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CONSTRUCTION OF A *COL11A1* TRANSGENE
VECTOR

by
Cameron M. Beck

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

Department of Physiology and Developmental Biology
Brigham Young University
December 2006

BRIGHAM YOUNG UNIVERSITY

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BRIGHAM YOUNG UNIVERSITY

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ABSTRACT

CONSTRUCTION OF A *COL11A1* TRANSGENE VECTOR

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Master of Science

Background: Cartilage disorders affect millions of people in the United States alone, with effects ranging from poor skeletal development and joint pain to shortened lifespan and perinatal lethality. Many of these disorders have their root in defects of collagen, type XI collagen being among the most important. A mouse model of such a type XI collagen defect is the *chondrodysplasia (cho)* mutant. Mice homozygous for this null mutation in the *Col11a1* gene do not express the $\alpha 1$ chain of type XI collagen. This results in a functional knockout of type XI collagen, leading to insufficient skeletal development and perinatal lethality. **Objective:** 1) To construct a transgenic expression vector designed to express a human *COL11A1* cDNA in a cartilage-specific manner. This transgene will be used in future studies to correct the type XI collagen defect in homozygous *cho* mice. 2) To place the cDNA in an *in vitro* expression vector to be used for *in vitro* transcription/translation assays. **Methods and**

Results: Through the relatively new approach of “recombineering”, the coding sequence of a human *COL11A1* cDNA was constructed from two cloned cDNA fragments. A copy of the cDNA was inserted into the pcDNA3.1 expression vector for *in vitro* transcription/translation assays.

Another copy of the cDNA was fused with a genomic mouse α -globin fragment to provide a polyadenylation signal. The resulting cDNA/ α -globin segment was inserted into p1757, the expression vector to be used for future transgenic studies. p1757 contains a *Col2a1* promoter, a β -globin splice sequence and a *Col2a1* enhancer. The cDNA/ α -globin segment was inserted between the splice sequence and the enhancer. With the cDNA in this expression cassette *COL11A1* can be expressed in a chondrocyte-specific manner in transgenic studies of the *cho* mouse model.

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Introduction

Importance of cartilage

Cartilage plays a fundamental role in skeletal development, as well as in other structural settings including the lining of articular surfaces within many synovial joints. For these reasons, hereditary and acquired defects in this important connective tissue can have severe consequences on human development and health. Millions in the United States alone suffer from disorders such as osteoarthritis, achondrogenesis, and Stickler Syndrome, which have their root in insufficient, malformed, or damaged cartilage¹. Mutations in collagen type XI, an essential component of hyaline cartilage, have been implicated in such disorders².

Structure and function of type XI collagen

Type XI collagen, along with collagen type II, forms the collagen fibrils that give hyaline cartilage its characteristic tensile strength and durability³. Though collagen XI is a quantitatively minor component of these collagen fibrils, its unique morphology allows it to perform an essential regulatory function in the fibril.

Like all collagens, type XI collagen is a trimeric molecule. The collagen XI molecule is a heterotrimer comprised of three α polypeptide chains, $\alpha 1$, $\alpha 2$ and $\alpha 3$, the products of three distinct genes, *Col11a1*, *Col11a2*, and *Col2a1*, respectively. As with other fibril-forming collagens, type XI collagen is secreted from chondrocytes as a procollagen containing several characteristic domains: an amino propeptide containing a short triple helical segment, followed by an extended triple helical domain, and terminating with a carboxy propeptide. While all fibrillar collagens undergo

proteolytic processing in the extracellular matrix to produce the mature form of the molecule, collagen XI is processed more slowly and incompletely, allowing it to retain most of its N-terminal domain⁴. When incorporated into growing fibrils, this domain of collagen XI is excluded from the interior of the fibril, where the majority of the molecule is found^{5,6}. This globular domain, residing on the surface of the collagen fibril, is thought to limit the diameter of growing fibrils, most likely by sterically blocking the accumulation of additional collagen molecules past a certain fibril diameter⁷. Fibrils without collagen XI, containing only type II collagen, become much thicker and spindle-shaped without this regulation of collagen accretion⁸. The altered shape and content of these fibrils appear to adversely affect their interaction with the other components of the ECM and inhibit their ability to serve the structural functions of normal heterotypic fibrils.

Hyaline cartilage and endochondral bone formation

Hyaline cartilage, containing type II/XI collagen fibrils, is crucial to endochondral bone formation during fetal development. In this process, chondrocytes, the main cellular component of cartilage, first lay down a cartilaginous matrix, forming a precursor model of the bone. This cartilage model later serves as scaffolding for osteoblast invasion and calcium deposition during ossification. Apart from its role in bone formation, non-osteogenic hyaline cartilage can also be found as a structural component of the larynx, trachea, and the articular surface of bones within joint capsules.

Effects of cartilage disorders

Disorders such as mutations in collagen XI genes, affecting the body's ability to create or maintain sufficient cartilaginous tissue, can have severe consequences on human development and health. Without adequate cartilage scaffolding or proper morphology of the precursor model, bone formation may proceed abnormally, resulting in shortened or unusually thick bones and appendages². In cases where the cartilage is most severely affected, bone and structural development is almost completely abrogated and death can occur, often due to secondary complications such as pulmonary hypoplasia⁹.

Potential for gene therapy for cartilage disorders

The ability to correct such conditions through gene therapy, both *in utero* and in children and adults, would be of inestimable benefit. Such treatment could allow individuals with genetically-based cartilage disorders to receive functional genes that would produce the necessary products to halt and possibly reverse progression of the disorder. For such therapies to succeed, however, transgenic constructs must be created that will function in the correct tissues and provide the needed mitigation of the targeted disorder.

The *chondrodysplasia* mouse model

The feasibility of gene therapy for correction of cartilage defects can first be tested in mouse models of these disorders. One illustrative model of a genetic cartilage disorder is the murine *chondrodysplasia* (*cho*) mutant phenotype. *Cho* mice possess a frameshift mutation in the *Coll1a1* gene, which encodes $\alpha 1(XI)$, the $\alpha 1$ chain of the collagen XI heterotrimer. The mutation introduces a premature stop codon and is

believed to cause instability of the mRNA transcript, resulting in a functional knockout of the $\alpha 1(XI)$ protein⁸ (Fig. 1). With no $\alpha 1$ chain to complete the heterotrimer, type XI collagen is not formed in homozygous mutant fetuses, and the fetuses exhibit stunted bone development, apparent in their shortened appendages, poor vertebral and rib development, and micrognathia¹⁰. Skeletal staining in these animals shows that long bones and ribs are not only shortened, but also fail to ossify correctly, confirming the requirement of collagen XI for proper bone formation (Fig. 2). Mutant fetuses are believed to die at birth from asphyxiation due to pulmonary and tracheal hypoplasia¹⁰. Many structural characteristics of this animal model are similar to human chondrodysplastic disorders such as Stickler Syndrome, some cases of which have been linked to *COL11A1* mutations^{11, 12}.

Disorders resulting from a null mutation of a collagen gene, such as the chondrodysplasia of the *cho* mice, might feasibly be corrected through gene therapy. Introduction of a functional gene expressing the missing protein under tissue-specific transcriptional regulation could substitute for the null allele and allow for sufficient collagen formation, thereby rescuing the mutant phenotype.

COL11A1 transgene development

To test the efficacy of such a treatment we have developed a transgenic construct, called pCOL11A1, containing a human *COL11A1* cDNA under the transcriptional control of a rat *Col2a1* promoter and enhancer in the p1757 expression vector (Fig. 3). The p1757 vector was kindly provided by Dr. Y Yamada (National Institute of Dental Research, National Institutes of Health, Bethesda, MD)¹³. This

<i>wt</i>	CCC	CTC	GAT	AGA	AGT	GAG	AGA	TCG	ATA	GTT
	P	L	D	R	S	E	R	S	I	D
	P	S	I	E	V	R	D	R	Ter	
<i>cho</i>	CCC	TCG	ATA	GAA	GTG	AGA	GAT	CGA	TAG	TTG

Figure 1: The *cho* mutation introduces a premature termination codon. The deletion of a cytosine ~570 nucleotides downstream of the translation initiation site causes a frame shift, resulting in a premature stop codon shortly after the mutation site.



Figure 2: The *cho* mutation leads to stunted skeletal development. The *cho* mutant has a shortened vertebral column, smaller thoracic volume, a shortened lower jaw and shorter, thicker long bones than wild-type. Red staining (Alizarin red) indicates calcified bone; blue-green staining indicates uncalcified cartilage (Alcian blue).

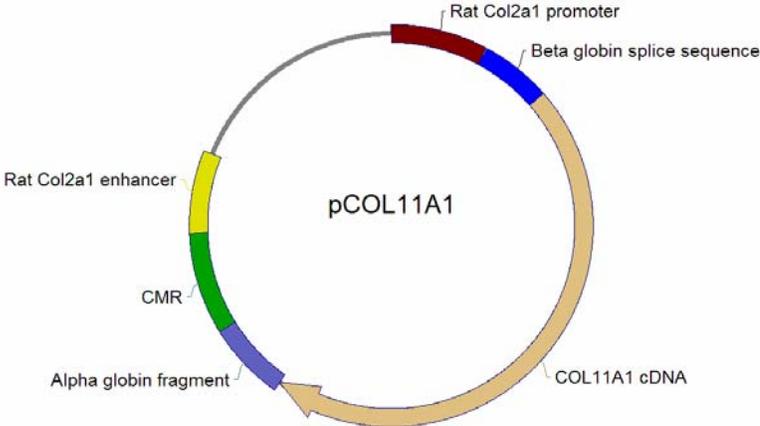


Figure 3: Design of the transgenic expression vector for the *COL11A1* cDNA. Details of the design are discussed in Materials and Methods.

expression vector has been used previously to drive chondrocyte-specific expression of transgenic constructs¹⁴.

