Microarray Analysis of Defective Cartilage in Hoxc8- and Hoxd4-Transgenic Mice

Claudia Kruger and Claudia Kappen

Abstract

Objective: Homeobox genes of the Hox class are required for proper patterning of skeletal elements and play a role in cartilage differentiation. In transgenic mice with overexpression of Hoxc8 and Hoxd4 during cartilage development, the authors observed severe defects, namely, physical instability of cartilage, accumulation of immature chondrocytes, and decreased maturation to hypertrophy. To define the molecular basis underlying these defects, the authors performed gene expression profiling using the Affymetrix microarray platform. Results: Primary chondrocytes were isolated from Hoxc8- and Hoxd4-transgenic mouse embryo rib cartilage at 18.5 days of gestation. In both cases, differentially expressed genes were identified that have a role in cell proliferation and cell cycle regulation. A comparison between the controls for both experimental groups did not reveal significant differences, as expected. However, the repertoires of differentially expressed genes were found not to overlap between Hoxc8- and Hoxd4-transgenic cartilage. This included different Wnt genes, cell cycle, and apoptosis regulators. Conclusion: Overexpression of Hoxc8 and Hoxd4 transcription factors alters transcriptional profiles in chondrocytes at E18.5. The differences in repertoires of altered gene expression between the 2 transgenic conditions suggest that the molecular mechanisms underlying the cartilage defects may be different in both transgenic paradigms, despite apparently similar phenotypes.

Keywords

primary chondrocytes, transgenic mice, transcription factor, Hox gene, Hoxc8, Hoxd4, cell cycle, differentiation, proliferation, cartilage defect, microarray, differential gene expression

Introduction

Bone formation is the process by which mesenchymal cells condense at specific sites and differentiate into chondrocytes, forming the cartilage anlagen that are the model for future bone. These cells undergo an ordered differentiation program: The chondrocytes proliferate, become prehypertrophic, and then undergo hypertrophy. The mature cartilage undergoes calcification, known as ossification. Each step of cartilage maturation occurs in a precise and tightly regulated manner, and disruption of this process can cause abnormalities in cartilage and bone formation.1,2

Homeobox genes of the Hox class are required for proper patterning of elements in the developing skeleton.3-5 They also play a role in the regulation of cartilage differentiation prior to overt bone formation.6-8 Misexpression and overexpression studies suggested that Hox genes affect the size of cartilage condensations and chondrocyte proliferation.3,8-10 We recently demonstrated a role for Hoxc8 in cell cycle regulation in primary chondrocytes.11

Transgenic mice with overexpression of Hoxc8 and Hoxd4 under control of the Hoxc8 promoter exhibit profound cartilage defects, predominately in the ribs and vertebral column, and the severity of defects is dependent on transgene dosage.10 The abnormal cartilage is characterized by an accumulation of proliferating chondrocytes and reduced cartilage maturation. The cartilage of the ribs in transgenic mice remains weak and is structurally insufficient, resulting in pulmonary failure and death shortly after birth.8,10 Thus, Hox genes are important regulators of chondrocyte proliferation and maturation.

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Developmental Biology, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana, USA

Corresponding Author:
Claudia Kappen, Developmental Biology, Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Road, Baton Rouge, LA 70808, USA
E-mail: claudia.kappen@pbrc.edu
However, it is not well understood how Hox transcription factors control the process of chondrogenesis or which target genes they may regulate in chondrocytes. The aim of these studies was to identify genes with altered expression in the Hoxc8- and Hoxd4-transgenic paradigms as a first step toward determining the transcriptional targets of Hox transcription factors in cartilage differentiation and maturation.

Materials and Methods

Animals

Animals used in this work were created by the VP16-dependent binary system for expression of Hoxc8 and Hoxd4 transgenes. In brief, the binary transgenic system is based on the potent transcriptional activator VP16 of herpes simplex virus (HSV). One line, the transactivator (TA), harbors the transgene encoding VP16 under the control of the promoter from the Hoxc8 gene, which is active in cartilage precursor cells (C. Kappen, unpublished data). The other line, the transresponder (TR), harbors a Hox transgene under the control of the HSV ICP4 gene immediate early promoter. Activation of the immediate early promoter requires the presence of VP16 protein; hence, transgene expression is achieved only in individuals carrying both the TA and TR transgenes. Here, we classify progeny by 2 genotypes: the control genotype (TA), containing at least one TA and no TR transgene, and the experimental genotype (TA+TR), containing at least one TA and one TR transgene. All transgenes were maintained on an FVB inbred genetic background. The characterization of the phenotypes of both transgenic lines and the levels of expression for Hox transgenes in transgenic chondrocytes have been published.

Pregnant dams were sacrificed at 18.5 days of gestation. Double transgenic embryos are phenotypically identifiable by failure of eyelid closure, and for confirmation, genotyping was performed on DNA isolated from tails of individual specimen. Transgene expression in all samples was demonstrated by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) as described earlier.

RNA and cDNA Preparation

Embryos were collected at day 18.5 of gestation, and individual rib cages were dissected. Rib cages from the same litter were pooled according to genotype, and rib chondrocytes were prepared as described. Freshly isolated cells were immediately transferred into Trizol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated and purified as described previously. Quality of RNA was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA), and quantity was determined in the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE). Complementary DNA was obtained by reverse transcription (SuperScript III First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA) of 3 μg of RNA from each sample. This reaction used both Oligo(dT) and random hexamers as primers; all further steps were done following the supplier’s instructions (Invitrogen). Purification of cDNA was performed using QIAquick PCR purification columns (Qiagen, Valencia, CA).

Microarray Analysis

A total of 16 samples (4 controls and 4 Hoxc8-transgenic samples, respectively) were hybridized to individual Affymetrix GeneChip Mouse Genome 430 2.0 arrays. Probe labeling, hybridization, washing, and scanning were performed according to Affymetrix’s protocol using a GenePix4000 scanner. Data sets were analyzed using GCOS software for background normalization, and each probe set (entity) was assigned an expression call (P = present, M = marginal, A = absent). CHP files were loaded into GeneSpring GX10 software (Agilent Technologies) using default parameters. Entities that were assigned “present” or “marginal” for 5 of 8 samples underwent an unpaired t-test with false discovery rate set at 0.05 (the Gene Spring default setting was used for multiple testing correction). Entities satisfying the P-value cutoff of $P \leq 0.05$ and fold-change $\geq 1.5$ were saved in separate lists for further analysis. In parallel, the microarray results were analyzed using CyberT (http://cybert.microarray.ics.uci.edu/), which gave essentially identical results. Hierarchical clustering was performed in GeneSpring GX10, using the K-means method with Euclidean distance metric; 3 clusters were revealed after 50 iterations.

Annotations for probe set ID numbers were taken as provided by Affymetrix and were further hand curated by comparison to the Mouse Genome Informatics database (http://www.informatics.jax.org)

Quantitative Real-Time PCR Assays

The MultiPROBE II PLUS HT EX robot (Perkin Elmer, Shelton, CT) was programmed to pipette 10-µL reactions into an Applied Biosystems (Foster City, CA) 384-well plate. The robot adds 3 µL template (1.6 ng cDNA) and 7 µL Master Mix (5 µL iTaq SYBR Green Supermix with ROX, 0.1 µL forward primer 10 µM, 0.1 µL reverse primer 10 µM, 1.8 µL NanoPure water) per reaction. The iTaq SYBR Green Supermix with ROX (2X) was obtained from Bio-Rad Laboratories (Hercules, CA). The cDNA template and the Master Mix were provided in a 96-well optical plate. Gene expression levels were measured using the
ABI PRISM 7900HT Sequence Detection System with SDS 2.2.2 software version (Applied Biosystems). Individual samples were run in triplicate. The thermal cycler conditions consisted of 1 cycle of 2 min at 50 °C, one cycle of 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 1 min at 60 °C.

Primers for amplification were designed using Primer Express 3 software (Applied Biosystems) with default settings. Primers for the gene Gapdh were used as provided by Applied Biosystems. The sequences of primer pairs used in this work are listed in Supplemental Table S1. To exclude amplification of potentially contaminating genomic DNA, primers were designed to span an exon/exon junction where possible. Each primer pair was validated by melting point analysis under conditions of a programmed temperature ramp of 15 s at 95 °C, 15 s at 60 °C, and 15 s at 95 °C, monitoring the hybridization activity of nucleic acids present in the sample, and by PCR on cDNA derived from pooled RNA of E10.5 mouse embryos.

