

Faithful tissue-specific expression of the human chromosome 21-linked *COL6A1* gene in BAC-transgenic mice

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Abstract

We created transgenic mice with a bacterial artificial chromosome (BAC) containing the human *COL6A1* gene. In high-copy and low-copy transgenic lines, we found correct temporal and spatial expression of *COL6A1* mRNA, paralleling the expression of the murine *Col6a1* gene in a panel of nine adult and four fetal organs. The only exception was the fetal lung, in which the transgene was expressed poorly compared with the endogenous gene. Expression of *COL6A1* mRNA from the transgene was copy number-dependent, and the increased gene dosage correlated with increased production of collagen VI alpha 1 in skin and heart, as indicated by Western blotting and immunohistochemistry. *COL6A1* maps to Chromosome 21 and this gene has been a candidate for contributing to cardiac defects and skin abnormalities in Down syndrome. The low-copy and high-copy *COL6A1* transgenics were born and survived in normal Mendelian proportions, without cardiac malformations or altered skin histology. These data indicate that the major promoter and enhancer sequences regulating *COL6A1* expression are present in this 167-kb BAC clone. The lack of a strong cardiac or skin phenotype in the *COL6A1* BAC-transgenic mice suggests that the increased expression of this gene does not, by itself, account for these phenotypes in Down syndrome.

Introduction

The *COL6A1* gene, on human Chromosome 21, encodes one of the three polypeptide chains in trimeric alpha (VI) collagen, a major component of extracellular microfibrils in various fetal and adult organs, including heart, skeletal muscle, skin, and others. This gene is of interest for the phenotypic consequences of both its deficiency and its overexpression. Mutations in *COL6A1* or in the other two genes encoding polypeptides in alpha (VI) collagen (*COL6A2* and *COL6A3*) cause two musculoskeletal disorders: Ullrich congenital muscular dystrophy and Bethlem myopathy. These genetic diseases are often inherited in a dominant pattern via transmission of missense mutations, and their pathophysiology involves a deficiency of functional alpha (VI) collagen trimers (Jobsis et al. 1996). Mice with a germline deletion (knockout; KO) of the *Col6a1* gene have skeletal muscle defects and are an animal model for Bethlem myopathy (Bonaldo et al. 1998).

The opposite situation, gain of gene dosage and overexpression of *COL6A1* and the closely linked *COL6A2* gene, occurs in Down syndrome (DS) due to trisomy 21. Overexpression of *COL6A1* mRNA and alpha (VI) collagen protein has been found in fetal hearts from DS patients, particularly when the region of the endocardial cushions is specifically examined (Gittenberger-de Groot et al. 2003). The preferential expression of *COL6A1* in the region of the endocardial cushions has motivated the hypothesis that increased dosage of this gene might contribute to the high frequency (~20%) of atrioventricular septal defect (AVSD) in people with DS

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(Antonarakis et al. 2004; Epstein 2001). While susceptibility to another cardiac malformation, tetralogy of Fallot, also associated with DS, has been mapped to a region of Chromosome 21 that excludes *COL6A1* (Kosaki et al. 2005), this gene remains a candidate for contributing to AVSD. A second category of DS phenotype, altered structure of the skin, specifically a paucity of cellular elements and a relative increase in extracellular matrix in the dermis, has also been circumstantially though not functionally linked to overexpression of alpha (VI) collagen (Brand-Saberi et al. 1994a, 1994b; von Kaisenberg et al. 1998a, 1998b). To address these issues and to study the regulation of the *COL6A1* gene, we have created transgenic mice with genomic integrations of BACs containing the human *COL6A1* gene. In this article we describe the stage-specific, tissue-specific, and copy number-dependent expression of the *COL6A1* transgene in low-copy and high-copy BAC-transgenic lines, and we assess the structure and histology of the heart and skin in these mice.

Materials and methods

BAC DNA and creation of transgenic mice. BAC DNA was prepared by double KAc precipitation followed by CsCl gradient separation (Gong et al. 2003). BAC host cells were streaked on an agar plate with chloramphenicol (20 µg/ml). A single colony was picked and inoculated with 3 ml of Luria Broth medium containing chloramphenicol and incubated at 37°C overnight, followed by transfer of 50 µl of the inoculated broth into 500 ml of Luria Broth containing chloramphenicol, and grown at 30°C to a final optical density (OD₆₀₀) of 1.0. The cells were harvested by centrifugation at 4000 rpm (Sorvall SLA3000 rotor) for 20 min at 4°C. The pellet was suspended with 40 ml of 10 mM EDTA, pH 8.0, with 100 µg/ml RNAase and 5 mg/ml lysozyme. Lysis solution (0.2 N NaOH, 1% SDS), 80 ml, was added, mixed, and incubated for 5 min at room temperature. Cold 2 M KAc, 60 ml, was added, mixed, and incubated on ice for 5 min. The lysate was centrifuged at 10,000 rpm for 30 min at 4°C in a Sorvall SLA3000 rotor. The supernatant was collected and mixed with an equal volume of isopropanol, then centrifuged at 5000 rpm (Sorvall SLA3000 rotor) for 30 min at 4°C. The DNA pellet was dissolved in 18 ml of 10:50 TE (10 mM Tris, 50 mM EDTA), and 9 ml of 7.5 M KAc was added, mixed, and incubated at -70°C for 30 min. The solution was thawed and centrifuged at 6000 rpm (Sorvall SS34) for 10 min at 4°C. The supernatant was collected and mixed with 2.5 volumes of ethanol and centrifuged at 12,000 rpm (Sorvall SS34) for 30 min at 4°C to precipitate the