The transgenic construct is based on a design from previous unpublished work in our laboratory¹⁵ with modifications made to accommodate a new method of construction and to potentially enhance expression. This construct will be used in future studies in the *cho* mouse model to assess the extent to which the mutant defects can be corrected through transgenic $\alpha 1(XI)$ chain production. We anticipate that the introduction of a functional *COL11A1* transgene into homozygous mutant embryos will ameliorate the *cho* phenotype by expressing the missing chain and allowing skeletal development to proceed normally.

This purpose of this research was to build a *COL11A1* transgenic vector through a relatively new method known as “recombineering”¹⁶. Through this method, the coding region of a human *COL11A1* cDNA was constructed and fused with necessary regulatory elements before being inserted into the final expression vector. A vector was also prepared to test the recombinant cDNA through *in vitro* assays for expression of the correct protein product.

Materials and Methods

cDNA GenBank accession numbers

Base pair (bp) identifications for the *COL11A1* cDNA refer to GenBank Accession #NM_001854. This entry describes the *COL11A1* mRNA transcript splice variant A, which includes the alternatively spliced exons 6A, 7, and 8.

Base pair identifications for mouse α -globin gene refer to a portion of the sequence found under GenBank Accession #NM_039515. This entry describes a large segment of mouse chromosome 11, part of which contains a complex of globin genes. The alpha globin gene sequence, comprising bp 29183674-29184493 of this contig, is numbered herein with the transcriptional start site as bp 1.

Plasmids used

pCR2.1 (Invitrogen): TA cloning vector for ligation and maintenance of Taq polymerase-amplified PCR product. Contains ampicillin and kanamycin resistance genes (Amp and Kan, respectively).

pACT2 (Clontech): Plasmid containing the 3' fragment of human *COL11A1* cDNA (bp 1124-6035) screened from a cDNA library pooled from human knee cartilage¹⁵. Contains Amp.

pBC (Stratagene): Plasmid containing a chloramphenicol resistance gene (CMR); used as template for amplification of CMR for recombination.

pcDNA3.1 (Invitrogen): In vitro expression vector for transcription/translation assay of cDNA. Contains Amp and Kan.

p1757 (Dr. Yoshihiko Yamada, NIH): Transgenic expression vector containing the *Col2a1* promoter, β -globin splice sequence, and *Col2a1* enhancer. Contains Amp.

Cloning of 5' *Coll1a1* cDNA fragment

Human chondrosarcoma mRNA was kindly provided by Dr. Joel Block (Rush Medical College). RT-PCR was performed using Qiagen OneStep RT-PCR kit and primers previously designed¹⁵ to amplify the 1506 base pairs of human *Coll1a1* mRNA beginning at the translation start codon and running to the end of the listed sequence (bp 319-1824) [Forward sequence: GCGGGATCCATGGAGCCGTGGTCC TCTAG; Reverse sequence: ATCACCACCATAACGGAACGGTAACATCAACAT AG]. The primers also introduced a *BamHI* restriction site at the 5'-most end of the PCR product. The resulting product was then ligated into pCR2.1 for subsequent maintenance and use.

Cloning of α -globin gene

PCR was performed on mouse genomic DNA with primers designed to amplify the last 776 bp of the mouse α -globin gene (GenBank Accession #NT_039515). [Forward sequence: 5'-CTGGGGAAGACAAAAGCAAC-3'; Reverse sequence: 5'-GCAGGCTTCTTCCT ACTCAG-3'] This region of the α -globin gene contains a splice sequence and a polyadenylation signal, both of which aid in optimal expression of cDNA transgenes¹⁷. The PCR product was then ligated into pCR2.1 for subsequent maintenance and use.

General recombinering protocols

Recombinogenic engineering, also known as “recombineering”, is a term which describes the process of directed DNA recombination reactions,

Gene name	Product	Function
<i>exo</i>	Exo (RecT analog)	Digests 5' ends of PCR product through endonuclease activity
<i>bet</i>	Beta (RecE analog)	Stabilizes single stranded DNA and facilitates annealing to complementary sequence in the cell
<i>gam</i>	Gam	Inhibits endogenous nuclease activity

utilizing E. coli bacteria with a defective λ -prophage stably

Table 1: λ -prophage gene products and functions in recombineering. Three inducible prophage genes, *bet*, *gam*, and *exo*, express proteins that aid in recombination.

incorporated into the genome. The prophage genes can be induced through heat shock to express proteins which aid in the recombination process (Table 1)¹⁶. Samples of one such strain of E. coli, known as SW102¹⁸, were kindly provided by the Dr. Donald L. Court (National Cancer Institute). The general protocol for initiating recombination events using this strain was as follows:

Preparation of electrocompetent cells

A liquid culture of SW102 cells in LB broth containing 12.5 μ g/ml tetracycline (SW102 are engineered to be tetracycline-resistant) was incubated overnight at 32°C. At this temperature, temperature sensitive repressors of the prophage genes are active. The next day, 1.5 ml of this culture was diluted in 30 ml of LB broth with 12.5 μ g/ml tetracycline and grown at 32°C until OD₆₀₀~0.5. 20 ml of this culture was cooled completely on ice and centrifuged at 4000 rpm for 10 minutes at 4°C to pellet cells. The supernatant was discarded and the cells resuspended in 2ml of ice-cold Nanopure

water. The resuspended cells were transferred to two cooled 1.5ml eppendorf tubes, 1ml per tube, and centrifuged at ~13500 rpm for 25 seconds at room temperature. The supernatant was again discarded, and the cells were washed twice more in this manner. After the final centrifugation, the cells, now electrocompetent, were resuspended in ~50 μ l of water and kept on ice until used. ~5-30 ng of plasmid was mixed with the cells and the cell/plasmid mixture placed in cooled electroporation cuvettes (1 mm). Plasmid was electroporated into the competent cells (1800 V, 25 μ F, 200 Ω), and the cells were retrieved in 1ml of LB and allowed to incubate for 1 hr at 32°C in a shaking incubator. Between 50 μ l and 150 μ l of cells were plated on LB/agar medium containing the appropriate antibiotic for selection and grown overnight.

Design of PCR primers for recombineering

PCR was performed using Platinum Pfx DNA polymerase kit (Invitrogen) and specially designed primer sets. Each primer in a set was composed of two fused elements: 1) a typical 19-to-22-bp primer specific to the PCR template for amplifying the sequence of interest; and 2) a 50-bp sequence homologous to one of two sequences on the recombineering target plasmid (on the reverse primer, this sequence was the reverse complement of the actual target sequence.) On the target plasmid, these sequences, known as regions of homology (RHs), flanked the spot into which the amplified sequence was inserted. The product of PCR using such primers contained the amplified sequence flanked by two 50-base-pair RHs to the recombineering target plasmid. These regions paired with homologous sequences on the target plasmid during recombination, allowing for specific, directed recombination to take place.

Thermal cycler programs for each PCR reaction were created such that the first five cycles employed a primer annealing temperature approximately 3-5°C below the melting temperatures of the template-specific segment of the primers (e.g. 52°C for a primer set with melting temperatures for the template-specific segment of 55°C and 56°C, respectively). This allowed the template-specific region a better opportunity to bind its target template sequence. The last 30 cycles utilized a primer annealing temperature of 68°C (the extension temperature for Pfx) with the annealing time simply added to the extension time at the same temperature (e.g. 1 min 30 sec at 68°C, instead of a 30-sec step for primer annealing followed by a separate 1 min step for extension). This allowed for better specificity of the full-length primer when amplifying template containing both the RHs and the targeted sequence. An example of a thermal cycler program with these specifications is listed below (Table 2).

Step	Temperature	Time(s)
1. PCR initiation	94°C	2 min
2. Denaturation	94°C	15 sec
3. Primer Annealing #1	52°C	30 sec
4. Extension	68°C (for Pfx)	1 min (for 1 kb product)
5. Go to step 2	N/A	4 times
6. Denaturation	94°C	15 sec
7. Primer Annealing #2 and Extension	68°C (for Pfx)	1 min 30 sec (for 1 kb product)
8. Go to step 6	N/A	29 times

Table 2: Sample PCR thermal cycler program for recombineering. The temperature for Step 3 can be adjusted based on the melting temperature of the template-specific primer set. The temperature for Step 7 should remain 68°C (if using Pfx polymerase) regardless of the primer properties. The melting temperature for full-length recombineering primers is usually significantly higher than 68°C, so this temperature will work for any primer set.

Another element essential to screening for correct recombination is the presence of a unique antibiotic resistance gene (i.e. one that is not contained in the target plasmid) in the PCR product along with the sequence of interest. Incorporation of the resistance gene into the target plasmid through recombination allows for selection of recombinant colonies with appropriate antibiotic.

PCR product was purified either through electrophoresis and extraction using QIAEX Gel Extraction Kit (QIAGEN) or with QIAquick PCR Purification Kit (QIAGEN), both according to supplied protocol.