Amplification efficiencies were determined for each gene-specific reaction over the first 3 cycles above the threshold of detection by using the formula $\Delta R_{\text{cycle}(n)} / \Delta R_{\text{cycle}(n-1)}$, averaged over the triplicates for each sample. Relative quantification was done using the Comparative $C_T$ method with actual amplification efficiency to produce the relative fold-change in expression level between transgenic and control.14,16 For quantitative RT-PCR, at least 6 samples each were used for the Hoxc8- and Hoxd4-transgensics and their control littermates, respectively ($n = 6$ for each condition).

**Statistical Analysis**

Statistical analysis was performed by $t$-tests and analysis of variance to evaluate significance of differences in gene expression between controls and Hoxc8-transgenic or controls and Hoxd4-transgenic samples, respectively. $P$ values of < 0.05 were considered statistically significant.

**Results**

**Gene Expression in Hoxc8-Transgenic Cartilage**

To discover genes that potentially exhibit deregulated expression in cartilage of mice overexpressing Hoxc8, we conducted genomewide expression profiling in primary chondrocytes using the Affymetrix Mouse 430 2.0 platform. Of 45,101 entities arrayed on the chips, 58 entities passed the fold-change ≥ 1.5 and $P$ value < 0.05 criteria when samples from transgenic mice were compared with controls (Table 1). Two probe sets lack annotation for gene or locus. The expression level of 27 entities (26 genes) was significantly elevated in Hoxc8-transgenic samples compared with the control group, whereas 31 entities (containing 2 probe sets for Zbtb3; 29 genes) exhibited decreased expression in Hoxc8-transgenic samples. Differences in expression levels ranged between 1.5- and 2.5-fold. An independent statistical analysis of our Hoxc8 data sets was performed using CyberT on raw hybridization intensity values; this identified the same group of genes found by GeneSpring.

For visualization of gene expression profiles, we used the K-means clustering algorithm. The analysis grouped the entities listed in Table 1 into 3 clusters, pictured in Figure 1 (Fig. 1A). The first 2 clusters contain genes with expression levels higher (red) or lower (blue) than the mean (white) over all 8 samples. Fourteen entities fall in the 3rd cluster, which represents moderate changes in expression level.

Of 55 genes, 18 genes with a moderate to strong hybridization signal were chosen for validation by quantitative RT-PCR (Table 2). A $P$ value lower than 0.05 was found only for Gpaa1, but decreased expression in transgenic samples by more than 1.5-fold, as found by the microarray study, could not be confirmed.

**Gene Expression in Hoxd4-Transgenic Cartilage**

To identify genes whose expression may be deregulated by overexpression of Hoxd4 in cartilage, we conducted a separate genomewide expression profiling experiment using the same platform as before. Eighty-five entities displayed expression levels that were significantly changed by more than 1.5-fold in Hoxd4-transgenic chondrocytes relative to controls (Table 3). These findings were confirmed when using CyberT as a statistical analysis tool. Two probe sets lack annotation. The majority of entities (50 genes) we identified were expressed at lower levels in Hoxd4-transgenic chondrocytes; 35 entities (28 genes) exhibited significantly elevated expression in Hoxd4-transgenic chondrocytes compared with control samples. Among this group, 2 genes (Ddx3y and Eif2s3y) were represented by 2 probe sets and Uty by 4 probe sets. Among the 78 differentially expressed transcripts, we identified 3 members of the solute carrier family (Slc25a32, Slc34a2, Slc46a1), 2 zinc finger proteins (Zfp69, Zfp316), 2 protein tyrosine phosphatases (Ptprb, Ptprd), and 2 cadherins (Chd5, Cdh10).

Cluster analysis (Fig. 1B) revealed 43 transcripts with moderate expression levels: 33 transcripts with high (red) and 9 transcripts with lower expression levels (blue) in Hoxd4-transgenic chondrocytes and the control group.

Gene expression levels were validated by quantitative RT-PCR for a total of 18 genes, as shown in Table 2. Uty (ubiquitously transcribed tetratricopeptide repeat gene, Y-chromosome) was the only gene for which the differential expression detected by microarray experiment was confirmed by RT-PCR when using the criteria of $P < 0.05$ and
Table 1. Differentially Expressed Genes in Hoxc8-Transgenic Chondrocytes

| Probe set ID | Gene symbol | Gene title | Fold-change | Transgenic/control | P value |
|--------------|-------------|------------|-------------|-------------------|--------|
| 1430756_at   | 5430427G11Rik | RIKEN cDNA 5430427G11 gene | 1.97 | Up | 0.0000931 |
| 1433777_at   | 583044SD09Rik | RIKEN cDNA 583044SD09 gene | 1.73 | Up | 0.0439948 |
| 1437076_at   | A930017M01Rik | RIKEN cDNA A930017M01 gene | 1.60 | Up | 0.0040773 |
| 1446095_at   | Airn        | Antisense Igf2r RNA | 1.51 | Up | 0.0407707 |
| 1417470_at   | Apobec3     | Apolipoprotein B editing complex 3 | 1.66 | Up | 0.0385239 |
| 1420120_at   | AU020177    | Expressed sequence AU020177 | 1.79 | Up | 0.0268048 |
| 1435909_at   | C030034I22Rik | RIKEN cDNA C030034I22 gene | 1.55 | Up | 0.0157238 |
| 1457749_at   | Cc2d1b      | Coiled-coil and C2 domain containing 1B | 1.67 | Up | 0.0259771 |
| 1417936_at   | Ccl9        | Chemokine (C-C motif) ligand 9 | 1.61 | Up | 0.0071056 |
| 1429976_at   | Clasp2      | CLIP associating protein 2 | 1.58 | Up | 0.0231768 |
| 1435754_at   | Fam35a      | Family with sequence similarity 35, member A | 1.53 | Up | 0.0354986 |
| 1443628_at   | Fam82b      | Family with sequence similarity 82, member B | 1.79 | Up | 0.0257141 |
| 1457228_x_at | Glei        | GLE1 RNA export mediator (yeast) | 1.52 | Up | 0.0200380 |
| 1438555_at   | Mxra7       | Matrix-remodeling associated 7 | 1.80 | Up | 0.0276739 |
| 1439999_at   | NA          | NA | 1.75 | Up | 0.0055803 |
| 1457117_at   | Nfe2l2      | Nuclear factor, erythroid derived 2, like 2 | 1.62 | Up | 0.0351502 |
| 1432539_a_at | Nup54       | Nucleoporin 54 | 1.51 | Up | 0.0157982 |
| 1455145_at   | Pcdh19      | Protocadherin 19 | 1.53 | Up | 0.0408313 |
| 1456955_at   | Ppde1       | PPPDE peptidase domain containing 1 | 1.67 | Up | 0.0098931 |
| 1436569_at   | Prex2       | Pphosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2 | 1.55 | Up | 0.0192962 |
| 1451560_at   | Prr12       | Prolinrich 12 | 1.53 | Up | 0.0217165 |
| 1443043_a_at | Repin1      | Replication initiator 1 | 1.69 | Up | 0.0474017 |
| 1442044_at   | Rps6        | Ribosomal protein S6 | 1.56 | Up | 0.0169256 |
| 1428216_s_at | Tomm7       | Translocase of outer mitochondrial membrane 7 homolog (yeast) | 1.52 | Up | 0.0391876 |
| 1459672_at   | Top1        | Topoisomerase (DNA) 1 | 1.68 | Up | 0.0010994 |
| 1455722_at   | Wafs3       | WAS protein family | 1.51 | Up | 0.0030891 |
| 1429474_at   | Zadh1       | Zinc binding alcohol dehydrogenase | 1.64 | Up | 0.0445114 |
| 1443902_at   | 6430573F11Rik | RIKEN cDNA 6430573F11 gene | 1.72 | Down | 0.0341196 |
| 1437940_at   | Apba1       | Amyloid beta (A4) precursor protein binding | 1.55 | Down | 0.0198718 |
| 1449356_at   | Asb5        | Ankyrin repeat and SOCs box-containing protein 5 | 1.56 | Down | 0.0289805 |
| 1442207_at   | Atg16l2     | Autophagy related 16 like 2 (S. cerevisiae) | 1.55 | Down | 0.0119739 |
| 1443337_at   | B130020M22Rik | 0 day neonate lung cDNA | 1.56 | Down | 0.0227655 |
| 1452966_at   | Bcl11b      | B-cell leukemia/lymphoma 11B | 1.77 | Down | 0.0115030 |
| 1418777_at   | Ccl25       | Chemokine (C-C motif) ligand 25 | 1.58 | Down | 0.0356987 |
| 1443746_x_at | Dmp1        | Dentin matrix protein 1 | 1.92 | Down | 0.0021768 |
| 1446431_at   | Dnml4       | Dnml | 1.70 | Down | 0.0378060 |
| 1434714_at   | Erg1b       | ERO1-like beta (S. cerevisiae) | 1.53 | Down | 0.0312197 |
| 1440359_at   | Fam110b     | Family with sequence similarity 110, member B | 1.54 | Down | 0.0340775 |
| 1453689_at   | Fance       | Fanconi anemia | 1.84 | Down | 0.0054524 |
| 1450319_at   | Gabrb2      | Gamma-amino butyric acid (GABA-A) receptor | 1.51 | Down | 0.0367941 |
| 1438512_at   | Gapgr2      | GPI anchor attachment protein 1 | 2.00 | Down | 0.0375435 |
| 1419196_at   | Hamp        | Hecpicid antimicrobial peptide | 2.16 | Down | 0.0399568 |
| 1444709_at   | Invs        | Inversin | 1.93 | Down | 0.0246062 |
| 1446131_at   | Jam3        | Junction adhesion molecule 3 | 1.58 | Down | 0.0347649 |
| 1425104_at   | Kctd1       | Potassium channel tetramerisation domain containing 1 | 2.12 | Down | 0.0020032 |
| 1454845_x_at | Mchr1       | Melanin-concentrating hormone receptor 1 | 1.66 | Down | 0.0210230 |
| 1443267_at   | NA          | NA | 1.57 | Down | 0.0435891 |

