$-CEBP$α_{TET}$$BMP2_{PIP}$ was produced by transfecting pCF240 (P_{PIR8}-BMP-HA-pA_I-P_{PGK}-zeo-pA_{II}) into $C2C12_{PIT/tTA}$-CEBP$α_{TET}$ followed by a 3-week expansion of a stable mixed population in medium supplemented with various selective and regulating antibiotics (800 μg/ml G418, 1 μg/ml puromycin, 40 μg/ml zeocin, 5 μg/ml tetracycline and 2 μg/ml pristinamycin) prior to FACS-meditated single-cell cloning.

Western blot analysis

For preparation of whole cell extracts, cells were washed with ice-cold PBS and scraped into lysis buffer (Roche Diagnostics AG, Basel, Switzerland). Cells were allowed to lyse for 15 min prior to centrifugation of the lysates for 20 s at 23 000 *g*. Protein concentrations of whole cell extracts were determined using a BCA™ Protein Assay (SOCOCHIM, Lausanne, Switzerland). Prior to resolution on a 12% polyacrylamide gel, proteins mixtures contained in whole cell extracts and cell culture supernatants were denatured by boiling for 10 min in sample buffer (10% glycerol, 50 mM Tris–HCl, 1% SDS and 0.005% bromophenol blue). Proteins were then blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), which were subsequently blocked using 5% non-fat dry milk in TTBS (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20). Blots were probed with specific primary antibodies (C/EBPα: Santa Cruz, 14AA, catalog no. sc-61, lot

no. F080; HA-probe: Santa Cruz, Y-11; catalog no. sc-805, lot no. G070) and visualized using an appropriate horseradish peroxidase-coupled secondary antibody (Amersham, catalog no. NA934V, lot no. 211112) linked to an ECL detection readout (Amersham, Buckinghamshire, UK; catalog no. RPN 2106).

Characterization of cell phenotypes

In order to visualize adipogenic cell phenotypes, cells were cultured to 80% confluence in basic medium and then transferred to medium supplemented with 1 μM dexamethasone, 5 μg/ml insulin and 50 μM ETYA [Dex/ETYA/Insulin, DEI; (19)]. Fourteen days post-DEI induction, the cells were fixed in a 3.7% aqueous formaldehyde solution 1 h. Excess formaldehyde was removed by rinsing three times 30 s with ddH_2O prior to immersion of cells for 30 min with an Oil Red O working solution. Subsequently, the cells were washed three times with ddH_2O for 30 s and the nuclei were stained for 60 s with Mayer's haematoxylin (Sigma Chem. Co., St Louis, MO). Finally, the cells were rinsed in ddH_2O for 10 min and covered with a coverslip using 10% glycerol in PBS (43). Alkaline phosphatase-specific staining of osteogenic cell phenotypes was visualized following a 5-day cultivation using the Sigma Kit 85 according to the manufacturer's instructions (Sigma Chem. Co., St Louis, MO).

Antibiotics and chemicals

Pyostacin® pills (500 mg; Aventis Inc., France; lot no. 27404) were ground in a mortar and dissolved in DMSO (50 mg/ml). Alternatively, antibiotic discs containing 15 μg pristinamycin (bioMérieux, Geneva, Switzerland) were soaked in 7.5 ml 4°C cell culture medium for 30 min. Pristinamycin I (PI) (2 μg/ml) was routinely used for regulation studies in cell culture and calculated based on the fixed 70:30 ratio (w/w) of the non-regulating pristinamycin II and PI in the composite antibiotic pristinamycin (44). Pyostacin- or antibiotic disc-derived PI was as efficient in regulating streptogramin-responsive expression systems as the pure PI reference compound kindly provided by Aventis Inc. (RP27404, lot no. SOU2890IICA; France). G418 was obtained form Calbiochem-Novabiochem Inc. (La Jolla, CA), puromycin from ALEXIS Inc. (San Diego, CA), zeocin from Invitrogen (Carlsbad, CA) and tetracycline from Sigma Chemicals (St Louis, MO). Dexamethasone was purchased from Fluka Chemie (Buchs SG, Switzerland), insulin and ETYA from Sigma Chemicals (St Louis, MO). For an Oil Red O (Fluka Chemie, Buchs SG, Switzerland) stock solution 500 mg Oil Red O were dissolved in 100 ml 60% triethyl-phosphate (Fluka Chemie, Buchs SG, Switzerland). Prior to staining, a 36% triethyl-phosphate working solution containing 12 ml Oil Red O stock solution and 8 ml ddH_20 was prepared. Purified human BMP-2 was purchased from PeproTech EC Ltd (London, UK) and used at concentrations of 3 ng/ml.

RESULTS

Production of C2C12 cells engineered for tetracycline-responsive C/EBP-α expression

In order to enable tetracycline-responsive expression of C/EBP-α in C2C12 cells we transduced this cell line with pRetroTWIN3-derived retroviral particles encoding the tetracycline-dependent as well as the pristinamycin-dependent transactivators. Resulting $C2C12_{PIT/tTA}$ was stably transfected with pCF141, which contains human C/EBP-α under control of the tetracycline-responsive promoter ($P_{hCMV*-1}$) and encodes a puromycin resistance-conferring gene driven by the constitutive phosphoglycerate kinase promoter (P_{PGK}) (Fig. 1A). In order to prevent any differentiation/growth bias, C/EBP-α expression was repressed by addition of tetracycline during the entire selection and cloning procedures. Two randomly chosen $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$ clones (numbers 1 and 45) showed tetracycline-responsive C/EBP-α expression profiles (Fig. 1B). Also, both cell clones differentiated into myotubes when C/EBP-α was repressed by addition of tetracycline while myotube formation was repressed following cultivation of $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$ under C/EBP-α-induced and low serum conditions (data not shown). These findings support an earlier report correlating C/EBP-α expression and low-serum cultivation of G8 myoblasts with inhibition of myotube-specific differentiation (19).

