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Dlx5 Regulation of Mouse Osteoblast Differentiation Mediated by Avian Retrovirus Vector

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Aim. To study the effect of Dlx5 introduced by replication-competent avian splice-acceptor (RCAS) in mouse calvarial and bone marrow stromal cells, and to demonstrate that RCAS vector can be a useful system for studying gene expression in mammalian cells derived from ß-AKE mouse.

Method. ß-AKE mouse used in experiments is a transgenic mouse line expressing the receptor for the Bryant polymerase subgroup A of RCAS vector (RCAS-BP(A) vector). Primary calvarial osteoblast cultures were obtained from 7-day-old ß-AKE mice. Bone marrow stromal cells were derived from the long bones of 8-week-old ß-AKE mice. Expression of genes cloned into RCAS vector in mouse cells was first established by detecting green fluorescent protein (GFP) in cells infected with RCAS-BP(A)-GFP sapphire by using fluorescence microscopy. Cells were then infected with RCAS-BP(A)-Dlx5 or RCAS-BP(A) alone as a control, for three days. After differentiation, cells were harvested for mRNA analysis at different time points (day 6 or 7, 11 or 12, 14 or 18, and 21 or 25). The cells were cultured in the presence of ascorbic acid and ß-glycerophosphate, which promotes osteoblastic differentiation.

Results. Mouse calvarial and bone marrow stromal cells infected with RCAS-BP(A)-GFP sapphire were fluorescent compared with the controls. Both types of cells infected with RCAS-BP(A)Dlx5 consistently expressed increased levels of bone differentiation markers – type 1 collagen (Col1α1), osteocalcin, and bone sialoprotein mRNA.

Conclusion. RCAS-BP(A) vector transduction of cells from ß-AKE mice is a useful system for studying the role of gene expression in mouse osteoblastic cells. Dlx5 overexpression mediated by an RCAS-BP(A) vector stimulates mouse osteoblastic differentiation in ß-AKE transgenic mice. Dlx5 induces osteoblast differentiation from bones formed either by endochondral or by membranous ossification.

Key words: *bone and bones; homeodomain proteins; mice, transgenic; osteoblasts; retroviridae*

Induction of ectopic expression in non-immortalized primary osteoblastic cells is often important for a complete understanding of protein function. Standard transfection methods are usually not efficient enough to induce protein expression in a high proportion of cultured cells. Retroviral vectors can efficiently induce stable protein expression in cultures of dividing cells and are very suitable for gene therapeutic approaches based on permanent expression of therapeutic genes (1-3). There are two main types of retroviral vectors, replication-competent and replication-defective. To achieve vector virus titers of 107 transducing particles per milliliter, replication-defective vectors require highly efficient transfection of multiple DNA constructs into a helper cell line, followed by ultracentrifuge concentration (4). Alternatively, multiple rounds of transfection and transduction of helper cells have to be carried out, with selection and evaluation of vector producer cells to identify clones that produce high titers of the vector virus. In contrast, replication-competent vectors require only transfection of fibroblasts and subculturing the cells 2-3 times to routinely produce titers of 107 transducing particles per milliliter.

To date, one of the most valuable replication-competent vectors are the replication-competent avian splice (RCAS)-acceptor series (4). Originally, the use of these vectors was restricted to avian species, because the viral envelope coat protein, which is necessary for cell attachment and internalization, binds to a cell surface receptor protein restricted to avian cells (4). Because of the availability of murine osteoblastic cell culture systems, knockout and transgenic mice, and the large number of sequenced mouse genes, it would be advantageous to use the RCAS system with murine cells. Thus, a ß-AKE transgenic mouse line has been developed, with the gene for the cell surface receptor for the subgroup A RCAS-RCAS-BP(A) vector driven by the ß–actin promoter (5).