(continued)
fold-change $\geq 1.5$. The genes encoding Uty, as well as Eif2s3y (eukaryotic translation initiation factor 2) and Ddx3y (DEAD box polypeptide 3), which are also represented in the gene list of interest (Table 3), are known to be Y-linked. Given the unequal representation of Y-linked gene expression between controls and transgenic samples, we hypothesized that male embryos were overrepresented in the Hoxd4-transgenic samples, which was confirmed by PCR on genomic DNA. Such differential expression of Y-linked genes is thus likely an indicator of sex status of the samples and unrelated to overexpression of any Hox transgene.

**Differential Expression between Hoxc8- and Hoxd4-Transgenic Mice**

The VP16-dependent binary system allowed us to transactivate the Hoxc8 and Hoxd4 transgenes in exactly the same fashion with regard to temporal and tissue specificity, because both transgenes are expressed under control of the same chondrocyte-specific enhancer.\(^\text{10}\) Thus, we would expect that a comparison between the Hoxc8- and Hoxd4-transgenic cartilage should enable us to determine whether both models of defective cartilage exhibit the same underlying molecular alterations. We therefore compared the data sets from both microarray experiments to screen for differential gene expression between Hoxc8- and Hoxd4-transgenic chondrocytes and their control groups, respectively.

The comparison between the 2 control groups (Table 4) revealed only minor differences in gene expression levels, as would be expected given that the genetic background of all samples is the inbred FVB strain. Out of 49 entities with a fold-change $\geq 2$, most (39 genes) exhibited higher expression in the controls compared to the Hoxc8-transgenic group, whereas 7 genes exhibited higher expression levels in the controls to the Hoxd4-transgenic animals. The transcript

### Table 1. (continued)

| Probe set ID | Gene symbol | Gene title                          | Fold-change | Transgenic/control | P value |
|--------------|-------------|-------------------------------------|-------------|--------------------|---------|
| 1438614_x_at| Osbp9       | Oxysterol binding protein-like 9    | 2.04        | Down               | 0.0058520 |
| 1426753_at  | Phf17       | PHD finger protein 17               | 1.66        | Down               | 0.0481360 |
| 1439508_at  | Rab11b      | RAB11B                              | 1.71        | Down               | 0.0289390 |
| 1459315_at  | Rab5c       | RAB5C, member RAS oncogene family   | 1.92        | Down               | 0.0172757 |
| 1452862_at  | Rreb1       | ras responsive element binding protein 1 | 1.53     | Down               | 0.0402444 |
| 1428794_at  | SpecI       | Sperm antigen with calponin homology and coiled-coil domains I | 1.69 | Down | 0.0072438 |
| 1446680_at  | Stag1       | Stromal antigen I                   | 2.48        | Down               | 0.0070552 |
| 1416927_at  | Trp53inpI   | Transformation related protein 53 inducible nuclear protein I | 1.60 | Down | 0.0446587 |
| 1447894_x_at| Vps52       | Vacuolar protein sorting 52 (yeast) | 1.56        | Down               | 0.0230638 |
| 1427106_at  | Zbtb3       | Zinc finger and BTB domain containing 3 | 1.61 | Down | 0.0168218 |
| 1440180_x_at| Zbtb3       | Zinc finger and BTB domain containing 3 | 1.58 | Down | 0.0101039 |

Note: Affymetrix probe set ID numbers are given for representative probe sets; Zbfb3 is represented by 2 probe sets, and 1 probe set has no annotation. The comparison of transgenic/control indicates elevation or reduction of expression in Hoxc8-transgenic chondrocytes relative to controls. Fifty-eight entities (57 genes) are differentially expressed (unpaired t-test; fold-change $\geq 1.5$; P value < 0.05) in Hoxc8-transgenic chondrocytes compared with controls.

Figure 1. Differential gene expression in Hoxc8- and Hoxd4-transgenic cartilage. K-means clustering algorithm and Euclidean distance metric (as implemented in GeneSpring) were used to visualize the different expression profiles for (A) Hoxc8 and (B) Hoxd4 transgenic chondrocytes relative to their controls. Columns 1 to 4 represent the control groups (transactivator-containing samples), and columns 5 to 8 display the transgenic groups (transresponder-containing samples). Fifty-eight entities for Hoxc8 and 85 entities for Hoxd4 passed the unpaired t-test (‘present’ or ‘marginal’ flag in 5 of 8 samples, fold-change $\geq 1.5$ and P value < 0.05).
Table 2. Validation of Gene Expression by Quantitative Reverse Transcriptase Polymerase Chain Reaction CR in Hoxc8– and Hoxd4– Transgenic Chondrocytes

| Probe set ID | Gene symbol | ΔCt ± SD control | ΔCt ± SD Hoxc8–transgenic | Fold–change transgenic/control | P value |
|--------------|-------------|-----------------|---------------------------|-------------------------------|---------|
| 1430756_at  | 5430427G1Rik| 11.56 ± 0.40    | 11.51 ± 0.45              | 1.03                          | 0.85403 |
| 1433777_at  | 5830445D09Rik| 13.35 ± 0.34    | 13.64 ± 0.84              | -1.18                         | 0.45344 |
| 1417407_at  | Apobec3     | 7.58 ± 0.34     | 7.82 ± 0.22               | -1.16                         | 0.17429 |
| 1418777_at  | Ccl25       | 9.74 ± 0.49     | 9.78 ± 0.36               | -1.03                         | 0.86563 |
| 1446431_at  | Dnmt3       | 7.25 ± 0.28     | 7.36 ± 0.42               | -1.05                         | 0.71857 |
| 1437654_at  | Fam35a      | 10.26 ± 0.64    | 10.36 ± 0.56              | -1.07                         | 0.76193 |
| 1453689_at  | Fance       | 7.27 ± 0.27     | 7.27 ± 0.33               | 1.00                          | 0.98538 |
| 1457228_x_at| Gle1        | 4.94 ± 0.24     | 5.06 ± 0.32               | -1.08                         | 0.48505 |
| 1438152_at  | Gpaa1       | 4.70 ± 0.32     | 4.91 ± 0.47               | -1.15                         | 0.04020 |
| 1450104_at  | Kctd1       | 13.07 ± 0.42    | 12.81 ± 1.00              | 1.16                          | 0.57281 |
| 1454845_x_at| Mcr1        | 13.54 ± 0.95    | 12.91 ± 0.64              | 1.41                          | 0.24740 |
| 1436569_at  | Prex2       | 9.35 ± 0.39     | 9.17 ± 0.61               | 1.12                          | 0.54688 |
| 1451560_at  | Prr12       | 5.23 ± 0.60     | 5.37 ± 0.57               | -1.09                         | 0.68803 |
| 1459315_at  | Rab5c       | 12.55 ± 0.31    | 12.81 ± 0.62              | -1.18                         | 0.37860 |
| 1434043_a_at| Rep1        | 7.94 ± 0.25     | 8.05 ± 0.16               | -1.08                         | 0.39754 |
| 1428794_at  | Spec1       | 9.05 ± 0.44     | 9.28 ± 0.36               | -1.16                         | 0.34507 |
| 1446680_at  | Stag1       | 4.94 ± 0.38     | 5.05 ± 0.27               | -1.07                         | 0.58376 |
| 1427106_at  | Zbtb3       | 7.58 ± 0.34     | 7.82 ± 0.22               | -1.16                         | 0.17429 |