C/EBP-α is sufficient to convert C2C12 cells into adipocytes

Previous initiatives to differentiate G8 myoblasts into adipocytes required concomitant expression of C/EBP-α and PPARγ as well as addition of DEI to the culture medium. Either of the two determinants alone was not sufficient for adipocyte commitment of this muscle cell type (19). In order to assess the potential of $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$ cells to differentiate into adipocytes we cultivated this cell line for 14 days in DEI-containing medium in the presence (+Tet; C/EBP-α repressed) and absence of tetracycline (–Tet; C/EBP-α induced) prior to Oil Red O-mediated staining of adipocyte-specific triglyceride droplets. Both $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$ clones showed increased adipocytic lineage commitment following sustained expression of C/EBP-α (Fig. 1C). In the control settings consisting of $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$ cultivated in the presence of tetracycline or the parental cell line $C2C12_{PIT/tTA}$ no adipogenic cell phenotypes could be observed (Fig. 1C). Likewise, transduction of C2C12 wild-type cells with lentiviral particles engineered for constitutive C/EBP-α expression induced adipocyte-specific differentiation in contrast to control transductions using isogenic lentiviral particles (Fig. 1D). Despite their equivalence, clone $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET45}$ was used for further analysis.

Design of a C2C12-derived cell line transgenic for dual-regulated expression of human CEBP-α and BMP-2

Dual-regulated expression technology enables independent control of two different transgenes (34,36,37). We have engineered C2C12 cells for tetracycline-responsive expression of human CEBP-α as well as streptogramin-responsive expression of the human BMP-2. Therefore, $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET45}$ was stably transfected with pCF240 which provides streptogramin-responsive expression of BMP-2-HA and enables selection for zeocin (Fig. 2A). Two randomly chosen cell clones $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-$BMP2_{PIP2}$ and $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-$BMP2_{PIP6}$ showed independent regulation of BMP-2 and CEBP-α following 5-day cultivation of engineered cells in media supplemented with different

