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Expression of Folate Pathway Genes in the Cartilage of *Hoxd4* and *Hoxc8* Transgenic Mice

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Abstract

BACKGROUND—Hox transcription factors are well known for their role in skeletal patterning in vertebrates. They regulate gene expression during the development of cartilage, the precursor to mature bone. We previously reported that overexpression of the homeobox genes *Hoxc8* and *Hoxd4* results in severe cartilage defects, reduced proteoglycan content, accumulation of immature chondrocytes, and decreased maturation to hypertrophy. We have also shown that *Hoxd4* transgenic mice whose diets were supplemented with folate had their skeletal development restored. Since folate is required for growth and differentiation of chondrocytes, we hypothesized that the beneficial effect of folate in *Hoxd4* transgenic mice might indicate a local deficiency in folate utilization, possibly caused by deregulation of genes encoding folate transport proteins or folate metabolic enzymes.

METHODS—We assayed the prevalence of transcripts for 22 folate transport proteins and metabolizing enzymes, here collectively referred to as folate pathway genes. Quantitative real-time PCR was performed on cDNA samples derived from RNA isolated from primary chondrocytes of individual rib cartilages from *Hoxd4* and *Hoxc8* transgenic mice, respectively.

RESULTS—This study shows that the *Hox* transgenes produce overexpression of *Hoxd4* and *Hoxc8* in primary chondrocytes from perinatal transgenic mice. However, no differences were found in expression levels of the folate pathway genes in transgenic cells compared to littermate controls.

CONCLUSIONS—Our results provide evidence that folate pathway genes are only indirect targets of *Hox* transgene overexpression in our transgenic animals. These expression studies provide a baseline for future studies into the role of folate metabolism in chondrocyte differentiation.

Keywords

chondrocytes; homeobox genes; hypertrophy; nutritional supplementation; gene-environment interaction; cartilage maturation; transcription factor; folic acid; expression profiling; embryonic development; cell differentiation; extracellular matrix; proteoglycan; rib cage

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INTRODUCTION

Hox genes encode transcription factors that are involved in patterning the individual elements of the developing skeleton (Capecchi, 1996). They also play a role in the regulation of cartilage differentiation prior to overt bone formation (Yokouchi et al., 1995; Knezevic et al., 1997; Yueh et al., 1998), and in gene regulation in osteoblastic cells (Shi et al., 1999; Yang et al., 2000).

We analyzed cartilage from *Hoxc8* and *Hoxd4* transgenic mice generated in the VP16-based binary system for trans-gene activation (Kappen, 1999). Overexpression of the homeobox genes *Hoxd4* and *Hoxc8* results in severe cartilage defects and perinatal lethality (Yueh et al., 1998; Kappen et al., 2004). The cartilage of the ribs in transgenic animals is weak and structurally insufficient, resulting in pulmonary failure and death shortly after birth. Vertebral and rib cartilage contain an accumulation of proliferating chondrocytes, indicating that cartilage maturation is affected by overexpression of *Hoxc8* and *Hoxd4*, respectively, with reduced progression of chondrocytes to hypertrophy. These results implicate *Hox* genes as important regulators of cartilage development and differentiation.

Folic acid is a water-soluble vitamin in the B-complex group and an essential nutrient. Supplementation of folate in the diet of pregnant mothers is known to reduce the risk for neural tube defects (Czeizel, 1996) and craniofacial anomalies (Czeizel et al., 1999). The effects of folate on skeletal development are poorly understood. Cell culture experiments show that primary chondrocytes isolated from neonatal ribs need folate to proliferate and differentiate to hypertrophy (Kappen et al., 2004). We previously showed that the cartilage defects induced by overexpression of *Hoxd4* can be rescued by folate supplementation (Kappen et al., 2004). Alcian Blue staining of cartilage in ribs and vertebral column was restored, and rigidity of the skeleton was improved (Kappen et al., 2004). Taken together with an earlier report of beneficial action of folate on cranial bone development in the *Cart-1*-mutant mouse (Zhao et al., 1996), these are the first reports implicating folate in skeletal development in mice genetically predisposed to defects in the skeletal system.

Given that the *Hoxd4*-induced cartilage defects respond to folate supplementation and that chondrocytes require folate for proliferation and differentiation, we hypothesized that the observed defects in chondrocyte proliferation and differentiation in *Hoxd4* and *Hoxc8* transgenic mice might be due to local folate deficiency. Local folate deficiency could arise in transgenic chondrocytes if the expression of enzymes that metabolize folate is altered by the overexpression of the *Hox* transgenes. The metabolism of folate affects multiple cellular processes, including methylation of DNA and proteins, and their synthesis. Any one of the cellular processes involving folate or its metabolites (for a schematic, see Fig. 1) might be affected in delayed maturation of chondrocytes. For example, defective DNA synthesis could be responsible for the apparently longer cell cycle in *Hoxc8* transgenic chondrocytes (Cormier et al., 2003); similarly, reduced protein synthesis would be consistent with reduced proteoglycan content of *Hoxc8* transgenic cartilage (Yueh et al., 1998), and other transcriptional changes could be associated with altered DNA or protein methylation. Finally, an elevated rate of elimination of folate from the metabolic cycle could also account for reduced availability of folate in chondrocytes. To investigate the proposition that folate pathway genes might be directly regulated by Hox transcription factors, we assayed the expression of folate pathway genes in chondrocytes from *Hoxd4* and *Hoxc8* transgenic mice.

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MATERIALS AND METHODS

Transgenic Mice

We used mice created by the VP16-dependent binary system (Kappen, 1999) for expression of *Hox* transgenes. The characterization of the phenotypes and similarities of defects in *Hoxd4* and *Hoxc8* transgenic mice have been published (Yueh et al., 1998; Kappen et al., 2004). In brief, the transactivator transgenic line (TA) harbors the transgene encoding VP16 under the control of the developmentally regulated promoter from the *Hoxc8* gene. The other line, the transresponder (TR), harbors a *Hox* transgene under the control of an immediate early (IE) promoter from the *ICP4* gene of herpes simplex virus 1. Activation of the IE promoter of the TR transgene requires the presence of VP16 protein. All transgenes were on a homogenous FVB inbred genetic background.

Hoxc8 transgenic mice are obtained from crosses of the T239 transactivator line (TA/TA +/+) to mice carrying both the *Hoxc8*-VP16 TA and the IE-*Hoxc8* TR transgenes in hemizygous configuration (TA/+ TR/+). From these matings, we obtained 4 different genotypes: TA/+ +/+, TA/TA +/+, TA/+ TR/+, and TA/TA TR/+, the first 2 of which do not contain the *Hoxc8* TR transgene and therefore served as littermate controls. The *Hoxc8* transgenic mice are born with open eyes, a condition already evident at the time of collection, providing an easy visual ascertainment criterion for the expression of the *Hoxc8* TR transgene (Yueh et al., 1998). *Hoxd4* transgenic mice were generated by crossing the same transactivator strain (T239) to an IE-*Hoxd4* hemizygous transresponder strain (+/+ TR/+). Lack of eyelid closure is also fully concordant with *Hoxd4* transgene status (Kappen et al., 2004). The results presented here are classified by the control genotype containing at least one TA but *no* TR locus (TA/+ +/+, or TA/TA +/+, also referred to as TA only), and the experimental genotype containing at least one TA and one TR locus (TA/+ TR/+ or TA/TA TR/+, also referred to as TA + TR).

Genotyping was performed by semiquantitative PCR (Rundle et al., 1998; Yueh et al., 1998) on DNA isolated from tails of individual animal specimen. All samples were processed with tracking of information on individual and family of origin.

Preparation of Primary Chondrocytes from Transgenic Mice

At 18.5 days of gestation (the morning with presence of a vaginal plug being counted as 0.5 days), pregnant dams were sacrificed following standard laboratory procedures, and the embryos (day 18.5 of gestation) were collected. Individual rib cages were dissected as described previously (Cormier et al., 2003). Cells were transferred into Trizol reagent (Invitrogen, Carlsbad, CA) and RNA was extracted as follows: 20% vol chloroform was added, and samples were shaken vigorously by hand for 15 sec, followed by centrifugation at 12,000 rpm for 15 min at 4°C. The upper aqueous layer was removed to fresh tubes, and 5 μg glycogen was added to assist precipitation, which was performed with 0.5 ml isopropyl alcohol/ml of Trizol. Tubes were placed at −20°C for 20 min followed by centrifugation at 12,000 rpm for 15 min at 4°C. The supernatant was removed and the pellet was washed with 500 μl of 70% isopropyl alcohol. After final centrifugation at 7500 rpm for 5 min at 4°C, the supernatant was removed, and the pellet was air dried for 5 to 10 min and dissolved in 100μl of ultra pure DNase/RNase-free water.

Complementary DNA was obtained by reverse transcription (1st Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA) of at least 5 μg of RNA of each sample, following the supplier's instructions. Purification of cDNA was accomplished using QIAquick PCR purification columns (Qiagen, Valencia, CA). RNA as well as cDNA concentrations were

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measured with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE).

Primers

Primers for amplification were designed using Primer Express software (Applied Biosystems, Foster City, CA). For all primer pairs, the same parameters were used: Tm (melting temperature) requirements: minimum Tm 58°C; maximum Tm 60°C; optimal Tm 59°C; GC content requirements: minimum GC content 30%; maximum GC content 80%; length requirements: minimum length 9; maximum length 40; optimal length 20 base pairs; amplicon requirements: minimum Tm 0°C; maximum Tm 85°C; minimum length 50; maximum length 150 base pairs. Primers for the gene *Gapdh* were used as provided by Applied Biosystems. The locations and sequences of primers are listed in Table 1. Where possible, the expected product amplicon was designed to span an exon/exon junction so that amplification of potentially contaminating genomic DNA would be excluded.