|            | Control | Hoxd4–transgenic | Transgenic/control |
|------------|---------|------------------|--------------------|
| 1453338_s_at | Amn1    | 8.89 ± 0.43      | 8.94 ± 0.54        | -1.03                          | 0.88327 |
| 1421392_a_at| Birc3    | 7.75 ± 0.39      | 7.54 ± 0.30        | 1.15                          | 0.31514 |
| 1439327_at  | Ccbe1    | 9.98 ± 1.02      | 10.15 ± 0.91       | -1.12                         | 0.76701 |
| 1433956_at  | Cdh5     | 6.73 ± 0.90      | 6.59 ± 0.75        | 1.09                          | 0.78587 |
| 1452077_at  | Ddx3y    | 5.89 ± 0.75      | 5.12 ± 0.78        | -1.35                         | 0.76750 |
| 1427462_at  | E2f3     | 6.72 ± 0.42      | 6.51 ± 0.13        | 1.15                          | 0.26012 |
| 1417210_at  | Elf2s3y  | 6.56 ± 0.77      | 5.85 ± 0.77        | 1.58                          | 0.14367 |
| 1416916_at  | Elf3     | 11.13 ± 0.25     | 11.26 ± 0.29       | -1.09                         | 0.42656 |
| 1445191_at  | Exd1     | 11.37 ± 0.48     | 11.68 ± 0.64       | -1.24                         | 0.36159 |
| 1437106_at  | Kdm5a    | 5.04 ± 0.24      | 5.09 ± 0.16        | -1.04                         | 0.64603 |
| 1456618_at  | Mark4    | 7.03 ± 0.50      | 6.76 ± 0.27        | 1.19                          | 0.27688 |
| 1429715_at  | Ppp2r2a  | 6.98 ± 0.45      | 6.79 ± 0.23        | 1.12                          | 0.38864 |
| 1460419_a_at| Prkcb    | 12.36 ± 0.44     | 12.85 ± 0.66       | -1.38                         | 0.15940 |
| 1451995_at  | Taf1l    | 6.78 ± 0.50      | 6.87 ± 0.74        | -1.06                         | 0.81399 |
| 1445668_at  | Tbec     | 6.55 ± 0.20      | 6.35 ± 0.26        | 1.14                          | 0.17103 |
| 1450038_s_at| Usp9x    | 3.73 ± 0.25      | 3.71 ± 0.22        | 1.01                          | 0.88108 |
| 1426598_at  | Uty      | 8.73 ± 0.79      | 7.47 ± 0.63        | 2.27                          | 0.01184 |
| 1450151_at  | Zfp316   | 9.15 ± 0.36      | 9.40 ± 0.86        | -1.18                         | 0.52654 |

Note: Six transgenic chondrocyte samples were compared with 6 control samples, and reactions were done in triplicates. ΔCt values were determined relative to the Ct value for Gapdh in the same sample. For each gene, the fold–change was calculated using the formula fold–change = AE/ΔCt (AE = amplification efficiency; see Supplemental Table S1), where AE was calculated using the formula AE = ΔRn cycle(n)/ΔRn cycle(n–1) over 3 cycles in the linear range of the reaction.

with the highest expression difference, 1446680_at, is lacking any annotation, as do 2 other transcripts in this list. A graphic representation of the respective cluster analysis is shown in Figure 2A.

When we compared the group of Hoxc8-transgenic samples to the group of Hoxd4-transgenic samples, this yielded 72 entities with differential expression greater than 2-fold (Table 5). Three probe sets lack annotation, and several genes (Mt1: metallothionein 1, Akap9: kinase anchor protein 9, and Ddit3: DNA-damage inducible transcript 3) are represented with 2 probe sets. Only 7 of the transcripts on this list exhibited decreased expression levels in Hoxc8-transgenic samples, whereas the majority (59 genes) displayed elevated expression in Hoxc8-transgenic animals. Most notably, Xist (inactive X-specific transcript) levels were higher in the group of Hoxc8-transgenic samples, likely reflecting a higher ratio of female-derived samples as compared with the Hoxd4-transgenic condition, consistent with elevated expression of Y-linked genes in the Hoxd4-transgenic samples. Figure 2B
## Table 3. Differentially Expressed Genes in Hoxd4-Transgenic Chondrocytes

| Probe set ID     | Gene symbol | Gene title                                                                 | Fold-change | Transgenic/control | P value  |
|------------------|-------------|------------------------------------------------------------------------------|-------------|--------------------|----------|
| 1443346_at       | 2700007P21Rik | RIKEN cDNA 2700007P21 gene                                                   | 1.62        | Up                 | 0.0043344 |
| 1429510_at       | 2810410L24Rik | RIKEN cDNA 2810410L24 gene                                                    | 1.77        | Up                 | 0.0466781 |
| 1459145_at       | A930033H14Rik | RIKEN cDNA A930033H14 gene                                                    | 1.51        | Up                 | 0.0015565 |
| 149641_at        | Adk         | Adenosine kinase                                                            | 1.63        | Up                 | 0.0294934 |
| 1434296_at       | BC049349     | cDNA sequence BC049349                                                      | 1.54        | Up                 | 0.0099971 |
| 1452503_a_at     | Brwd1       | Bromodomain and WD repeat domain containing 1                               | 2.00        | Up                 | 0.0234259 |
| 1447803_x_at     | Capg        | Capping protein (actin filament)                                             | 1.64        | Up                 | 0.0271682 |
| 1430605_at       | Ccc101      | Coiled-coil domain containing 10                                             | 1.51        | Up                 | 0.0483277 |
| 1435574_at       | Chordc1     | Cysteine and histidine-rich domain (CHORD)-containing                        | 1.92        | Up                 | 0.0068320 |
| 142167_a_at      | Cited2      | Cbp/p300-interacting transactivator                                          | 1.55        | Up                 | 0.0249589 |
| 1426438_at       | Ddx3y       | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3                                    | 1.82        | Up                 | 0.0410493 |
| 1452077_at       | Ddx3y       | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3                                    | 1.81        | Up                 | 0.0428568 |
| 1434789_at       | Depdc1b     | DEP domain containing 1B                                                    | 1.51        | Up                 | 0.0075120 |
| 1417210_at       | Eif2s3y     | Eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked | 1.95        | Up                 | 0.0113469 |
| 1457945_at       | Eif2s3y     | Eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked | 1.80        | Up                 | 0.0285858 |
| 1437791_s_at     | Eml5        | Echinoderm microtubule associated protein like 5                            | 1.62        | Up                 | 0.0244599 |
| 1441543_at       | Eya3        | Eyes absent 3 homolog (Drosophila)                                          | 2.05        | Up                 | 0.0207881 |
| 1460021_at       | Gm6658      | Predicted gene 6658                                                        | 1.50        | Up                 | 0.0043136 |
| 1449954_at       | Hyal1       | Hyaluronoglucosaminidase 1                                                  | 1.67        | Up                 | 0.0171650 |
| 1456618_at       | Mark4       | MAP/microtubule affinity-regulating kinase 4                                | 2.20        | Up                 | 0.0250543 |
| 1440924_at       | Mphosph1    | M-phase phosphoprotein 1                                                    | 1.51        | Up                 | 0.0244418 |
| 1442153_at       | NA          | NA                                                                          | 1.87        | Up                 | 0.0377096 |
| 1438907_at       | NA          | NA                                                                          | 1.75        | Up                 | 0.0080244 |
| 1453139_at       | Nudt12      | Nudix (nucleoside diphosphate linked moiety X)-type motif 12                 | 1.61        | Up                 | 0.0044737 |
| 1424605_at       | Pcsk5       | Proprotein convertase subtilisin/kexin type 5                               | 1.59        | Up                 | 0.0161911 |
| 1429715_at       | Ppp2r2a     | Protein phosphatase 2 (formerly 2A)                                          | 1.74        | Up                 | 0.0266788 |
| 1439960_at       | Rpsd2       | RNA pseudouridylate synthase domain containing 2                            | 1.75        | Up                 | 0.0102783 |
| 1445668_at       | Tbce        | Tubulin-specific chaperone E                                                | 1.82        | Up                 | 0.0259176 |
| 1450038_s_at     | Usp9x       | Ubiquitin specific peptidase 9                                              | 1.65        | Up                 | 0.0004397 |
| 1459565_at       | Uty         | Ubiquitously transcribed tetratricopeptide repeat gene                      | 2.18        | Up                 | 0.0019270 |
| 1457582_at       | Uty         | Ubiquitously transcribed tetratricopeptide repeat gene                      | 1.95        | Up                 | 0.0087905 |
| 1426598_at       | Uty         | Ubiquitously transcribed tetratricopeptide repeat gene                      | 1.91        | Up                 | 0.0122254 |
| 1422247_a_at     | Uty         | Ubiquitously transcribed tetratricopeptide repeat gene                      | 1.53        | Up                 | 0.0251188 |
| 1458274_at       | Zfp69       | Zinc finger protein 69                                                      | 1.71        | Up                 | 0.0088002 |
| 1443105_at       | Zfp398      | Zinc finger protein 398                                                     | 1.69        | Up                 | 0.0085782 |
| 1422107_at       | 2410066E13Rik | RIKEN cDNA 2410066E13 gene                                                   | 1.63        | Down               | 0.0343780 |
| 1442237_at       | 3010026O09Rik | RIKEN cDNA 3010026O09 gene                                                   | 1.61        | Down               | 0.0298389 |
| 1430940_at       | 3110045A19Rik | RIKEN cDNA 3110045A19 gene                                                   | 1.58        | Down               | 0.0413230 |
| 1431566_at       | 9030622O22Rik | RIKEN cDNA 9030622O22 gene                                                   | 1.64        | Down               | 0.0111243 |
| 1432798_at       | 9530023I19Rik | RIKEN cDNA 9530023I19 gene                                                   | 1.96        | Down               | 0.0341054 |
| 1453538_s_at     | Amn1        | Antagonist of mitotic exit network 1 homolog (S. cerevisiae)                 | 1.55        | Down               | 0.0251336 |
| 1443551_at       | Atp2a2      | ATPase                                                                       | 1.88        | Down               | 0.0125872 |
| 1437310_at       | Bbs1        | Bardet-Biedl syndrome 1 homolog (human)                                     | 1.50        | Down               | 0.0079099 |
| 1421392_a_at     | Birc3       | Baculoviral IAP repeat-containing 3                                          | 1.68        | Down               | 0.0047363 |
| 1439327_at       | Ccb1        | Collagen and calcium binding EGF domains 1                                   | 1.70        | Down               | 0.0161750 |
| 1425092_at       | Cdh10       | Cadherin 10                                                                  | 1.55        | Down               | 0.0017864 |
| 1433956_at       | Cdh5        | Cadherin 5                                                                   | 1.63        | Down               | 0.0036152 |
| 1428574_a_at     | Chn2        | Chimerin (chimaerin) 2                                                       | 1.68        | Down               | 0.0445669 |
| 1430173_x_at     | Cyp4f16     | Cytochrome P450                                                              | 1.64        | Down               | 0.0099976 |
| 1459280_at       | D1Ertd185e  | DNA segment                                                                  | 1.71        | Down               | 0.0239316 |
| 1436480_at       | Dpp7        | Dipeptidylpeptidase 7                                                        | 1.61        | Down               | 0.0365102 |