There are several different proteins involved in bone development. Dlx5 is a homeodomain protein that binds to a site in the col1a1 promoter of the collagen gene required for deposition of the bone matrix and is of fundamental importance for osteoblast differentiation (6). The vertebrate Dlx genes are related to the distal-less gene, which is involved in limb development in Drosophila and other invertebrates (7). There are six to seven vertebrate Dlx genes, which are expressed in overlapping, but significantly divergent, protein domains in the craniofacial region and limbs during development (8,9). Dlx5 and Dlx6 are expressed in almost every developing skeletal element, including endochondral and membranous bone (8-10). In the chick limb, Dlx5 is found in the developing cartilaginous skeletal elements and expressed most strongly in the cells that will become the initial bony collar surrounding the cartilage (11). Thus, the expression pattern of Dlx5 is consistent with its stimulative role in skeletal development. Dlx5 mRNA levels increase during in vitro rat calvarial osteoblast differentiation, although overexpression of Dlx5 in 17/2.8 rat osteosarcoma cells (17/2.8 ROS) inhibited the expression of osteocalcin, a marker of differentiated osteoblasts (12). Dlx5 is induced by bone morphogenetic proteins in the immortalized osteoblastic cell line MC3T3-E1, and overexpression of the protein stimulates expression of osteoblastic markers in these cells (13). Mutation of murine Dlx5 by homologous recombination causes craniofacial and sensory capsule skeletal defects (14,15). In addition, calvarial ossification is delayed and diaphyseal cortical bone less well organized in Dlx5 knockout mice (15). A homeodomain protein-binding site within the mouse bone sialoprotein promoter is important for osteoblast-specific expression in transfected cell lines. This site binds Dlx5 in gel shift analysis, and Dlx5 stimulates bone sialoprotein promoter in cotransfected ROS simian virus 40 (SV40)-transformed cells of African green monkey kidney (16).

The goal of this study was to determine whether RCAS system could be used to study the effect of a gene expression on mouse osteoblast differentiation, to investigate whether Dlx5 induces osteoblastic differentiation in mouse cells like it was shown with chick calvarial osteoblasts (6), and to determine whether Dlx5 induces osteoblastic differentiation of bone marrow stromal cells, which are multipotent stem cells that can be induced to take part in the formation of endochondral bone.

Material and Methods

Transgenic mouse line (ß-AKE) has the gene for the cell surface receptor for RCAS-BP(A) vector driven by the ß–actin promoter (5).

Construction of RCAS-BP(A) Retroviral Vectors

The RCAS vectors (RCAS-replication-competent avian sarcoma-leukosis virus long terminal repeat with a splice acceptor) are a family of retroviral vectors derived from the SR-A strain of Rous sarcoma virus, a member of the avian sarcoma-leukosis virus family. RCAS-BP(A) is Bryant polymerase subgroup A of RCAS vectors, produced by substituting the pol region from the Bryant high-titer strain of Rous sarcoma virus into the RCAS virus. It replicates better than RCAS (17).

The coding sequence of the green fluorescent protein (GFP) or chicken Dlx5 was cloned into the ClaI site of the RCAS-BP(A) helper independent retroviral vector. GFP sapphire dye (Packard Instruments, Meriden, CT, USA) cloned into RCAS-BP(A) was used to visualize expression of protein mediated by RCAS vectors in mouse cells.

Control virus was RCAS-BP(A) vector without inserted cDNA. Vectors were transfected into producer cells – chicken embryonal fibroblasts, by using the calcium phosphate method (18). Reverse transcriptase activity was determined in the producer cell media as a measure of virus production (4). Conditioned media with high reverse transcriptase activity were collected and stored at -70 °C until use.

Primary Mouse Calvarial Osteoblast Culture

Calvaria were dissected from 7-day-old pups. Calvarial cells were isolated by four sequential 15-minute digestions in an enzyme mixture containing 0.05% trypsin (Gibco BRL, Rockville, MD, USA) and 0.1% collagenase P (Boehringer Mannheim, Indianapolis, IN, USA) at 37 °C, on a rocking platform. Fractions 2-4 were collected, resuspended in media, and plated at $5\times10^3/cm^2$ in 6-well culture plates (Costar, Cambridge, MA, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 10% fetal calf serum (FCS; Gibco BRL). After the cells reached a confluent monolayer at day 7, α-MEM (Gibco BRL) containing 10% FCS, 50 μg/mL ascorbic acid (Gibco BRL), and 5 mmol ß-glycerophosphate (Gibco BRL) was used to maintain the cells for the duration of the experiment. Cells were harvested for analysis at different time points: day 6 or 7, 12, 18, or 25.