DNA. The DNA pellet was resuspended in 4.4 ml of TE, mixed with another 4.4 ml of TE containing 10.2 g of dissolved CsCl, and 0.2 ml of ethidium bromide solution (10 mg/ml) was added. The solution was centrifuged at 65,000 rpm in a Beckman VTI 65 rotor overnight at 18°C. The BAC DNA band (bottom band) was removed with an 18-gauge needle and was brought up to 2 ml with TE. The solution was extracted 4-5 times with NaCl-saturated butanol and the DNA was precipitated with ethanol. The DNA was resuspended in 0.5 ml of 0.3 M sodium acetate and precipitated with ethanol once more, followed by washing with 70% ethanol. The DNA was dialysed on a 25-mm, 0.025-µm filter (Millipore) by floating it on oocyte injection buffer (5 mM Tris, pH 7.4, 0.2 mM EDTA, 100 mM NaCl). The BAC DNA (1 ng/µl) was injected into 200 pronuclei of fertilized oocytes of B6CBA mice, and the oocytes were transferred to pseudopregnant Swiss Webster foster mothers. The transgenic lines were expanded by crossing to C57BL6 and were maintained as both heterozygotes and homozygous lines.

Southern and Northern blotting. Total RNA was prepared after solubilizing tissues in Trizol reagent (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. The RNA was electrophoresed on 1.0% agarose gels containing formaldehyde and then transferred to Nytran membranes (Schleicher & Schuell, Keene, NH). The blots were hybridized at 42°C in Ultrahyb solution (Ambion, Austin, TX), with DNA probes specific for the last exon of human *COL6A1* or mouse *Col6a1*, and washed at high stringency in 0.1% SDS and 0.1× SSC for 1 h at 64°C. The blots were stripped and rehybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a loading control when required. Genomic DNA was prepared by lysing the tissue in SDS/proteinase K and incubating for several hours at 50°C, followed by phenol/chloroform extraction and precipitation in ethanol. The DNA, 2.5 µg, was digested overnight with restriction enzymes, and the digested DNA was resolved on 0.7% agarose gels, denatured, and neutralized under standard conditions and transferred to Nytran membranes. The Southern blots were hybridized with genomic probes for human *COL6A1* or mouse *Col6a1*. Primers for synthesizing the human *COL6A1* 5' region, intron region, and 3' region probes and the mouse *Col6a1* probe were as follows:
 5termFor, 5'-GCTCTGAATCCCCTCGGTA-3';
 5termRev, 5'-GCCACTGATGTCATCCACAC-3';
 intronFor, 5'-CACGGCTTTCTGTCTCTTCC-3';
 intronRev, 5'-AGCTACCAAGGTCTGGAGCA-3';
 3termFor, 5'-CTCCTTCCCTAGGCACCTCT-3';

3termRev, 5'-ATGGATGGACAGCTGTAGCC-3'; ms-For, 5'-AGCCACAACCTTCGAAACCAC-3'; ms-Rev, 5'-CCTTCCTGGAGACTGTCTGG-3'. The primers for generating the Northern probes were as follows: mouseFor, 5'-TGACCCAACTGGTCAACTCA-3'; mouseRev, 5'-AGCAGAGATAGC TGGCT TGG-3'; humanFor, 5'-GCCCTGAGCTAGTGTCA CCT-3'; humanRev, 5'-GGAGAGGTT TGC GTTGT TTC-3'.

Western blotting. Tissues were lysed in cold RIPA buffer and centrifuged to remove the debris. Total cell extracts normalized for protein content were boiled at 100°C for 5 min in a denaturing solution containing 12 mmol/L Tris (pH 6.8), 5% glycerol, 0.4% SDS, 3 mmol/L 2-mercaptomethanol, and 0.02% bromophenol blue. Total protein lysates, 50 µg, were electrophoresed on 8% polyacrylamide gradient/SDS gels (Invitrogen). After transferring to Immobilon membranes (Millipore, Bedford, MA) and blocking by 5% milk in 1× TBST, the membranes were hybridized with a polyclonal antibody (H-200, sc-20649, Santa Cruz Biotechnology) that recognizes the N-terminus of collagen VI alpha 1 protein of both human and mouse origin, or by a mouse monoclonal antibody against α-actin (Sigma, St. Louis, MO) as a loading control, in 1× TBST containing 5% dry milk overnight at 4°C. After washing, the signal was amplified and detected using a peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (IgG; Amersham Pharmacia Biotech, Piscataway, NJ) and ECL-Plus detection system (Amersham Pharmacia Biotech).

