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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[12] 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,[7,10] 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.[13] 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 characterizations of the phenotypes of both transgenic lines[8,10] and the levels of expression for Hox transgenes in transgenic chondrocytes[14] have been published.

Pregnant dams were sacrificed at 18.5 days of gestation. Double transgenic embryos are phenotypically identifiable by failure of eyelid closure,[8,10] and for confirmation, genotyping was performed on DNA isolated from tails of individual specimen.[13] Transgene expression in all samples was demonstrated by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) as described earlier.[14]

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.[15] Freshly isolated cells were immediately transferred into Trizol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated and purified as described previously.[14] 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$(dT)_{20}$ 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, and 4 controls and 4 Hoxd4-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 ≥ 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 $\Delta Rn_{cycle(n)}/\Delta Rn_{cycle(n-1)}$, 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-transgenics 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
1433377_at	5830445D09Rik	RIKEN cDNA 5830445D09 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
1437654_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	Gle1	GLE1 RNA export mediator (yeast)	1.52	Up	0.0200380
1453855_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	Pppde1	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	Pproline rich 12	1.53	Up	0.0217165
1434043_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) I	1.68	Up	0.0010994
1455722_at	Wasf3	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	0.0201768
1446431_at	Dnm3	Dynamin	1.70	Down	0.0378060
1434714_at	Ero1lb	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-aminobutyric acid (GABA-A) receptor	1.51	Down	0.0367941
1438152_at	Gpaa1	GPI anchor attachment protein 1	2.00	Down	0.0375435
1419196_at	Hamp	Hepcidin 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	0.0433589

(continued)

Table 1. (continued)

Probe set ID	Gene symbol	Gene title	Fold-change	Transgenic/control	*P* value
1438614_x_at	Osbpl9	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	Specc1	Sperm antigen with calponin homology and coiled-coil domains 1	1.69	Down	0.0072438
1446680_at	Stag1	Stromal antigen 1	2.48	Down	0.0070552
1416927_at	Trp53inp1	Transformation related protein 53 inducible nuclear protein 1	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 ≥1.5; *P* value < 0.05) in Hoxc8-transgenic chondrocytes compared with controls.

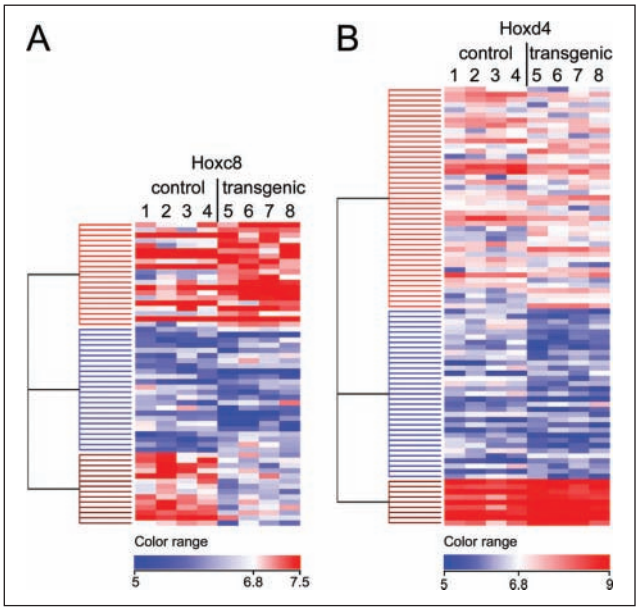

Figure 1. Differential gene expression in Hoxc8- and Hoxd4-transgenic cartilage. K-means clustering algorithm and Euclidean distance metric (as implemented in GeneSpring) were used to visualize the different expression profiles for (**A**) Hoxc8 and (**B**) Hoxd4 transgenic chondrocytes relative to their 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 ≥ 1.5 and *P* value < 0.05).