Quantitative Real-Time PCR

Gene expression was evaluated in independent families of *Hoxc8* and *Hoxd4* transgenic mice and their nontransgenic littermates using the ABI Prism 7000 Instrument (Applied Biosystems). Each PCR reaction (25 μl) was performed on 4 ng of template cDNA, 100 nM each primer, and SYBR Green Master Mix (Applied Biosystems). The PCR reactions consisted of a denaturation step of 15 sec at 95°C, annealing for 2 min at 50°C, and extension for 1 min at 60°C, for a total of 40 cycles. Each cDNA sample was assayed in triplicate, and the cycle number at first detection of signal above threshold (Ct) was determined. Analysis was performed with ABI Prism 7000 SDS Software Version 1.0 (Applied Biosystems). Triplicate measurements for a given sample were averaged, and the values for each gene were then normalized to measurements for *Gapdh* cDNA in the same sample applying the formulas: $Ct_{gene} - Ct_{Gapdh}$ ΔΔCt. Comparison of transgenic samples to nontransgenic littermate controls was achieved in a second subtraction that yielded the ΔΔCt values: ΔΔCt $\Delta\Delta Ct_{transgenic} - \Delta Ct_{control}$. Amplification efficiencies were determined for each gene-specific reaction from the slope of the linear portion of the amplification reaction averaged over at least 10 samples and yielded the efficiencies and amplification rates shown in Table 1. Amplification rates did not differ between controls and transgenic samples; we thus did not see a need for further correction of the normalized ΔCt or ΔΔCt values, as this would only affect estimations of absolute levels of gene expression but not the relative levels in a comparison of experimental and control samples. Consequently, all data presented here have to be considered relative quantifications, and the results are therefore presented as ΔCt or ΔΔCt units. In order to estimate the “relative fold change” in expression level for comparisons of select samples, we used the formula $f = r^{\Delta\Delta Ct}$, with r representing amplification rate (r = amplification efficiency e + 1).

Statistical Evaluation

For statistical analyses, we used the software modules implemented in Microsoft Excel (Microsoft, Redmond, WA) as well as S-PLUS 6.2 for Windows (S-PLUS; Insightful Corp., Seattle, WA). Student *t* tests were conducted to analyze difference in gene expression between the controls and TR-containing transgenic mice.

RESULTS

In the first assays, we measured the levels of expression of *Hoxc8* and *Hoxd4* in respective control and transgenic animals. *Hoxd4*-transgenic and control animals were generated in crosses of parents with the genotypes TA/TA +/+ and +/+ TR/+. Six different families with a total of 29 TR transgenic progeny (TA/+ TR/+) and 26 control progeny (TA/+ +/+) were

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investigated. The mean cycle number above the detection threshold level (Ct) for *Hoxd4* expression in control samples was 35.8 ± 1.7 (mean ± SD); this relatively high number indicates relatively low to absent expression of *Hoxd4* in non-transgenic control chondrocytes. The mean Ct number in TR-containing samples was 25.3 ± 1.8. This was lower than in controls, indicating that the expression level of *Hoxd4* is higher in these transgenic samples. Normalized to *Gapdh* expression in each sample (Fig. 2A), the mean of the ΔCt was 15.2 ± 1.7 for transgenic *Hoxd4* samples, compared to 5.6 ± 0.9 in the controls (Fig. 2C), demonstrating a statistically significantly elevated expression of the *Hoxd4* gene in transgenic animals ($P < .0001$). On average, *Hoxd4* transgene activation leads to 15.4-fold higher levels of *Hoxd4* expression in transgenic chondrocytes with very low expression in control samples. To visualize the distribution of values in each experimental group, we ordered ΔCt values into bins of equal intervals and plotted the fraction of observations that fell into each bin interval in histograms (Fig. 2E and G). As shown in Figure 2E, 73% of the ΔCt values in control samples were between 14 and 17. In transgenic samples (Fig. 2G), the distribution is centered in the 4 to 6 interval (72% of ΔCt values), illustrating the elevated *Hoxd4* expression levels in *Hoxd4* transgenic samples. Overlaid on the histograms are nonparametric estimates of the probability density functions. Each density curve describes the overall pattern of a distribution that has an area of exactly 1 underneath it. From the density graphs for *Hoxd4* transgenic and control samples (Fig. 2E and G), it is evident that there is no overlap of *Hoxd4* gene expression levels between controls and transgenic mice, demonstrating that *Hoxd4* is overexpressed only in TR-containing mice.

Results for 23 families with 85 *Hoxc8* transgenic (TA + TR) and 78 control individuals (TA only) also showed a significant overexpression of the *Hoxc8* transgene (Fig. 2B, D, and H). The Ct number for the *Hoxc8* gene in controls was 26.8 ±2.2, representing the normal level of *Hoxc8* expression in chondrocytes, compared to 24.5 ±1.7 in TR-carrying samples. Normalized to the expression levels of *Gapdh* in each sample, the mean ΔCt value was 3.3 ±1.2 for the *Hoxc8* transgenic animals compared to 0.78 ±0.94 for the control samples ($P < .0001$), demonstrating significantly increased expression of *Hoxc8* in these transgenic mice (Fig. 2D). On average, *Hoxc8* expression in *Hoxc8* transgenic primary chondrocytes is elevated by 2.52 cycles, or 4.6-fold over the mean of the controls. The significant differences of the ΔCt values between transgenics and controls demonstrate that our RT-PCR system is able to identify increased expression of genes of interest (Table 2) in our animal models.

The histograms in Figure 2E, G, F, and H reveal, for each control or transgenic group, that individual measurements of ΔCt were distributed over a range of values. Quantile-quantile plots (Q-Q plots) were used to assess normality of the distributions. For *Hoxd4* transgenic as well as control samples, the points on a Q-Q plot were close to a straight line (Fig. 2I and K), indicating that the data are distributed normally. The Q-Q plots for *Hoxc8* transgenic and respective control samples (Fig. 2J and L) again showed that the results followed a normal distribution.

The histograms for *Hoxc8* transgenic and control progeny (Fig. 2F and H) show that there was some overlap of *Hoxc8* gene expression levels between the controls and transgenic samples. This was expected, since the *Hoxc8* gene is normally expressed in rib chondrocytes, and *Hoxc8* transgene activation elevates the overall *Hoxc8* expression levels (Rundle et al., 1998). If animals were grouped strictly based upon the levels of *Hoxc8* expression, the overlap between the groups of nontransgenic and transgenic animals would cause concern about potential misclassification. If a cutoff value of 2 is used to divide the mice into control and transgenic group, 9 control mice, and 5 TR-containing mice would be misassigned, representing fractions of 12% and 6%, respectively. Conversely, 88% and 94% of animals, respectively, would be classified correctly, providing a 90% confidence level to

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classification solely on the basis of *Hoxc8* gene expression levels. In contrast, the *Hoxd4* transgene is activated in cells that do not normally express *Hoxd4*. Consequently, discrimination between controls and *Hoxd4* transgenic individuals by *Hoxd4* expression levels was absolute. Nevertheless, we used genotyping for transgene status to unequivocally classify control and transgenic samples. Since it is conceivable that any changes in folate pathway gene expression are dependent on the relative level of transgene expression, we correlated all measurements to transgene expression levels in each individual sample.

Genes of the folate pathway investigated in this study are listed in Table 2. Expression of each gene was assayed by quantitative real-time PCR in triplicates on chondrocytes from individual rib cages of our *Hoxd4* and *Hoxc8* transgenic animals. For the *Hoxd4* transgenic line, we investigated at least 3 different control and 8 TR-containing samples (Fig. 3A). At least 5 controls and 6 TR-containing samples were investigated for the *Hoxc8* transgenic line (Fig. 3B). The results are plotted as ΔCt (expression level for each gene normalized to *Gapdh*) relative to the ΔCt values for *Hoxd4* or *Hoxc8* gene expression, in each sample. Lower ΔCt values indicate higher gene expression levels; high ΔCt values correspond to lower expression levels. Each data point represents the average of triplicate measurements for an individual animal. Animals carrying a TR transgene (Fig. 3A and B; filled symbols) always had a lower ΔCt value for expression of the *Hoxd4* and *Hoxc8* transgene, indicating that *Hoxd4* and *Hoxc8* gene expression is higher than in non-transgenic littermate controls (Fig. 3A and B; open symbols). Individual genes in the folate pathway were found expressed at different relative levels; for example, the low ΔCt values for *Dhfr* indicate high overall expression, the high ΔCt values for *Cbs* and *Nat3* indicate that expression of these genes is generally at very low levels or absent in chondrocytes. These results show that the quantitative PCR assay detects folate pathway gene expression in primary chondrocytes with high fidelity. The close clustering of data points for each gene in the X-axis dimension demonstrates high consistency of measurements between animals, with higher levels of expression for *Dhfr, Tysy, Sahh*, and *Mat2a*, intermediate expression for *Dnmt, Nat1*, and *Nat2*, and low expression or absence of *Folr1, Nat3, Mat1a*, and *Cbs*.

The comparison of folate pathway gene expression between transgenic and control animals revealed no significant differences for the ΔCt values of folate pathway genes. All 22 genes investigated were expressed at the same relative levels in *Hoxd4* transgenics compared to nontransgenic controls; the same observation pertains to *Hoxc8* transgenic animals. Table 3 summarizes all results as means of ΔCt values. No significant differences in expression of folate pathway genes were detected between transgenics and respective controls; there were also no differences between just the 2 control groups or the transgenic groups from the 2 strains.

