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Cell-Type-Specific Modulation of Hox Gene Expression by Members of the TGF-β Superfamily: A Comparison between Human Osteosarcoma and Neuroblastoma Cell Lines

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Homeobox gene expression in osteoblast-like cells was investigated using the polymerase chain reaction (PCR). A total of 13 homeobox genes was detected in U-2 OS (human osteosarcoma) and MC3T3-E1 (mouse osteoblast) cells by sequencing cloned PCR products. Using specific primers, a different pattern of Hox gene expression was shown for the neuroblastoma cell line SK-N-SH relative to U-2 OS and MC3T3-E1. Additionally, we showed that expression of HOXC6 in U-2 OS and SK-N-SH was differentially regulated by rhBMP-2, TGF-β and activin-A. This suggests that specific Hox genes may be target genes for TGF-β superfamily members, and allows us to further understand the complex functions of these growth factors and how they relate to growth and development.

The vertebrate Hox genes encode a closely related subset of homeobox containing transcription factors, consisting of at least 38 members that are conserved over many species. The homeobox of these transcription factors encodes for a 61 amino acid homeodomain that binds specifically to DNA. Mammalian Hox genes are physically linked in various chromosomal clusters (Hox loci A-D), located on chromosomes 2, 7, 12, and 17 respectively (1, 2). Their position along the 5′-3′ transcriptional axis is related to their location along the anteroposterior axis of the embryo (3), as well as to their appearance in time (4) (spatiotemporal colinearity (5)). The transcriptional regulation of homeobox genes is complex and still poorly understood, although it is clear that many factors are involved. Hox genes have been shown to be responsive to retinoic acid (RA) (4) and basic fibroblast growth factor (bFGF) (6). Members of the transforming growth factor-β (TGF-β) superfamily, such as bone morphogenetic proteins (BMPs), activins, and TGF-β, are also possible regulators of homeobox genes, given their pivotal roles in normal growth and development. The Drosophila homologue of BMP-2 and BMP-4, decapentaplegic protein (DPP), enhances the expression of the homeobox gene labial (7) and ectopic expression of DPP alters the expression of various homeobox genes both at ectopic and adjacent sites (8). A close correlation has been shown between expression of BMP-2 and particular Hox genes in the developing limb (9). In addition, several Hox genes are expressed during BMP-induced ectopic bone formation (10). Based on these studies we hypothesized that homeobox gene expression in cells representative of the skeletal system is regulated by TGF-β superfamily members.

We initially investigated the expression of Hox genes in osteoblast-like cells using the polymerase chain reaction (PCR), based on a strategy described by Murtha et al. (11). Osteoblast-like cell lines used were the human osteosarcoma cell line (U-2 OS) and the mouse osteoblast cell line MC3T3-E1 (12). Next, we studied whether expression is modulated by 3 members of the TGF-β superfamily (BMP-2, TGF-β1, and activin-A) in U-2 OS. To study whether expression and regulation of Hox genes was cell type-specific, Hox gene expression and effects of BMP-2 and TGF-β on Hox gene expression were also studied in the human neuroblastoma cell line SK-N-SH.

MATERIAL AND METHODS

Cell lines and growth factors. U-2 OS, a human osteosarcoma cell line, and SK-N-SH, a human neuroblastoma cell line (both from

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American Type Culture Collection), were subcultured in Dulbecco's Modified Eagle Medium (DMEM) containing penicillin (50 IU/ml), streptomycin (50 µg/ml), and 10% newborn calf serum (NCS). The mouse osteoblast cell line MC3T3-E1 (12) was grown in α-minimal essential medium (αMEM) containing penicillin (50 IU/ml), streptomycin (50 µg/ml), and 10% fetal calf serum (FCS). Recombinant bone morphogenetic protein-2 (rhBMP-2) was a generous gift from the Genetics Institute (Cambridge, MA, USA). Human TGF-β1 was purchased from R&D Systems (UK), and human activin-A was a generous gift from D Haylebroek, Celagen, University of Leuven, Belgium.