Recombination into target plasmid

With PCR product in hand, overnight cultures of SW102 colonies containing the target plasmid were prepared for electroporation as described above, with the exception of a 42°C incubation for 15 minutes in a shaking water bath prior to cooling on ice. This incubation induces the expression of recombination proteins encoded by the prophage genes. Following the final resuspension of cells in ~50 µl of water, ~250-500 ng of purified PCR product was mixed with the cells, and the cell/PCR product mixture was placed in cooled electroporation cuvettes (1 mm). After electroporation, ~500 µl of cells were plated on two LB/agar plates with the appropriate antibiotic selection for overnight growth.

Purification of recombinant plasmid

Colonies resulting from this selection step still contained a mixture of recombinant and nonrecombinant plasmid, as confirmed by restriction digest. To further isolate recombinant plasmid, resistant colonies were grown overnight in liquid

culture, and minipreps were made using QIAGEN Miniprep kit. Isolated plasmid was diluted to ~0.5 ng/μl, and chemically competent cells were transformed with 1μl of the dilute plasmid preparation. The majority of the colonies resulting from this transformation contained only recombinant plasmid, confirmed by restriction digest and subsequent sequencing.

Primers for *COL11A1* transgene recombineering

The following table lists the primer sequences used in recombineering the *COL11A1* transgene (Table 3).

Primer Set	Sequences
CMR #1	Forward – 5'-ATCCCGCTTAACCCGGGAGATCTACGTACGAGCTCGCCGGCGGTACACTGGGATTTTGGTCATGAGATTATC-3'; Reverse – 5'-GCCACCGTTTCGTTTTCCACCTAGAGGACCACGGCTCCATGGATCCCGCCGTTTAAGGGCACCAATAACT-3'
CMR/5'	Forward – 5'-GAGATCTGTATGGCTTACCCATACGATGTTCCAGATTACGCTAGCTTGGGGGATTTTGGTCATGAGATTATC-3'; Reverse – 5'-GACCAGGTAGACCATCAGCCCCCTGGTAA GCCAGGACGTCCTGGGGGGCCCCCTATCGCCAGGGTCACCAG-3'
Kan	Forward – 5'-GAGATCTGTATGGCTTACCCATACGATGTTCCAGATTACGCTAGCTTGGGGG GCTATCTGGACAAGGGAA-3'; Reverse – 5'-AGCCACCGTTTCGTTTTCCACCTAGAGGACCACGGCTCCATGGATCCCGCTCAGAAGAAGCTCGTCAAGAAGGC-3'
CMR #2	Forward – 5'- TGGTGTGGCTAAGATGGAACAGGGCTGATTCTTGATTCCCAATTCTCAAG CTCAGTGAACGAAAAC-3'; Reverse – 5'- AGATGGTGCACGATGCACAGTTGAAGTG AACTTGCGGGTTTTTCAGTATGTAGCAACCAGGCGTTAAG-3'
cDNA/ CMR	Forward – 5'-GCTGGCTAGCGTTTAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTAT GGAGCCGTGGTCCTCTA-3'; Reverse – 5'- GCCCTCTAGACTCGAGCGGCCGCACTGTGCT GGATATCTGCAGAATCCGTAGCAACCAGGCGTTAAG -3'
CMR #3	Forward – 5'-GGTCTTGAATAAAGCCTGAGTAGGAAGAAGCCTGCAAGCCGAATTCTGCG CTCAGTGAACGAAAAC-3'; Reverse – 5'-AGGGCGAATTGGGCCCTCTAGATGCATGCT CGAGCGGCCCCAGTGTGATGTAGCAACCAGGCGTTAAG-3'
Alpha/ CMR	Forward – 5'-TGGTGTGGCTAAGATGGAACAGGGCTGATTCTTGATTCCCAATTCTCAACT GGGGAAGACAAAAGCAAC-3'; Reverse – 5'-AGATGGTGCACGATGCACAGTTGAAGTGAA CTTGCGGGTTTTTCAGTATGTAGCAACCAGGCGTTAA G-3'
cDNA/ alpha/ CMR	Forward – 5'-CTTCTTTTCTACAGCTCCTGGGCAACGTGCTGGTTGTTGTGCGGAATG GAGCCGTGGTCCTCTA-3'; Reverse – 5'-CGGAGCCCCATCCACAAAGAGTCAGCCAGCAGC TCTCACACCCGGCTGGAGTAGCAACCAGGCGTTAAG-3'

Table 3: Primer sets used in *COL11A1* transgene recombineering.

Results

Specific recombination steps for creation of *COL11A1* transgenic vector

The following recombineering steps created the fusion of the *COL11A1* cDNA and α -globin gene fragment prior to recombination into the p1757 transgenic expression

vector and pcDNA3.1 *in vitro*

expression vector (Figs. 4-11). Each step

was confirmed with restriction digest

and/or cycle sequencing at the BYU

Sequencing Center using plasmid

specific primers (Table 4). Final

products were verified through

restriction digest, PCR with primers for

the full cDNA, and cycle sequencing.

Plasmid	Primer Set
pCR2.1	Forward: 5'-CAGGAAACAGCTATGAC-3' Reverse: 5'-GTAAAACGACGGCCAG-3'
pACT2	Forward: 5'-TACCCATACGATGTTCCAGATTACGC-3' Reverse: 5'-GAACTGCGGGGTTTTTCAGTATCTA-3'
pcDNA3.1	Forward: 5'-TAATACGACTCACTAT-3' Reverse: 5'-TAGAAGGCACAGTCGAGGC-3'
p1757	Forward: 5'-CCTCTGCTAACCATGTTTCAT-3' Reverse: 5'-GTCTGTCTGATGGAGGGCTG-3'

1. Using primer set CMR #1, a chloramphenicol resistance gene

(CMR) was amplified from pBC and introduced into pCR2.1 vector containing the 5' fragment of the *Col11a1* cDNA (TA5'), immediately upstream of the cDNA insert (Fig. 4).

2. Using primer set CMR/5', the resulting CMR/5' cDNA region was amplified and introduced into pACT2 vector containing the 3' fragment of the *Col11a1* cDNA. The resulting pACT2 plasmid contained CMR immediately followed by the complete *COL11A1* cDNA (Fig. 5).

Table 4: Plasmid-specific primers for sequencing inserts.

3. Using primer set Kan, a kanamycin resistance gene (Kan) was amplified from pCR2.1, and the CMR upstream of the *COL11A1* cDNA was replaced with Kan (Fig. 6).

The final steps of recombineering followed two different paths. Steps 4a and 5a created the *in vitro* expression vector for testing the *COL11A1* cDNA for correct protein expression. Steps 4b through 6b created the final transgenic vector containing the complete *COL11A1* expression cassette for future transgenic experiments.

pcDNA recombineering final steps

- 4a. Using primer set CMR #2, CMR was amplified from pBC and reintroduced into pACT2 immediately downstream of the *COL11A1* cDNA (Fig. 7).
- 5a. Using primer set cDNA/CMR, the cDNA/CMR region was amplified and introduced into pcDNA3.1 within the multiple cloning site, completing the expression vector for *in vitro* transcription/translation assays (hereafter called simply pcDNA) (Fig. 8).

pCOL11A1 recombineering final steps

- 4b. Using primer set CMR #3, CMR was amplified from pBC and introduced into pCR2.1 vector containing the α -globin PCR product, immediately downstream of the insert (Fig. 9).
- 5b. Using primer set Alpha/CMR, the α -globin/CMR region was amplified and introduced into the pACT2 plasmid from step 3, immediately downstream of the cDNA (Fig. 10).
- 6b. Using primer set cDNA/ α -globin/CMR #2, the cDNA/ α -globin/CMR region was amplified and introduced into p1757, immediately following the β -globin

splice sequence and immediately preceding the *Col2a1* enhancer. The completed vector (herein called pCOL11A1) contains, sequentially, the *Col2a1* promoter, a β -globin splice sequence, the *COL11A1* cDNA, the α -globin gene fragment, CMR, and the *Col2a1* enhancer (Fig. 11).