Histology and immunohistochemistry. Mouse embryos at 13.5 and 14.5 days post coitum (dpc) were fixed in formalin and processed by standard paraffin embedding. Tissue sections of heart and skin were stained with hematoxylin and eosin (H&E). Procedures for immunohistochemistry were essentially as previously described (Li et al. 2002). Antigen retrieval was done by boiling the deparaffinized sections on slides for 10 min in 1 mM EDTA (pH 8.0) in a microwave oven. The anti-collagen VI alpha 1 polyclonal antibody (H-200, sc-20649, Santa Cruz Biotechnology) was used at a dilution of 1:500. The secondary antibody (biotinylated horse anti-mouse; Vector Laboratories, Burlingame, CA) was used at a dilution of 1:400.

Chromosomal fluorescence in situ hybridization (FISH). Bone marrow cells obtained from femur of the transgenic mice were washed with RPMI medium, dissolved in RPMI complete bone marrow medium, and exposed to colcemid for 2 h. Pelleted

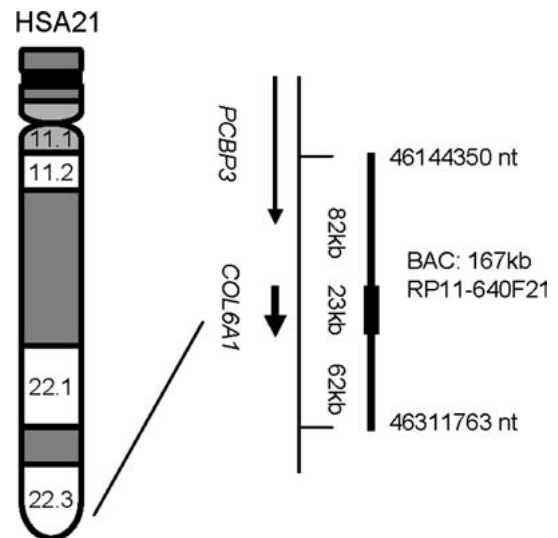


Fig. 1. Diagram of the *COL6A1* gene on human Chromosome 21 and the BAC clone used in this study. The RP11-640F21 BAC contains the complete *COL6A1* gene and one third of the 3' end of the *PCBP3* gene. *COL6A1* maps to the distal portion of chromosome band 21q22.3.

cells were treated with 0.56% KCl hypotonic solution for 20 min, and fixed in three parts methanol and one part acetic acid. Metaphase spreads were prepared by standard methods. Human BAC clone RP11-640F21 containing the *COL6A1* gene used in transgenic mice was used to generate a FISH probe. The BAC DNA was labeled by nick-translation using spectrum green dUTP fluorochrome (Vysis, Downers Grove, IL). Cy3-labeled mouse pan-centromeric probe (Cambio, UK) was utilized as control to identify chromosomes. FISH was performed using standard methods and hybridization signals were scored for at least 20 metaphase spreads on DAPI-stained slides.

Results

Creation of BAC-transgenic mice carrying the human *COL6A1* locus. As shown in Fig. 1, the RP11-640F21 BAC, from the library described by Osoegawa et al. (2001), is a 167-kb clone that contains the entire *COL6A1* gene, flanked by 82 kb of upstream DNA and 62 kb of downstream DNA. The BAC does not include any other complete genes, but it does include one third of the 3' end of *PCBP3* (Poly-rC Binding Protein 3) gene. Based on this configuration, in which *PCBP3* is severely truncated and promoterless, the only gene expected to be expressed from the RP11-640F21 BAC is *COL6A1*. We prepared the BAC DNA by a double acetate precipitation/CsCl gradient protocol (Gong et al. 2003) and created BAC-transgenic mice by injecting the BAC DNA into male pronuclei of fertilized B6CBA oocytes. Among the resulting progeny, we used *COL6A1*-specific

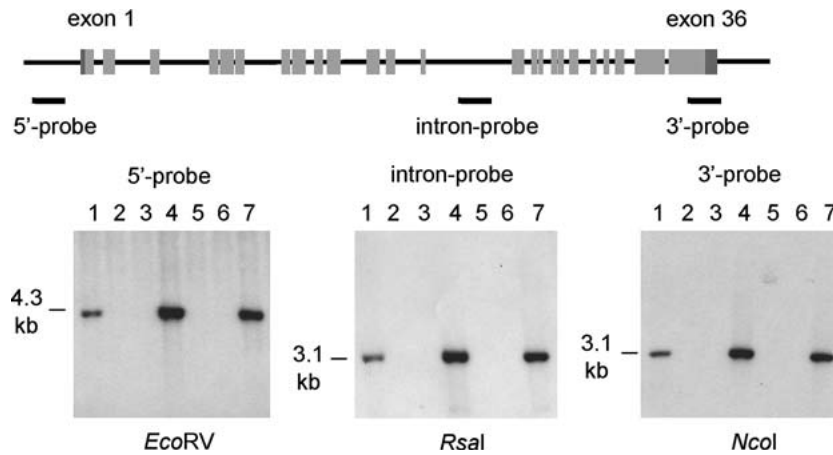


Fig. 2. Southern blots showing that the human *COL6A1* transgene is intact in three BAC-transgenic founder mice. Mouse tail DNA was digested with the indicated restriction enzymes and analyzed by Southern blotting. The blots were hybridized with human *COL6A1*-specific probes, as indicated on the map (top panel). The 5' probe, intron probe, and 3' probe are predicted based on the human genome sequence to recognize 4.3 kb, 3.1 kb, and 3.1 kb restriction fragments, respectively, and these fragments are seen in the blots. The lines derived from No. 1 and No. 7 founders are *COL6A1*^{low} and *COL6A1*^{high}, respectively. The lanes without specific bands contain DNA from wild-type littermates.

