Mutations in Collagen Genes

Consequences for Rare and Common Diseases

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Introduction

As readers of this journal are well aware, the technologies of gene cloning and gene analysis have recently lead to dramatic discoveries about the molecular basis of heritable disorders such as sickle cell disease, thalassemias, phenylketonuria, and the Lesch-Nyhan syndrome. In many instances the discoveries have provided the basis for new DNA tests for the early, prenatal diagnosis of serious forms of the diseases. Recent work on human genes for collagen is proceeding along a similar path and has now defined the molecular basis for a number of heritable disorders of connective tissue (for reviews, see references 1–5). It is very likely that DNA tests for the prenatal diagnosis of a few of these conditions will soon be available. The recent work on mutations in collagen genes, however, has provided several surprises as to the kinds of mutations and some of the consequences of these mutations.

Structure and function of the protein

Collagen is among the most abundant proteins in the body, and it is also among the largest of proteins. In several respects, however, it is one of the simplest (for review, see reference 1). The molecule of type I collagen, the most common form of collagen, is a long, thin rod (Fig. 1). It is comprised of two identical polypeptide chains called α1(I) and a third chain with a slightly different amino acid sequence called α2(I). The three chains are wrapped around each other in a coillike, three-stranded conformation that is similar to a three-stranded rope. The major biological property of the protein is that it spontaneously self-assembles under physiological conditions into long, thin fibrils that have about the same tensile strength as steel wires. Collagen in fact provides one of the simplest examples of how large structures in biological systems are formed through the principle of self-assembly of macromolecules. The structure of the protein is also relatively simple in the sense that it is highly repetitive. Glycine, the smallest amino acid, is every third amino acid in the three polypeptide chains of the protein. Therefore, the amino acid sequence of the α-chains, which are $\sim$1,000 amino acids long, is commonly represented as $(Gly\text{-}X\text{-}Y)_{333}$. Many of the X-positions in this sequence are occupied by the rigid, ring amino acid proline. Many of the Y-positions are occupied by the analogous ring amino acid 4-hydroxyproline. Studies with synthetic peptides have conclusively demonstrated that the proline and hydroxyproline residues, particularly in sequences of -Gly-Pro-Hyp-, direct folding of the α-chains of procollagen into a triple-helical conformation and give the helix its characteristic, ropelike structure. The remaining X- and Y-positions in the α-chains are largely occupied by charged and hydrophobic amino acids. These amino acids occur in clusters along the surface of the molecule and direct a self-assembly of a collagen molecule into a quarter-staggered array which accounts for the characteristic cross-striations of collagen fibrils.

After fibrils are assembled, covalent crosslinks are formed among adjacent molecules. The crosslinks are essential to give collagen fibrils the required tensile strength. Formation of the crosslinks is initiated by oxidative de-amination of selective lysine and hydroxylysine residues in the protein followed by complex interactions of the resulting aldehyde groups with side chains of amino acids on adjacent molecules.

To date, some 10 different types of collagens have been identified (see reference 1). All the collagens have repeating amino acid sequences of -Gly-X-Y- and therefore have regions in which the protein is triple-helical. They differ, however, in the precise amino acid sequences and as to whether they contain globular domains in addition to collagenous domains. Type I, II, and III collagens form readily discernable fibrils and therefore are referred to as fibrillar collagens. Type I collagen is the major constituent of skin, tendons, ligaments, bone, and many other tissues. Type II collagen is the predominant collagen of cartilage. Type III is a less abundant collagen but is usually found in association with type I. Type IV collagen is the most common nonfibrillar collagen and is the major constituent of all basement membranes. Type V is found in association with blood vessels and smooth muscle cells. Type VI and the family of other collagens are found in small amounts in specific tissues. Therefore, it is apparent that collagens constitute a family of closely related proteins, each of which probably have a specific role in defining the structure of connective tissues.