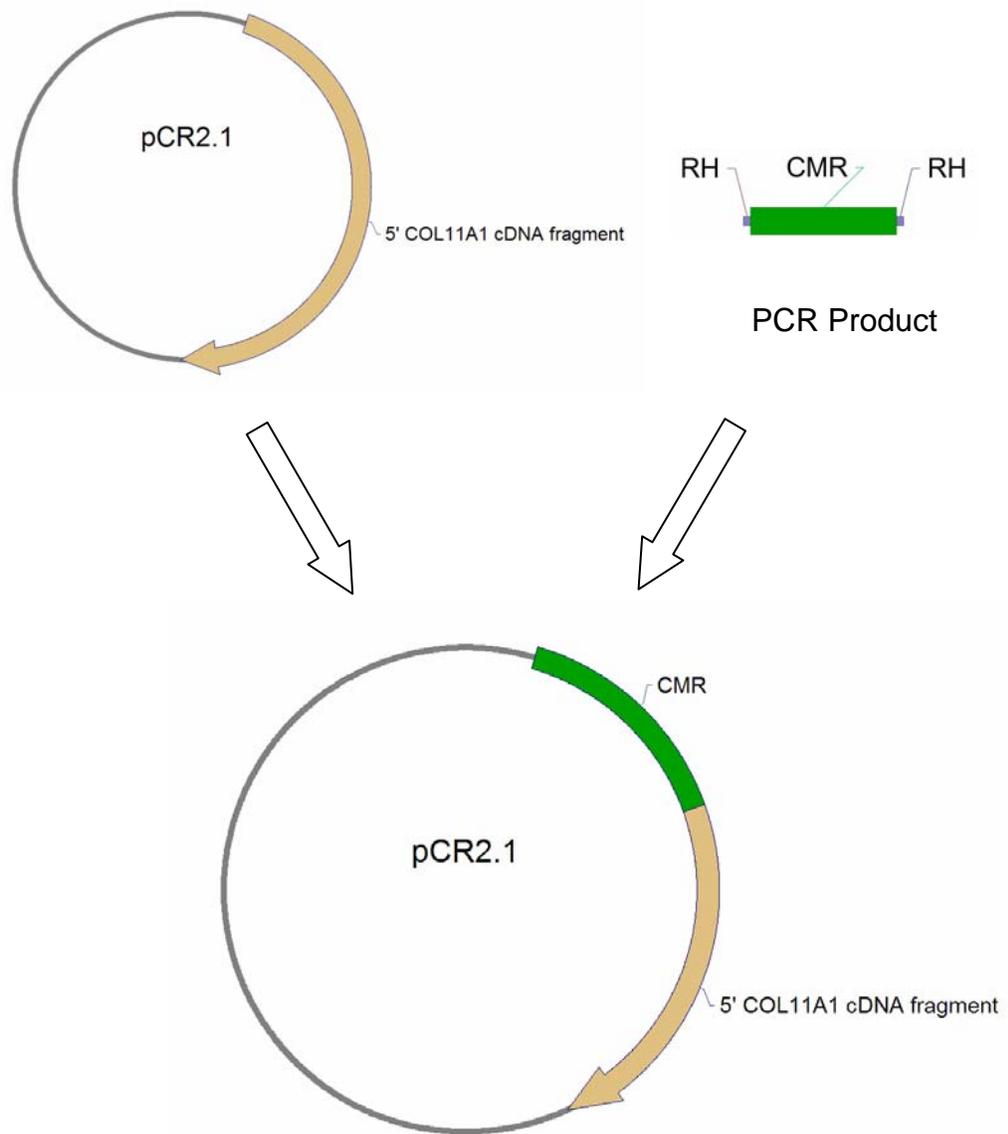


Figure 4: Recombineering step 1. A chloramphenicol resistance gene (CMR) was inserted immediately upstream of the 5' fragment of the *COL11A1* cDNA in pCR2.1.

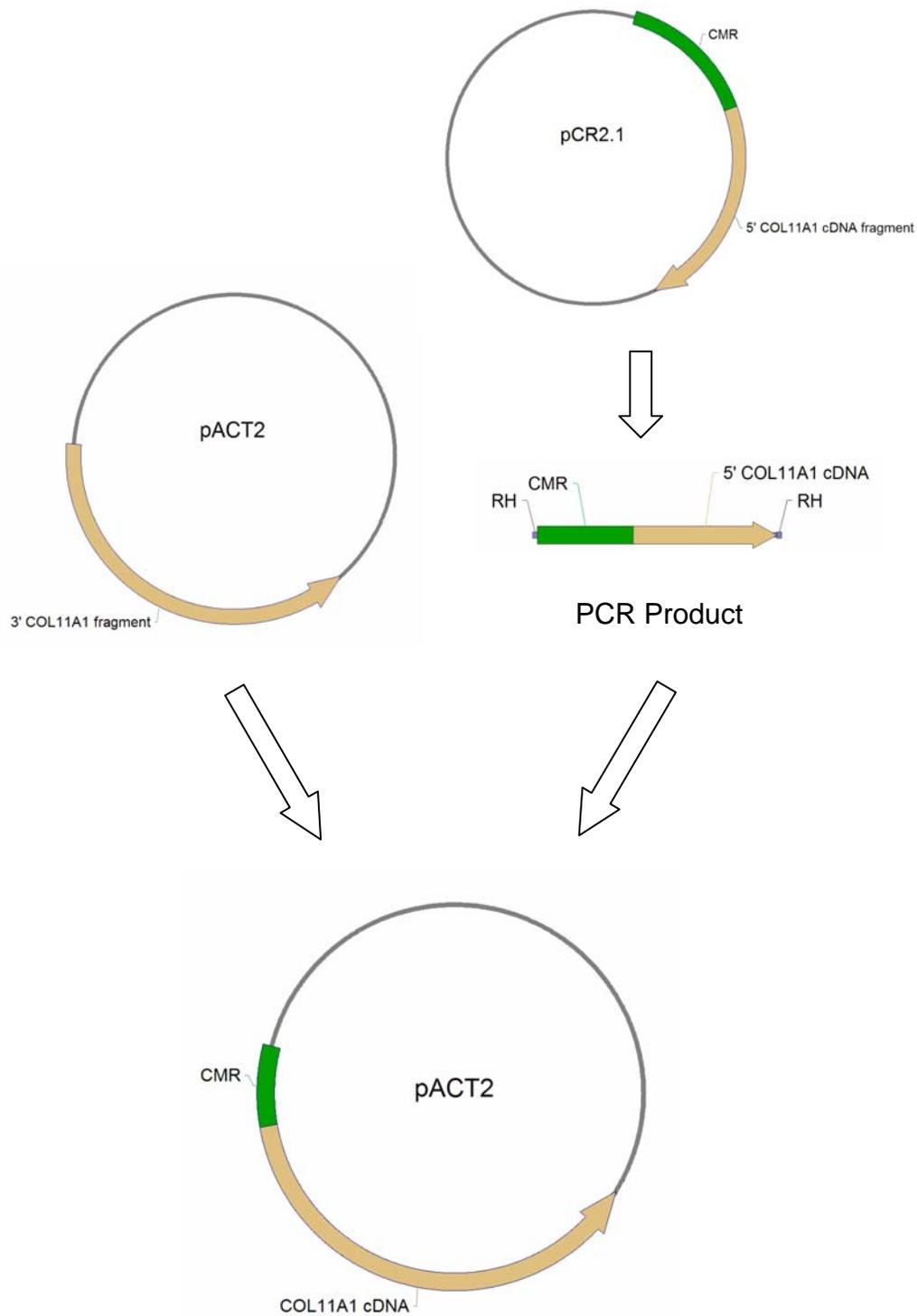


Figure 5: Recombineering step 2. CMR/5' cDNA fragment was inserted into pACT2 containing the 3' *COL11A1* fragment, completing the *COL11A1* cDNA coding sequence.

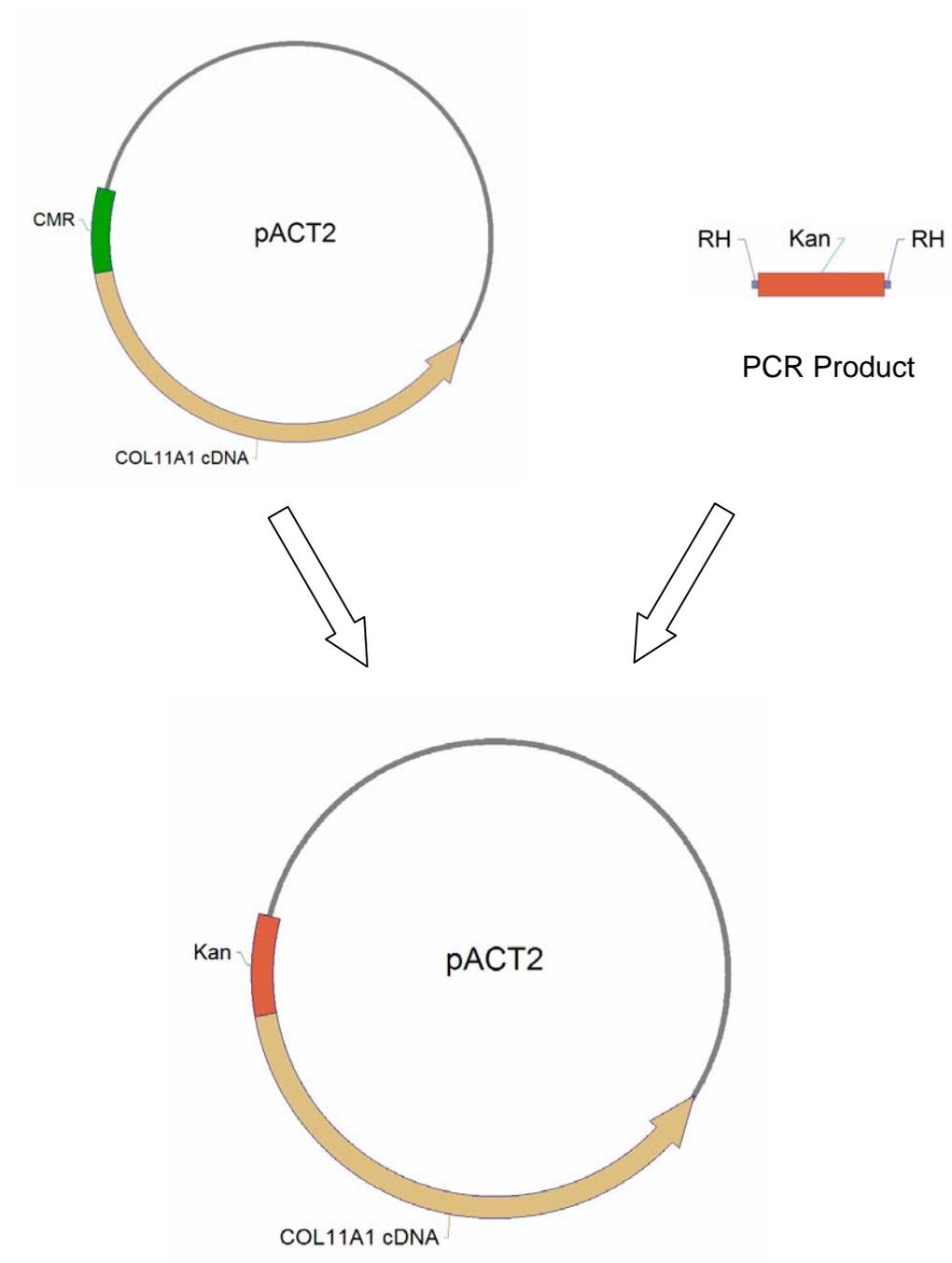


Figure 6: Recombineering step 3. CMR was replaced by Kanamycin resistance gene (Kan) immediately upstream of the *COL11A1* cDNA.