genomic PCR and Southern blotting to identify three transgenic founders. As shown by Southern blotting with probes for the 5', middle, and 3' portions of the

COL6A1 gene, in all three founders the BAC DNA was intact and appeared nonrearranged (Fig. 2). Of these founders, No. 4 died during the first pregnancy

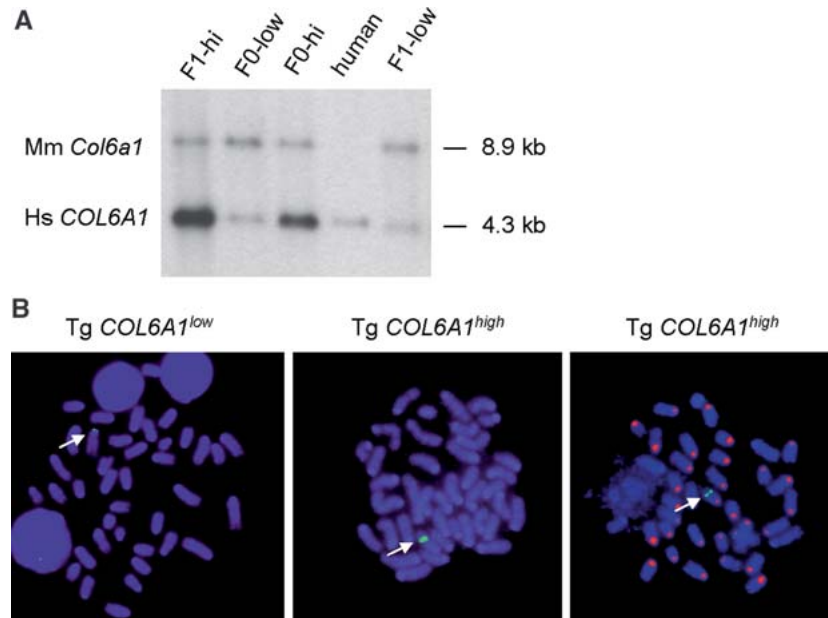


Fig. 3. Copy number analysis of the BAC transgene in F₁ progeny. **(A)** Mouse tail DNA and control human genomic DNA were digested with *EcoRV* and used for Southern blotting. Human *COL6A1*- and mouse *Col6a1*-specific probes were used together to hybridize the blot. The human probe (767 bp long; 57% GC content) recognizes a 4.3-kb restriction fragment, while the mouse probe (550 bp long; 58% GC) recognizes an 8.9-kb restriction fragment. F0-low: low-copy founder DNA (No. 1); F0-hi: high-copy founder DNA (No. 7); F1-low: low-copy F₁ DNA; F1-hi: high-copy F₁ DNA. The F₁ DNAs are from progeny of Tg × wild-type crosses. Phosphorimaging of the blot allowed an estimate of the BAC copy number as 1 for the *COL6A1*^{low} line and approximately 15 for the *COL6A1*^{high} line. **(B)** FISH analysis of metaphase preparations using the *COL6A1* BAC as a probe. The cells are from heterozygotes, and the results indicate that the *COL6A1*-containing BAC (white arrows; green signals) has undergone single-site integration in both lines. Consistent with the copy-number analysis by Southern blotting, the FISH signals are stronger in the high-copy line than in the low-copy line. To orient the chromosomes and to control for uniformity of the hybridization, the FISH procedure was repeated for the high-copy line with a pan-centromere probe (red signal).

and therefore did not transmit the transgene, while founders No. 1 and No. 7 transmitted the transgene, allowing the establishment of two mouse lines. As shown in Fig. 3A, phosphorimaging of Southern blots simultaneously hybridized with similarly sized DNA probes specific for the human *COL6A1* and mouse *Col6a1* genes allowed us to estimate that the BAC copy number in the line derived from founder No. 1 was 1, while the copy number in the line derived from founder No. 7 was approximately 15. Below we refer to these transgenic lines as *COL6A1^{low}* and *COL6A1^{high}*, respectively. For our interpretation of transmission in these mice, by performing FISH with the *COL6A1* BAC DNA as a probe we found that in each line the transgene was inserted at a single site. A single FISH signal was found on only one homolog of the chromosome detected in metaphase preparations of heterozygous cells, in both lines, with the site of insertion being near a chromosome telomere for the *COL6A1^{low}* line and in the midregion of a chromosome in the *COL6A1^{high}* line (Fig. 3B). We have not determined the specific identities of these chromosomes. As shown in Fig. 3, the relative intensities of the FISH signals qualitatively confirmed the differences in transgene copy number between the *COL6A1^{low}* and *COL6A1^{high}* lines (Fig. 3B). As shown in Table 1, both lines transmitted the transgene in Mendelian proportions, with no evidence of embryonic mortality. We have not observed any deaths or morbidity in cohorts of ten adult mice from each line that we have maintained up to 10 months of age.