Biosynthesis

In contrast to the relative simplicity of the structure and function of collagen, its biosynthesis presents a complex picture (Fig. 1). The collagen molecule is first synthesized as a larger precursor called "procollagen," which must be enzymically cleaved at both its amino- and carboxyl-terminal ends to generate collagen. One enzyme, procollagen N-proteinase, cleaves the amino-terminal propeptides. A second enzyme, procollagen C-proteinase, cleaves the C-terminal propeptides. If the amino-propeptides are not cleaved from the molecule, the protein can form fibrils, but the fibrils are thin, irregular, and do not become adequately crosslinked. If the carboxyl-terminal propeptide is not cleaved, the large C-terminal propeptides completely prevent the protein from assembling into fibrils. Cleavage of the propeptides may occur within crypts or folds of fibroblasts as suggested by the schematic diagram in

J. Clin. Invest.

0021-9738/85/03/0783/05 $1.00
Volume 75, March 1985, 783–787

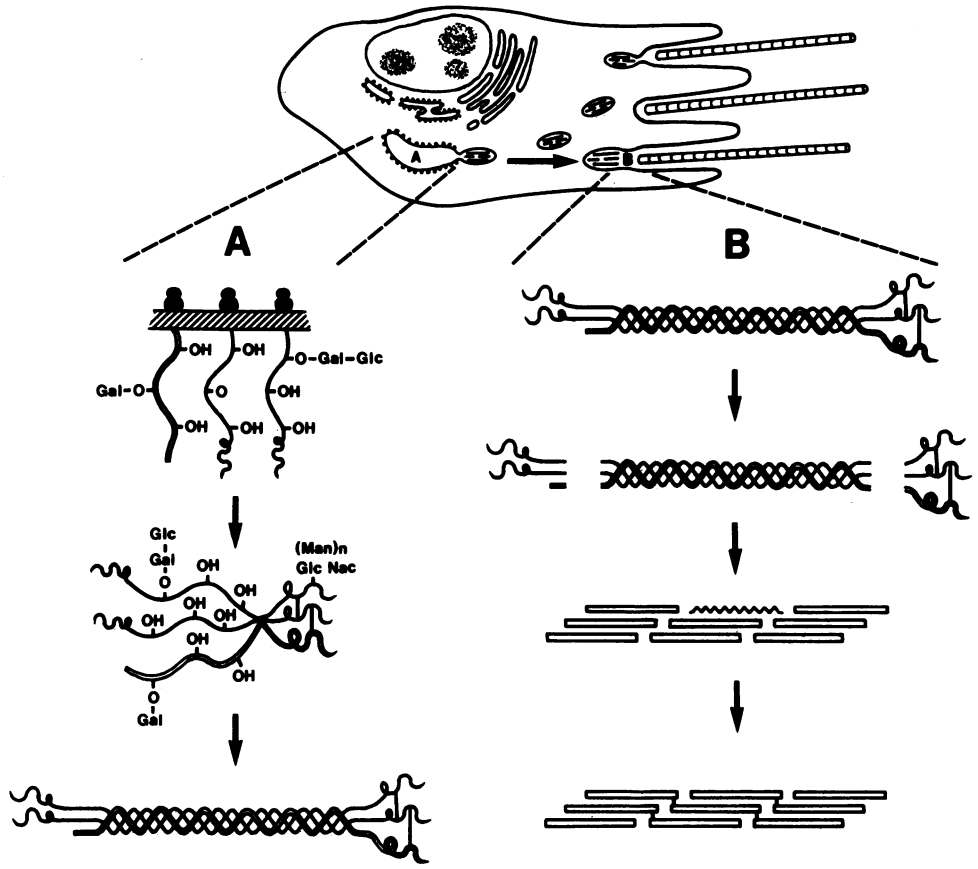

Figure 1. Fibroblast assembly of collagen fibrils. *A* shows intracellular post-translational modifications of the proα-chains, association of the carboxyl-propeptide domains, and folding into the triple-helical conformation. Gal, galactose; Glc, glucose; GlcNaC, Nacetylglucosamine; and $(Man)_n$, mannose residues. *B* shows enzymic cleavage of procollagen to collagen, self-assembly of collagen monomers into fibrils, and cross-linking of fibrils. Reproduced with permission (1)

Fig. 1. In some circumstances, however, the cleavage of procollagen and assembly of collagen into fibrils may occur at some distance from the biosynthetic cells.