fold-change ≥ 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.[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 ≥ 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 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	5430427G11Rik	11.56 ± 0.40	11.51 ± 0.45	1.03	0.85403
1433377_at	5830445D09Rik	13.35 ± 0.34	13.64 ± 0.84	–1.18	0.45344
1417470_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	Dnm3	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
1425104_at	Kctd1	13.07 ± 0.42	12.81 ± 1.00	1.16	0.57281
1454845_x_at	Mchr1	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	Repin1	7.94 ± 0.25	8.05 ± 0.16	–1.08	0.37954
1428794_at	Specc1	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	
1453358_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	Eif2s3y	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	Taf11	6.78 ± 0.50	6.87 ± 0.74	–1.06	0.81399
1445668_at	Tbce	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 = $AE^{-\Delta\Delta Ct}$ (AE = amplification efficiency; see Supplemental Table S1), where AE was calculated using the formula AE = $\Delta Rn_{cycle(n)}/\Delta Rn_{cycle(n-1)}$ 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
1449641_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	Ccdc101	Coiled-coil domain containing 101	1.51	Up	0.0483277
1435574_at	Chordc1	Cysteine and histidine-rich domain (CHORD)-containing	1.92	Up	0.0068320
1421267_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	Rpusd2	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
1453358_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	Ccbe1	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)

Table 3. (continued)

Probe set ID	Gene symbol	Gene title	Fold-change	Transgenic/control	*P* value
1443772_at	Dzip1	DAZ interacting protein 1	1.56	Down	0.0054066
1427462_at	E2f3	E2F transcription factor 3	1.54	Down	0.0248094
1416916_at	Elf3	E74-like factor 3	1.57	Down	0.0159981
1437020_at	Ep400	E1A binding protein p400	1.54	Down	0.0347020
1445191_at	Exdl1	Exonuclease 3′-5′ domain-like 1	1.97	Down	0.0053002
1440063_at	Farsa	Pphenylalanyl-tRNA synthetase	1.64	Down	0.0250529
1419378_a_at	Fxyd2	FXYD domain-containing ion transport regulator 2	1.65	Down	0.0331975
1437106_at	Kdm5a	Lysine (K)-specific demethylase 5A	1.54	Down	0.0325942
1428174_x_at	Khsrp	KH-type splicing regulatory protein	1.56	Down	0.0364211
1428980_at	Kprp	Keratinocyte expressed	1.71	Down	0.0279730
1441526_at	Mbtd1	mbt domain containing 1	1.59	Down	0.0238720
1426557_at	Mesp1	Mesoderm posterior 1	1.61	Down	0.0337495
1443165_at	Mrps31	Mitochondrial ribosomal protein S31	1.52	Down	0.0362433
1416839_at	Mut	Methylmalonyl-Coenzyme A mutase	1.67	Down	0.0387990
1460159_at	Mysm1	myb-like	1.85	Down	0.0334813
1455343_at	Plekha7	Pleckstrin homology domain containing	1.59	Down	0.0331890
1460419_a_at	Prkcb	Protein kinase C, beta	1.95	Down	0.0010795
1429474_at	Ptgr2	Prostaglandin reductase 2	1.54	Down	0.0374520
1443162_at	Ptpn3	Protein tyrosine phosphatase, non-receptor type 3	2.47	Down	0.0147085
1427486_at	Ptprb	Protein tyrosine phosphatase, receptor type B	1.61	Down	0.0172879
1443860_at	Ptprd	Protein tyrosine phosphatase, receptor type D	1.70	Down	0.0254090
1457488_at	Ralgapb	Ral GTPase activating protein, beta subunit (non-catalytic)	1.61	Down	0.0010210
1429462_at	Slc25a32	Solute carrier family 25	1.66	Down	0.0135294
1416854_at	Slc34a2	Solute carrier family 34 (sodium phosphate)	1.73	Down	0.0441978
1426715_s_at	Slc46a1	Solute carrier family 46	1.60	Down	0.0267808
1451938_a_at	Sntb1	Syntrophin	1.55	Down	0.0125492
1421472_at	Srm3	Serine/arginine repetitive matrix 3	1.61	Down	0.0277383
1456363_at	St7l	Supression of tumorigenicity 7-like	1.84	Down	0.0015698
1451995_at	Taf11	TAF11 RNA polymerase II	1.51	Down	0.0037954
1422973_a_at	Thrsp	Thyroid hormone responsive SPOT14 homolog (Rattus)	1.61	Down	0.0480069
1419949_at	Tmem38b	Transmembrane protein 38B	1.58	Down	0.0456857
1436108_at	Txndc9	Thioredoxin domain containing 9	1.53	Down	0.0196796
1429971_at	Txnrd2	Thioredoxin reductase 2	1.66	Down	0.0306703
1450151_at	Zfp316	Zinc finger protein 316	1.84	Down	0.0000381

Note: Affymetrix Probe set ID numbers are given; Ddx3y, Eif2s3y, and Uty are represented by multiple probe sets, and 2 probe sets have no annotation. The comparison of transgenic/control indicates elevation or reduction of expression in Hoxd4-transgenic chondrocytes relative to controls. Eighty-five entities (80 genes) are differentially expressed (unpaired *t*-test; fold-change ≥1.5; *P* value < 0.05) in Hoxd4-transgenic chondrocytes compared with controls.