(continued)
is a graphic representation of the corresponding cluster analysis.

It is of interest to note here that 12 differentially expressed entities were identified both in the comparison of samples between the transgenic conditions and in the comparison between the control groups. These entities are all decreased in expression levels in samples from the Hoxd4-transgenic animals and their littermate controls, regardless of whether the Hoxd4 transgene is expressed (as in mice doubly transgenic for TA and TR transgenes, the Hoxd4-transgenics) or not (as in the controls). This finding would suggest that progeny in such litters may be different from those in the Hoxc8-transgene–related crosses. Indeed, in contrast to the Hoxc8-transgene, the Hoxd4-transgene is inherited only through the female germline (C. Kappen et al., unpublished data). The deregulation of these 12 entities in all progeny (controls and transgenics) from Hoxd4-transgenic dams could thus be associated with a transgene-locus–specific maternal effect but is likely independent of transgene expression in the progeny cartilage.

Discussion

This article reports genomewide expression profiling in primary chondrocytes of Hoxc8- and Hoxd4-transgenic mice. Our aim was to use differential expression as a means to identify genes whose transcription may be under control of Hox transcription factors. Among such targets of the Hox transcription factors in cartilage could be new genes that might play important roles in cartilage development.
Table 4. Genes Differentially Expressed between Control Groups to the Hoxc8- and Hoxd4-Transgenic Chondrocytes

| Probe set ID   | Gene symbol | Gene title                                                                 | Fold-change | C_01/C_04 | P value |
|---------------|-------------|-----------------------------------------------------------------------------|-------------|-----------|---------|
| 1446904_at    | 4933430H15Rik | RIKEN CDNA 4933430H15 GENE                                                  | 2.20        | Up        | 0.0267147 |
| 1441372_at    | 5930405F01Rik | RIKEN cDNA 5930405F01 gene                                                  | 2.03        | Up        | 0.0106855 |
| 1459878_a_at  | A430107O13Rik | RIKEN cDNA A430107O13 gene                                                  | 2.05        | Up        | 0.0146135 |
| 1449785_at    | AA414993     | Expressed sequence AA414993                                               | 2.23        | Up        | 0.0066515 |
| 1444518_at    | Acox1       | Acyl-Coenzyme A oxidase 1                                                   | 2.15        | Up        | 0.0427789 |
| 1457548_at    | Adamts6     | A disintegrin-like and metalloproteinase with thrombospondin motif 6       | 2.05        | Up        | 0.0048153 |
| 1442331_at    | Alas1       | Aminolevulinic acid synthase 1                                             | 2.39        | Up        | 0.0309663 |
| 1442750_at    | B3galnt2    | UDP-GalNAc:betaGalNAc beta                                                  | 2.11        | Up        | 0.0003865 |
|               |             | 1,3-galactosaminytrnasferase 2                                              |             |           |         |
| 1443837_x_at  | Bcl2        | B-cell leukemia/lymphoma 2                                                  | 2.14        | Up        | 0.0278712 |
| 1460005_at    | Bod1I       | Biorientation of chromosomes in cell division 1-like                       | 2.59        | Up        | 0.0019409 |
| 1425556_at    | Crkr5       | Cdc2-related kinase                                                        | 2.10        | Up        | 0.0212575 |
| 1419209_at    | Cxcl1       | Chemokine (C-X-C motif) ligand 1                                            | 2.26        | Up        | 0.0418118 |
| 1443068_at    | D130084N16Rik | RIKEN cDNA D130084N16 gene                                                  | 2.03        | Up        | 0.0155479 |
| 1458924_at    | D430013B06Rik | RIKEN cDNA D430013B06 gene                                                  | 2.18        | Up        | 0.0038493 |
| 1439972_at    | Etnk1       | Ethanolamine kinase 1                                                       | 2.15        | Up        | 0.0072570 |
| 1441543_at    | Eya3        | Eyes absent 3 homolog (Drosophila)                                          | 2.20        | Up        | 0.0125155 |
| 1424155_at    | Fabp4       | Fatty acid binding protein 4                                                | 2.82        | Up        | 0.0387713 |
| 1459140_at    | Fam172a     | Family with sequence similarity 172, member A                               | 2.15        | Up        | 0.0045271 |
| 1450297_at    | Il6         | Interleukin 6                                                               | 2.27        | Up        | 0.0181221 |
| 1438519_at    | LOC100042938 | Hypothetical protein LOC100042938                                           | 2.93        | Up        | 0.0068033 |
| 1440365_at    | Lrcc58      | Leucine rich repeat containing 58                                            | 2.01        | Up        | 0.0024045 |
| 1446680_at    | NA          | NA                                                                          | 3.10        | Up        | 0.0048833 |
| 1443267_at    | NA          | NA                                                                          | 2.21        | Up        | 0.0058328 |
| 1457020_at    | NA          | NA                                                                          | 2.12        | Up        | 0.0491902 |
| 1447863_s_at  | Nrar2       | Nuclear receptor subfamily 4, group A, member 2 (Nurr1)                     | 2.07        | Up        | 0.0356418 |
| 1442700_at    | Pde4b       | Phosphodiesterase 4B                                                        | 2.02        | Up        | 0.0129483 |
| 1444817_at    | Plekhk2     | Pleckstrin homology domain containing                                       | 2.08        | Up        | 0.0045902 |
| 1444288_at    | Pnpt1       | Polyribonucleotide nucleotidytransferase I                                  | 2.22        | Up        | 0.0026789 |
| 1456506_at    | Pprf38b     | PRP38 pre-mRNA processing factor                                            | 2.32        | Up        | 0.0054556 |
|               |             | 38 domain containing B                                                       |             |           |         |
| 1456262_at    | Rbm5        | RNA binding motif protein 5                                                 | 2.13        | Up        | 0.013445 |
| 1419247_at    | Rgs2        | Regulator of G-protein signaling 2                                           | 2.02        | Up        | 0.0149932 |
| 1429810_at    | Rictor      | RPTOR independent companion of MTOR, complex 2                             | 2.47        | Up        | 0.0033017 |
| 1459627_at    | Sc4mol      | Sterol-C4-methyl oxidase-like                                               | 2.02        | Up        | 0.0150953 |
| 1444811_at    | Sec62       | SEC62 homolog (S. cerevisiae)                                               | 2.52        | Up        | 0.0008590 |
| 1444006_at    | Setd2       | SET domain containing 2                                                    | 2.55        | Up        | 0.0039997 |
| 1441417_at    | Sst3a       | STT3 homolog A (S. cerevisiae)                                              | 2.08        | Up        | 0.0039461 |
| 1456710_at    | Tead1       | TEA domain family member 1                                                  | 2.57        | Up        | 0.0068079 |
| 1440314_at    | Trip12      | Thyroid hormone receptor interactor 12                                     | 2.27        | Up        | 0.0025044 |
| 1456843_at    | Yes1        | Yamaguchi sarcoma viral (-yes) oncogene homolog 1                          | 2.19        | Up        | 0.0119575 |
| 1441701_at    | Zfp148      | Zinc finger protein 148                                                     | 3.05        | Up        | 0.0008374 |
| 1457908_at    | Zfp407      | Zinc finger protein 407                                                     | 2.61        | Up        | 0.0053867 |
| 1442270_at    | Zfp521      | Zinc finger protein 521                                                     | 2.21        | Up        | 0.0085250 |
| 1425092_at    | Cdh10       | Cadherin 10                                                                 | 2.27        | Down      | 0.0412955 |
| 1453931_at    | Col14a1     | Collagen, type XIV, alpha 1                                                 | 2.19        | Down      | 0.0310586 |
| 1430369_at    | Ebp4.1      | Erythrocyte protein band 4.1                                               | 2.17        | Down      | 0.0019551 |
| 1443716_at    | LOC100039210 | Hypothetical protein LOC100039210                                          | 2.70        | Down      | 0.0227941 |
| 1438239_at    | Mid1        | Midline 1                                                                   | 2.66        | Down      | 0.0023385 |
| 1455591_at    | Zfp618      | Zinc finger protein 618                                                     | 2.01        | Down      | 0.0040700 |
| 1435031_at    | Zikscan1    | Zinc finger with KRAB and SCAN domains 1                                   | 2.13        | Down      | 0.0003634 |