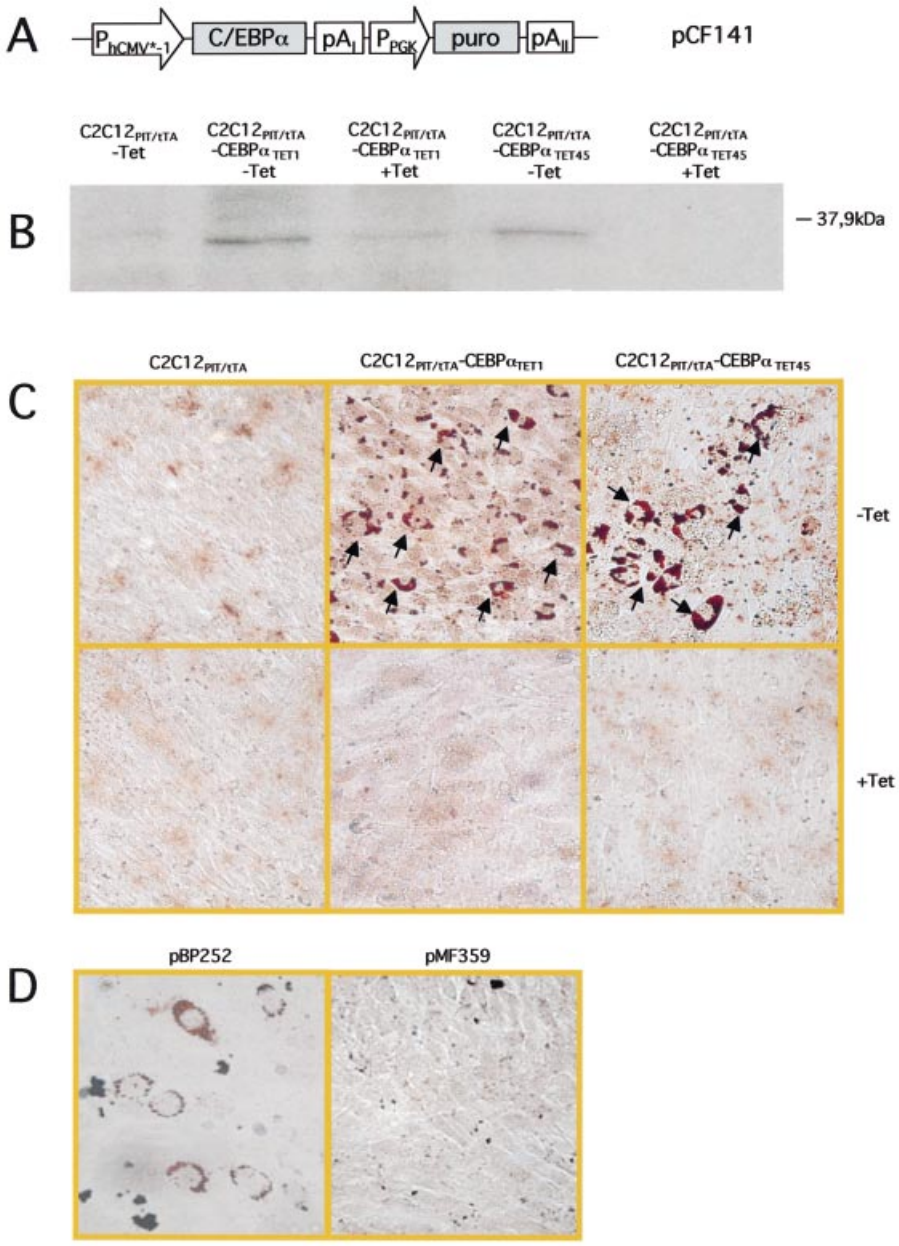

Figure 1. C/EBPα expression of $C2C12_{PIT/tTA}$ cells stably transfected with pCF141 ($C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$). (**A**) Schematic representation of pCF141. pCF141 encodes human C/EBP-α under control of the tetracycline-responsive promoter ($P_{hCMV*-1}$). The gene conferring resistance to puromycin (puro) is driven by the constitutive phosphoglycerate kinase promoter (P_{PGK}). Polyadenylation sites are indicated (pA_I, pA_{II}). (**B**) Western blot analysis of whole-cell extracts (20 µg total protein) of $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET1}$ and $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET45}$ grown for 5 days in the presence (+Tet) and absence (–Tet) of tetracycline. The parental cell line $C2C12_{PIT/tTA}$ was used as a control. (**C**) Adipocyte-specific differentiation of C2C12 cell derivatives following tetracycline-responsive expression of C/EBP-α. (**D**) Adipocyte-specific differentiation of C2C12 wild-type cells after transduction of lentiviral particles engineered for constitutive C/EBP-α expression (pBP252) or isogenic control lentiviruses (pMF359). Oil Red O-mediated staining of triclyceride droplets of C2C12 derivatives cultivated for 14 days in DEI-containing medium supplemented with tetracycline where indicated (C and D).

combinations of regulating pristinamycin and tetracycline antibiotics (Fig. 2B).

BMP-2 cooperates with C/EBP-α on conversion of C2C12 into adipocytes

Recent reports associated BMP-2 with promoting adipogenic differentiation of the murine pre-adipocyte cell line 3T3-L1 following coexpression with peroxisome proliferator-activated receptor gamma (PPARγ) (30). In order to evaluate the potential of BMP-2 to differentiate C2C12 cells into adipocytes, $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-BMP_{PIP2} and $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-BMP_{PIP6} ($C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-$BMP_{PIP2/6}$) were cultivated for 12 days in DEI-containing medium supplemented with different cocktails of regulating antibiotics: –Tet/–PI, –Tet/+PI, +Tet/–PI and +Tet/+PI. Adipocyte-specific Oil Red O staining revealed increased adipocyte formation in the –Tet/–PI combination (C/EBP-α and BMP-2 expressed; Fig. 3A and E) compared to

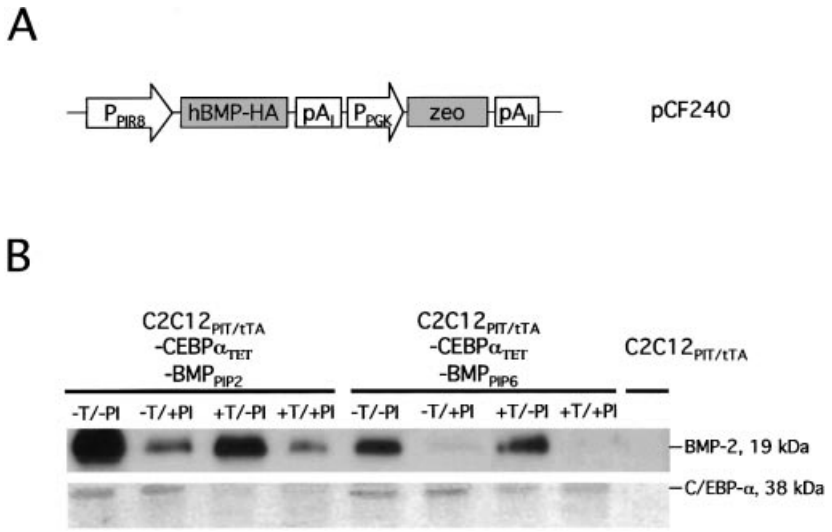

Figure 2. C2C12 cells (C2C12$_{PIT/tTA}$-CEBPα$_{TET}$-BMP$_{PIP}$) engineered for pristinamycin-responsive expression of human BMP-2. (**A**) Diagram pCF240 encoding HA-tagged BMP-2 (BMP2-HA) under control of an optimized streptogramin-responsive promoter (P_{PIR8}) and a zeocin resistance-conferring gene (zeo) driven by the phosphoglycerate kinase promoter (P_{PGK}). (**B**) BMP-2- and C/EBP-α-specific western blot analysis of 20 µl culture supernatants (60/20 µg total protein [BMP-2/C/EBP-α]) derived from C2C12$_{PIT/tTA}$-CEBPα$_{TET}$-BMP$_{PIP2}$ and C2C12$_{PIT/tTA}$-CEBPα$_{TET}$-BMP$_{PIP6}$ clones cultivated for 5 days in the presence (+) or absence (–) of different combinations of tetracycline (T) and pristinamycin (PI) antibiotics. The parental cell line C2C12$_{PIT/tTA}$ was used as control.

the –Tet/+PI (only C/EBP-α expressed; Fig. 3B and F) configuration suggesting that BMP-2 cooperates with C/EBP-α for maximum adipocyte commitment compared to –Tet/+PI (only C/EBP-α expressed; Fig. 3B and F). Clone C2C12$_{PIT/tTA}$-CEBPα$_{TET}$-BMP$_{PIP6}$ showed less prominent adipocyte differentiation compared to C2C12$_{PIT/tTA}$-CEBPα$_{TET}$-BMP$_{PIP2}$ which correlates with its lower maximum BMP-2 expression levels (see Fig. 2B). Interestingly, following exclusive BMP-2 expression no adipogenic differentiation could be observed in either of the two cell clones (Fig. 3C and G). Likewise, no fat cells were detected for the +Tet/+PI setting when both genes were not expressed (Fig. 3D and H).