Faithful tissue-specific expression of the human *COL6A1* transgene in high- and low-copy *COL6A1* BAC-transgenic mice. To ask whether the major regulatory elements for *COL6A1* expression are present on the RP11-640F21 BAC transgene, we surveyed mRNA expression in multiple adult and fetal tissues of the *COL6A1^{low}* and *COL6A1^{high}* transgenic mice. We hybridized Northern blots sequentially with partial-length cDNA probes matching the human (Hs) and murine (Mm) *COL6A1* and *Col6a1* genes (since we designed the probes to span regions of these genes that have divergent nucleotide sequences, there was no cross-hybridization on the Northern blots). As shown in Fig. 4 for the *COL6A1^{low}* line and in Fig. 5 for the *COL6A1^{high}* line, the mRNA expression pattern of the human *COL6A1* gene in the BAC-transgenic mice was found to be very similar to that of the endogenous murine *Col6a1* gene in all organs except the fetal lung, which showed proportionately less expression of the transgene compared with the endogenous gene. From these data we conclude that the major regulatory

Table 1. Transmission of the *COL6A1* BAC transgene in Tg × wild-type crosses

<i>F</i> ₁ generation	Copy number	Transgenic pups/ total <i>F</i> ₂ pups
<i>COL6A1^{low}</i>	1X	23/44 (52%)
<i>COL6A1^{high}</i>	15X	30/62 (48%)

As a further control, in these crosses we did not observe any difference in the frequency of transmission of the transgenes via the female vs. the male germline. For example, in the high-copy line, 8/17 offspring inherited the transgene via maternal transmission and 22/45 offspring inherited the transgene via paternal transmission.

elements (promoter and enhancer sequences) required for tissue-specific expression of *COL6A1* are present on the RP11-640F21 BAC, i.e., these elements are all encompassed within the chromosomal region including the gene itself and 82 kb of upstream DNA and 62 kb of downstream DNA. The discordant expression seen in the fetal lung suggests that an additional lung-specific enhancer element may be present outside of this region.

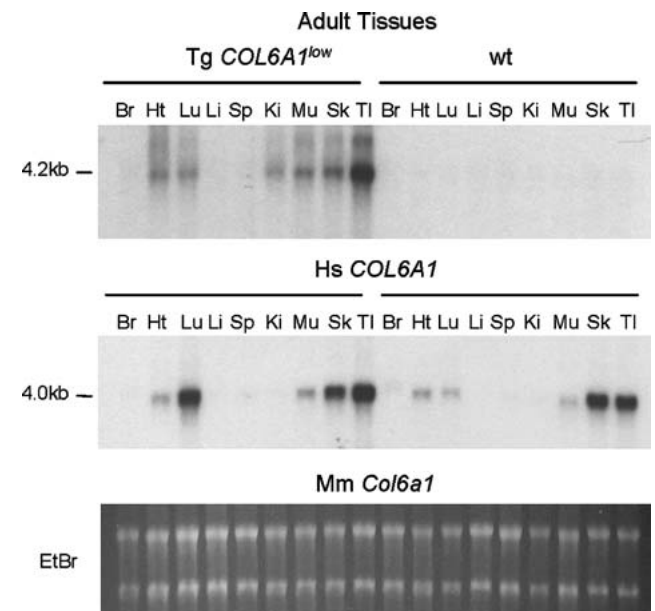


Fig. 4. Faithful tissue-specific expression of human *COL6A1* in adult low-copy BAC-transgenic mice. Total RNA was prepared from the indicated organs of the low-copy BAC-transgenic and wild-type littermate adult mice and used for Northern blotting. Human *COL6A1*- and mouse *Col6a1*-specific probes were used to recognize human *COL6A1* mRNA and endogenous mouse *Col6a1* mRNA, respectively. The blot was exposed for four days after hybridization with the human probe and one day after hybridization with the murine probe. The expression pattern of the human gene carried on the BAC is similar to that of the endogenous murine gene. Ethidium bromide staining of 28S and 18S rRNA is shown as a loading control. Br: brain; Ht: heart; Lu: lung; Li: liver; Sp: spleen; Ki: kidney; Mu: muscle; Sk: skin; TI: tail.