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_{c8}/C_{d4}	*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 metallopeptidase 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:betaGlcNAc beta 1,3-galactosaminyltransferase 2	2.11	Up	0.0003865
1443837_x_at	Bcl2	B-cell leukemia/lymphoma 2	2.14	Up	0.0278712
1460005_at	Bod1l	Biorientation of chromosomes in cell division 1-like	2.59	Up	0.0019409[a]
1425556_at	Crkrs	Cdc2-related kinase	2.10	Up	0.0212575
1419209_at	Cxcl1	Chemokine (C-X-C motif) ligand 1	2.26	Up	0.0418118[a]
1443068_at	D130084N16Rik	RIKEN cDNA D130084N16 gene	2.03	Up	0.0155479
1458924_at	D430013B06Rik	RIKEN cDNA D430013B06 gene	2.18	Up	0.0038493[a]
1439972_at	Etnk1	Ethanolamine kinase 1	2.15	Up	0.0072570[a]
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[a]
1438519_at	LOC100042938	Hypothetical protein LOC100042938	2.93	Up	0.0006803[a]
1440365_at	Lrrc58	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[a]
1447863_s_at	Nr4a2	Nuclear receptor subfamily 4, group A, member 2 (Nurr1)	2.07	Up	0.0356418
1442700_at	Pde4b	Phosphodiesterase 4B	2.02	Up	0.0129483[a]
1444817_at	Plekhh2	Pleckstrin homology domain containing	2.08	Up	0.0045902
1444288_at	Pnpt1	Polyribonucleotide nucleotidyltransferase 1	2.22	Up	0.0026789
1456506_at	Prpf38b	PRP38 pre-mRNA processing factor 38 domain containing B	2.32	Up	0.0054556
1456262_at	Rbm5	RNA binding motif protein 5	2.13	Up	0.0013445
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[a]
1444811_at	Sec62	SEC62 homolog (S. cerevisiae)	2.52	Up	0.0008590[a]
1444006_at	Setd2	SET domain containing 2	2.55	Up	0.0039997
1441417_at	Stt3a	STT3 homolog A (S. cerevisiae)	2.08	Up	0.0039461
1456717_at	Tead1	TEA domain family member 1	2.57	Up	0.0068079[a]
1440314_at	Trip12	Thyroid hormone receptor interactor 12	2.27	Up	0.0025044[a]
1456843_at	Yes1	Yamaguchi sarcoma viral (v-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
1442709_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	Epb4.1	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
1453051_at	Zkscan1	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.

[a]Probe sets that are also represented after comparative analysis of Hoxc8- and Hoxd4-transgenic chondrocytes (**Table 5**).

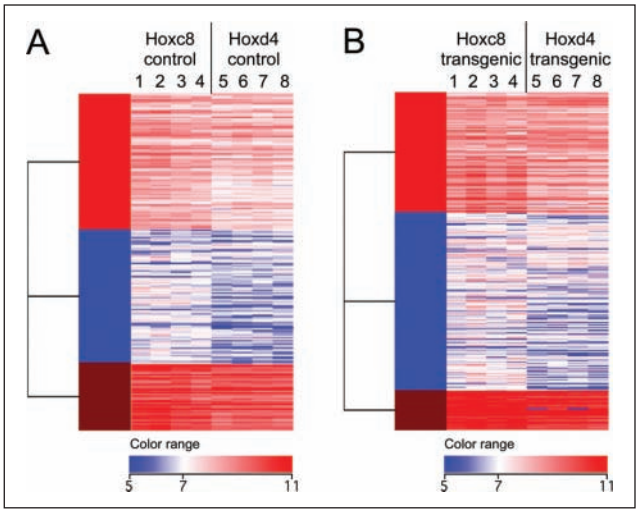

Figure 2. Differential gene expression between control groups and transgenic groups. Entities revealed using the unpaired *t*-test (present or marginal flag in 5 of 8 samples, fold-change ≥ 1.5 and *P* value < 0.05) were clustered according to the K-means algorithm with Euclidean distance metric. (**A**) Cluster analysis for 445 entities with significantly different expression levels between control groups to the transgenic conditions; 387 entities had a differential in expression between 1.5- and 2-fold. (**B**) Cluster analysis for 532 entities with significantly different expression levels in comparison with Hoxc8-transgenic versus Hoxd4-transgenic samples; 460 entities had a differential in expression between 1.5- and 2-fold.