We also assessed the expression of each *Hox* gene in the rib cage chondrocytes from the reciprocal transgenic strain. *Hoxc8* gene expression in *Hoxd4* transgenic samples and littermate controls (Fig. 4A) did not reveal significant differences (P = .57; Fig. 4C). *Hoxd4* expression levels in *Hoxc8* transgenic mice were very low as indicated by a high mean ΔCt, and slightly lower on average in the transgenic chondrocytes compared to littermate controls (Fig. 4B and D). While this result reached statistical significance of P = .037, its biological significance is unclear at present given that the standard deviations for transgenic and control groups broadly overlap (Fig. 4C and D).

DISCUSSION

The goal of this study was to elucidate consequences of *Hox* transgene expression in chondrocytes at the transcriptional level. After discovering a beneficial effect of folate on cartilage development in *Hoxd4* transgenic animals, we hypothesized that *Hox* transgene

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expression might affect the expression of folate pathway genes in the chondrocytes of *Hoxd4* or *Hoxc8* transgenic mice. We therefore set out to quantitatively measure *Hox* gene and transgene expression levels in *Hoxc8* and *Hoxd4* transgenic chondrocytes from individual animals and correlate these with the levels of expression of folate pathway genes in the same samples.

Quantitative real-time PCR was used to measure the relative level of mRNA present in chondrocyte samples from individual rib cages of transgenic and control animals. All assays were done in triplicate, giving generally high reproducibility for each sample. Between samples, there was considerable variability in gene expression levels, even within the same family. Nevertheless, transgenic groups were clearly distinguishable from control groups, as they showed very little overlap (up to 8.6% of the samples) between ΔCt values. Thus, we establish highly significant correlations between genotype for the respective *Hox* transgene and its expression level.

For *Hoxc8* transgenic chondrocyte samples, we determined a 4.6-fold elevated level of *Hoxc8* expression compared to controls. This is in very good agreement with our previous estimates by semiquantitative PCR and reporter gene assays that showed overexpression of *Hoxc8* by 3.6-fold and 5.7-fold in the posterior region of *Hoxc8* transgenic embryos at midgestation (Rundle et al., 1998). Our current results thus support the conclusion that a similar level of overexpression is achieved in transgenic chondrocytes at late stages of development. In *Hoxd4* transgenic chondrocytes, 15.4-fold increased expression of *Hoxd4* was found compared to controls. This apparently higher magnitude of transgene overexpression compared to the *Hoxc8*-transgenic chondrocytes may result from the fact that *Hoxd4* expression levels in normal chondrocytes are exceedingly low. Alternatively, it is possible that the *Hoxd4* transresponder transgene could be activated to a greater degree than the *Hoxc8* transresponder transgene. However, in a previous study (Rundle et al., 1998), we have shown that efficacy of VP16-mediated transactivation is less dependent on the transresponder transgene than on the levels of activity of the transactivator and genotype status for the transactivator transgene (homozygous TA/TA versus hemizygous TA/+). The greater magnitude of *Hoxd4* transgene overexpression is consistent with the finding that the defective cartilage phenotype is more pronounced in *Hoxd4* transgenics that are hemizygous for the transactivator transgene (Kappen et al., 2004), whereas the most severe phenotype in *Hoxc8*-transgenic cartilage was only found in animals homozygous for the transactivator transgene locus (Yueh et al., 1998). Taken together, these considerations support the notion derived from genetic evidence (Condie and Capecchi, 1993, 1994; Fromental-Ramain et al., 1996) that the level of *Hox* gene expression is critical to normal as well as pathological development.

It is noteworthy that the measurements of *Hox* gene expression levels generally follow normal distributions. The only exceptions are a group of 3 samples (out of 29) in Figure 2G, and a group of 4 samples (out of 78) in Figure 2F; graphs in both figures display bimodal distributions that form a minor peak towards the direction of reduced expression. The 3 samples from Figure 2G are shown in Figure 2K to be outside of the regression line, and, correspondingly, the 4 samples from Figure 2F also deviate from the regression line in Figure 2J. The reasons for these deviations are unknown, but apply to only a small number of samples. The most plausible explanation involves technical considerations: for low gene expression levels in rib chondrocytes, experimental errors are expected to have larger impact on normalization calculations.

This proposition is borne out when we analyze the size of standard deviations for all gene measurements. For samples with very low mRNA prevalence of the gene of interest, and hence higher ΔCt values, we find larger standard deviations for the triplicate measurements

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for each sample (Fig. 5A and B, for *Hoxd4* and *Hoxc8*, respectively). This trend is also confirmed for the means of all individuals and the standard deviation for each folate pathway gene measurement (Fig. 5C and D).

The normal distributions of gene expression for transgene as well as endogenous *Hox* gene expression levels indicate that even in fully inbred genetic strains with defined genetic background (here FVB), there exists variability in gene expression levels. The range from lowest to highest ΔCt value covers 8 cycles within the control groups and 6 cycles within the transgenic groups. It should be noted, however, that each range includes the most extreme values. Considering the majority of data points (75%), the expression levels of *Hoxd4* in normal primary chondrocytes vary by 2.4-fold, and within transgenic samples, by 1.5-fold. Thus, even though the animals are genetically identical in their transgene configuration and inbred genetic background, the respective populations vary in *Hoxd4* expression levels. This is also evident for *Hoxc8* expression, both in control as well as in *Hoxc8* transgenic samples. In the control group, 75% of the samples fall in a range of up to 4.6-fold difference; measurements between transgenic samples vary by 3.4-fold. These measurements are, to our knowledge, the first demonstration of variation in *Hox* gene expression levels in populations of genetically identical animals.

It is unlikely that this variation is entirely due to potential technical error: although it is possible that our chondrocyte preparations could contain some nonchondrocytic cells, this proportion consistently was less than 5% (Cormier et al., 2003), and all cell preparations for this study were performed by the same experienced individual. Furthermore, all measurements were normalized to an internal reference gene, *Gapdh*, excluding differences in cell yield as a confounding variable. Therefore, we believe that the distributions of relative expression levels reflect genuine biological variability in gene expression or in the frequency of *Hoxc8*-expressing cells within the cartilage. It is noteworthy in this context that the range of variability (variance) in control groups statistically is not significantly different from the variances exhibited in transgenic groups (data not shown), further supporting the notion that variability is not associated with the technical system, but is rather a biological phenomenon. While in transgenic cartilage, the range of expression levels may be associated with the severity of cartilage defects, the functional relevance of such variation in *Hox* gene expression levels in normal mice remains to be investigated.

Our studies show that *Hoxd4* and *Hoxc8* gene expression levels were significantly upregulated in transgenic animals compared to controls, consistent with the cartilage defects that manifest postnatally (Yueh et al., 1998; Kappen et al., 2004). However, measuring the gene expression of 18 different enzymes involved in the folate pathway as well as the *Folate receptors 1, 2*, and *4* and the *Reduced folate carrier 1* (Table 2) in chondrocytes from rib cages of individual *Hoxd4* and *Hoxc8* transgenic mice, we found no significant differences in mRNA levels compared to littermate controls. Thus, our results do not provide evidence in support of the notion of local misregulation of folate pathway genes. This still leaves open the possibility that enzymatic activities affecting folate metabolism could be altered. Furthermore, it is conceivable that supplementation of folate affects pathways other than its own metabolism, a proposition we are currently investigating by performing quantitative RT-PCR assays for genes known to be involved in chondrocyte maturation as well as microarray studies.

Our *Hoxc8* transgenic mice overexpress *Hoxc8* under control of its own promoter. Thus, overexpression of the Hoxc8 transgene occurs in cells that express Hoxc8 normally during skeletal development, namely chondrocytes. *Hoxd4* transgene expression within the *Hoxc8* domain is achieved by using the same *Hoxc8* regulatory sequences as for the *Hoxc8* transgene. Transgenesis results in significantly elevated *Hoxd4* expression in *Hoxd4*

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transgenic chondrocytes. Yet, there is no effect on expression of the *Hoxc8* gene in these animals. Consistent with expression in normal chondrocytes, *Hoxc8* was detectable at normal levels in *Hoxd4* transgenic rib cage chondrocytes. In contrast, *Hoxd4* is expressed at very low levels in normal rib chondrocytes; in *Hoxc8* transgenic cells, *Hoxd4* levels appeared to be somewhat lower (albeit with marginal statistical significance). These results indicate that changes in *Hoxd4* expression have no effect on the level of *Hoxc8* expression, and vice versa, overexpression of *Hoxc8* does not affect *Hoxd4* mRNA levels to a considerable degree. Therefore, we conclude that it is unlike that *Hoxc8* and *Hoxd4* regulate each other's gene expression; instead, each transgene acts independently.

The expression of folate pathway enzymes in cartilage or chondrocytes has not previously been investigated. Specific folate transport proteins, the folate receptors, are required for the uptake of folate into cells (Antony, 1996). We showed recently that *Folate receptor 2* is expressed in mouse chondrocytes (Kappen et al., 2004). From the current data, mRNAs for *Folate receptors 1* and *4* are also detectable, albeit at very low levels. The reduced folate carrier (Rfc1) is a typical member of the major facilitator superfamily and mediates the transport of folate in mammalian cells (Sirotnak and Tolner, 1999). This carrier system generates uphill folate transport through an exchange with organic phosphates concentrated within the intracellular compartment (Henderson and Zevely, 1983; Yang et al., 1984). We find that the *Rfc* gene is transcribed in neonatal chondrocytes, consistent with the postulated widespread expression of *Rfc1* in the developing embryo (Maddox et al., 2003). Overall, we found no alterations in the expression of any of the folate transport proteins in *Hoxd4* or *Hoxc8* transgenic chondrocytes, suggesting that—at least at the developmental stage analyzed—folate transport in these cells is likely normal.