Differential lineage control of a C2C12-derived cell line engineered for independent control of C/EBP-α and BMP-2

BMP-2 has first been identified as a factor that induces bone and cartilage formation (23,26,45,46). We have assessed the potential of C2C12$_{PIT/tTA}$-CEBPα$_{TET}$-BMP$_{PIP2/6}$ to form osteoblasts following growth for 5 days in medium containing different combinations of regulating tetracycline and pristinamycin antibiotics (–Tet/–PI, –Tet/+PI, +Tet/–PI and +Tet/+PI). Specific staining for the osteogenic alkaline phosphatase (ALP) revealed highest osteoblast formation following exclusive expression of C/EBP-α (–Tet/+PI) (Fig. 4B and F). Coordinated expression of C/EBP-α and BMP-2 resulted in reduced osteoblast-specific differentiation (Fig. 4A and E). Expression of BMP-2 (+Tet/–PI) failed to induce osteogenic differentiation in C2C12$_{PIT/tTA}$-CEBPα$_{TET}$-BMP$_{PIP2/6}$ cells, yet prevented myotube formation (Fig. 4C and G). In the control configuration in which transgene expression was silenced by addition of both regulating antibiotics, C2C12$_{PIT/tTA}$-CEBPα$_{TET}$-BMP$_{PIP2/6}$ retained its C2C12 typical cell morphology (+Tet/+PI) (Fig. 4D and H). In order to confirm our finding that BMP-2 blocks myotube formation in C2C12 cells we cultivated C2C12 wild-type cells for 5 days in medium containing 100 µl/ml supernatant of C2C12$_{PIT/tTA}$-CEBPα$_{TET}$-BMP$_{PIP2/6}$ cultivated under BMP-2-induced conditions or of C2C12 wild type as control. BMP2-mediated inhibition of myotube formation was also observed following transduction of C2C12 with BMP-2-encoding lentiviral particles or addition of purified BMP-2 to C2C12 wild-type cultures (Fig. 5). BMP-2 seems to set C2C12 cells for adipocyte differentiation as it blocks or reduces commitment of myoblasts for myotubes and osteoblasts.

Dose-dependence of C/EBP-α-induced adipocyte versus osteoblast differentiation

The capacity of C/EBP-α to induce adipocytes as well as osteoblasts raised the question of how lineage decision is implemented. In order to establish a direct correlation between C/EBP-α levels and osteoblast as well as adipocyte formation we cultivated C2C12$_{PIT/tTA}$-CEBPα$_{TET45}$ for 5 days in medium supplemented with increasing tetracycline concentrations and scored adipocyte as well as osteoblast differentiation as a consequence of different C/EBP-α dosing. Figure 6 shows dose-dependent osteoblast formation starting at low C/EBP-α levels while adipogenesis requires maximum expression of this differentiation factor. This finding suggests that C/EBP-α mediates osteoblast-specific differentiation at low and differentiation of fat cells at high intracellular concentrations.

DISCUSSION

Mesenchymal precursor cells can differentiate into adipocytes, chondrocytes, myoblasts and osteoblasts following a program which remains elusive in many cell types. Based on C2C12 cells engineered for dual-regulated expression of C/EBP-α and BMP-2, we studied single as well as combinatorial gene expression impact on the differentiation

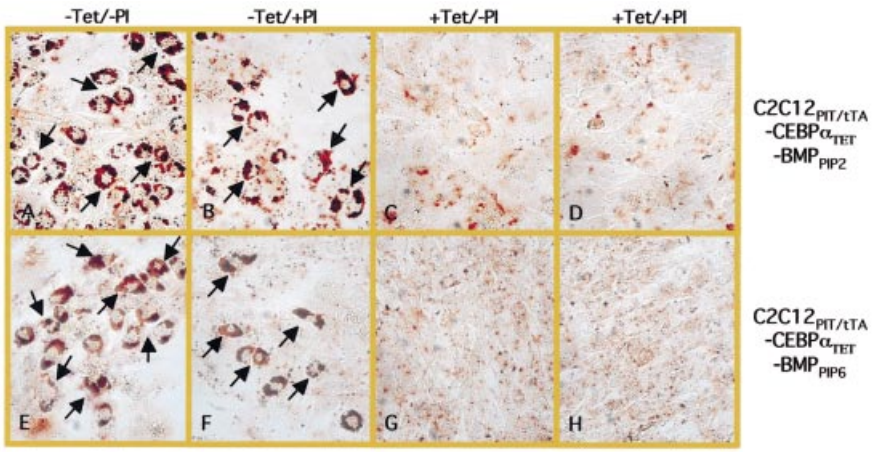

Figure 3. Adipogenic differentiation of C2C12 derivatives following different C/EBP-α and BMP-2 expression profiles. Adipocyte formation of $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-BMP_{PIP2} (A–D) and $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-BMP_{PIP6} (E–H) cell clones cultivated for 14 days in medium containing different combinations of regulating tetracycline (Tet) and streptogramin (PI) antibiotics. (**A** and **E**), –Tet/–PI; (**B** and **F**), –Tet/+PI; (**C** and **G**), +Tet/–PI; (**D** and **H**), +Tet/+PI.