Cells were grown at near-confluence by incubation with serum-free DMEM (U-2 OS), αMEM (MC3T3-E1) or DMEM with 1% NCS (SK-N-SH) for 72 h, after which growth factors were added: rhBMP-2 (50 ng/ml), TGF-β1 (2.5 ng/ml), and activin-A (25 ng/ml). After 24 h (U-2 OS) and after 48 h (U-2 OS, and SK-N-SH), cells were washed with phosphate-buffered saline (PBS) and stored as a pellet at −80°C until mRNA isolation.

mRNA isolation and RT-PCR. mRNA isolation and RT-PCR were performed as previously described (13) with minor modifications. mRNA was extracted using the Micro-Fast Track mRNA isolation kit (Invitrogen Corp., San Diego, CA). For each sample approximately 5 µg mRNA was reverse transcribed using 50 µM of oligonucleotide primer and 200 units SuperScript II (Gibco-BRL) in a total volume of 30 µl.

Thereafter, 2 µl of the reaction volume was amplified by PCR using 0.5 µM of each sense and anti-sense primer, 150 µM dNTPs, 1.5 mM MgCl₂ and one unit Taq polymerase (Gibco-BRL) in a total volume of 20 µl. PCR mixtures were transferred directly from ice to 96°C for 10 seconds, followed by a specific number of amplification cycles consisting of 96°C (10 seconds), annealing temperature (see Table 1) for 30 seconds, 72°C (1 minute), and a final extension step at 72°C for 7 minutes (Perkin-Elmer Geneamp PCR system 2400). The number of cycles was either saturating (45 cycles) or limiting (see semiquantitative RT-PCR).

Degenerate PCR primers were designed using the sequence of the conserved region of the homeobox domain, amplifying the repertoire of homeobox genes expressed in the cells. The primer sequences are CA(A/G) (A/G)/(G) TGG TT(T/C) CAA(G), and (A/G) A(G/C) GCTTT/GTG GA(A/G) AA (A/G) G respectively. The expected PCR product was 115 basepairs and was amplified during a 45 cycle PCR with an annealing temperature of 51°C. Products were visualized on 2% agarose gels after staining with ethidium bromide (0.5 µg/ml).

PCR products were directly cloned (PGEM-T-vector, Promega), and the sequences were analysed using the Pharmacia Sequencing Kit. Classification of the sequenced clones was based on homology with known human and murine Homeobox sequences (as published in the EMBL database) using the Fasta computer analysis program (Caos/Camn, the Netherlands). Based on sequences in the EMBL database, PCR primers were designed to specifically amplify some of the identified homeobox genes (Table 1). PCR conditions were optimized for each primer set.

Southern hybridization. After separation by agarose gel-electrophoresis, plasmid DNA or PCR fragments were transferred onto Hybond-N membranes (Amersham) and hybridized with 32P-labeled ssDNA probes in 5xSSC, 0.5% SDS, containing 0.1% laurylsarcosine sodium salt and 1% blocking reagent (Boehringer Mannheim) at 65°C overnight. Membranes were then washed under increasingly stringent conditions (last 15 minutes: 0.1x SSC with 0.5% SDS at 65°C) prior to autoradiography. Probes used were the isolated homeodomains of HoxB9, HoxC6, and HoxC9. For hybridization of the blotted semiquantitative RT-PCR products the complete coding sequence of HoxC6 was used.

Semiquantitative RT-PCR. A semiquantitative PCR was performed in two ways. First, 10-fold serial dilutions of cDNA template (1×1000/100×1000) were amplified through 45 cycles to identify changes in the expression of a specific homeobox gene in cells treated under different conditions. When differences were found, they were confirmed by reducing the number of PCR cycles, avoiding the plateau level for amplification. The number of cycles was determined empirically to result in product that was just visible on gel by ethidium bromide staining. To normalize the amounts of cDNA used in each PCR, porphobilinogen deaminase (PBGD), a housekeeping gene (14), served as internal control (Table 1).