Our data also provide no evidence that any of the transcripts encoding folate-metabolizing enzymes are affected in expression levels by *Hoxd4* or *Hoxc8* overexpression. Widespread expression in many tissues has been reported for *Dhfr, Sahh, Mthfr, Msr, Cbs, Ms, Tysy*, and *Amd*, with apparently differential levels of expression for the latter four genes in different adult tissues (Robert et al., 2003). Interestingly, targeted disruption of *Cbs* was recently reported to affect endochondral ossification (Robert et al., 2005) through impaired cartilage differentiation primarily of the long bones. We did not detect obvious changes in the expression of *Cbs* at the transcript level in our transgenic models, suggesting that the mechanisms for impaired cartilage differentiation are different from hyperhomocysteinemia. *Mat1* expression is believed to be restricted to liver, with *Mat2* expressed in the embryo and non-liver tissues (Horikawa et al., 1993; Gil et al., 1996). Consistent with this notion, although both *Mat1* and *Mat2* could be measured, the level of detection of *Mat1* in chondrocytes was very low. S-adenosylhomocysteine hydrolase (Sahh) is the only enzyme that cleaves S-adenosylhomocysteine, and detection of mRNA in chondrocytes is consistent with the reported ubiquitous expression (Hershfield and Krodich, 1987; Chiang et al., 1996) in proliferating cells (Radomski et al., 1999). *Bhmt* expression appears to be restricted to liver and kidney (Sunden et al., 1997), which also express *Bhmt 2* (Chadwick et al., 2000), although in embryos, somewhat wider distribution was noted (Chadwick et al., 2000). Both *Nat1* and *Nat2* genes are expressed early in mouse development in the embryo-placental complex, as well as in the placental tissue (Stanley et al., 1998; Mitchell et al., 1999), with overlapping expression in many adult tissues (Windmill et al., 2000). It is believed that these acetyltransferases are involved in folate catabolism (Upton et al., 2001). We detected transcripts for all of these folate pathway genes in primary chondrocytes. DNA methylation is essential for development, and Dnmt1, Dnmt3a, and Dnmt3b are responsible for de novo methylation of the mouse genome during early embryonic development (Okano et al., 1998).

Expression of all *Dnmt* transcripts was detected in our assays. Thymidylate synthase (Tysy) catalyses the conversion of dUMP and 5–10-methylene-tetrahydrofolate to dTMP and

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dihydrofolate. This is the de novo source of deoxythymidylate for DNA replication (Zhao and Goldman, 2003). The levels of cellular folate metabolites are thus important for nucleotide synthesis (Herbig et al., 2002). Tysy and dihydrofolate reductase (Dhfr) are expressed in many tissues (Baker, 1969; Priest et al., 1981), some at low levels (Mudd et al., 1965), and Tysy expression is cell-cycle dependent. In our transgenic mice, no changes were found in *Tysy* gene expression, making it unlikely that DNA synthesis is directly affected by *Hox* transgene expression. Expression levels for *Dhfr*, whose product reduces folate to tetrahydrofolate, as well as *Mthfr*—both important candidates in the folate pathway—or gene for enzymes involved in the methionine remethylation cycle, such as *Ms, Msr, Bhmt, Sahh*, and *Mat*, show no alterations in transgenic chondrocytes. Interestingly, both the Tysy and Dhfr enzymes can bind to their cognate mRNAs and in this way autoregulate expression via translational repression (Tai et al., 2004). Thus, in addition to transcriptional regulation, cell- or tissue-specific mechanisms control the availability of enzymes in the folate pathway for folate metabolism.

Targeted gene disruption in mice demonstrated that Folbp1 (Piedrahita et al., 1999), Rfc (Zhao et al., 2001), Amd (Nishimura et al., 2002), Dmnt1 (Li et al., 1992), Sahh (Miller et al., 1994), and Ms (Swanson et al., 2001) all are essential, while Folbp2 (Piedrahita et al., 1999), Nat1 and 2 (Sugamori et al., 2003), are not required for embryonic development. No skeletal abnormalities have been reported for those mutants that survive postnatally. We have previously shown (Kappen et al., 2004) that folate metabolism is required for chondrocyte proliferation and differentiation, and that defects in cartilage maturation in *Hoxd4* transgenic mice can be rescued by folate supplementation (Kappen et al., 2004), but which specific pathways involve folate in chondrocytes remains to be investigated. From our current studies, we conclude that overexpression of *Hoxd4* and *Hoxc8*, respectively, does not perturb folate pathway gene expression. The response to folate in *Hoxd4* transgenic mice therefore likely involves mechanisms other than the transcriptional regulation of folate metabolizing enzymes.

A second important implication from our results is that the expression of enzymes that metabolize folate does not appear to be regulated by *Hox* transcription factors. Thus, folate pathway genes may only be indirect targets of *Hox* transgene overexpression in our transgenic system, and other targets of Hox transcription factors must be responsible for the cartilage defects. Finally, our results allow us to determine whether *Hox* transgene expression affects the expression of other *Hox* genes. The measurements of *Hoxc8* and *Hoxd4* in the reciprocal transgenics strains do not provide evidence for such cross-regulation, and thus exclude *Hox* genes themselves as potential target genes.

To our knowledge, the analyses we present here constitute the first comprehensive assessment of folate pathway metabolic genes in primary mouse tissue of late stage embryos. Other tissues have not yet been evaluated in a systematic fashion, but the reagents and methodology established here make such assays straightforward. Our expression studies thus provide a baseline for future studies into the role of folate metabolism in skeletal development, and in embryonic tissues in general.

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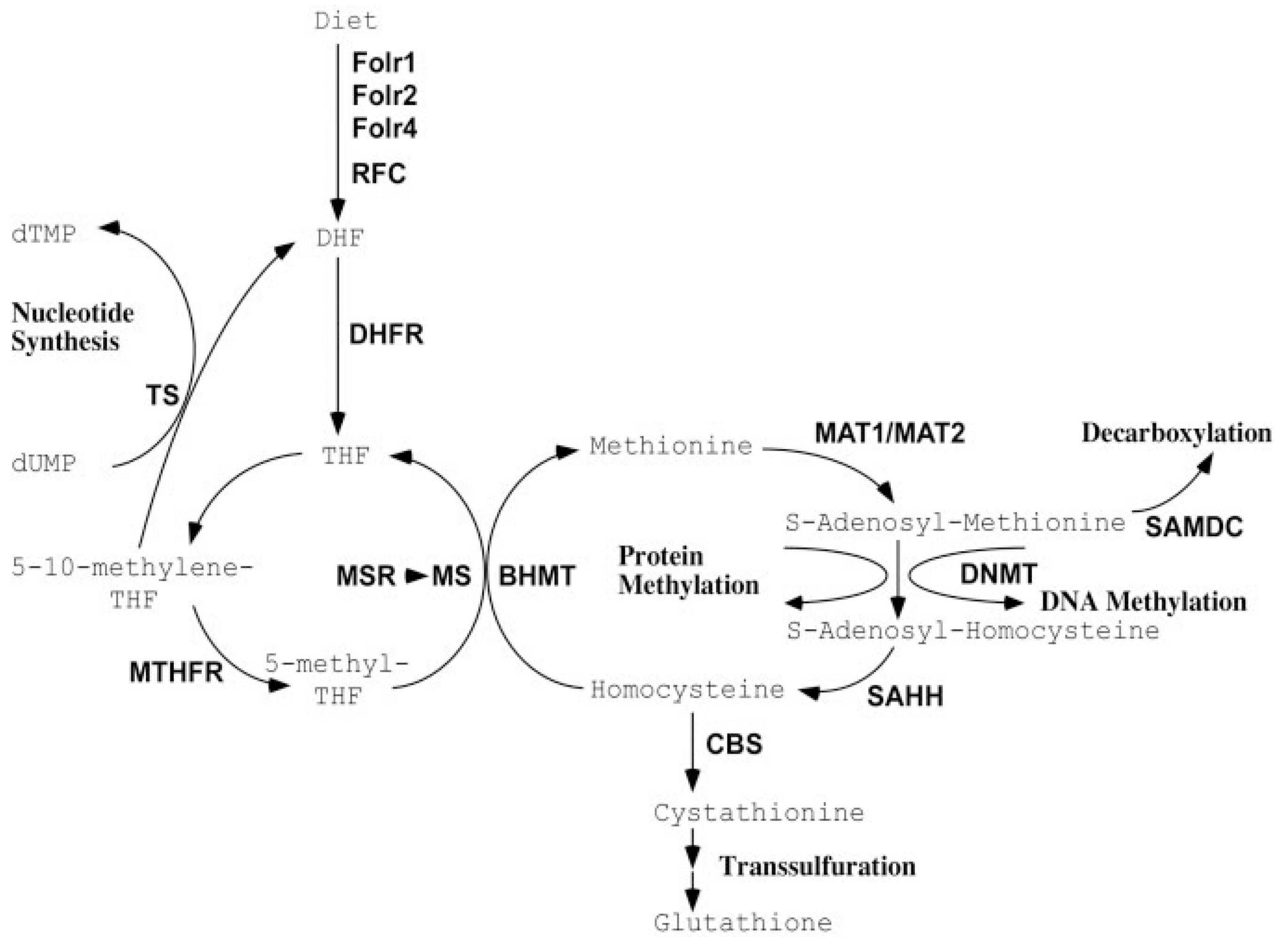

Figure 1.
Folate pathway. Diagram of folate metabolic pathway compiled from the literature.