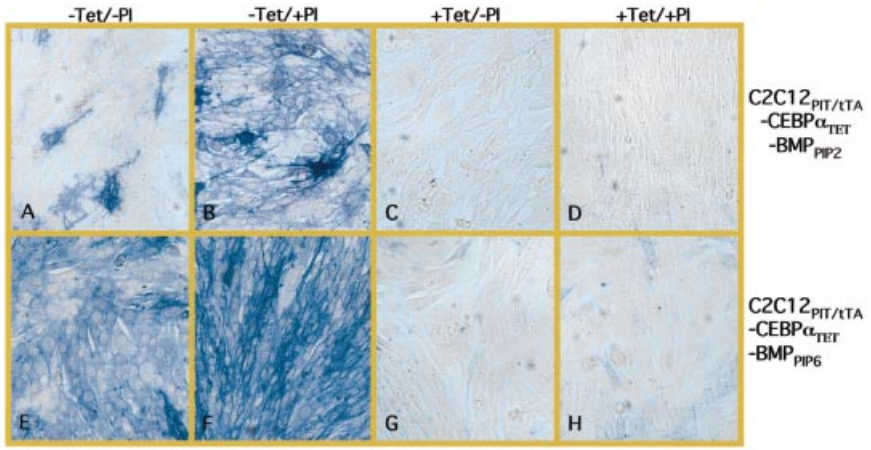

Figure 4. Osteoblast-specific differentiation of C2C12 clones expressing different combinations of C/EBP-α and BMP-2. $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-BMP_{PIP2} and $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-BMP_{PIP6} were grown for 5 days in medium supplemented with different combinations of regulating tetracycline (Tet) and streptogramin (PI) and stained for osteoblast-specific expression of alkaline phosphatase. (**A** and **E**), –Tet/–PI; (**B** and **F**), –Tet/+PI; (**C** and **G**), +Tet/–PI; (**D** and **H**), +Tet/+PI.

of myoblasts into adipocytes or osteoblasts. Dual-regulated expression enabled gene-function correlations of four different expression configurations ($C/EBP\text{-}\alpha_{ON}/BMP\text{-}2_{ON}$; $C/EBP\text{-}\alpha_{OFF}/BMP\text{-}2_{ON}$; $C/EBP\text{-}\alpha_{ON}/BMP\text{-}2_{OFF}$; $C/EBP\text{-}\alpha_{OFF}/BMP\text{-}2_{OFF}$).

Previously, differentiation of myoblasts (G8 type) into adipocytes required coordinated expression of C/EBP-α and PPARγ as well as addition of fatty acids and adipogenic hormones to the culture medium (19). G8 myoblasts exclusively expressing C/EBP-α failed to differentiate into adipocytes and muscle-specific differentiation was prevented by repression of myogenic factors. In contrast, the mutant C2C12 cell line C2C12N supported development of fat cell precursor cells following cultivation in medium supplemented with fatty acids and thialidinediones (47). To date, only fibroblasts differentiated into adipocytes following ectopic expression of C/EBP-α (16). We have demonstrated C/EBP-α-mediated myoblast to adipocyte transition of C2C12 in the presence of DEI.

Besides C/EBPs, BMPs play key roles during adipocyte- and osteoblast-specific differentiation processes (20,22,48). BMP-2 was reported to stimulate adipocyte differentiation of 2T3 and 3T3-L1 cells (5,30). In both cell lines BMP-2-mediated fat cell formation required activation of PPARγ induced by addition of PPARγ ligand to the culture medium. We have shown that BMP-2 cooperated with C/EBP-α for increased fat cell differentiation of C2C12 cell derivatives grown in DEI-containing medium. BMP-2-boosted adipocyte differentiation of C/EBP-α-expressing C2C12 cells was dose-dependent since clones expressing increased BMP-2 levels committed to fat cells at a higher frequency. Based on its name BMP-2 was expected to rather drive osteogenic differentiation of C2C12 than adipocyte lineage control (26). In fact, transgenic C2C12 cells set for BMP-2 expression did not differentiate into bone cells as did C2C12 cells grown in supernatants of BMP-2-producing cell lines. Yet, BMP-2 was active and inhibited myotube formation in C2C12 cells. This unexpected finding may either represent a new function of the