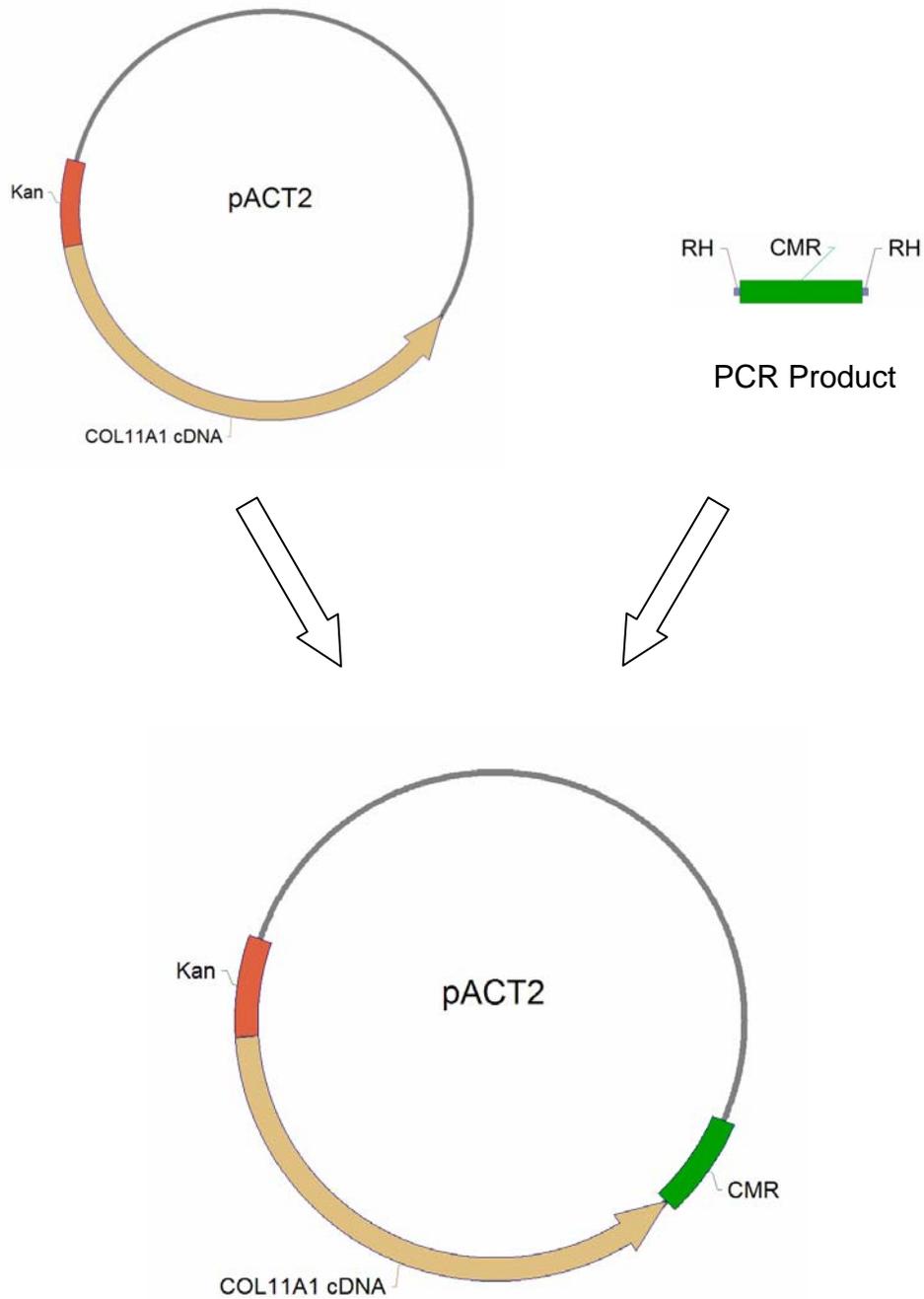


Figure 7: pcDNA recombineering step 4a. CMR was reinserted into the pACT2 plasmid immediately downstream of the *COL11A1* cDNA.

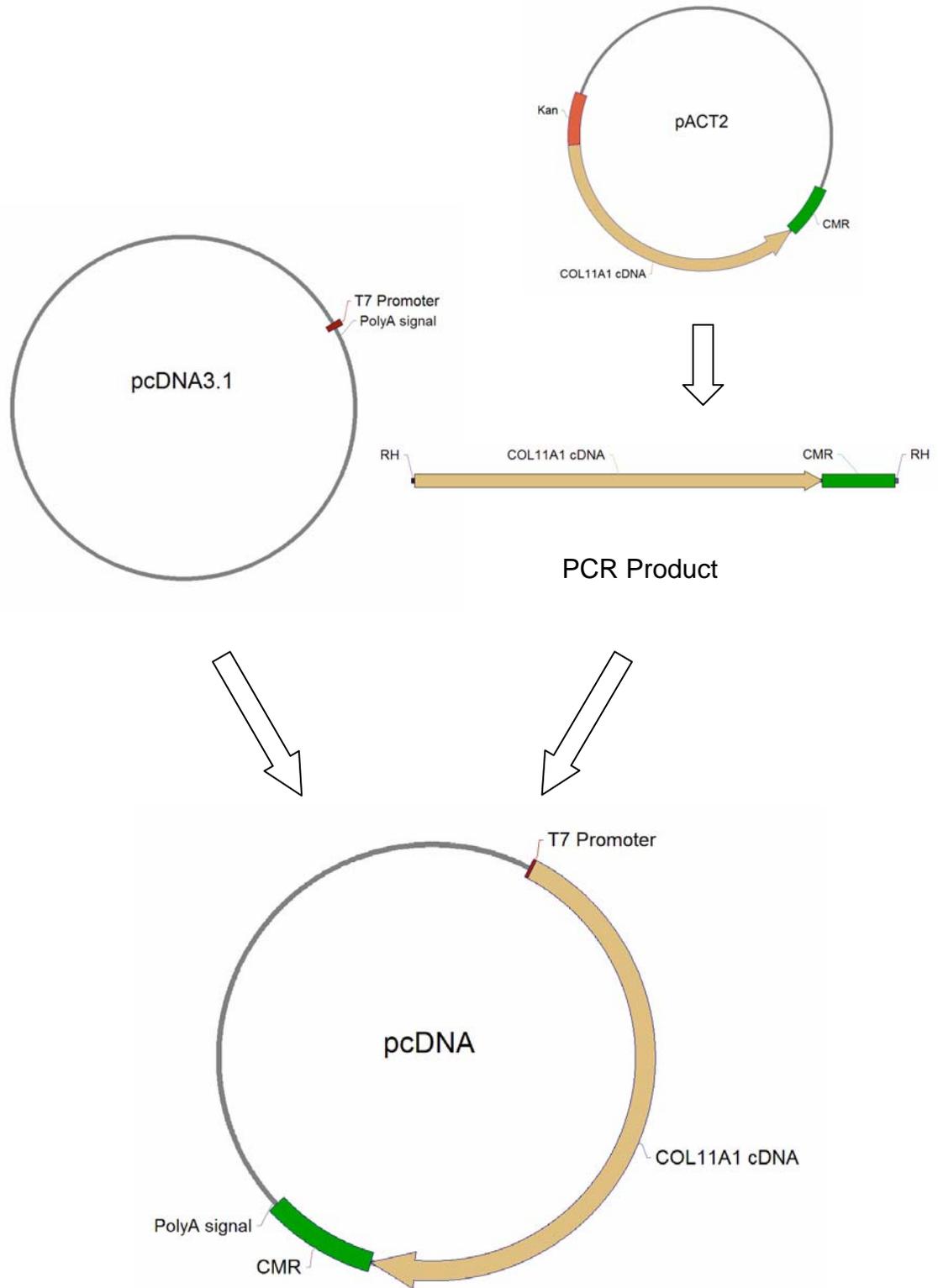


Figure 8: pcDNA recombineering Step 5a. The cDNA/CMR region was inserted into the pcDNA3.1 *in vitro* expression vector for testing cDNA protein expression.