RESULTS

Amplification of homeobox gene subsets in U-2 OS and MC3T3-E1. A total of 13 homeobox genes were detected in osteoblast-like cells based on sequencing of cloned PCR products that had been amplified from cDNA using degenerate homeobox primers (Figure 1). Initially 10-20 clones derived from each U-2 OS and MC3T3-E1 PCR products were sequenced. In both cell lines HoxC9 was most frequently detected. Alternatively, other clones representing U-2 OS cDNA were identified by means of hybridization with probes Hox genes that had been identified earlier (HOXB9, MC3T3-E1).

### TABLE 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>5' - 3' sequence</th>
<th>Annealing temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HoxA1-F</td>
<td>GGG AAA GTT GGA GAG TAC GGC</td>
<td>60°C</td>
</tr>
<tr>
<td>HoxA7-F</td>
<td>GCC GGA CAA CAA ATC ACA GCG</td>
<td>60°C</td>
</tr>
<tr>
<td>HoxA9-F</td>
<td>CCA AAT ACC CCG CTG CCA ACT G</td>
<td>60°C</td>
</tr>
<tr>
<td>HoxB9-F</td>
<td>TGC GAG GCG GGC GTT AAC</td>
<td>57°C</td>
</tr>
<tr>
<td>HoxC6-F</td>
<td>TCT TTA CCA GGA GAA AGA CAT G</td>
<td>60°C</td>
</tr>
<tr>
<td>HoxC8-F</td>
<td>ACA AAC TTA CAG CGG GTA TC</td>
<td>55°C</td>
</tr>
<tr>
<td>HoxC9-F</td>
<td>TTG ACG ATG TCG AGG AGC</td>
<td>60°C</td>
</tr>
<tr>
<td>Gbx1-F</td>
<td>GCC CCT ACG GCC ACC ACG</td>
<td>57°C</td>
</tr>
<tr>
<td>PBGD-F</td>
<td>TGT GGT AAC GGC AAT GCG</td>
<td>60°C</td>
</tr>
</tbody>
</table>

Accession numbers are: M20215, M15927, X06024 (hoxa-1); U104231 (HOXAI); M17192 (hox-7); M28449, M36677 (hox-9); X13537 (guinea pig HoxA9); M43857, M27244 (hox-9); X16172 (HOXB9); X00264 (hox-9); S69027, M16938 (HOXC6); X07439, Y00215 (hoxc-8); M62968 (cat HoxB9); X55718 (Hox-9); H83603 (HOX5); M81662, M81663 (gbx1): L12129 (Gbx1).

Sequence based on the murine gene sequence only. For HoxA9 and HoxC8, the most conserved regions were selected to alignment to other species. Murine Hox-9 was aligned with guinea pig HoxA9 and murine Hox-8 was aligned with cat and rat HoxC8.

3 As only a partial homeobox sequence of murine gbx1 has been published (15), the primers were designed to fit to the human GBX1.

4 Based on the human sequence only (14).

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HOXC6, and HOXC9). MC3T3-E1 clones were selected by means of digestion with Ava II, which digests the homeobox domain region of murine Hox-9 but not that of most other murine Hox genes. Of 40 U-2 OS clones, 11 that did not hybridize with HOXB9, HOXC6, or HOXC9 were further analyzed by sequencing. This approach led to the identification of an additional 4 Hox genes (HoxA1 1/11, HoxA4 2/11, HoxA9 1/11, and HoxB3 1/11) (Figure 1). The remaining clones represented HoxC6 (4/11), and HoxA10 (2/11), which had been identified in the first group of sequenced clones. The sequences of HOXA9 and HOXC9 homeodomains have not been published previously and are shown in Figure 2. Plasmid DNA of 8 out of 21 MC3T3-E1 clones was not digested with Ava II. Sequence analysis of these clones revealed another 5 Hox sequences (HoxB-6, Hoxb-8, Hoxc-6, Hoxd-8, and Gbx1).