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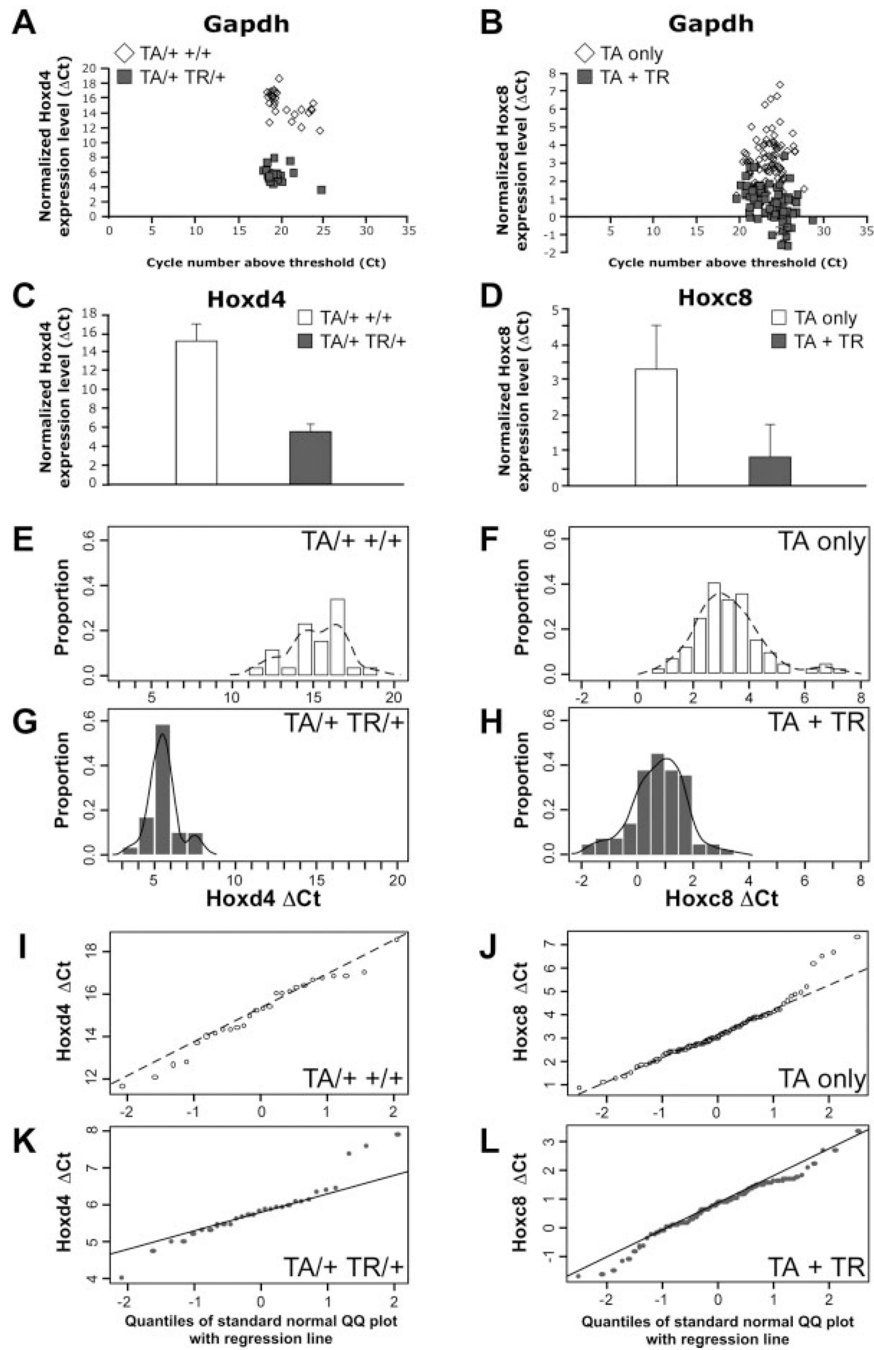

Figure 2.

Increased expression of the homeobox genes *Hoxd4* and *Hoxc8* in chondrocytes from transgenic mice. The expression of *Hoxd4* was measured by quantitative RT-PCR (qRT-PCR) in 56 samples from 6 different families, and expression of *Hoxc8* was assayed in 163 samples from 23 families. The Ct value is the cycle number for detection above threshold, and the ΔCt value is the Ct value normalized to concurrent determination of *Gapdh* expression in each sample. The Ct value for *Gapdh* does not differ between controls (open symbols, open bars) and transgenic (filled symbols, filled bars) samples in *Hoxd4* (**A**) and *Hoxc8* (**B**) families. A low ΔCt value indicates a high level of expression and a high ΔCt

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value indicates low level of expression, respectively, of *Hoxd4* (A,**C**) or *Hoxc8* (B,**D**) in these samples. Histograms show the distributions for the *Hoxd4* (**E,G**) and *Hoxc8* (**F,H**) expression levels, separately for the controls (TA only; E,F) and the transgenic mice (TR-containing; G,H). The histograms are scaled as proportions of total data points, depicting probability density; the sum of the bar heights multiplied by bar widths will equal 1 (note the different scale of x-axis between E/F and G/H, respectively). A nonparametric estimate of the probability density function is overlaid in solid line. Normal quantile plots (**I–L**) indicate that the data are distributed normally, as the points lie close to a straight line.

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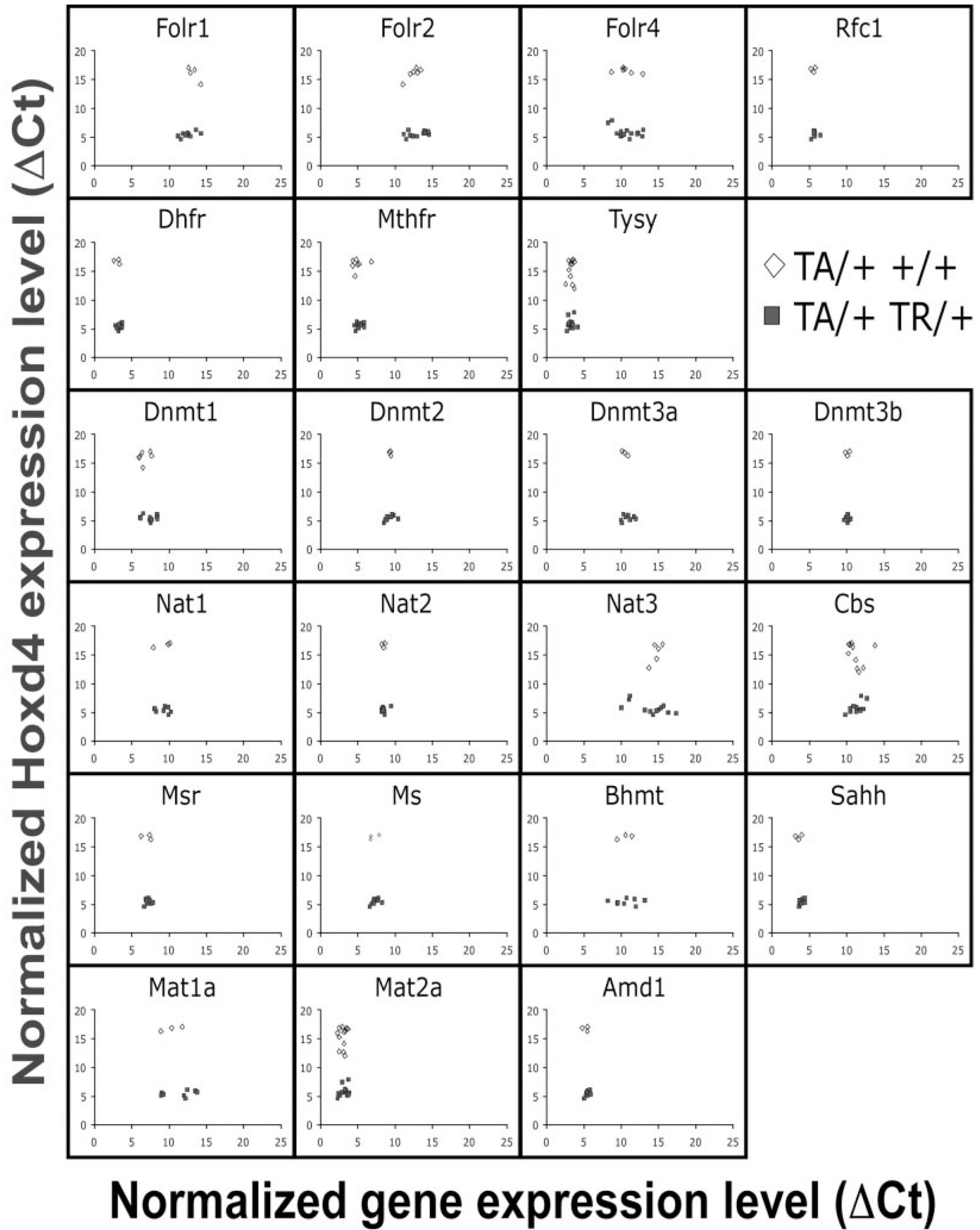