Note: The microarray results were compared between the respective control samples (n = 4 each) using the same criteria as before (unpaired t-test: fold-change ≥1.5; P value < 0.05); this listing contains 49 probe sets with an apparent expression difference greater than 2-fold. Three probe sets lacked any annotation.

*Probe sets that are also represented after comparative analysis of Hoxc8- and Hoxd4-transgenic chondrocytes (Table 5).
Using the Affymetrix microarray platform, we identified 57 genes with differential expression in Hoxc8-transgenic chondrocytes relative to controls. Of particular interest are the elevated expression levels of Replication initiator 1 (Rep11), Topoisomerase 1 (Top1), and Clip associating protein 2 (Clasp2), an M-phase expressed protein, and the decreased expression of Stag1, an inhibitor of cell growth. These results are consistent with the accumulation of proliferating cells in Hoxc8-transgenic cartilage and with a role for Hoxc8 in regulating cell cycle of chondrocytes in M-phase. The lower expression level of Inversin, which acts in the PCP pathway, is consistent with our earlier finding of reduced Wnt5a expression in Hoxc8-transgenic chondrocytes. In Hoxd4-transgenic chondrocytes, we identified 80 deregulated genes; the majority of these genes had lower expression compared with controls. Elevated expression was found for 2 genes with roles in cell proliferation, M-phase phosphoprotein 1 (Mphos1) and Protein phosphatase 2A (PP2A), which controls the G2/M checkpoint of the cell cycle. Antagonist of mitotic exit network 1 (Anm1), which is required for progression through the cell cycle, displays reduced expression in Hoxd4-transgenic chondrocytes. These results support the notion that cell cycle regulation and cell proliferation are altered in Hoxd4-transgenic cartilage, just as in Hoxc8-transgenic chondrocytes.

However, overexpression of Hoxc8 in chondrocytes appears to deregulate a different repertoire of genes compared with Hoxd4 overexpression. We therefore conclude that the 2 transcription factors affect proliferation and/or differentiation of chondrocytes through different molecular mechanisms. This is further supported by direct comparison of Hoxd4-transgenic to Hoxc8-transgenic chondrocytes; the latter exhibit higher expression of the apoptosis regulators Bel2 and Ccar1, prompting the speculation that, in addition to different Wnt signaling pathway activities, apoptosis regulation could be different between the 2 transgenic paradigms.

For the validation by quantitative RT-PCR, 18 transcripts were chosen from each transgenic condition, equally distributed over the range of expression levels. Statistical significance for differences between groups could not be confirmed in these assays. One technical limitation may be the small sample size of $n = 6$ per group. This would be particularly limiting if overexpression of the respective transgene induces a wider spread of gene expression levels (i.e., greater variability in gene expression) within the transgenic group compared with controls.

To investigate the extent of variation in expression levels on Hox transgene overexpression, raw signal intensity values from the microarray chips were obtained. Only entities with a “present” flag were included in this analysis. This was done by dividing the standard deviation of each individual measurement from the average by the mean over 4 samples; thus, variation is expressed in relation to the absolute expression level for each gene. The resulting values were grouped by $P$ value for the comparison ($P < 0.05$ = significant, or $P \geq 0.05$ = not significant) between controls and transgenic samples for each gene and sorted in descending order within the group of entities with significantly different gene expression levels and the group of nonsignificant comparisons, separately (Fig. 3A). Greater variation, as represented by higher CV values on the y-axis, was found for the group of entities with $P$ values $\geq 0.05$, as would be expected (large within-group variations tend to produce nonsignificant $P$ values in between-group comparisons). This applies to the CV values of control samples, as well as samples in the Hoxc8-transgenic group (Fig. 3B), and no difference in variation between control and transgenic group was detected for genes that are not differentially expressed ($P \geq 0.05$) between the 2 conditions. There was also no difference between controls and transgenic samples in the distribution of coefficients for the entities exhibiting differential expression ($P < 0.05$). This argues against the possibility that transgene overexpression increases overall variability of gene expression levels. Applied to the Hoxd4-transgenic condition (Figs. 3 C and D), this type of analysis...
Table 5. Genes Differentially Expressed between Hoxc8- and Hoxd4-Transgenic Chondrocytes