The intracellular assembly of the procollagen molecule is in itself a complex process. The proα-chains of procollagen are synthesized by translation of the appropriate mRNAs on ribosomes attached to the rough endoplasmic reticulum. As the newly assembled chains pass into the cisternae of the rough endoplasmic reticulum, a series of post-translational processing steps occur. "Signal" peptides are cleaved from the amino-termini of the three chains. Prolyl residues in the Y-position of the chains are enzymatically converted into hydroxyprolyl residues, and some of the lysyl residues in Y-positions are enzymatically converted into hydroxylysine. The hydroxylysyl residues are substituted with galactose or glucosylgalactose. In addition, a mannose-rich oligosaccharide is added to the carboxyl-terminal propeptides. After assembly of the chains is complete, the carboxyl-terminal propeptides associate and become disulfide linked. Post-translational modifications of prolyl and lysyl residues continue until a critical level of ~100 hydroxyprolyl residues per chain is reached. At this stage, the protein folds into a triple-helical conformation, first by formation of a "nucleus" of a triple-helix near the carboxyl-terminal ends of the chains, and then propagation of this helical structure toward the amino-terminus. In all, some eight separate enzymes are involved in the intracellular processing and assembly of procollagen, and these enzymes modify well over 100 sites in each chain. Some of the complexity in the biosynthesis is probably explained by the need to generate a molecule of the correct degree of rigidity. Human collagen and procollagen monomers in solution unfold into gelatin just 3–5°C above body temperature. Collagens and procollagens from other species with differing body temperatures also unfold 3–5°C above the body temperature of each species. Apparently, therefore, the molecule must be in a triple-helical conformation that is close to its unfolding temperature in order to form normal collagen fibrils in vivo.

Of special interest to medicine is the fact that ascorbic acid is an essential cofactor for the hydroxylation of both prolyl and lysine residues, and that deficiency of ascorbate leads to the synthesis of collagen monomers which are so deficient in hydroxyproline that they cannot appropriately fold into a triple-helix at body temperature. This set of facts explains why wounds do not heal normally in scurvy and why, in some instances, old wounds break down.

Genes for procollagens

The genes for procollagen also present a relatively complex picture. Since the 10 or more collagens have different amino acid sequences, they must be products of different genes. Several of these genes have now been cloned and characterized, and the genes for the fibrillar collagens (types I, II, and III) have now been extensively analyzed (see reference 1, 6–9). All these genes are large. For example, the gene for the proα1-chain of type I procollagen is ~18,000 bases, and the gene for the proα2 chain of type I procollagen is ~38,000 bases. The coding sequences for the α-chain domain of the genes is characteristically divided into short exons of 54 bases, which code for 18 amino acids with the sequence $(Gly\text{-}X\text{-}Y)_6$. A few of the exons are 45 base pairs, and a few are twice 54 or 108 base pairs. The discovery of the typical 54-base exon of the genes has lead to the suggestion that collagen genes may evolve

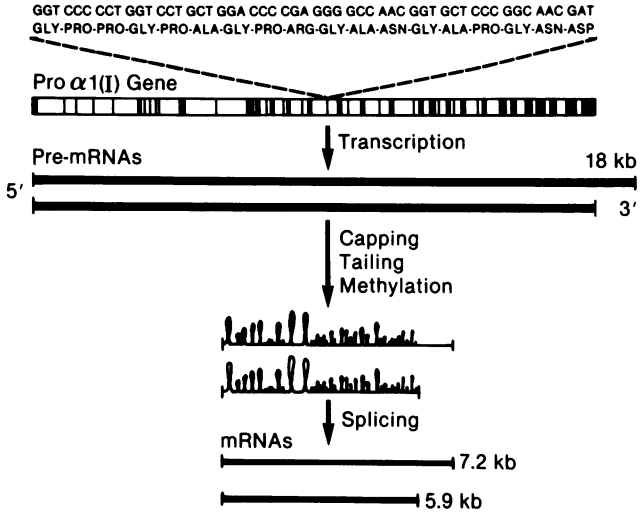

Figure 2. Structure and transcription of the proα1(I) gene. A typical 54-base pair exon encoding for a sequence of $(Gly\text{-}X\text{-}Y)_6$ is shown at the top of the figure. The pattern of exons and introns in the proα1(I) gene as represented here is based on a similar pattern of the chick proα2(I) gene. The gene is transcribed into two different pre-mRNAs, apparently because the first signal for termination of transcript is not entirely efficient. Each initial RNA transcript is ~18,000 kilobases (kb) long. Splicing out the intervening sequences reduces these transcripts to mRNAs of ~7.2 and 5.9 kilobases. The processing of the initial RNA transcripts also includes addition of a "cap" of the unusual base 7-methylguanylate at the 5′ end, addition of a "tail" of polyadenylate at the 3′ end, and methylation of a few bases. The two mRNAs with different length 3′-non-coding ends are both used on polysomes to synthesize proα1(I) chains with the same primary structure. Reproduced with permission (1)