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 (Repin1), 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[10] and with a role for Hoxc8 in regulating cell cycle of chondrocytes in M-phase.[11] 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.[16] 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 (Amn1), 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 Bcl2 and Ccar1, prompting the speculation that, in addition to different Wnt signaling pathway activities,[16] 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. For all gene expression measurements, the coefficient of variation (CV) was calculated for the groups of control samples and groups of transgenic samples, independently. 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 ≥ 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	0.0246180
1432600_at	2310061A09Rik	RIKEN cDNA 2310061A09 gene	2.04	Up	0.0120357
1437110_at	2810474O19Rik	RIKEN cDNA 2810474O19 gene	2.03	Up	0.0191083
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
1446068_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
1434988_x_at	Aldh2	Aldehyde dehydrogenase 2	2.00	Up	0.0050611
1420947_at	Atrx	Alpha thalassemia/mental retardation syndrome X-linked homolog	2.16	Up	0.0166582
1439216_at	BB211804	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	Bod1l	Biorientation of chromosomes in cell division 1	2.91	Up	0.0009071[a]
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	Chd2	Chromodomain helicase DNA binding protein 2	2.18	Up	0.0203581
1441726_at	Clasp2	CLIP associating protein 2	2.16	Up	0.0430622
1417496_at	Cp	Ceruloplasmin	2.10	Up	0.0467060
1437372_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[a]
1458924_at	D430013B06Rik	RIKEN cDNA D430013B06 gene	3.61	Up	0.0421971[a]
1454869_at	Dcaf12l1	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
1439972_at	Etnk1	Ethanolamine kinase 1	2.36	Up	0.0077480[a]
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	0.0074027
1429257_at	Gtl2	GTL2	2.03	Up	0.0174762
1450297_at	Il6	Interleukin 6	3.03	Up	0.0060546[a]
1438519_at	LOC100042938	Hypothetical protein LOC100042938	2.50	Up	0.0018819[a]
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[a]
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	Ophn1	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[a]
1446490_at	Ptbp2	Polypyrimidine tract binding protein 2	2.09	Up	0.0003002
1447164_at	Rlf	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[a]
1444811_at	Sec62	SEC62 homolog (S. cerevisiae)	2.84	Up	0.0030046[a]

(continued)

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	Tead1	TEA domain family member 1	2.43	Up	0.0040962[a]
1423405_at	Timp4	Tissue inhibitor of metalloproteinase 4	2.45	Up	0.0038079
1440314_at	Trip12	Thyroid hormone receptor interactor 12	2.05	Up	0.0418329[a]
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	D10Bwg1379e	DNA segment	2.05	Down	0.0088644
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_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.
[a]Probe sets also represented as differentially expressed in the comparison of controls to these transgenic samples (**Table 4**).

yielded 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 ΔCt 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 ($Ct_{Gapdh} = 18.89 \pm 0.52$ for Hoxc8-transgenic samples versus $Ct_{Gapdh} = 18.79 \pm 0.68$ for controls, and $Ct_{Gapdh} = 18.39 \pm 0.69$ for Hoxd4-transgenic samples versus $Ct_{Gapdh} = 18.29 \pm 0.66$ 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[14] and 37 cartilage developmental genes[16] 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.[16] 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.