| Probe set ID | Gene symbol | Gene title                                                                 | Fold-change | c8-transg/ d4-transg | P value |
|--------------|-------------|-----------------------------------------------------------------------------|-------------|----------------------|---------|
| 1443584_at   | 1110028C15Rik | RIKEN cDNA 1110028C15 gene                                                   | 2.10        | Up                   | 0.024618 |
| 1432600_at   | 2310061A09Rik | RIKEN cDNA 2310061A09 gene                                                   | 2.04        | Up                   | 0.012037 |
| 1437110_at   | 2810474O19Rik | RIKEN cDNA 2810474O19 gene                                                   | 2.03        | Up                   | 0.019108 |
| 1453595_at   | 2900064B18Rik | RIKEN cDNA 2900064B18 gene                                                   | 2.45        | Up                   | 0.0271002 |
| 1441331_at   | A230061C15Rik | RIKEN cDNA A230061C15 gene                                                   | 2.01        | Up                   | 0.0092051 |
| 1439143_at   | A930018M24Rik | RIKEN cDNA A930018M24 gene                                                   | 2.91        | Up                   | 0.0048276 |
| 1446608_at   | Adk         | Adenosine kinase                                                           | 2.23        | Up                   | 0.0243666 |
| 1455151_at   | Akap9       | A kinase (PRKA) anchor protein (yotiao) 9                                   | 2.20        | Up                   | 0.0276093 |
| 1437082_at   | Akap9       | A kinase (PRKA) anchor protein (yotiao) 9                                   | 2.15        | Up                   | 0.0430726 |
| 1449012_at   | Aldh2       | Aldehyde dehydrogenase 2                                                   | 2.00        | Up                   | 0.003061 |
| 1420947_at   | Atrx        | Alpha thalassemia/mental retardation syndrome X-linked homolog             | 2.16        | Up                   | 0.0166582 |
|              |             | Expressed sequence BB211804                                                | 2.01        | Up                   | 0.0106094 |
| 1458163_at   | BC066028    | cDNA sequence BC066028                                                      | 4.00        | Up                   | 0.0126564 |
| 1440770_at   | Bcl2        | B-cell leukemia/lymphoma 2                                                 | 2.16        | Up                   | 0.0007920 |
| 1460005_at   | Bodl        | Biobriorentation of chromosomes in cell division 1                         | 2.91        | Up                   | 0.0009071 |
| 1456050_at   | C80998      | Expressed sequence C80998                                                   | 2.19        | Up                   | 0.0019366 |
| 1453319_at   | Ccar1       | Cell division cycle and apoptosis regulator 1                               | 2.58        | Up                   | 0.0046564 |
| 1445843_at   | Chad2       | Chromodomain helicase DNA binding protein 2                                 | 2.18        | Up                   | 0.0203581 |
| 1441726_at   | Clasp2      | CLIP associating protein 2                                                  | 2.16        | Up                   | 0.0430662 |
| 1417946_at   | Cpsf6       | Cleavage and polyadenylation specific factor 6                              | 2.11        | Up                   | 0.0192667 |
| 1419038_A_at | Csnk2a1     | Casein kinase 2                                                             | 2.37        | Up                   | 0.0013194 |
| 1419209_at   | Cxcl1       | Chemokine (C-X-C motif) ligand 1                                            | 2.08        | Up                   | 0.0235844 |
| 1458924_at   | D430013B06Rik| RIKEN cDNA D430013B06 gene                                                  | 3.61        | Up                   | 0.0421971 |
| 1454869_at   | Dcaf12i1    | DDB1 and CUL4 associated factor 12-like 1                                   | 3.59        | Up                   | 0.0499441 |
| 1442329_at   | Dclre1a     | DNA cross-link repair 1A                                                     | 2.30        | Up                   | 0.0022126 |
| 1443897_at   | Ddit3       | DNA-damage inducible transcript 3                                           | 2.13        | Up                   | 0.0179668 |
| 1417516_at   | Ddit3       | DNA-damage inducible transcript 3                                           | 2.07        | Up                   | 0.0324307 |
| 1439977_at   | Etnkl       | Ethanolamine kinase 1                                                       | 2.36        | Up                   | 0.0077480 |
| 1443628_at   | Fam82b      | Family with sequence similarity 82, member B                               | 2.14        | Up                   | 0.0089534 |
| 1441548_at   | Frmd4b      | FERM domain containing 4B                                                   | 2.15        | Up                   | 0.0027981 |
| 1419378_A_at | Fxyd2       | FXYD domain-containing ion transport regulator 2                            | 2.58        | Up                   | 0.074027 |
| 1429257_at   | Gtl2        | GTL2                                                                        | 2.03        | Up                   | 0.0174762 |
| 1450297_at   | Il6         | Interleukin 6                                                               | 3.03        | Up                   | 0.0060546 |
| 1438519_at   | LOC100042938| Hypothetical protein LOC100042938                                          | 2.50        | Up                   | 0.0018819 |
| 1446316_at   | Lpin2       | Lipin 2                                                                     | 2.68        | Up                   | 0.0403329 |
| 1452592_at   | Mgst2       | Microsomal glutathione S-transferase 2                                      | 2.50        | Up                   | 0.0052901 |
| 1451612_at   | Mt1         | Metallothionein 1                                                           | 2.25        | Up                   | 0.0304642 |
| 1422557_S_at | Mt1         | Metallothionein 1                                                           | 2.01        | Up                   | 0.0252146 |
| 1428942_at   | Mt2         | Metallothionein 2                                                           | 2.20        | Up                   | 0.0241249 |
| 1429013_at   | Mtap7d2     | MAP7 domain containing 2                                                    | 2.25        | Up                   | 0.0085073 |
| 1440708_at   | Myh9        | Myosin                                                                      | 2.12        | Up                   | 0.0422888 |
| 1418366_at   | NA          | NA                                                                          | 2.09        | Up                   | 0.0224857 |
| 1446730_at   | NA          | NA                                                                          | 3.42        | Up                   | 0.0236191 |
| 1457020_at   | NA          | NA                                                                          | 2.21        | Up                   | 0.0399145 |
| 1457208_at   | Nfxl1       | Nuclear transcription factor                                                 | 2.81        | Up                   | 0.0056797 |
| 1430309_at   | Nipbl       | Nipped-B homolog (Drosophila)                                               | 2.02        | Up                   | 0.0056786 |
| 1419107_at   | Oph1        | Oligophrenin 1                                                              | 2.77        | Up                   | 0.0162735 |
| 1441026_at   | Parp4       | Poly (ADP-ribose) polymerase family                                         | 2.08        | Up                   | 0.0060589 |
| 1442700_at   | Pde4b       | Phosphodiesterase 4B                                                        | 2.14        | Up                   | 0.0027187 |
| 1446490_at   | Ptpb2       | Polypyrimidine tract binding protein 2                                       | 2.09        | Up                   | 0.0003002 |
| 1447164_at   | Rif         | Rearranged L-myc fusion sequence                                            | 2.21        | Up                   | 0.0029808 |
| 1437224_at   | Rtn4        | Reticulon 4                                                                 | 2.21        | Up                   | 0.0016998 |
| 1459627_at   | Sc4mol      | Sterol-C4-methyl oxidase-like                                               | 2.06        | Up                   | 0.0181274 |
| 1444811_at   | Sec62       | SEC62 homolog (S. cerevisiae)                                               | 2.84        | Up                   | 0.0030046 |

(continued)
yields the same conclusion, that is, lack of evidence for greater variability upon transgene overexpression. It should be noted, however, that this analysis is limited by the fact that the group sizes for \( P \geq 0.05 \) and \( P < 0.05 \) are necessarily different. Furthermore, we did not find differences in variation between control and transgenic samples when we applied these calculations to gene expression measurements (in form of \( \Delta Ct \) values) obtained from the quantitative RT-PCR assays (Figs. 3 E and F).

A 2nd possible technical reason for the lack of congruence of the RT-PCR results with the microarray data could be the location of the PCR amplicon for each gene. The microarray probes are designed to sample the far 3’ end of the gene transcript, whereas we designed primer pairs to span exon-exon boundaries, which are located further toward the 5’ end of the transcript.

A 3rd possibility is that the mRNA abundance in the samples is at the lower level of detection by RT-PCR. However, we selected both high- and low-abundance transcripts for our validation experiments and should have been able to detect such a phenomenon.

A 4th possibility bearing on the outcome of the PCR assays is the choice of reference gene. Because expression measurements for each gene of interest are normalized to Gapdh expression level, any changes in Gapdh expression as a consequence of Hox transgene overexpression would have a profound impact on the results. However, signals for Gapdh expression were not different between any of the experimental groups in the microarray assays.

Furthermore, the levels of Gapdh expression detected by quantitative RT-PCR were also within a narrow range for all groups (Ct_{Gapdh} = 18.89 ± 0.52 for Hoxc8-transgenic samples versus Ct_{Gapdh} = 18.79 ± 0.68 for controls, and Ct_{Gapdh} = 18.39 ± 0.69 for Hoxd4-transgenic samples versus Ct_{Gapdh} = 18.29 ± 0.66 for controls). These data confirm Gapdh as a suitable reference gene for our RT-PCR assays.

In earlier studies, we performed targeted gene expression studies by quantitative RT-PCR on 23 folate metabolism genes\(^4\) and 37 cartilage developmental genes\(^6\) in primary chondrocytes from Hoxc8- and Hoxd4-transgenic animals, respectively. The choice of targets for this prior work was guided by evidence from the literature, and assays were performed prior to the microarray analyses reported here. Among the 60 genes thus investigated, 9 genes (Bmp4, Fgf8, Fgf10, Mmp9, Mmp13, Nos3, Timp3, Wnt3a, and Wnt5a) exhibited differential expression in Hoxc8-transgenic cartilage and 4 genes (Fgfr3, Ihh, Mmp8, and Wnt3a) in Hoxd4-transgenic samples.\(^6\) Upon retroactive inspection of these 60 genes in our microarray analyses, we found that they are either not represented on the arrays, did not pass the signal requirement of “present” or “marginal” in 5 of 8 samples or more, or did not pass cutoff criteria for fold-change and \( P \)-value levels. The current study adds an additional 18 genes per condition to the list of genes whose expression was not altered by Hox transgene overexpression. Thus, of 78 candidate genes measured by RT-PCR, 9 genes (11.54%) exhibited altered levels in Hoxc8-transgenic chondrocytes and 4 (5.1%) in Hoxd4-transgenic cells.