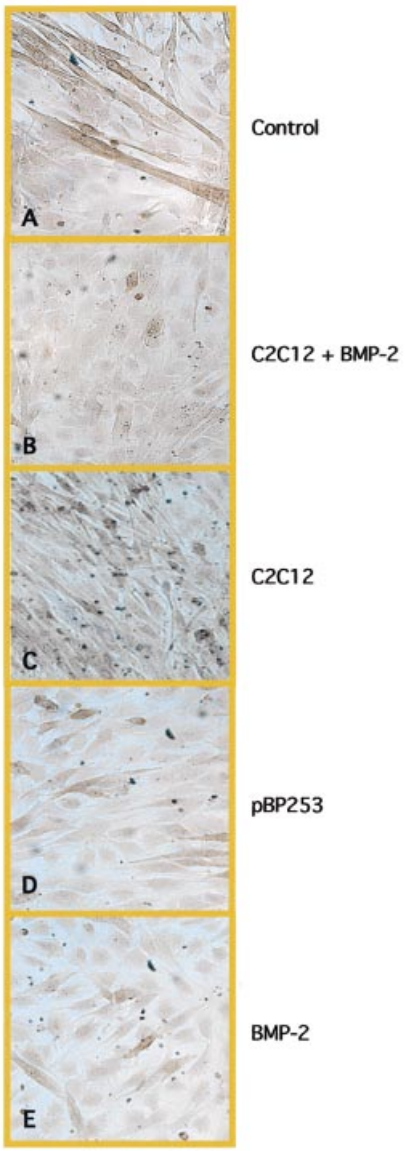

Figure 5. BMP-2-mediated inhibition of myotube formation in C2C12 cells. C2C12 wild-type cells were grown to confluence and then cultivated for 5 days in standard medium (**A**), in conditioned medium containing BMP-2 produced by $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-BMP_{PIP2} (**B**), in conditioned medium derived from $C2C12_{PIT/tTA}$ (**C**), followed by transduction with BMP-2-encoding lentiviral particles (**D**), supplemented with purified BMP-2 (3 ng/ml) (**E**). All cultures were stained for osteo-specific alkaline phosphatase expression. While BMP-2 fails to induce osteoblasts it inhibits myotube formation typical for C2C12 differentiation.

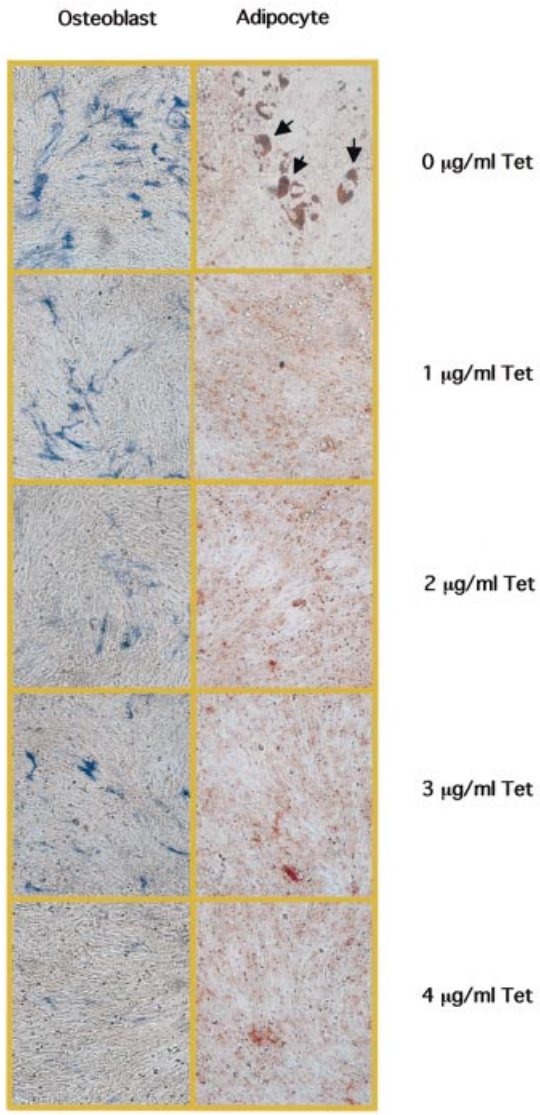

Figure 6. Dose-dependence of C/EBP-α-mediated osteoblast versus adipocyte lineage control. $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET45}$ was grown for 5 days at increasing tetracycline concentrations correlating with decreased C/EBP-α expression profiles. Osteoblast-specific differentiation was assessed by alkaline phosphatase staining while adipocytes were visualized by Oil Red O-mediated staining of triglycerides.

master differentiation regulator BMP-2 and/or results from integration of mutual interference between BMP-2-/C/EBP-α regulatory networks.

Recently, BMP-2 was reported to differentially trigger adipocyte and osteoblast differentiation via different BMP receptor isoforms IA and IB, respectively (5). The fact that C2C12 cells express type IA BMP receptors (49) substantiates our finding of synergistic BMP-2/C/EBP-α-mediated induction of fat cell formation in myoblasts.

Analysis of $C2C12_{PIT/tTA}$-$CEBP\alpha_{TET}$-$BMP_{PIP2/6}$ capacity to differentiate into bone cells when cultivated in different C/EBP-α and BMP-2 expression configurations revealed maximum osteoblast formation following exclusive expression of C/EBP-α, while the osteoblast frequency decreased following coexpression of BMP-2. Similar to the situation in ST-2 stromal cells, BMP-2 may reduce endogenous C/EBP-α levels and therefore bias differentiation against osteoblasts (50). Although expression of C/EBP family members has been reported for various cell lines, including adipocytes and osteoblasts in which they function as transactivators for bone-specific osteocalcin expression (11,50), this is, to our knowledge, the first time that C/EBP-α-induced osteoblast-specific differentiation could be shown. C/EBP-α's potential to induce differentiation into adipocytes as well as osteoblasts raises questions on how a particular lineage commitment is controlled. C/EBP-α dose-differentiation profiles revealed

that osteoblast-specific differentiation occurs at lower C/EBP-α levels while adipocyte formation only takes place at maximum C/EBP-α expression. This finding suggests that C/EBP-α triggers osteoblast versus adipocyte differentiation in a dose-dependent manner. In addition, BMP-2 may reduce C/EBP-α-mediated osteoblast differentiation and bias lineage control towards adipocytes.

Our study demonstrated that C/EBP-α plays an important role in adipocyte- and osteoblast-specific differentiation of myoblasts and that this lineage control switch is modulated by BMP-2. Novel insight into fat–muscle–bone transdifferentiation my pave the way towards rational reprogramming of desired cell phenotypes for gene therapy and tissue engineering.

ACKNOWLEDGEMENTS

We thank Valeria Nicolini for critical comments on the manuscript. This work was supported by the Swiss National Science Foundation (grant no. 631-065946), the Swiss Federal Office for Education and Science (BBW) within EC Framework 5, and Cistronics Cell Technology GmbH, Einsteinstrasse 1–5, CH-8093 Zurich, Switzerland.