by successive duplication of a primitive 54-base gene. However, recent studies on a collagen characteristically found in cartilage (type IX collagen) indicate that some collagen genes are not divided into 54-base pair exons (10). Therefore, it is possible that the unusual intron/exon pattern found in some collagen genes may reflect still undefined selective pressure exercized during evolution of the genes.

Search for mutations in collagen

A large number of heritable diseases have long been recognized to involve tissues rich in collagen. Therefore, it has been suggested for some time that many of these diseases are produced by mutations in the genes for one or more collagens. There have been a number of technical problems, however, in fully exploring the suggestion. Some of these technical problems continue to present formidable obstacles and therefore are probably worth noting here.

One of the technical problems in searching for mutations in collagen genes is the large size of the genes and their unusual exon/intron structure. Even with present day technologies, searching for small nucleotide alterations in genes with so many similar exons still presents a formidable challenge. A second technical problem lies in the complexity of procollagen biosynthesis. A defect in one of the eleven post-translational enzymes could well mimic a mutation which alters a structural gene for procollagen. Also, several different collagens may be present in the same tissue and vital for its integrity. Therefore, it is difficult at the outset to be certain which set of genes should be explored in examining any particular heritable disorder that might involve collagen.

For these and related reasons, our laboratory and several other laboratories have followed several special strategies to define mutations in procollagen genes. One of these strategies is to focus experimental efforts on mutations that produce a readily demonstrable change in structure of the procollagen molecule. To detect such mutations, the procollagen synthesized by cultured fibroblasts is examined for structural changes which alter the electrophoretic mobility of proα-chains or peptide fragments of the proα-chains. If the data from such experiments establish which proα-chain has a structural abnormality and where within the proα-chain the abnormality occurs, the appropriate region of the gene is analyzed. A second strategem is to identify restriction fragment length polymorphisms associated with a given gene to test for coinheritance (or cosegregation) of a specific allele for a procollagen gene and a heritable disease within a family (11). If coinheritance is found, the specific allele is examined in detail.

Mutations that alter the structure of type I procollagen molecule

About 10 mutations that change the structure of type I procollagen molecules have now been defined in patients with osteogenesis imperfecta, Ehlers-Danlos syndrome, or related disorders (for reviews, see references 1–5, 12). The number is small, but the data are beginning to provide the bases for several generalizations. One of these generalizations is that very similar structural alterations in different regions of the protein can produce very different clinical syndromes.

Mutations that change the structure of the amino-terminal propeptides appear to have their primary effect on the processing of the procollagen molecule by procollagen N-proteinase. In terms of their clinical characteristics, they are primarily associated with marked laxity of joints, and therefore generally classified as variants of Ehlers-Danlos syndrome. Procollagen N-proteinase is a large molecular weight, neutral metalloproteinase which has at least one unique property: it requires a procollagen substrate that not only has the correct amino acid sequence but also the correct three-dimensional conformation. In one patient, a still undefined mutation in one allele for proα2(I) chains produced an in-phase deletion of between 10 and 30 amino acids near the amino-terminus of half the α2(I) chain synthesized by fibroblasts (13). The deletion was ~100 amino acids removed from the site at which procollagen N-proteinase cleaves the type I procollagen molecule, but it produced a slippage of proα-chains in the molecule which distorted the conformation of the cleavage site sufficiently to make the procollagen resistant to the proteinase. The consequence in the patient was persistence of half the type I procollagen as pNcollagen, the intermediate in the conversion of procollagen to collagen containing an intact amino-terminal propeptide. Persistence of the pNcollagen, in turn, apparently accounted for the fact that the patient's joints were so loose that he had dislocations of the hips, knees, and other joints. Several other variants of Ehlers-Danlos syndrome also appear to have mutations that change amino acid sequences in or near the cleavage site for procollagen N-proteinase and thereby lead to persistence of pNcollagen in tissues (14, 15). Previous studies demonstrated that persistence of pNcollagen can also be produced by a genetic mutation that creates a deficiency of