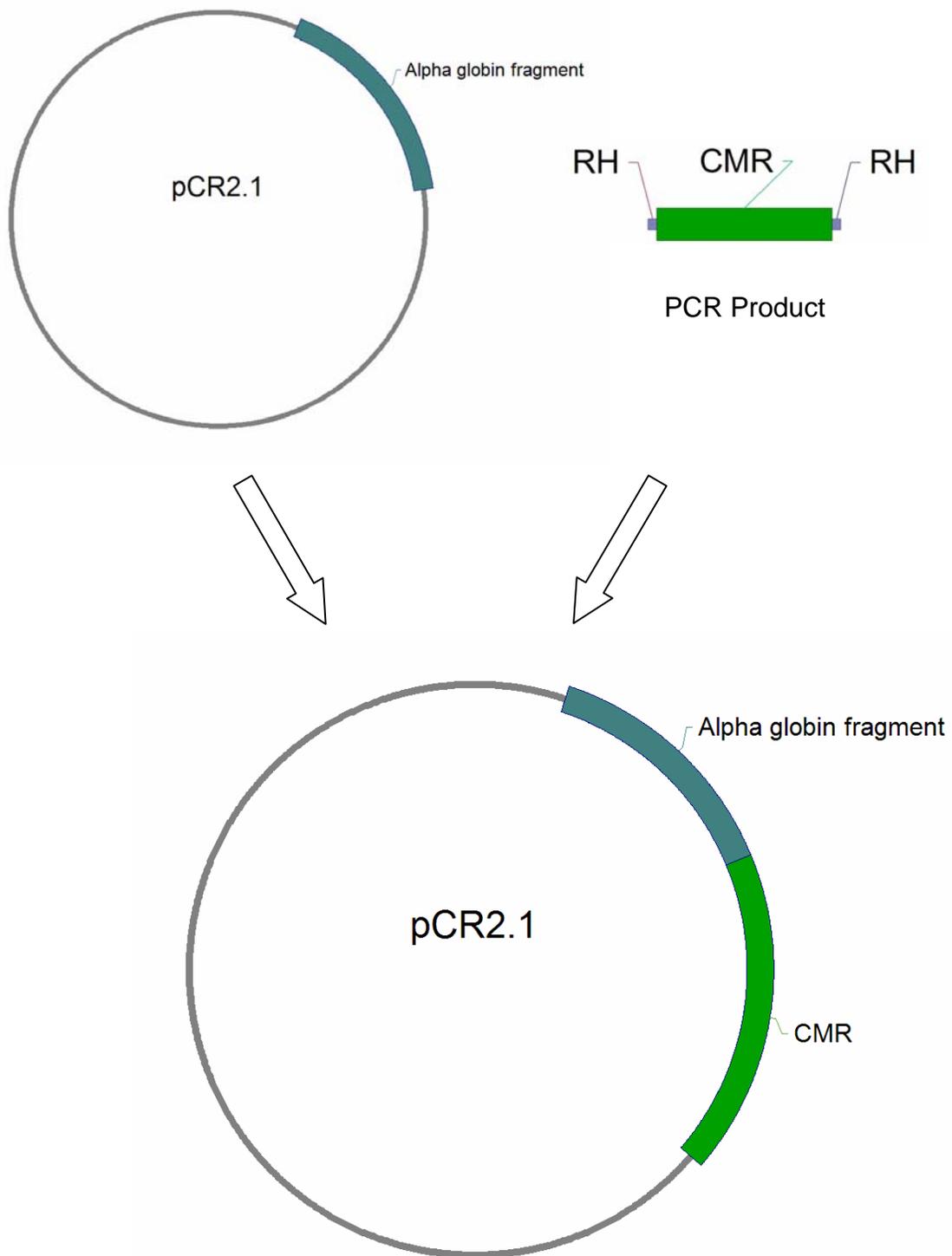


Figure 9: pCOL11A1 recombineering step 4b. CMR was inserted into pCR2.1 immediately downstream of the cloned α -globin fragment.

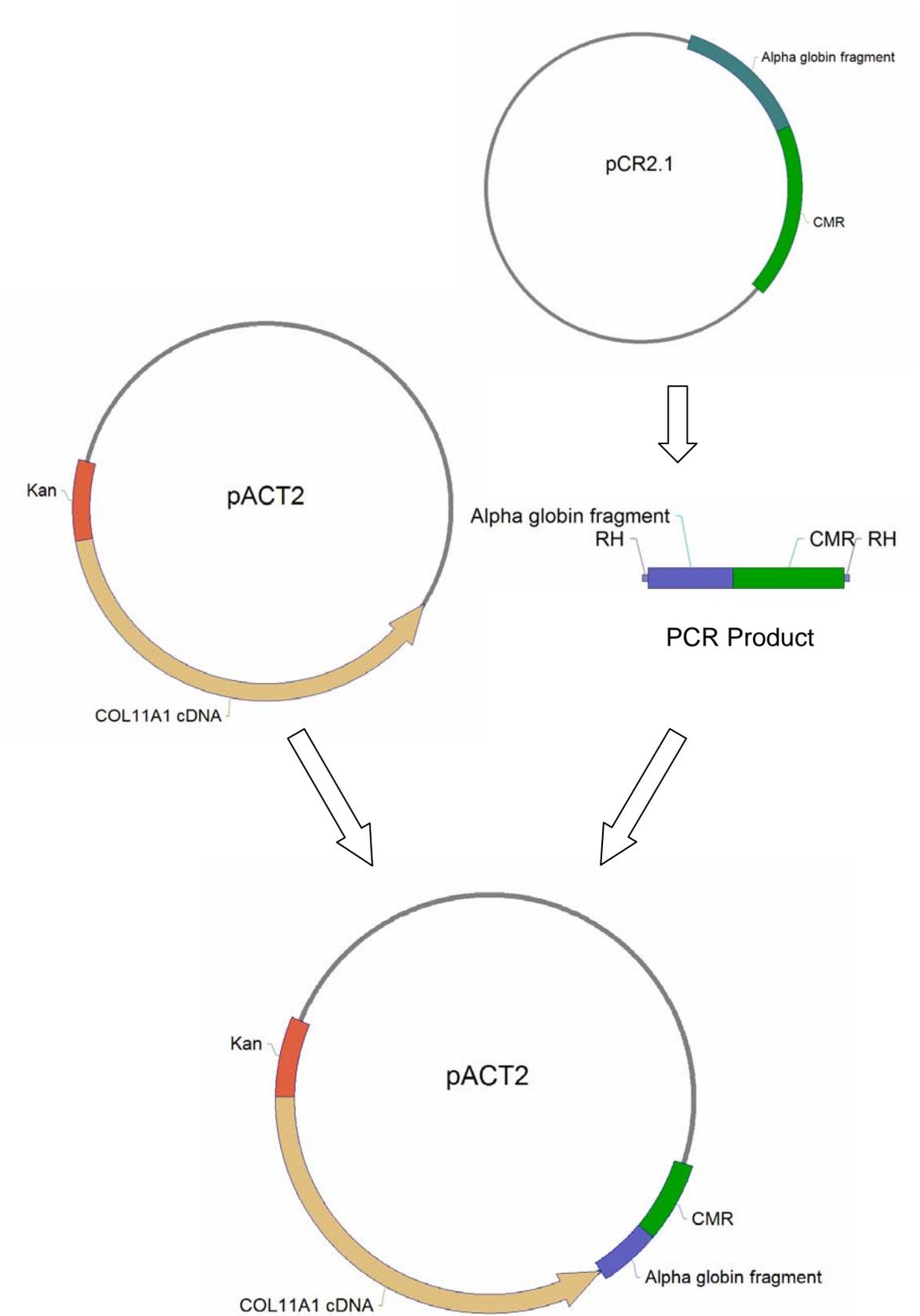


Figure 10: pCOL11A1 recombineering step 5b. The α -globin/CMR region was inserted into pACT2 immediately downstream of the *COL11A1* cDNA.