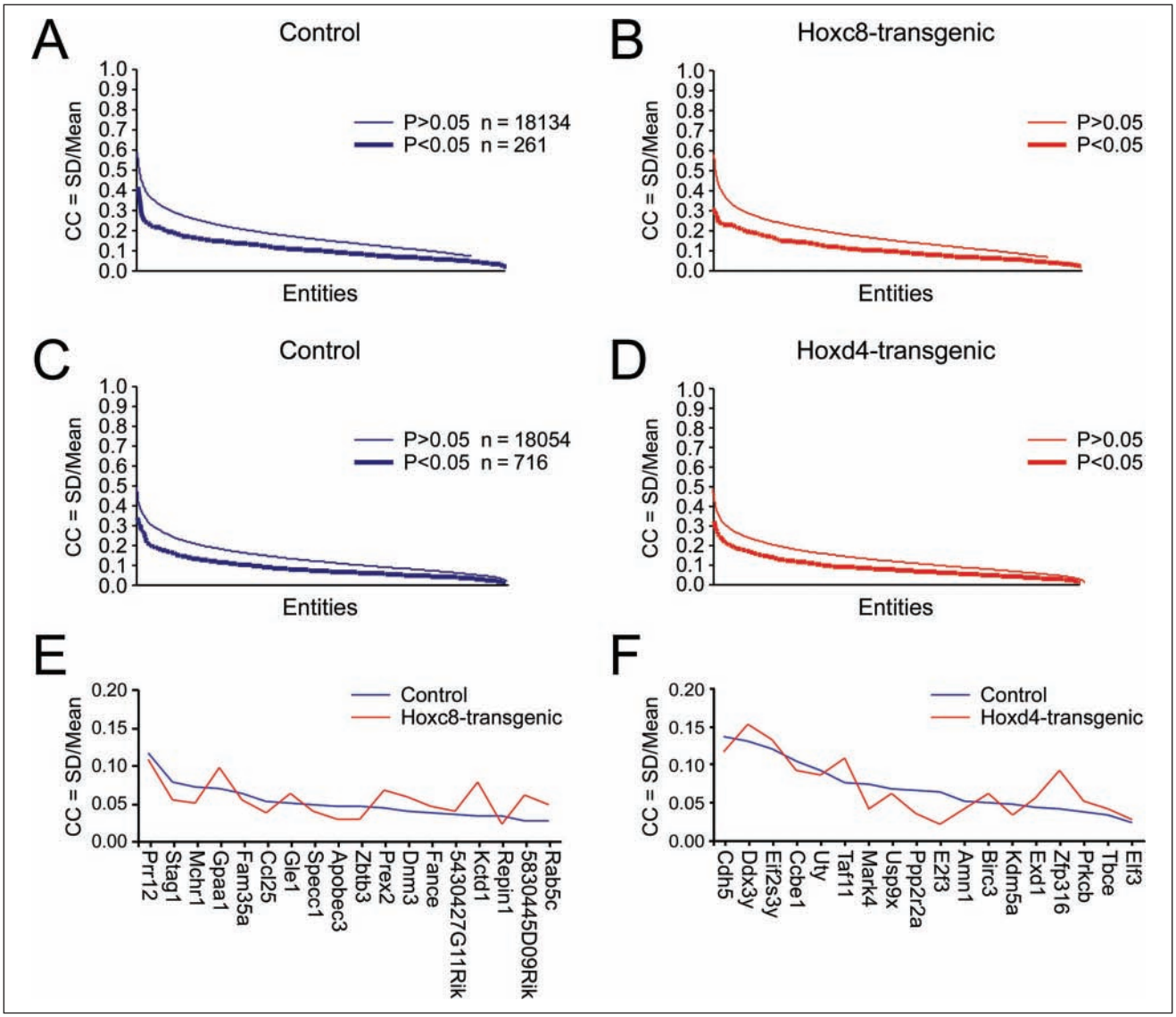

Figure 3. Variability of gene expression levels in Hoxc8- and Hoxd4-transgenic chondrocytes. Only entities with a "present" flag were included in the calculation. The microarray detection signals were averaged over the 4 control samples and the standard deviation calculated. The standard deviation was 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 ΔCt 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%).[17] Even so, this low number of potential transcriptional targets is perplexing, given the serious cartilage differentiation defects in the Hoxc8- and Hoxd4-transgenic animals.[8,10] 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.[14] 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.[13] 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.[10,13,14] We have also demonstrated that knockdown of Hoxc8 expression affects the proliferation and cell cycle progression of primary chondrocytes *in vitro*,[11] 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.[15] Hox transgene overexpression[10] 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.[7,10,12,18] 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,[14] we have previously demonstrated that some genes are indeed aberrantly expressed in Hox transgenic primary chondrocytes prepared at E18.5[16]; 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[19-21]; they form stable heterodimers that bind DNA cooperatively.[22-24] Both Hox and Pbx genes have been implicated in cell proliferation in leukemia[25-31] as well as in skeletal development.[32-35] 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.[36-39] 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,[8] 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.[15] 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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