Comparison of Hox genes detected in osteoblast-like cells by sequencing of cloned PCR products that had been amplified from cDNA using degenerate homeobox primers. Boxes are aligned vertically in their paralog subgroups, and horizontally based on their chromosomal position.

FIG. 1. Hox genes detected in osteoblast-like cells by sequencing of cloned PCR products that had been amplified from cDNA using degenerate homeobox primers. Boxes are aligned vertically in their paralog subgroups, and horizontally based on their chromosomal position.

Comparison of Hox Genes Detected in U-2 OS, MC3T3-E1, and SK-N-SH Cell Lines Using Specific PCR Primers

<table>
<thead>
<tr>
<th>Hox gene</th>
<th>U-2 OS</th>
<th>MC3T3-E1</th>
<th>SK-N-SH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A7</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A9</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B9</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C8</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C9</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gbx1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The specific primer for Gbx1 only recognizes the human sequence, whereas the HoxA7 primer only recognizes the mouse sequence.

The separate Hox genes in three different cell lines (see Table 1). Integrity of the PCR product was confirmed by Southern blot hybridization (data not shown). The Hox genes that were identified in the three cell lines using specific Hox primers are depicted in Table 2. An additional Hox gene was detected in U-2 OS and MC3T3-E1 cDNA using specific PCR primers for HoxC8, which had not been found to be expressed in U-2 OS and MC3T3-E1 in the initial cloning experiments. Unexpectedly, we observed Gbx1 expression in U-2 OS but not in MC3T3-E1 cells, while Gbx1 was originally only detected in clones containing amplified Hox domains from MC3T3-E1 cells. This may be due to the fact that primers were designed to the human sequence, because only the homeobox domain sequence of murine gbx1 has been described (15). Another striking result was that the Hoxa-7 expression was detected in murine MC3T3-E1 osteoblasts but not in human U-2 OS osteosarcoma cells. This may be due to the fact that only the homeomain of HOXA7 has been described. Therefore, primers were designed to regions upstream and downstream of the homeomain of murine Hoxa-7, selecting the most conserved regions by alignment to other species (Table 1). In a control RT-PCR experiment HOXA7 could not be amplified from normal human osteoblasts (data not shown). The apparent lack of expression of HOXA7 is likely due to species differences. In the neuroblastoma cell line SK-N-SH we found expression of HOXA1, HOXB9, HOXC6, HOXC8, and HOXC9 but no HOXA7, HOXA9 or Gbx1, showing that Hox expression in cell lines is clearly cell-type dependent.

TGF-β superfamily members modulate expression of Hox genes. To study possible modulation of Hox gene expression by activin-A, rhBMP-2, and TGF-β1, a semi-quantitative PCR approach was used. The cDNA of growth factor treated and untreated cell lines was initially screened for differences in Hox gene expression...
The regulation of Hox gene expression is still incompletely understood. Several studies have shown that RA is an important inducer of Hox genes in vitro (4,29). Its mechanism in embryonal carcinoma cells involves a cascade model of sequential transcriptional activation of Hox genes in a 5'-3' polarity (30). Its effects in vivo have also been shown in studies which document the induction of HoxC6 by placement of an RA-soaked bead in the anterior limb bud (31). In addition, homeobox genes may auto- and/or cross-regulate each other, or even share the same promoters (32), making their regulatory pathways even more complex. Only three studies have reported on modulation of homeobox genes by members of the TGF-β superfamily: activin-A induced expression of MIX-2 in Xenopus (33); BMPs induced several homeobox genes indirectly during BMP-induced ectopic bone formation (10); and BMPs were shown to induce the expression of the homeobox-containing genes Msx-1 and Msx-2 in developing teeth (34). These studies mostly imply differentiation effects during embryogenesis. Our studies have now identified differential effects of three members of the TGF-β superfamily on the expression of HOXC6 in U-2 OS and known what causes the aberrant expression of homeobox genes in growth disturbances such as limb malformations and tumors. One mechanism could be autocrine and/or paracrine effects of growth factors. With bone being a large producer and storage site for TGF-β and BMPs, the potential effects on growth, skeletal patterning, limb development, and skeletal neoplasia through modulation of Hox expression are significant. Having shown previously (27) that osteosarcoma cell lines, including U-2 OS, produce active and latent TGF-β1 at levels used in our current studies, in addition to BMPs (28), we speculate that modulation of expression of specific Hox genes by TGF-β1 superfamily members may affect the differentiation and neoplastic behavior in osteosarcoma cells.