Primary Mouse Marrow Stromal Culture

Mouse marrow stromal cells were obtained by flushing bone marrow from long bones of 8-week-old mice. Cells were plated at 10×10^6 cells/cm^2 in 6-well culture plates (Costar) and maintained in α-MEM containing 10% FCS, 50 μg/mL ascorbic acid, 5 mmol ß-glycerophosphate, and 10^{-8} mol/L dexamethasone (all from Gibco BRL). Cells were harvested for analysis at different time points: day 7, 11, 14, or 21.

Infection of Mouse Primary Osteoblast Cell Cultures

Mouse calvarial osteoblast cultures were infected for 3 days with 500 μL of conditioned media containing RCAS-BP(A)-Dlx5 or RCAS-BP(A) as a control, beginning on the second day after plating. The virus was added to 2 mL of fresh media in a 35-mm dish (Costar). Mouse marrow stromal cells were infected for 3 days in a same way, beginning on day 5.

Northern Blot Analysis

Experiments were performed 10 times and mRNA extraction was performed at different time points, depending on cell culture growth. RNA was isolated with TRI reagent (Molecular Research Center, Inc, Cincinnati, OH, USA) by the method of Chomczynski and Sacchi (19). Ten μg of total RNA was separated on 1% agarose /1.1 mol/L formaldehyde gel and transferred to an extra strength nylon membrane (Schleicher and Schuell, Keene, NH, USA) by positive pressure. Membranes were hybridized with a ^{32}P-labeled probes for Dlx5 (20), bone sialoprotein (21), and osteocalcin (22), which represented full length cDNA, and Col1α1 (23). Hybridization was performed in 50% formamide (Gibco BRL) and 6× standard saline phosphate with EDTA at 42 °C, then washed and exposed to X-ray film with an intensifying screen at -70 °C. Filters were stripped and rehybridized to an 18S RNA probe to assess RNA quantitation and standardization.

Results

Mouse calvarial osteoblasts and mouse bone marrow stromal cells derived from transgenic ß-AKE mouse were infected first with RCAS-BP(A)-GFP sapphire because we wanted to test if it was possible for avian retrovirus to infect mouse cells. Approximately 50% of the cells were fluorescent in mouse calvarial and marrow stromal cells (Figs. 1 and 2). These results indicated that RCAS-BP(A) vector could infect cells derived from ß-AKE mice with high efficiency.

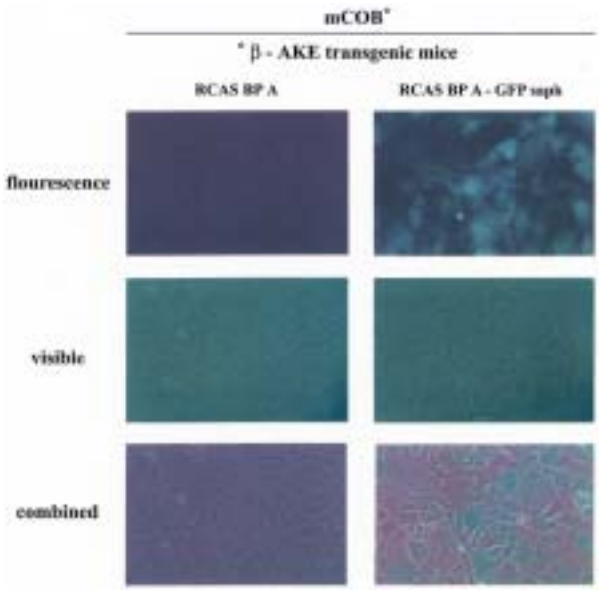

Figure 1. RCAS-BP(A)-GFP sapphire expression in mouse calvarial osteoblasts derived from ß-AKE mice. Fluorescent images were taken through a GFP sapphire specific filter. Digital images were taken with a Kodak DCS 420 camera. For combined (fluorescence plus visible) images, the phase contrast image was converted to grey scale in Adobe Photoshop, and the fluorescent image was merged with it. RCAS – replication-competent avian splice-acceptor; BP(A) – Bryant polymerase subgroup A of RCAS vectors; GFP – green fluorescent protein.