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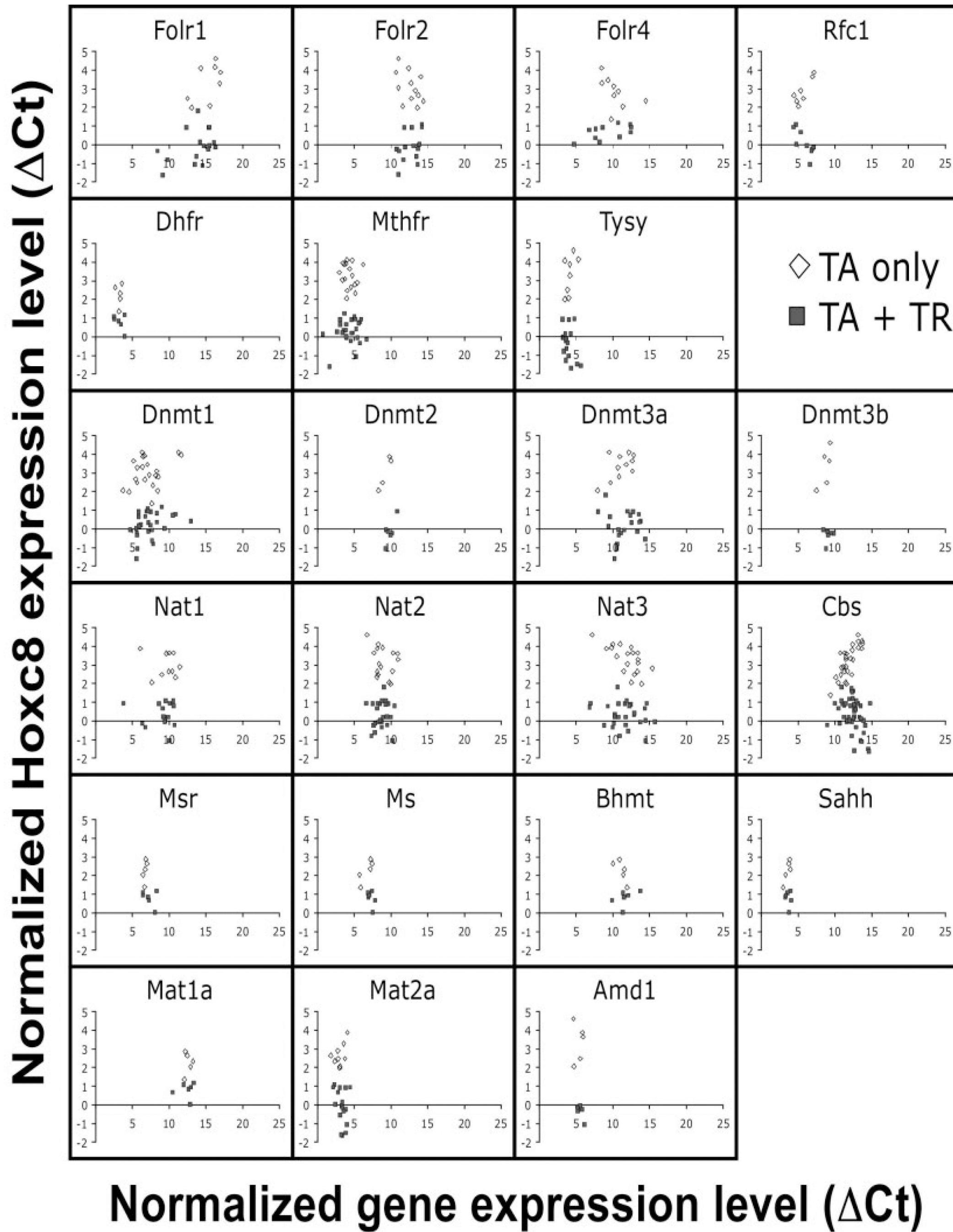

Figure 3.

Expression of folate pathway genes in *Hoxd4* and *Hoxc8* transgenic chondrocytes. Quantitative RT-PCR was performed in triplicate on mRNA of primary chondrocytes from rib cages of individual *Hoxd4* (**A**) and *Hoxc8* (**B**) transgenic mouse embryos isolated at 18.5 days of gestation. *Gapdh* cDNA levels in each sample were used to standardize measurements. The results are plotted as cycle number above the detection threshold (Ct) for each primer pair relative to *Gapdh*-normalized *Hoxd4* and *Hoxc8* gene expression (ΔCt) in each sample. Low ΔCt values reflect higher relative expression levels, and high ΔCt values

reflect low relative levels of gene expression. Each symbol represents an individual animal (open symbols = controls; filled symbols = TR-containing samples).

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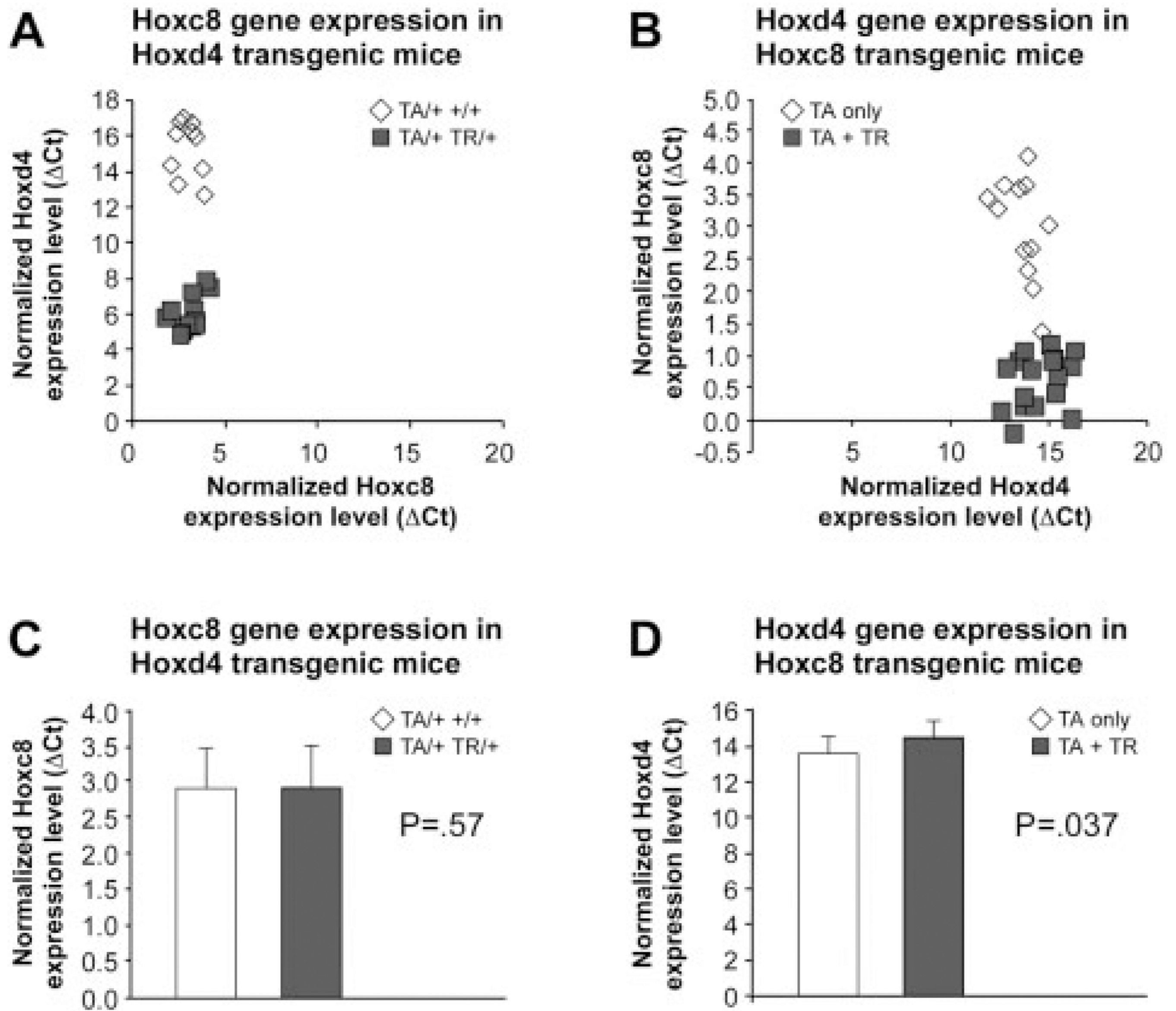

Figure 4.

Expression of *Hoxd4* and *Hoxc8* in transgenic chondrocytes. Expression of the *Hoxc8* gene was measured in individual *Hoxd4* transgenic and control samples (**A**), and *Hoxd4* expression was assayed in individual *Hoxc8* transgenic and control chondrocytes (**B**). Open symbols = controls; filled symbols = TR-containing samples. The results are plotted as *Gapdh*normalized *Hoxc8* and *Hoxd4* expression levels relative to normalized levels of *Hoxd4* and *Hoxc8*, respectively. Statistical evaluation by Student *t* test of control and transgenic groups (**C, D**) indicates a possible difference of *Hoxd4* levels ($P = .037$) in *Hoxc8* transgenic samples.