This study documents for the first time that at least 13 homeobox genes are expressed in osteoblast-like cells. The total number of homeobox genes expressed in both cell lines might be larger than the 13 that we detected, since our degenerate primers do not recognize all homeobox sequences, and a limited number of clones was screened. This was further demonstrated by the fact that although HoxC8 was not detected by cloning of the homeobox region as amplified with the degenerate primers, HoxC8 could be detected at rather low levels in all three cell lines if specific HoxC8 primers were used.

RT-PCR amplification of Hox gene transcripts with primers that recognize specific Hox genes showed that the same Hox genes were expressed in osteosarcoma cells as in the osteoblast cell line MC3T3-E1. The only difference observed (HoxA7) appeared to be due to a specific difference in sequence or expression of HoxA7, as also in osteoblasts isolated from human bone HoxA7 was not detected (results not shown).

FIG. 3. Modulation of Hox gene expression by TGF-β superfAMILY members in (A) U-2 OS cells, and (B) SK-N-SH cells. The top row of each figure shows duplicate experiments of cDNA template of U-2 OS (A) or SK-N-SH (B) amplified using HOXC6 primers. cDNA was obtained from cells treated with different growth factors, and a control. The bottom row represents 10-fold serial dilutions of cDNA template amplified with PBGD primers, as an internal experimental control for the amount of template used.

using increasing dilutions of template with saturating PCR conditions. Based on this we observed clear differences in expression of HoxC6. Minimal or no effects were seen on the expression of HoxA1, HoxA9, and HoxC8 in both cell lines (data not shown). By reducing the number of PCR cycles to unsaturating conditions we could more precisely define the modulating effect of each growth factor on the expression of a particular Hox gene. Differential effects of rhBMP-2, TGF-β1, and activin-A were found on the expression of HOXC6 in both U-2 OS and SK-N-SH. HOXC6 expression in U-2 OS cells was down-regulated by rhBMP-2 and activin-A (Figure 3A), whereas in SK-N-SH cells HOXC6 was unaffected by rhBMP-2 and up-regulated by TGF-β1 (Figure 3B). Effects were present after 24 hours as well as after 48 hours treatment.

**DISCUSSION**

Since a relationship between normal growth/differentiation and tumorigenesis has been well established, it is not surprising that abnormal homeobox expression has been linked to neoplasia (16). The oncogenic potential of Hox genes in mouse fibroblasts has been described (17, 18), and altered expression of several homeobox proteins has been found in tumors (19-25) and haematopoietic malignancies (26). It is still largely un-
SK-N-SH. The fact that we see regulation of Hox genes in cell lines by members of the TGF-β superfamily (after relatively short incubation times), suggests a much more direct role of Hox genes in the signal transduction of these growth factors. The finding that regulation of HoxC6 expression by members of the TGF-β family is clearly cell type-specific is very interesting with regard to the role that Hox-genes are supposed to have in cell and tissue differentiation and determination of cell-fate. Our observation that HoxC6 is regulated by rhBMP-2, activin-A, and TGF-β is perfectly in line with studies demonstrating that members of the TGF-β superfamily regulate neural cell adhesion molecule (N-CAM) expression (35-37) and with other studies showing that HoxC6 itself is a potent regulator of N-CAM expression. N-CAM and other cell adhesion and substrate adhesion molecules modulate cell surface events, cellular responses and cellular differentiation through adhesion, a mechanism implicated in metastasis.

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