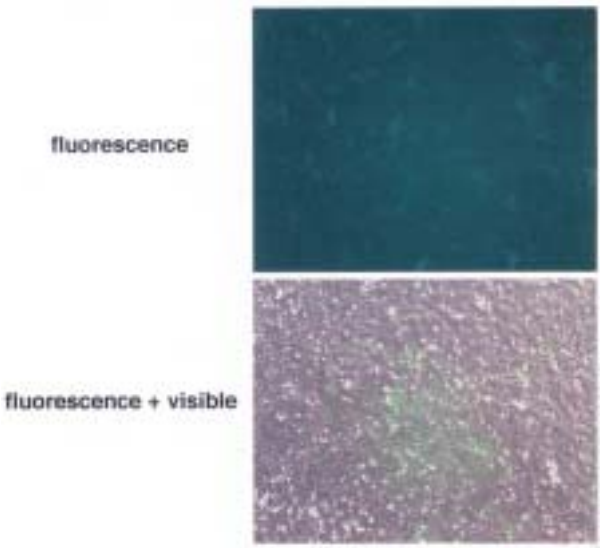

Figure 2. RCAS-BP(A)-GFP sapphire expression in mouse bone marrow stromal cells derived from ß-AKE mice. RCAS – replication-competent avian splice-acceptor; BP(A) – Bryant polymerase subgroup A of RCAS vectors; GFP - green fluorescent protein.

Next we used RCAS-BP(A)-Dlx5 to study the effect of Dlx5 on mouse osteoblast differentiation. Northern blot hybridization of mRNA from mouse calvarial cells with chick Dlx5 probe showed that chick Dlx5 was expressed in mouse calvarial osteoblasts (Fig. 3).

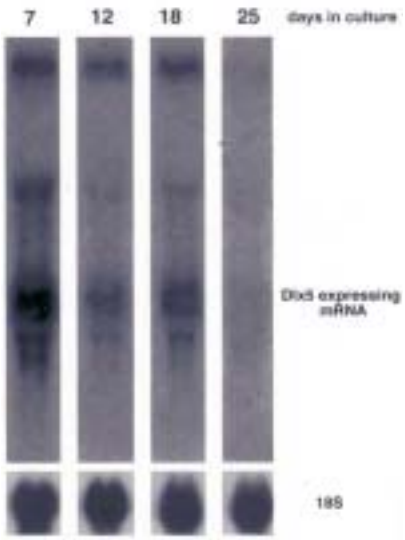

Figure 3. Northern blot hybridization demonstrates expression of chick Dlx5 in mouse calvarial osteoblasts at days 7, 12, 18 and 25 of culture.

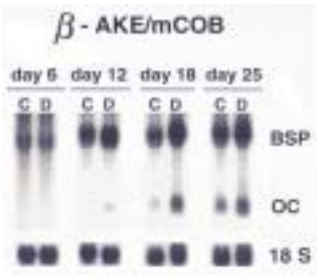

Figure 4. RCAS-BP(A)-Dlx5 infection of mouse calvarial osteoblasts (mCOB) stimulates bone sialoprotein (BSP) and osteocalcin (OC) synthesis. Cultures were harvested on days 6, 12, 18 and 25 after plating. RCAS – replication-competent avian splice-acceptor; BP(A) – Bryant polymerase subgroup A of RCAS vectors; C – control cultures; D – RCAS-BP(A)-Dlx5 infected cultures.

Parallel cultures of mouse calvarial osteoblast cells were infected with RCAS-BP(A)-Dlx5 or RCAS-BP(A) alone as a control. Northern blot analysis of mRNA extracted from mouse calvarial cells with Dlx5 overexpression showed increased levels of osteocalcin and bone sialoprotein bone markers in calvarial osteoblasts compared with control cells infected with RCAS-BP(A) virus without Dlx5 (Fig. 4).

Northern blot analysis of mRNA from bone marrow stromal cells infected with RCAS-BP(A)-Dlx5 showed increased concentration of bone-specific markers Col1α1 and osteocalcin mRNA compared with control cells (Fig. 5).

These results implied that Dlx5 stimulated mouse osteoblast differentiation.