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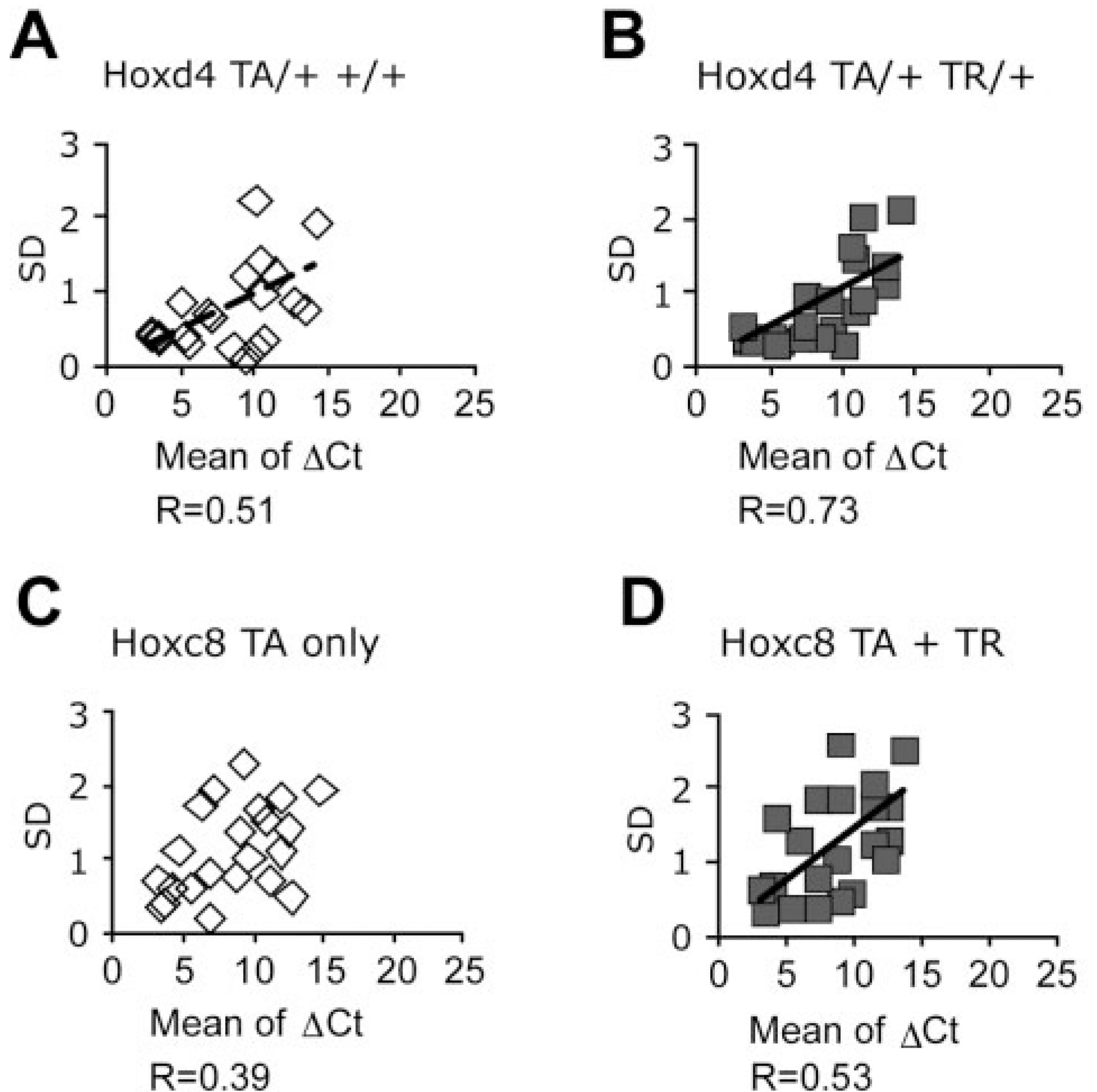

Figure 5.

Biological variability in folate pathway gene expression is similar in control and *Hox* transgenic animals. Graphic display of results in Table 3. Generally, larger standard deviations were found in cases of lower expression level (high ΔCt value) with correlation coefficients noted below each panel.

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Table 1

Genes Investigated in This Study: Primer Location, Sequences, and Efficiency*

Abbreviation	Full name (alternative abbreviation)	Accession #	Forward primer position	Reverse primer position	Exon-exon boundary?
Bhmt	Betaine-homocysteine methyltransferase	NM_016668	458–478	545–523	NO
Cbs	Cystathionine beta-synthase	NM_144855	1086–1106	1161–1184	YES
Dhfr	Dihydrofolate reductase	NM_010049	438–460	558–538	YES
Dnmt1	DNA methyltransferase (cytosine-5) 1	NM_010066	384–413	493–470	NO
Dnmt2	DNA methyltransferase 2	NM_010067	208–236	287–268	YES
Dnmt3a	DNA methyltransferase 3A	NM_007872	751–769	874–849	YES
Dnmt3b	DNA methyltransferase 3B	NM_010068	1542–1563	1646–1622	YES
Folr1	Folate receptor 1	NM_008034	519–536	585–606	YES
Folr2	Folate receptor 2	NM_008035	402–424	473–492	YES
Folr4	Folate receptor 4	NM_022888	946–968	1022–1003	NO
Gapd	Glyceraldehyde-3-phosphate dehydrogenase	NM_008084	649–669	751–733	NO
Hoxc8	Homeobox c8	NM_010466	610–631	725–702	YES
Hoxd4	Homeobox d4	NM_010469	1658–1678	1748–1722	YES
Mat1a	Methionine adenosyl-transferase 1, alpha	NM_133653	1162–1182	1246–1226	YES
Mat2a	Methionine adenosyl-transferase 2, alpha	NM_145569	500–525	580–560	NO
Ms	Methionine synthase (Mtr)	XM_138431	2309–2329	2459–2439	YES
Msr	Methionine synthase reductase (Mtrr)	NM_172480	444–462	522–502	YES
Mthfr	5, 10-methyleneletrahydrofolate reductase	NM_010840	1608–1627	1690–1674	YES
Nat1	N-acetyl transferase 1	NM_008673	921–943	1024–997	NO
Nat2	N-acetyl transferase 2	NM_010874	194–218	276–257	NO
Nat3	N-acetyl transferase 3	NM_008674	598–622	717–693	NO
Rfc1	Reduced folate carrier (Slc19a1)	NM_031196	294–315	344–322	YES
Amd1	S-adenosylmethionine decarboxylase 1	NM_009665	650–674	745–719	NO
Sahh	S-adenosyl homocysteine hydrolase (Ahcy)	L32836	555–578	622–605	YES
Tysy	Thymidylate synthase (Tyms)	NM_021288	234–255	313–291	YES

Abbreviation	Forward primer; sequence	Reverse primer; sequence	Amplification rate	Efficiency (±SD)	Number of samples

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Abbreviation	Full name (alternative abbreviation)	Accession #	Forward primer position	Reverse primer position	Exon-exon boundary?
Bhmt	GAACGTGGACTTCCTCATTGC	GGGCTTACCAGATGCTTTTAAGG	1.95	0.95 (±0.24)	21
Cbs	GACGGAGCAAACAGCCTATGA	CATTGCTCTTGAACCACCTATCC	1.59	0.59 (±0.22)	24
Dhfr	AACCAGGCCACCTTAGACTCTTT	GAGAGGACGCCTGGGTATTCT	2.20	1.20 (±0.07)	22
Dnmt1	GGAAACCAAATTACATAAAGAGGAATTATC	GAGTGAGAGTGTGTGTTCCGTTCT	1.99	0.99 (±0.19)	21
Dnmt2	AGACTTTGACAAGCTATCTTTCAATATGA	CCCCCTGTAGGCCAATTCTT	1.66	0.66 (±0.12)	22
Dnmt3a	CGACCCATGCCAAGACTCA	TTACTGCAATTACCTTGGCTTTCTTC	1.32	0.32 (±0.10)	18
Dnmt3b	AGAAGAACCCTGTGTCCTTCCA	GATAGCCGTCCTCATCATACATGTA	1.78	0.78 (±0.10)	21
Folr1	CGGGCCCTGAGGACAATT	TTATGTGCTTCCTGGCTTGTGT	1.38	0.38 (±0.27)	32
Folr2	GACAAGCTGCATGACCAGTGTAG	GACGGGAGTCAGCCTTGTGT	1.63	0.63 (±0.31)	38
Folr4	CCTTCTCTCTGTGCCTGTTGTTC	GGCCCAGAAGGATCAGGAAA	1.82	0.82 (±0.15)	41
Gapd	CCAGAACATCATCCCTGCATC	GGTAGGAACACGGAAGGCC	1.83	0.83 (±0.11)	78
Hoxc8	CGAAGGACAAGGCCACTTAAAT	AGGTCTGATACCGGCTGTAAGTTT	1.84	0.84 (±0.18)	62
Hoxd4	TTCGGTGAACCCCAACTACAC	AAATTCCTTTTCCAGTTCTAGGACTTG	1.33	0.33 (±0.13)	55
Mat1a	CAGGTGTCCTATGCCATTGGT	GCTCCCGCTCAGTCTTATTGG	1.80	0.80 (±0.23)	21
Mat2a	TGAAGAGTGTATGCCTTTAACCATTG	AATGTACCATTGCGGCGTAGT	1.66	0.66 (±0.17)	40
Ms	AAGCTGCTCTGGACCACAAAG	TGGGTTCTTGAAGTGGTTGCT	1.78	0.78 (±0.12)	22
Msr	CTTGGAGCCCAGCGTTTCT	CAATCCACGGCTCTACCACAA	1.98	0.98 (±0.08)	22
Mthfr	TGCGGGTCAACTACCACATC	CCACGTCACGGCATTGG	1.71	0.71 (±0.16)	30
Nat1	GGGCTCCACCTTTACAAGTAGGA	CGGTTTTCAGTACATCTTCTATTTCTTC	2.05	1.05 (±0.25)	26
Nat2	TGAAAACATTAACCGAAATCCTTCA	CATGGATTCCCCACAATGGA	1.74	0.74 (±0.13)	34
Nat3	TGTAGATAGTGCATTCCCATTTTCC	TACCAGGTTCCATTCTCTTCTCTCA	1.46	0.46 (±0.21)	37
Rfc1	TGGAACGTAAATTCACCAAGGA	GCATCGGAATGATCTCGTTAGTC	1.85	0.85 (±0.14)	26
Amd1	GGAAGAAATCGAGTTTCTTAATGCA	TCCAAAGTATACAAGTACCAGCAGTCA	1.76	0.76 (±0.11)	22
Sahh	GTCACCAAGAGCAAGTTTGACAAC	GCCCGTTTGATGCCATCT	1.94	0.94 (±0.07)	22
Tysy	TTTTGGAGGAGTTGTTGTGGTTT	CATCCCAGATTCTCACTCCCTTT	1.36	0.36 (±0.15)	37

Messenger RNA sequences for the investigated genes were taken from GenBank (accession numbers), and the positions corresponding to primer sequences are listed. Where possible, the amplicon spanned across an exon-exon junction. Sequences of the gene-specific primers are given in the lower half of the table (in 5′ to 3′ direction), as is the amplification rate achieved with each primer set. The amplification rate was calculated from the formula $r = e + 1$ (see Methods), where e was determined from the slope of the detection curve in the linear range. Mean e values (± SDs) were determined from the number of samples given in the last column.