REFERENCES

1. Pereira,R.F., Halford,K.W., O'Hara,M.D., Leeper,D.B., Sokolov,B.P., Pollard,M.D., Bagasra,O. and Prockop,D.J. (1995) Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage and lung in irradiated mice. *Proc. Natl Acad. Sci. USA*, **92**, 4857–4861.
2. Taylor,S.M. and Jones,P.A. (1979) Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell*, **17**, 771–779.
3. Friedenstein,A.J., Chailakhyan,R.K. and Gerasimov,U.V. (1987) Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet.*, **20**, 263–272.
4. Prockop,D.J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science*, **276**, 71–74.
5. Chen,D., Ji,X., Harris,M.A., Feng,J.Q., Karsenty,G., Celeste,A.J., Rosen,V., Mundy,G.R. and Harris,S.E. (1998) Differential roles for bone morphogenetic protein (BMP) receptor type IB and IA in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages. *J. Cell Biol.*, **142**, 295–305.
6. Hanson,R.W. (1998) Biological role of the isoforms of C/EBP minireview series. *J. Biol. Chem.*, **273**, 28543.
7. Johnson,P.F. and McKnight,S.L. (1989) Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.*, **58**, 799–839.
8. Yamanaka,R., Lekstrom-Himes,J., Barlow,C., Wynshaw-Boris,A. and Xanthopoulos,K.G. (1998) CCAAT/enhancer binding proteins are critical components of the transcriptional regulation of hematopoiesis (Review). *Int. J. Mol. Med.*, **1**, 213–221.
9. Darlington,G.J., Ross,S.E. and MacDougald,O.A. (1998) The role of C/EBP genes in adipocyte differentiation. *J. Biol. Chem.*, **273**, 30057–30060.
10. Lane,M.D., Tang,Q.Q. and Jiang,M.S. (1999) Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. *Biochem. Biophys. Res. Commun.*, **266**, 677–683.
11. Gutierrez,S., Javed,A., Tennant,D.K., van Rees,M., Montecino,M., Stein,G.S., Stein,J.L. and Lian,J.B. (2002) CCAAT/enhancer-binding proteins (C/EBP) beta and delta activate osteocalcin gene transcription and synergize with Runx2 at the C/EBP element to regulate bone-specific expression. *J. Biol. Chem.*, **277**, 1316–1323.
12. Vinson,C.R., Hai,T. and Boyd,S.M. (1993) Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. *Genes Dev.*, **7**, 1047–1058.
13. Lekstrom-Himes,J. and Xanthopoulos,K.G. (1998) Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J. Biol. Chem.*, **273**, 28545–28548.
14. Landschulz,W.H., Johnson,P.F. and McKnight,S.L. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science*, **240**, 1759–1764.
15. Agre,P., Johnson,P.F. and McKnight,S.L. (1989) Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP. *Science*, **246**, 922–926.
16. Freytag,S.O., Paielli,D.L. and Gilbert,J.D. (1994) Ectopic expression of the CCAAT/enhancer-binding protein alpha promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes Dev.*, **8**, 1654–1663.
17. Keller,H., Dreyer,C., Medin,J., Mahfoudi,A., Ozato,K. and Wahli,W. (1993) Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc. Natl Acad. Sci. USA*, **90**, 2160–2164.
18. Issemann,I. and Green,S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*, **347**, 645–650.
19. Hu,E., Tontonoz,P. and Spiegelman,B.M. (1995) Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. *Proc. Natl Acad. Sci. USA*, **92**, 9856–9860.
20. Lee,M.H., Kwon,T.G., Park,H.S., Wozney,J.M. and Ryoo,H.M. (2003) BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. *Biochem. Biophys. Res. Commun.*, **309**, 689–694.
21. Musgrave,D.S., Bosch,P., Ghivizzani,S., Robbins,P.D., Evans,C.H. and Huard,J. (1999) Adenovirus-mediated direct gene therapy with bone morphogenetic protein-2 produces bone. *Bone*, **24**, 541–547.
22. Nakashima,K., Zhou,X., Kunkel,G., Zhang,Z., Deng,J.M., Behringer,R.R. and de Crombrugghe,B. (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell*, **108**, 17–29.
23. Okubo,Y., Bessho,K., Fujimura,K., Iizuka,T. and Miyatake,S. (1999) Expression of bone morphogenetic protein-2 via adenoviral vector in C2C12 myoblasts induces differentiation into the osteoblast lineage. *Biochem. Biophys. Res. Commun.*, **262**, 739–743.
24. Reddi,A.H. (1997) Bone morphogenetic proteins: an unconventional approach to isolation of first mammalian morphogens. *Cytokine Growth Factor Rev.*, **8**, 11–20.
25. Reddi,A.H. and Cunningham,N.S. (1993) Initiation and promotion of bone differentiation by bone morphogenetic proteins. *J. Bone Miner. Res.*, **8** (Suppl. 2), S499–S502.
26. Katagiri,T., Yamaguchi,A., Komaki,M., Abe,E., Takahashi,N., Ikeda,T., Rosen,V., Wozney,J.M., Fujisawa-Sehara,A. and Suda,T. (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J. Cell Biol.*, **127**, 1755–1766.
27. Wang,E.A., Israel,D.I., Kelly,S. and Luxenberg,D.P. (1993) Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. *Growth Factors*, **9**, 57–71.
28. Ahrens,M., Ankenbauer,T., Schroder,D., Hollnagel,A., Mayer,H. and Gross,G. (1993) Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. *DNA Cell Biol.*, **12**, 871–880.
29. Asahina,I., Sampath,T.K., Nishimura,I. and Hauschka,P.V. (1993) Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. *J. Cell Biol.*, **123**, 921–933.
30. Sottile,V. and Seuwen,K. (2000) Bone morphogenetic protein-2 stimulates adipogenic differentiation of mesenchymal precursor cells in synergy with BRL 49653 (rosiglitazone). *FEBS Lett.*, **475**, 201–204.
31. Weber,W., Fux,C., Daoud-el Baba,M., Keller,B., Weber,C.C., Kramer,B.P., Heinzen,C., Aubel,D., Bailey,J.E. and Fussenegger,M. (2002) Macrolide-based transgene control in mammalian cells and mice. *Nat. Biotechnol.*, **20**, 901–907.
32. Weber,W., Schoenmarkers,R., Spielmann,M., Daoud-el Baba,M., Folcher,M., Keller,B., Weber,C.C., Link,N., van de Wetering,P., Heinzen,C., Jolivet,B., Séquin,U., Aubel,D., Thompson,C.J. and Fussenegger,M. (2003) Streptomyces-derived quorum-sensing systems engineered for adjustable transgene expression in mammalian cells and mice. *Nucleic Acids Res.*, **31**, e71.
33. Weber,W., Marty,R.R., Link,N., Ehrbar,M., Keller,B., Weber,C.C., Zisch,A.H., Heinzen,C., Djonov,V. and Fussenegger,M. (2003) Conditional human VEGF-mediated vascularization in chicken embryos using a novel temperature-inducible gene regulation (TIGR) system. *Nucleic Acids Res.*, **31**, e69.