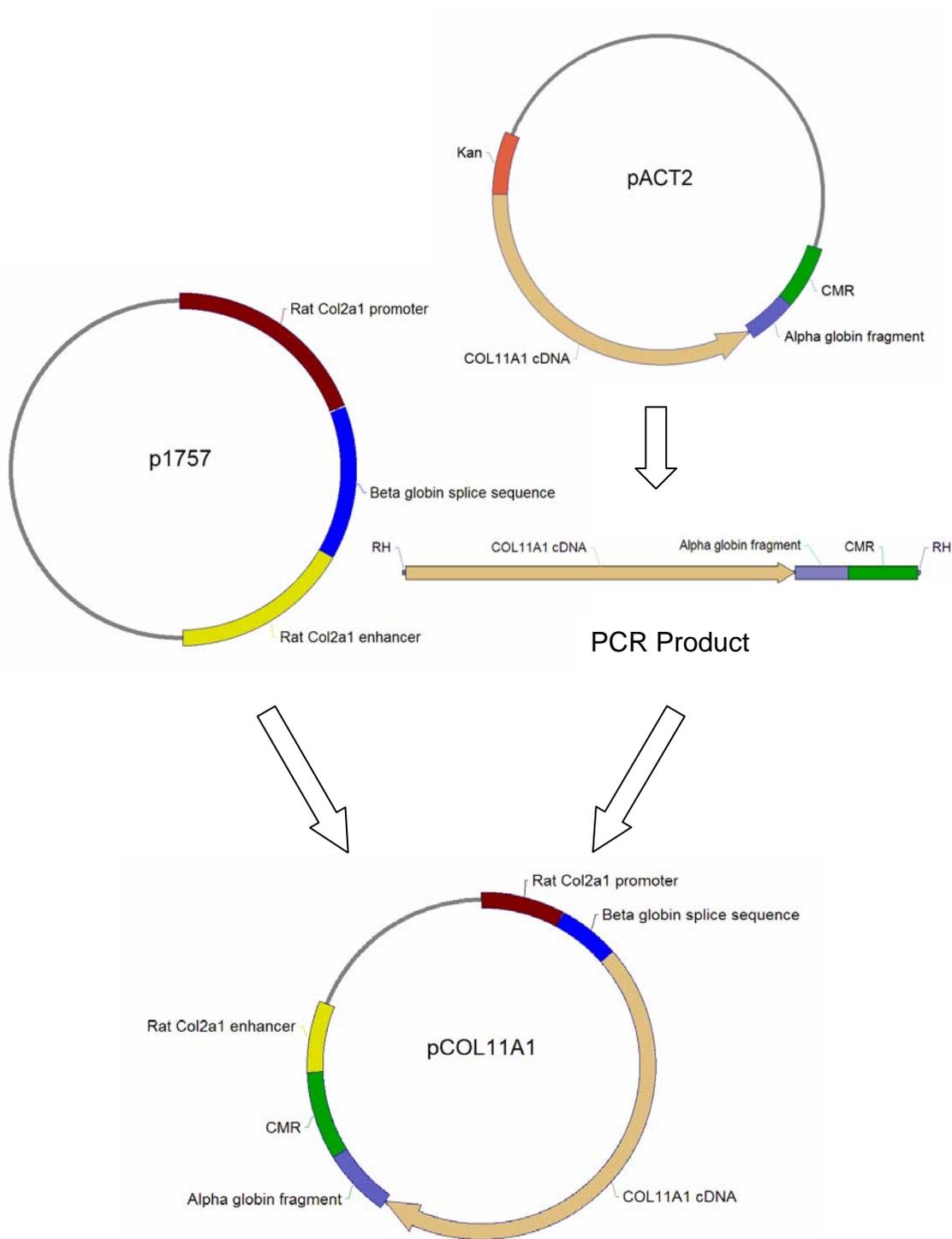


Figure 11: pCOL11A1 recombineering step 6b. The cDNA/ α -globin/CMR region was inserted into the p1757 transgenic expression vector immediately downstream of the β -globin splice sequence and immediately upstream of the rat *Col2a1* enhancer. This is the completed vector that will be used to create transgenic *cho* mice in future studies.

Sequencing analysis of COL11A1 cDNA

The *COL11A1* cDNA used in construction of the transgene vector was sequenced using a series of primers, most of which were previously designed¹⁵ to provide overlapping results (Table 4). From these results, a definitive determination of the entire sequence of the recombinant cDNA could be obtained. The results showed identity with the sequence under GenBank Accession #NM_001854 along the entire length of the cDNA, except for four single base substitutions (Table 5). These substitutions should not affect the functioning of the protein as the resulting codons specify either the same amino acid (in three of the substitutions) or an amino acid with similar properties (in the remaining substitution).

Digestion analysis of pcDNA and pCOL11A1

Following the final recombineering step, minipreps of pCOL11A1 and the pcDNA vectors contained a mixture of the final vectors and nonrecombinant p1757 or pcDNA3.1, respectively. The final vectors have not yet been completely isolated from nonrecombinant plasmid. Minipreps of the final recombineering mixture were subjected to restriction digest analysis as a preliminary test for correct recombination. With proper insertion of cDNA/ α -globin/CMR in p1757, digestion with *Bam*HI would produce two bands of ~275 bp and ~12.6 kb. *Bam*HI digestion of nonrecombinant p1757 would add a single band at ~5.4 kb. Electrophoresis of this digest showed the correct series of bands for the recombinant vector, but lacked the correct band for the non recombinant plasmid. It included instead an additional band of ~7-8 kb (Fig. 12a).

This unexpected band is as yet unidentified but is probably a recombination product of p1757.

With the cDNA/CMR insert in the correct location and orientation in pcDNA3.1, digestion with *Bam*HI would produce one band of ~12.4 kb. *Bam*HI digestion of nonrecombinant pcDNA3.1 would add a band of ~5.4 kb. Electrophoresis of this digest showed the correct size of band for the recombinant vector, but lacked the correct band for the nonrecombinant plasmid. It included instead an additional band of ~2.0-2.5 kb (Fig. 12b). The smaller band is as yet unidentified.

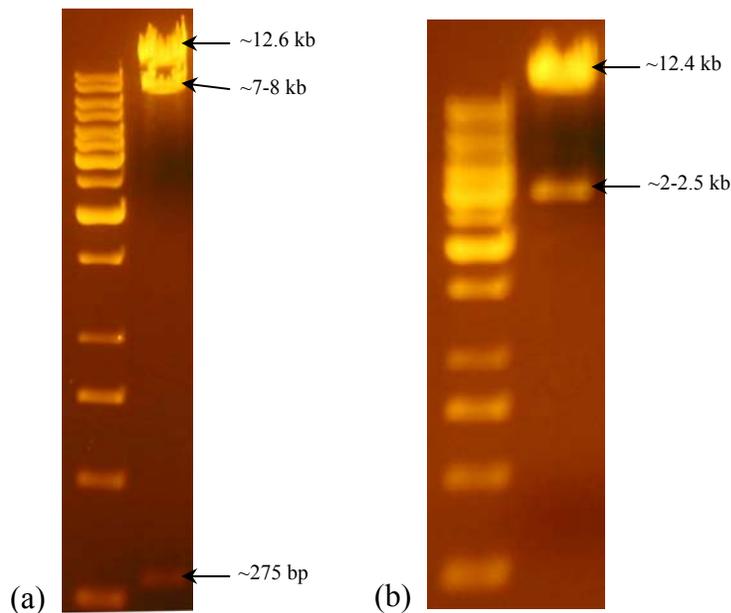


Figure 12: *Bam*HI restriction digests of pCOL11A1 and pcDNA. Top and bottom bands in (a) indicate recombinant vector pCOL11A1. Top band in (b) represents recombinant vector pcDNA. Addition band in (a) and (b) are unidentified product.

Sequencing analysis for correct insertion position and orientation

Primers specific to p1757 and pcDNA3.1 (see table 4 above) flanking the recombineering insertion positions were used to determine proper position and

orientation of the cDNA insert in pCOL11A1 and pcDNA, respectively. Sequencing results confirmed the correct insertion position and orientation of each insert.

Primer name	Sequence
<i>COL11A1</i> Forward	5' – ATGGAGCCGTGGTCCTCTA – 3'
<i>COL11A1</i> Sequencing #2	5' – AGGGGGACATTCAGCAGTTTTTTG – 3'
<i>COL11A1</i> Sequencing #3	5' – ATGAAGATAAACCAACAAGCC – 3'
<i>COL11A1</i> Sequencing #4	5' – AAAGGACCAACCATCTCTGCTCA – 3'
<i>COL11A1</i> Sequencing #5	5' – ACGAGGTTTGCTGGGTCC – 3'
<i>COL11A1</i> Sequencing #6	5' – TAAAAGGTGACAGAGGAGAAGTTGGT – 3'
<i>COL11A1</i> Sequencing #7	5' – GGATTCCCTGGACCAAAAG – 3'
<i>COL11A1</i> Sequencing #8	5' – ACCAGGTCCAGTTGGCTCAC – 3'
<i>COL11A1</i> Sequencing #9	5' – CTCCTGGTCCAATAGGTCTTC – 3'
<i>COL11A1</i> Sequencing #10	5' – GGTCTGTGGTTTTCTGG – 3'
<i>COL11A1</i> Sequencing #11	5' – GAACAAGGGGAAAAAGGTGAC – 3'
<i>COL11A1</i> Sequencing #12	5' – AGACCAATCCAGCCCGAACTT – 3'

Table 5: Primers used to sequence the entire *COL11A1* cDNA. All primers were designed previously (Murphy, 2000) except for “*COL11A1* Forward”.

Base number	Base Change		Amino Acid Change	
	From	To	From	To
462	A	G	Histidine (basic)	Arginine (basic)
3372	G	A	Glycine	Glycine (conserved)
4830	T	C	Glycine	Glycine (conserved)
5088	T	C	Isoleucine	Isoleucine (conserved)

Table 6: Nucleotide changes and resulting amino acids in the reconstructed *COL11A1* cDNA. Only four bases differ from the sequence under GenBank Accession #NM_001854. Three of the four changes code for the same amino acid as in the listed sequence, while the final change codes for an amino acid with similar properties.

Discussion

The *COL11A1* transgene vector has now been successfully assembled and, with a few more steps as discussed below, will be ready for use in the transgenic rescue of the *cho* mouse. Prior to using the construct for its designated purpose, however, a few concerns regarding the design of the transgene should be addressed.

Potential problems with the *COL11A1* transgene

Several aspects of the *COL11A1* transgene as currently constructed could potentially limit its effectiveness in a transgenic rescue experiment. Potential interspecies incompatibility, the utilization of a single *COL11A1* splice variant, and the regulation of *COL11A1* by a *Col2a1* promoter each raise concerns about the usefulness of this particular transgene design in rescuing the phenotype of the homozygous *cho* mouse.