### Table 5. (continued)

| Probe set ID | Gene symbol | Gene title | Fold-change | c8-transg/ d4-transg | \( P \)-value |
|-------------|-------------|------------|-------------|---------------------|-------------|
| 1421564_at  | Serpina3c   | Serine (or cysteine) peptidase inhibitor | 2.26 | Up | 0.0199104 |
| 1459571_at  | Sh3bgrl     | SH3-binding domain glutamic acid-rich protein like | 3.81 | Up | 0.0290655 |
| 1456717_at  | Ted1        | TEA domain family member 1 | 2.43 | Up | 0.0040962* |
| 1423405_at  | Timp4       | Tissue inhibitor of metalloproteinase 4 | 2.45 | Up | 0.0038079 |
| 1440314_at  | Trip12      | Thyroid hormone receptor interactor 12 | 2.05 | Up | 0.0413829* |
| 1447266_at  | Utp18       | UTP18 | 2.09 | Up | 0.0005509 |
| 1434433_x_at | Wdr61      | WD repeat domain 61 | 2.00 | Up | 0.0061046 |
| 1436746_at  | Wnk1        | WNK lysine deficient protein kinase 1 | 2.01 | Up | 0.0223951 |
| 1427262_at  | Xist        | Inactive X specific transcripts | 22.35 | Up | 0.0431562 |
| 1436429_at  | Zfp606      | Zinc finger protein 606 | 2.87 | Up | 0.0018548 |
| 1435050_at  | D10Bwg1397e | DNA segment | 2.05 | Down | 0.0086644 |
| 1445605_s_at | Fam135a    | Family with sequence similarity 135, member A | 2.23 | Down | 0.0019277 |
| 1419139_at  | Gdf5        | Growth differentiation factor 5 | 2.59 | Down | 0.0425737 |
| 1444657_at  | N4bp2       | NEDD4 binding protein 2 | 2.43 | Down | 0.0005223 |
| 1415893_at  | Sgpl1       | Sphingosine phosphate lyase 1 | 2.25 | Down | 0.0139590 |
| 1429979_a_at | Slc38a10    | Solute carrier family 38, member 10 | 2.11 | Down | 0.0116045 |
| 1419913_a_at | Strap       | Serine/threonine kinase receptor associated protein | 2.14 | Down | 0.0332865 |

Note: Comparison of microarray results between Hoxc8- and Hoxd4-transgenic samples (unpaired t-test; fold-change ≥ 1.5; \( P \)-value < 0.05); 72 entities exhibit expression differences of greater than 2-fold. Akap9, Ddit3, and Mt1 are represented by multiple probe sets; 3 probe sets lacked annotations.

*Probeset also represented as differentially expressed in the comparison of controls to these transgenic samples (Table 4).
Figure 3. Variability of gene expression levels in Hoxc8- and Hoxd4-transgenic chondrocytes. Only entities with a "present" flag were included in the calculation. The microarray detection signals were averaged over the 4 control samples and the standard deviation calculated. The standard deviation was then divided by the mean to obtain the coefficient of variation; values were sorted in descending order in both groups ($P < 0.05$ and $P \geq 0.05$). Parallel calculations were done for the transgenic animals. As expected, we found higher variability of expression levels in samples with $P$ values greater than 0.05 for Hoxc8 animals relative to samples (C, D). For polymerase chain reaction (PCR)–validated gene expression levels, calculations were performed as described above using $\Delta Ct$ values. (E, F) Variability in relative expression levels (measured by reverse transcriptase PCR) in the comparison between control ($n = 6$) and Hoxc8-transgenic samples ($n = 6$) and between controls and Hoxd4-transgenic samples, respectively.

Likewise, the results from microarray assays presented here identify only a relatively small (less than 100 per condition) number of genes with differential expression in transgenic chondrocytes. Similarly low yields were reported for cDNA microarray studies on Hoxd10 mutant spinal cord cells, which confirmed 9 genes by PCR of the 69 identified from the arrays (13%). Even so, this low number of potential transcriptional targets is perplexing, given the serious cartilage differentiation defects in the Hoxc8- and Hoxd4-transgenic animals. We also showed earlier, by RT-PCR assays with primer sets that amplify a part of the coding sequence, that the transgenes are overexpressed on average by 4.6-fold (in the case of Hoxc8) and close to 15-fold (for Hoxd4) when compared with respective littermate controls. In the microarray assays employed here, only 3′ regions of Hoxc8 and Hoxd4 are sampled. However,
the native 3′ regions of Hoxc8 and Hoxd4 are substituted by heterologous (SV40-derived) noncoding sequences in the Hoxc8- and Hoxd4-transgenes, respectively. A number of conceivable biological scenarios may limit our ability to define transcriptional consequences of Hox transgene overexpression in chondrocytes by the gene expression–profiling approaches we have employed:

1. The actions of the overexpressed Hox transcription factors are not occurring in chondrocytes themselves but nonautonomously through undefined mechanisms. This is unlikely, given that we have shown the transgenes to be expressed in developing cartilage by virtue of VP16-mediated transactivation. We have also demonstrated that knockdown of Hoxc8 expression affects the proliferation and cell cycle progression of primary chondrocytes in vitro, implicating a cell-autonomous action for Hoxc8. It is nevertheless possible that the fraction of cells with Hox transgene overexpression is rather small in the rib cages from which the chondrocytes were prepared, and thus, strong effects in transgene-expressing cells could be diluted by a larger fraction of unaffected cells; contamination with nonchondrogenic cells, however, is unlikely. Hox transgene overexpression is expected to be greatest in immature and proliferating cartilage precursor cells (C. Kappen unpublished), and presently, we do not have detailed information on the relative proportion of such cells versus more mature chondrocytes in our samples.

2. The time point of sampling, embryonic day 18.5, might affect the outcome of this study as well. Chondrocyte maturation is a continuous process commencing from the appearance of chondrogenic condensations at E12.5, and the Hox transgenes are activated at this stage and even earlier. Thus, if the major transcriptional effects of transgene overexpression occur earlier than E18.5, the altered expression of Hox target genes may not be maintained into later time points. Apart from the measurements of elevated expression of the transgenes themselves, we have previously demonstrated that some genes are indeed aberrantly expressed in Hox transgenic primary chondrocytes prepared at E18.5; these genes are known to be involved in cartilage development (see above) and are currently under investigation in the cartilage defects in our Hox transgenic paradigms. Nonetheless, it may be necessary to better define the critical time windows of Hox gene actions in the transgenic cartilage and extend the analysis to such time points.

3. The action of overexpressed Hox transcription factors in developing cartilage may not be primarily at the transcriptional level but through protein–protein interactions, which in turn may be involved in regulating chondrocyte proliferation and/or differentiation. Interactions with protein cofactors are thought to modulate the affinity and specificity of DNA binding by Hox proteins. Meis and Pbx are the best-studied Hox cofactors in mammals; they form stable heterodimers that bind DNA cooperatively. Both Hox and Pbx genes have been implicated in cell proliferation in leukemia as well as in skeletal development. Thus, in cartilage differentiation, the role of Hox transcription factors is likely to be modulated by protein interactions as well, and such interactions may even supersede transcriptional activity. Recently emerging evidence implicates the Smads, which are known to play roles in BMP and Tgfβ signal transduction, as another class of interactors with Hox proteins in various tissue systems. However, the role of such interactions in cartilage development under conditions of Hoxc8 or Hoxd4 overexpression remains to be investigated.

Intriguingly, the detrimental effects of Hox transgene overexpression can be ameliorated by supplementation of folate to the maternal diet, indicating that at least some of the cellular abnormalities are reversible. This is further highlighted by our finding that chondrocytes from Hoxc8-transgenic mice, when placed into primary cell culture, are able to proliferate and differentiate apparently normally. Thus, the in vivo conditions in the transgenic cartilage contribute to the action of overexpressed Hox transcription factors, possibly through cell communication, signaling, or cell-matrix interactions. The nutritional and cellular context may also influence the propensity for cartilage defects on Hox transcription factor misexpression. The genes we have identified in this and our earlier studies will help us elucidate the molecular and cellular basis for proliferation and differentiation defects in Hox transgenic cartilage.

**Conclusions**

We used genomewide expression profiling to identify genes with altered expression in primary chondrocytes from transgenic mice with overexpression of Hoxc8 and Hoxd4, respectively. In each transgenic paradigm, genes were found misexpressed that are consistent with the interpretation of altered cell proliferation in transgenic cartilage. Intriguingly, the repertoires of differentially expressed genes did not overlap between both conditions, indicating that the 2 Hox transcription factors employ distinct molecular mechanisms...
in the pathogenesis of defective cartilage. The relatively low number of independently validated misregulated transcripts, however, suggests that the phenotypic abnormalities may also be mediated by nontranscriptional mechanisms downstream of Hox transgene overexpression in developing cartilage.

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Declaration of Conflicting Interests
The authors declare that they have no competing interests.

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