34. Fussenegger,M., Morris,R.P., Fux,C., Rimann,M., von Stockar,B., Thompson,C.J. and Bailey,J.E. (2000) Streptogramin-based gene regulation systems for mammalian cells. *Nat. Biotechnol.*, **18**, 1203–1208.
35. Fussenegger,M. (2001) The impact of mammalian gene regulation concepts on functional genomic research, metabolic engineering and advanced gene therapies. *Biotechnol. Prog.*, **17**, 1–51.
36. Fux,C., Moser,S., Schlatter,S., Rimann,M., Bailey,J.E. and Fussenegger,M. (2001) Streptogramin- and tetracycline-responsive dual regulated expression of p27(Kip1) sense and antisense enables positive and negative growth control of Chinese hamster ovary cells. *Nucleic Acids Res.*, **29**, e19.
37. Fux,C. and Fussenegger,M. (2003) Toward higher order control modalities in Mammalian cells-independent adjustment of two different gene activities. *Biotechnol. Prog.*, **19**, 109–120.
38. Gossen,M. and Bujard,H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl Acad. Sci. USA*, **89**, 5547–5551.
39. Triezenberg,S.J., Kingsbury,R.C. and McKnight,S.L. (1988) Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev.*, **2**, 718–729.
40. Moser,S., Rimann,M., Fux,C., Schlatter,S., Bailey,J.E. and Fussenegger,M. (2001) Dual-regulated expression technology: a new era in the adjustment of heterologous gene expression in mammalian cells. *J. Gene Med.*, **3**, 529–549.
41. Timchenko,N.A., Wilde,M., Nakanishi,M., Smith,J.R. and Darlington,G.J. (1996) CCAAT/enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. *Genes Dev.*, **10**, 804–815.
42. Mitta,B., Rimann,M., Ehrengruber,M.U., Ehrbar,M., Djonov,V., Kelm,J. and Fussenegger,M. (2002) Advanced modular self-inactivating lentiviral expression vectors for multigene interventions in mammalian cells and *in vivo* transduction. *Nucleic Acids Res.*, **30**, e113.
43. Koopman,R., Schaart,G. and Hesselink,M.K. (2001) Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem. Cell Biol.*, **116**, 63–68.
44. Fussenegger,M., Bailey,J.E. and Varner,J. (2000) A mathematical model of caspase function in apoptosis. *Nat. Biotechnol.*, **18**, 768–774.
45. Lutolf,M.P., Weber,F.E., Schmoekel,H.G., Schense,J.C., Kohler,T., Muller,R. and Hubbell,J.A. (2003) Repair of bone defects using synthetic mimetics of collagenous extracellular matrices. *Nat. Biotechnol.*, **21**, 513–518.
46. Hogan,B.L. (1999) Morphogenesis. *Cell*, **96**, 225–233.
47. Teboul,L., Gaillard,D., Staccini,L., Inadera,H., Amri,E.Z. and Grimaldi,P.A. (1995) Thiazolidinediones and fatty acids convert myogenic cells into adipose-like cells. *J. Biol. Chem.*, **270**, 28183–28187.
48. Aubin,J.E., Liu,F., Malaval,L. and Gupta,A.K. (1995) Osteoblast and chondroblast differentiation. *Bone*, **17**, 77S–83S.
49. Namiki,M., Akiyama,S., Katagiri,T., Suzuki,A., Ueno,N., Yamaji,N., Rosen,V., Wozney,J.M. and Suda,T. (1997) A kinase domain-truncated type I receptor blocks bone morphogenetic protein-2-induced signal transduction in C2C12 myoblasts. *J. Biol. Chem.*, **272**, 22046–22052.
50. Pereira,R.C., Delany,A.M. and Canalis,E. (2002) Effects of cortisol and bone morphogenetic protein-2 on stromal cell differentiation: correlation with CCAAT-enhancer binding protein expression. *Bone*, **30**, 685–691.