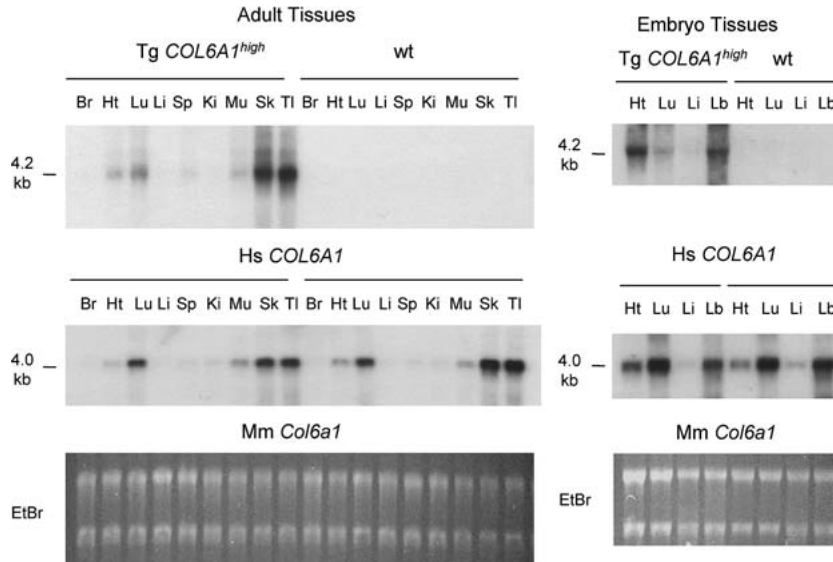


Fig. 5. Faithful tissue-specific expression of human *COL6A1* in adult high-copy BAC-transgenic mice and in most organs of late-stage (E17) mouse embryos. Total RNA was prepared from the indicated organs of high-copy BAC-transgenic and wild-type littermate adult and late-stage embryonic (E17) mice and used for Northern blotting. Human *COL6A1*- and mouse *Col6a1*-specific probes were used to recognize human *COL6A1* mRNA and endogenous mouse *Col6a1* mRNA, respectively. The blot was exposed for two days after hybridization with the human probe and six days after hybridization with the murine probe. The expression pattern of the human gene carried on the BAC is similar to that of the endogenous murine gene in all organs except the fetal lung, which shows proportionately less expression of the transgene. Ethidium bromide staining of 28S and 18S rRNA is shown as a loading control. Lb: limb; other organs abbreviated as in Fig. 4.

Copy number-dependent expression of *COL6A1* in the BAC-transgenic mice. Another criterion for testing the inclusion of key regulatory elements in a transgenic construct is copy number-dependent expression of the gene under consideration. If the observed expression increases with increased copy number, this suggests that the transgene is at least partially insulated from the *cis* effects of chromatin at its sites of integration. By Northern blotting of total RNA from adult hearts, we in fact observed copy number-dependent expression, with the steady-state *COL6A1* mRNA levels entirely consistent with the known BAC copy numbers (1X, 15X) in these two lines (Fig. 6A). Comparison of the expression with the endogenous mouse *Col6a1* mRNA further supported this conclusion, with roughly equal expression of the human and mouse genes found in the low-copy line and greater relative expression of the human gene in the high-copy line (data not shown).

Increased collagen VI alpha 1 protein expression in *COL6A1*^{high} BAC-transgenic mice. To verify that the observed expression of *COL6A1* mRNA in the transgenic mice in fact led to overproduction of collagen VI alpha 1 polypeptide, we performed Western blots using total protein lysates from adult heart

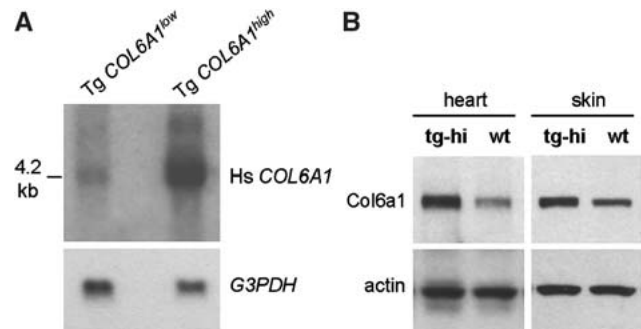


Fig. 6. (A) Transgene mRNA and protein expression level is positively correlated with copy number. Total RNA was prepared from both low-copy and high-copy adult BAC-transgenic mouse hearts and probed for human *COL6A1* mRNA. The middle lane of this gel was deliberately left blank to avoid overlap between the very strong signal from the high-copy transgenic with the low signal from the low-copy transgenic. The *Gapdh* (*G3PDH*) probe was used as an internal control for mRNA loading. (B) Western blot analysis confirming human collagen VI alpha 1 protein expression in heart and skin of high-copy adult transgenic mice. Total protein lysates were prepared from transgenic (heterozygote) and wild-type littermate heart and skin from Tg-heterozygote \times wild-type crosses. The anti-Col6a1 antibody, which detects collagen VI alpha 1 of both human and mouse, reveals greater total amounts of this protein in the transgenic tissues. Antibody against beta-actin was used to verify equal loading of wild-type and transgenic lanes.