Problem #1: Will using a human protein in a mouse embryo lead to interspecies incompatibility?

The *COL11A1* cDNA used in construction of the transgene was made from mRNA extracted from human knee cartilage¹⁵. In future experiments the transgene will be used in *cho* mouse embryos in an attempt to correct the defects caused by lack of $\alpha 1(XI)$ chain. While human and mouse $\alpha 1(XI)$ chains share 91.9% identity of amino acid sequence, according to NCBI's HomoloGene, and the same structural domains are conserved, there is still the possibility that some of the amino acids at the

points of disparity allow for species-specific functions or interactions. This might limit the functionality of the transgenically expressed protein in the mouse embryo and make it difficult to conclusively interpret the results of the transgenic rescue.

However, many of the amino acids that differ between the two analogs are similar in chemical properties, suggesting that the human and mouse $\alpha 1(XI)$ chains could likely be interchangeable. In addition, the other chains of the human type XI collagen molecule, $\alpha 2(XI)$ and $\alpha 3(XI)$, have 93.2% and 95.3% identity, respectively, with their mouse homologs. The fact that the $\alpha 1(XI)$ chain in humans normally functions with other proteins possessing such high sequence identity with mouse adds support to the probability that the transgenic $\alpha 1(XI)$ protein will also function well in mice.

Other transgenic mouse rescue experiments using human proteins with lower amino acid sequence identities have been performed successfully^{19, 20, 21}. Supporting the feasibility of a collagen rescue with human protein, *Col2a1* knockout mice have been rescued with a human *COL2A1* transgene²², indicating that a cross-species rescue involving collagen is possible. With this encouraging evidence, it appears that a transgenic rescue using human *COL11A1* has a high probability of success.

Problem #2: Will expression of a single *COL11A1* mRNA isoform result in insufficient functionality?

Aside from differences between species, multiple splice variants of the *COL11A1* transcript are expressed even within the same animal. Differential splicing of *COL11A1* occurs in chondrocytes and other tissues in which it is expressed,

yielding several different isoforms, each coding for a slightly different protein. Exons 6A, 6B, 7 and 8 are variably spliced, with 6A and 6B never occurring in the same mRNA and exon 7 contained in all variants. Exons 6A and 8 code for acidic peptides, while 6B codes for a basic peptide. Thus it is evident that the $\alpha 1(XI)$ chains resulting from the different splice variants would have slightly different molecular properties depending on which exons are included in the final product.

While unique functions have not yet been associated with individual isoforms, the location of the spliced exons makes it likely that they play an important role. The variable region of *COL1A1* is found within the globular N-terminal domain of the $\alpha 1(XI)$ chain, which resides on the surface of collagen fibrils. In addition to regulating fibril growth, it has been suggested that this domain might also provide a point of interaction with surrounding ECM components. If this is true, then it is possible that different splice variants might fill specific structural niches, interacting with unique ECM components to maintain a strong, complete and fully functional tissue.

The *COL1A1* cDNA used in the transgene codes for the 6A-7-8 splice variant. This splice variant is one of the most abundantly and widely expressed²³ and thus should be able to function in all *COL1A1*-expressing tissues. Though the transgenic animal will not receive the benefit of the full spectrum of isoform expression, expression of even a single isoform should be sufficient to attenuate the chondrodysplasia, even if only partially. Should the cartilage in *cho/cho* mice regain even a fraction of wildtype functionality, significant and measurable growth beyond the usual stunted phenotype should be readily observable. Any improvement from the typical homozygous phenotype would qualify the transgenic rescue as a success.

Problem #3: Will expressing COL11A1 under Col2a1 regulation lead to type XI collagen overexpression?

Assuming that the protein for which the transgenic cDNA codes is fully functional in the mouse embryos, the question still remains: what effect will the transgenic *Col2a1* promoter/enhancer have on the *quantity* of collagen XI expression? The typical ratio of type II collagen to type XI collagen in fibrils is approximately 8:1. When this ratio is increased, *in vitro* as well as in the *cho* homozygotes, the fibrils become shorter, thicker, and spindle-shaped^{7, 10}; when the ratio is significantly decreased *in vitro*, the fibrils become longer and thinner than normal. With *COL11A1* expressed under the control of the *Col2a1* promoter, it is likely that the quantity of $\alpha 1(\text{XI})$ chain will be increased from normal, more closely resembling the expression of $\alpha 1(\text{II})$ chain. With extra $\alpha 1(\text{XI})$ chain available, more type XI collagen than normal could potentially be produced, possibly leading to an *in vivo* phenotype similar to the thin, elongated fibrils of the *in vitro* results mentioned above. The physiological effects of such altered fibrils in adult cartilage are unknown. Thus overexpression of type XI collagen as a result of the transgene could be instructive, although unanticipated.

While some extra type XI collagen could potentially be formed with overexpression of *COL11A1*, it seems unlikely that the amount would reach overwhelming proportions. It should be remembered that the other two chains of the collagen XI heterotrimer, $\alpha 2(\text{XI})$ and $\alpha 3(\text{XI})$, would still be expressed at normal levels. It seems likely that these chains would act to some extent as limiting reagents

in the formation of collagen XI molecules, although no previous studies have tested this. The potential fate of the excess $\alpha 1(XI)$ chain is uncertain; it might be degraded soon after translation or perhaps simply accumulate within the endoplasmic reticulum of the chondrocytes. The collagen fibrils in the extracellular matrix of transgenic animals, however, would likely remain similar in phenotype to those in wild-type matrix.

Regardless of the final amount of type XI collagen and its resulting ratio with type II collagen, a transgenic *cho* mouse expressing any functional collagen XI will be an improvement over non-transgenic homozygote expression. Any negative effects from type XI over-expression would presumably be less severe than the complete lack of type XI in *cho* mice. Consequently, even if collagen XI is over-expressed, there should still be marked improvement in *cho* skeletal growth and postnatal survival which would indicate a successful transgenic rescue.

Despite these idiosyncrasies of the transgene construct, all probabilities point to a successful transgenic rescue. While the final product of the rescue may not mimic the wild-type phenotype in all aspects, it is anticipated that this construct will provide for significant improvement in fetal skeletal development and subsequent survival of transgenic homozygotes after birth. These will be the hallmarks of a successfully realized transgenic rescue of the *cho* phenotype.

Future work with the *COL11A1* transgene

Construction of the *in vitro* and transgenic vectors at this point in time is complete. The recombinant vectors containing the full expression cassettes are still unisolated

from the nonrecombinant pcDNA3.1 and p1757 vectors. This purification has proven difficult through the usual method described in “Materials and Methods” above. Two approaches present themselves as possible methods for isolating the recombinant plasmids:

1. The vector mixture could be digested with a restriction enzyme which will only cut once in the final vector, thus linearizing the vector. Digested vector could be separated on an agarose gel, the correct vector extracted and purified from contaminants, and recircularized through a self-ligation reaction. This isolated, religated vector could then be transformed and amplified for future use. For pcDNA *BamHI* could be used for linearization. For pCOL11A1, *BamHI* could also be used; however, the digest would remove an ~275 bp fragment from the internal sequence of the α -globin gene segment. The polyadenylation signal would be preserved, which is the most important feature of the α -globin gene segment.
2. The undigested vector mixture could be separated on an agarose gel, and the correct vector extracted and isolated in circular form. This extract would be immediately ready for transformation and amplification in bacteria. This procedure could be complicated should the recombined and non-recombined plasmid ring exist linked together. This would require the linearization and electrophoretic separation described above to isolate the recombinant plasmid. Barring linkage of the two plasmids, either of these methods should yield purified vector,

although the second method appears on the surface to be simpler and more straightforward.

Following isolation, the plasmids should be fully sequenced again to ensure that no mutations have been introduced through the construction process.

Once isolated and sequenced, these constructs must then be tested for proper expression prior to their use in transgenic experiments. The pcDNA plasmid containing the cDNA will be used in *in vitro* transcription/translation assays to look at the size and identity of protein coded for by the cDNA. The expressed protein should be ~181 kD and could be further identified by using $\alpha 1(XI)$ -specific antibody.

The pCOL11A1 transgenic vector will also be tested for expression in cultured chondrocytes before being used to produce transgenic animals. Rat chondrosarcoma cells will be transfected with the vector and isolated mRNA probed for *COL11A1*-specific sequences. Northern blots should show a band of ~6.4 kb, corresponding to the fusion mRNA of *COL11A1* cDNA and the α -globin fragment, in addition to the endogenous transcript of ~6 kb or less. Demonstrated expression of this unique mRNA in chondrocytes, in conjunction with *in vitro* transcription/translation assays of the cDNA itself, would confirm that the transgene possesses the ability to express the expected protein in the desired location. The vector could then be confidently used for creating transgenic *cho* animals and evaluating the rescue of the mutant phenotype.

Despite possible difficulties with the *COL11A1* transgene as discussed above, a transgenic rescue of the *cho* mouse using the p*COL11A1* cassette will be greatly instructive. Information gleaned from the rescue of this mouse model would provide a basis for efforts to correct similar human disorders. With the advance of delivery

methods for therapeutic genetic constructs, an understanding of the principles of gene therapy for cartilage disorders will pave the way for a cure for many who suffer from these disorders.

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