Discussion

Overexpression of Dlx5 protein obtained with an RCAS-BP(A) vector resulted in the stimulation of osteoblastic differentiation in mouse calvarial osteoblast cultures and mouse bone marrow stromal cell

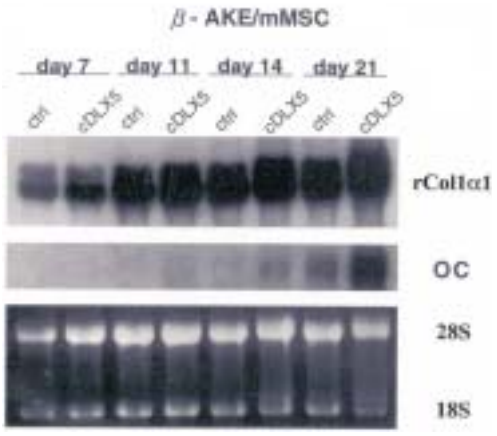

Figure 5. RCAS-BP(A)-Dlx5 infection of mouse bone marrow stromal cells (mMSC) stimulates Col1a1 and osteocalcin (OC) synthesis. Cultures were harvested on days 7, 11, 14 and 21 after plating. The Col1a1 signal is overexposed. RCAS – replication-competent avian splice-acceptor; BP(A) – Bryant polymerase subgroup A of RCAS vectors; Ctrl – control cultures; cDlx5 – RCAS-BP(A)-Dlx5 infected cultures.

cultures. However, the magnitude of the effect was less than that observed when the same vectors were used in chick calvarial osteoblasts (6).

In previous studies, the role of Dlx5 in osteoblastic differentiation was assessed by using a primary chick calvarial osteoblast culture system. In chick calvarial osteoblast culture system, Dlx5 expression increased simultaneously with the markers of osteoblastic differentiation, whereas Dlx5 overexpression stimulated osteoblastic differentiation (6). In addition, Dlx5-induced osteoblastic differentiation of cultures enriched in periosteal cells, which do not undergo osteoblastic differentiation under the usual conditions used in our experiments if Dlx5 is not added (6).

The differentiation of preosteoblasts to mature osteoblasts is a complex, multistage process whose control at the transcriptional level is not very well understood. The recent observations that Cbfa1 and osterix are required for bone development are significant discoveries (24). However, it may also be expected that other transcription factors are involved in regulating this process (25).

Previous data from our laboratory and others have shown that a homeodomain protein binding site in the Col1α1 promoter is necessary for high-level expression of the gene in differentiated osteoblasts of transgenic mice (26). However, this site is less important in tendon or periosteal fibroblasts (26,27). Since high-level expression of the Col1a1 gene is required for the deposition of bone matrix, proteins that regulate its expression are of fundamental importance for osteoblast differentiation.

In situ hybridization studies on mineralized tissue have shown bone sialoprotein expression (28), which is considered a bone differentiation marker as important as osteocalcin (29). This was the reason why we decided to evaluate the expression of those two bone-specific markers to confirm positive effect of Dlx5 on bone differentiation in mammalian cells from our transgenic mouse.

The RCAS-BP(A) system combined with the ß-AKE mouse line should be a convenient and useful system for protein function studies in primary mouse osteoblastic cell cultures.

Replication-competent vectors contain all the genes necessary for viral replication within the vector (4). In contrast, replication-defective vectors do not contain the genes coding for the viral proteins, thus these proteins must be provided by a helper cell line (4). Furthermore, to produce a large quantity of a retroviral vector with a high concentration of infectious particles requires less time and effort for replication-competent vectors than for producing replication-defective vectors by standard methods (4). As ß-AKE transgenic mouse line expresses the receptor for RCAS-BP(A) vectors driven by the chicken ß-actin promoter, most cell types from this mouse can be transduced with these vectors (5). However, the transducibility of osteoblastic precursors has not been evaluated. Our study showed that mouse calvarial osteoblasts and mouse bone marrow stromal cells from ß-AKE mice could be infected with RCAS-BP(A) virus vectors, although the expression was not as high as that observed in chicken cells.

In conclusion, our results showed that RCAS-BP(A) vector transduction of cells from ß-AKE mice was a useful system for studying the role of proteins in mouse osteoblast differentiation. We also confirmed and extended our previous studies by using mammalian cells, with Dlx5 overexpression mediated by an RCAS-BP(A) vector, which stimulates osteoblastic differentiation in osteoblasts derived from bones formed either by membranous or by endochondral ossification.

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