and skin of the *COL6A1^{high}* transgenic mice and their wild-type littermates. We probed these blots with a polyclonal antibody recognizing the N-terminus of collagen VI alpha 1 of both human and mouse origin. Comparing the intensities of the specific bands on these blots confirmed the increase in net collagen VI alpha 1 in both of these organs from the transgenic mice (Fig. 6B). Moreover, by immunohistochemistry we observed stronger collagen alpha VI immunoreactivity in the extracellular matrix of the mitral valves and in or immediately below the basement lamina as well as in the dermis of the high-copy transgenic fetuses, compared with their wild-type littermates (Fig. 7A-D). While more intense, the immunostaining was present in a pattern identical to that of the native collagen alpha VI. This finding, and the strong similarity between the amino acid sequences of human and mouse collagen VI alpha 1 polypeptides, suggests that the overexpressed human collagen VI alpha 1 chain is probably participating in collagen fibril formation with the other two isoforms (alpha 2 and alpha 3) of mouse collagen VI. However, the extent of protein overexpression appeared less strong than would be predicted from the degree of mRNA overexpression in these mice. This finding suggests that there may be a post-transcriptional mechanism for regulating the levels of collagen VI alpha 1 polypeptide, perhaps related to the biological necessity for forming stoichiometric trimers of the three polypeptide chains, each of which is encoded by a separate gene.

Normal histology of heart and skin in *COL6A1^{high}* BAC-transgenic mice. We compared heart development in BAC-transgenic embryos and their wild-type littermates at 13.5 and 14.5 dpc and found no cardiac defects or other qualitative anatomical differences in 14 *COL6A1^{high}* transgenic vs. 10 wild-type control mouse embryos at 14.5 dpc (Fig. 7C) and no defects or differences in two transgenic vs. two wild-type 13.5 dpc embryos (data not shown). We also examined nuchal and chest skin in these embryos at both stages, focusing on possible abnormalities of the thickness or staining properties of the dermis, and found no genotype-specific differences.

Discussion

Type VI collagen forms poorly structured fibrils in many tissues, including skin, heart, and skeletal muscle, and mutations in the polypeptides of this heterotrimeric protein produce myopathies—Bethlem myopathy and Ullrich muscular dystrophy—in both humans and mice (Lampe and Bushby 2005). The *COL6A1* gene, which encodes one of the collagen VI chains, is implicated by linkage and genetic

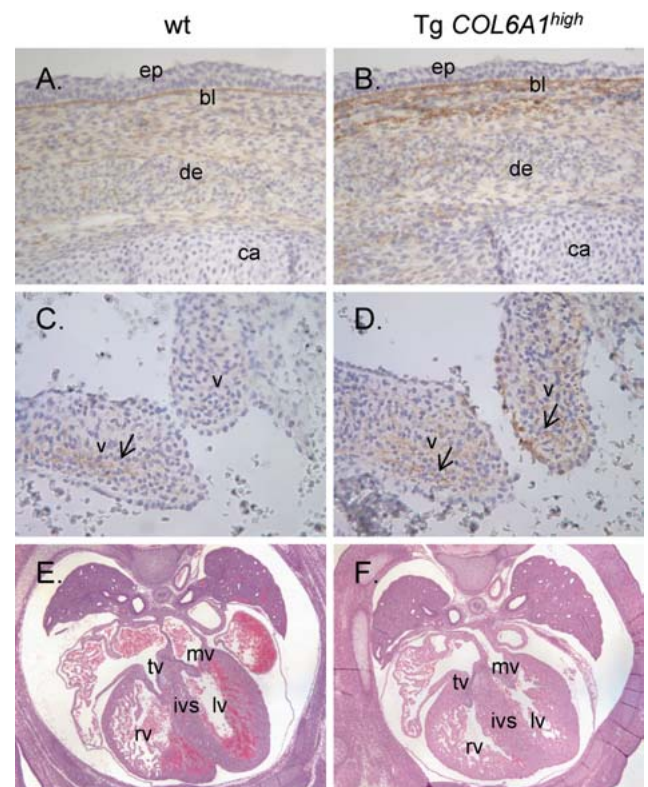


Fig. 7. Immunohistochemistry and histology of fetal skin and heart from *COL6A1^{high}* transgenic and wild-type littermate embryos. (A, B) Immunohistochemistry of chest skin from a Tg^{+/+} (homozygous transgenic) conceptus and a wild-type littermate conceptus at 14.5 dpc. Stronger collagen VI immunostaining is observed in the transgenic skin, but the spatial pattern of immunoreactivity remains faithful to that seen in the wild-type control, with strongest staining in or immediately below the basement lamina and moderate staining of the intercellular matrix in the dermis. (C, D) Immunohistochemistry on the mitral valves at 14.5 dpc. There is diffuse positive staining in the extracellular matrix of the valves (arrows). Stronger collagen VI staining is observed diffusely in the transgenic valves, which additionally show subendocardial concentrations of collagen VI. (E, F) Histological sections (H&E) of hearts at 14.5 dpc. There is no difference in the anatomy or histological features of the heart in the transgenic embryo compared to the wild-type littermate. A total of 14 transgenics and 10 wild-type littermates were examined at 14.5 dpc. A similar survey at 13.5 dpc also showed no genotype-specific abnormalities in cardiac anatomy. bl, basement lamina; ca, cartilage; ep, epidermis; de, dermis; ivs, inter-ventricular septum; lv, left ventricle; mv, mitral valve; rv, right ventricle; tv, tricuspid valve; v, developing mitral valve leaflet.