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Table 2

Functional Role of Folate Pathway Genes*

Abbreviation	Enzyme	EC	Reaction catalyzed	Pathway
Bhmt	Betaine-homocysteine S-methyltransferase	2.1.1.5	Trimethylammonioacetate + L-homocysteine = dimethylglycine + L-methionine	Methyl transfer
Cbs	Cystathionine beta-synthase	4.2.1.22	L-serine + L-homocysteine = cystathionine + H_2O	Transsulfuration
Dhfr	Dihydrofolate reductase	1.5.1.3	5,6,7,8-tetrahydrofolate + NADP(+) = 7,7-dihydrofolate + NADPH	Folate metabolism
Dnmt	DNA methyltransferase	2.1.1.37	S-adenosyl-L-methionine + DNA = S-adenosyl-L-homocysteine + DNA containing 5-methylcytosine	DNA Methylation
Mat	Methionine adenosyltransferase	2.5.1.6	ATP + L-methionine + H_2O = phosphate + diphosphate + S-adenosyl-L-methionine	Methyl transfer
Ms	Methionine synthase	2.1.1.13	5-methyltetrahydrofolate + L-homocysteine = tetrahydrofolate + L-methionine	Methylation
Msr	Methionine synthase reductase	1.16.1.8	2 [methionine synthase]-methylcob(l)alamin + 2 S-adenosylhomocysteine + NADP (+) = 2 [methionine synthase]-cob (l)alamin + NADPH + 2 S-adenosyl-L-methionine	Methylation
Mthfr	Methylenetetrahydrofolate reductase	1.5.1.20	5-methyltetrahydrofolate + NADP(+) = 5, 10-methylenetetrahydrofolate + NADPH	Folate metabolism
Nat	Arylamine N-acetyltransferase	2.3.1.5	Acetyl-CoA + an arylamine = CoA + an N-acetylarylamine	Acetyl transfer
Amd	S-adenosylmethionine decarboxylase	4.1.1.50	S-adenosyl-L-methionine = (5-deoxy-5-adenosyl) (3-aminopropyl) methylsulfonium salt + CO_2	Decarboxylation
Sahh	S-adenosyl-L-homocysteine hydrolase	3.3.1.1	S-adenosyl-L-homocysteine + H_2O = adenosine + L-homocysteine	Methionine cycle
Tysy	Thymidylate synthase	2.1.1.45	5, 10-methylenetetrahydrofolate + dUMP = dihydrofolate + dTMP	Nucleotide synthesis
Folr	Folate receptor		Binds to folate and reduced folic acid derivates and mediates delivery of 5-methyltetrahydrofolate to interior of cells	Folate metabolism
Rfc	Reduced folate carrier		Transporter for the intake of folate	Folate transport

*Information on folate pathway genes assayed in this study was assembled from the NCBI (www.ncbi.nlm.nih.gov) and BRENDA (www.brenda.uni-koeln.de) databases.

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Table 3

Normalized Expression of Folate Pathway Genes in *Hoxd4* and *Hoxc8* Transgenic Animals*

	Hoxd4		*Hoxc8*	
Gene	**TA/+ +/+**	**TA/+ TR/+**	**TA only**	**TA + TR**
Bhmt	10.4 ± 0.99 (*n* = 3)	10.6 ± 1.61 (*n* = 8)	11.1 ± 0.74 (*n* = 5)	11.6 ± 1.25 (*n* = 6)
Cbs	11.41 ± 1.25 (*n* = 12)	11.38 ± 0.88 (*n* = 11)	11.77 ± 1.11 (*n* = 27)	12.50 ± 1.28 (*n* = 47)
Dhfr	3.01 ± 0.41 (*n* = 3)	3.3 ± 0.33 (*n* = 8)	3.2 ± 0.35 (*n* = 5)	3.23 ± 0.65 (*n* = 6)
Dnmt	8.19 ± 0.65 (*n* = 3)	8.95 ± 0.56 (*n* = 8)	8.11 ± 0.47 (*n* = 5)	8.48 ± 0.57 (*n* = 6)
Dnmt1	6.1 ± 0.49 (*n* = 6)	6.62 ± 0.55 (*n* = 12)	6.74 ± 2.0 (*n* = 18)	6.89 ± 1.88 (*n* = 28)
Dnmt2	9.31 ± 0.11 (*n* = 3)	9.23 ± 0.48 (*n* = 8)	9.55 ± 1.05 (*n* = 5)	9.82 ± 0.59 (*n* = 6)
Dnmt3a	10.46 ± 0.36 (*n* = 3)	10.79 ± 0.74 (*n* = 8)	10.85 ± 1.54 (*n* = 13)	11.45 ± 1.78 (*n* = 20)
Dnmt3b	10.07 ± 0.27 (*n* = 3)	10.07 ± 0.27 (*n* = 8)	8.67 ± 0.75 (*n* = 5)	9.05 ± 0.48 (*n* = 6)
Folr1	13.33 ± 0.78 (*n* = 4)	12.73 ± 1.10 (*n* = 10)	14.68 ± 1.98 (*n* = 8)	13.83 ± 2.54 (*n* = 17)
Folr2	12.57 ± 0.85 (*n* = 7)	12.75 ± 1.37 (*n* = 12)	12.45 ± 1.42 (*n* = 12)	12.52 ± 1.74 (*n* = 17)
Folr4	9.91 ± 2.24 (*n* = 7)	10.68 ± 1.46 (*n* = 14)	10.34 ± 1.72 (*n* = 10)	9.07 ± 2.59 (*n* = 12)
Mat1a	10.3 ± 1.42 (*n* = 3)	11.35 ± 2.01 (*n* = 8)	12.55 ± 0.49 (*n* = 5)	12.35 ± 1.03 (*n* = 6)
Mat2a	3.01 ± 0.46 (*n* = 12)	3.18 ± 0.52 (*n* = 14)	3.08 ± 0.72 (*n* = 10)	3.17 ± 0.64 (*n* = 18)
Ms	7.04 ± 0.65 (*n* = 3)	7.28 ± 0.54 (*n* = 8)	6.67 ± 0.83 (*n* = 5)	7.24 ± 0.37 (*n* = 6)
Msr	7.02 ± 0.68 (*n* = 3)	7.17 ± 0.39 (*n* = 8)	6.72 ± 0.21 (*n* = 5)	7.26 ± 0.8 (*n* = 6)
Mthfr	4.95 ± 0.86 (*n* = 7)	5.1 ± 0.39 (*n* = 12)	4.44 ± 1.14 (*n* = 19)	4.4 ± 1.58 (*n* = 28)
Nat1	9.22 ± 1.22 (*n* = 3)	9.1 ± 0.89 (*n* = 8)	9.08 ± 2.3 (*n* = 11)	9.05 ± 1.84 (*n* = 16)
Nat2	8.41 ± 0.23 (*n* = 3)	8.48 ± 0.4 (*n* = 8)	8.95 ± 1.37 (*n* = 16)	8.78 ± 1.03 (*n* = 25)
Nat3	14.13 ± 1.93 (*n* = 6)	14.01 ± 2.13 (*n* = 12)	11.97 ± 1.86 (*n* = 19)	11.54 ± 2.06 (*n* = 27)
Rfc	5.48 ± 0.3 (*n* = 3)	5.68 ± 0.35 (*n* = 8)	6.1 ± 1.74 (*n* = 8)	6.07 ± 1.28 (*n* = 10)
Amd1	5.19 ± 0.4 (*n* = 3)	5.42 ± 0.28 (*n* = 8)	5.29 ± 0.63 (*n* = 5)	5.48 ± 0.39 (*n* = 6)
Sahh	3.51 ± 0.42 (*n* = 3)	3.88 ± 0.32 (*n* = 8)	3.51 ± 0.41 (*n* = 5)	3.62 ± 0.31 (*n* = 6)
Tysy	3.28 ± 0.37 (*n* = 11)	3.23 ± 0.37 (*n* = 14)	4.09 ± 0.62 (*n* = 8)	3.9 ± 0.68 (*n* = 18)
Hoxd4	15.2 ± 1.7 (*n* = 26)	5.6 ± 0.9 (*n* = 29)	13.7 ± 0.83 (*n* = 14)	14.5 ± 1.2 (*n* = 20)
Hoxc8	2.9 ± 0.61 (*n* = 11)	3.0 ± 0.65 (*n* = 15)	3.3 ± 1.2 (*n* = 78)	0.78 ± 0.94 (*n* = 85)

*ΔCt values were determined separately for all controls (TA only) and TR-containing *Hoxd4* and *Hoxc8* transgenic animals, and means were calculated for all animals tested in each group (*n* = from 3 to 85) as indicated. Generally, larger standard deviations were found in cases of lower expression level (high ΔCt value) with correlation coefficients noted where significant at *P* = .05 (Fig. 5).