association data in a rare inherited syndrome of ossification of the posterior spinal ligament in the Japanese population (Tanaka et al. 2003; Tsukahara et al. 2005), and it has been considered a candidate for contributing to cardiac defects and skin abnormalities in DS. Such defects, frequently atrioventricular defects due to anomalous development of

Table 2. Mouse lines carrying Chromosome 21-linked genes as large-insert genomic transgenes for modeling the genetics of Down syndrome

<i>Transgene</i>	<i>Types of clones</i>	<i>Phenotype of transgenic mice</i>	<i>References</i>
<i>APP</i>	YAC	No abnormalities reported	Lamb et al. 1993; Murai et al. 1998; Pearson and Choi 1993 Smith et al. 1997
<i>DYRK1A</i> (minibrain kinase)	YAC	Behavioral abnormalities	Branchi et al. 2004
<i>DYRK1A</i> and adjacent genes ^a	multiple YACs	Behavioral abnormalities in <i>DYRK1A</i> Tg	Chrast et al., 2000
<i>Sim2</i> (mouse gene)	BAC	Behavioral abnormalities	Ligon et al., 2006
<i>OLIG2</i>	BAC	Rescue of glial cell deficiency due to <i>Olig2</i> gene knockout. Phenotype due to the transgene alone not yet described	
<i>SOD1</i>	λ phage clone	Thymus and bone marrow abnormalities	Epstein et al., 1987; Peled-Kamar et al., 1995
<i>COL6A1</i>	BAC	Normal cardiac and skin anatomy and histology	This report

^aSeries of four YAC transgenic lines containing C21orf18 → PSMD4, DSCR6 → TTC3, DSCR3+DYRK1A, KCNJ6.

structures derived from the endocardial cushions, occur more frequently in DS, but the basis for abnormal heart development in this condition has remained unclear. Molecular candidates include proteins expressed in cardiac development that are encoded by Chromosome 21 genes such as the adhesion protein gene *DSCAM* (Barlow et al. 2001; Kosaki et al. 2005), collagen VI (*COL6A1* and *COL6A2* genes), the calcineurin pathway modulator *DSCR1* (Arron et al. 2006; Lange et al. 2004, 2005), and the *SH3BGR* gene (Egeo et al. 2000). Another interesting Chromosome 21-linked gene that is circumstantially implicated as a candidate for contributing to heart anomalies is *GART*, which encodes a metabolic enzyme and is nonlinearly overexpressed at the mRNA level in whole fetal hearts with trisomy 21 (Li et al. 2006).

To test for phenotypes from overexpression of the human *COL6A1* gene in a controlled animal model, we created low-copy and high-copy lines of *COL6A1* BAC-transgenic mice. Our analysis of viability, gene expression, and anatomy in these mice, as reported here, leads us to two conclusions. First, most of the promoter/enhancer elements responsible for tissue-specific expression of *COL6A1* are within the BAC, thus mapping within a 167-kb region containing the gene itself plus 82 kb of upstream and 62 kb of downstream DNA. However, an enhancer element for expression in fetal lung may lie outside this region. In this study, with only two independent BAC-transgenic lines, it is not possible to make a definite conclusion about the presence of specific insulator sequences on the RP11-640F21 BAC. However, a finding of copy number-dependent expression would at least indicate that the *COL6A1* gene on the BAC is not affected strongly by adjacent

sequences from these two independent integration sites, thereby leaving open the possibility that insulators may exist in the DNA encompassed by this BAC clone. Second, overexpression of human collagen VI alpha 1 protein in mice due to the addition of the human BAC transgene does not cause major developmental abnormalities in the heart or skin, and it has no detrimental effect on pre- or postnatal viability.

These findings argue against a major role for *COL6A1* overexpression in producing cardiac defects in DS, but there are a number of important caveats. In our anatomical studies, we assessed a substantial number of conceptuses by manually viewing the histological sections of skin and heart; however, because we did not carry out morphometry, we cannot exclude a subtle quantitative phenotype. Although our findings with immunohistochemistry show that the transgene-derived collagen VI alpha 1 polypeptide is deposited in the extracellular matrix of the heart and skin, we have not proven that this polypeptide chain is giving rise to a stoichiometric excess of complete collagen VI heterotrimers. In addition, we cannot exclude early biological selection in our founder mice, such that viability was preserved in subsequent generations. More generally, our data do not exclude the possibility that an extra copy of *COL6A1* might contribute to cardiac or skin abnormalities in DS, but only in concert with extra copies of other genes on human chromosome 21. In the future it may be possible to test this possibility by crossing multiple BAC-transgenic lines. In fact, as highlighted in Table 2, the *COL6A1* BAC-transgenic lines described here add to a growing list of large-insert transgenic mice that carry Chromosome 21-linked

genes cloned in BAC, yeast artificial chromosome (YAC), or phage vectors, which can be used to dissect the contributions of individual Chromosome 21-linked genes to the various phenotypic features of Down syndrome. Additional lines are being made, including in our own laboratory, and crosses among these lines may be useful for creating phenotypes that depend on increased expression of multiple Chromosome 21-linked genes.

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