procollagen N-proteinase in tissues (16). Although all these variants have not been studied in detail, there is considerable evidence for the conclusion that a defect either in the enzyme or the substrate which decreases cleavage of amino-propeptide produces an Ehlers-Danlos-like syndrome in which the primary manifestation is laxness of joints. Interestingly, the patients generally do not have symptoms related to bone even though it is very likely that pNcollagen persists in bone as well as other tissues. Therefore, the results suggest that the removal of the amino-propeptide is not as critical a step for normal bone formation as it is for formation of normal ligaments and tendons.

Several mutations in the central, collagenous domain of the procollagen molecule have been shown to decrease markedly the thermal stability of the triple-helix. In one of the best studied variants of this type, a deletion of ~500 base pairs was produced by a sporatic mutation of one allele for the proα1(I) chain (17–21). The deletion excised three exons with 252 base pairs of coding sequences (21). The deletion led to the synthesis of proα-chains that were shortened by 84 amino acids, but in which amino acid sequences on either side of the deletion were normal (19, 21). Since half of the proα1(I) chains synthesized by the same fibroblasts were normal, it was initially difficult to explain the fact that the mutation was lethal. However, it was subsequently shown that because of the structure of the carboxyl-terminal propeptides was normal, the shortened proα1 chains associated with normal proα-chains synthesized by the same fibroblasts and became disulfide-linked to them (19). The shortening of the proα1 chains, however, was so large as to prevent trimers containing these chains from folding into a normal triple-helical conformation at body temperature. Therefore, the trimers containing either one or two shortened proα1 chains were degraded. The net effect was to make three-fourths of the proα-chains synthesized by the fibroblasts unavailable for fibril formation in the process which, by analogy of "suicide inhibitors" of enzymes, has been referred to as "protein suicide."

In addition to altering the stability of the triple-helix, mutations of the collagenous domain of procollagen could well alter the self-assembly of collagen or its crosslinking. No definitive evidence has been obtained for mutations which affect the protein at this level, but several likely candidates have been encountered. In one variant of osteogenesis imperfecta, a mutation in one allele for proα2 chains produced a deletion of ~20 amino acids in about the middle of the proα2 chains synthesized by fibroblasts (22). The other allele for proα2 chains was not functioning and, as a result, all the proα2 chains synthesized by fibroblasts were shorter than normal. The effect of the two mutations was a lethal event, but the reasons for the lethality was not entirely apparent. In another variant of osteogenesis imperfecta, a deletion in the protein of ~20 amino acids close to the amino terminus of the α2(I) chain produced a moderately severe form of osteogenesis imperfecta (23). In two variants of osteogenesis imperfecta (24, 25), an amino acid substitution was found that introduced a new cysteine residue into the α1(I) chain, a chain which normally does not contain cysteine. The presence of the cysteine residue in one patient led to an unusual intra-chain disulfide bond and a triple-helical collagen molecule with slightly decreased thermal stability (24). In a patient with an atypical variant of the Marfan syndrome, a mutation which produced a longer proα2 chain of type I procollagen was found (26). The mutation apparently involved insertion of additional base sequences into an intervening sequence of the gene (27). The consequences of this base change have not been fully defined, but it may produce an abnormal splicing of initial RNA transcripts of the gene. Some or all of these mutations which change the collagenous domain of the procollagen molecule probably change critical features of the molecule that are essential for normal self-assembly (Fig. 3). Others probably alter the structural properties of the fibrils formed in tissues.

To date, only two mutations have been found that alter the structure of the carboxyl-terminal propeptides. The data on these two patients (28–30), both with the clinical symptoms of osteogenesis imperfecta, indicate that structural changes of the carboxyl-terminal propeptides can change either processing of the protein or association of the proα-chains. In one incompletely studied variant (28), an alteration of the carboxyl-propeptides apparently led to increased addition of a mannose-rich carbohydrate to the carboxyl-terminal propeptide and decreased the solubility of the protein. In a much more fully defined variant (29–31), a mutation that altered the structure of the carboxy-terminal propeptides was shown to prevent association of proα2 chains with proα1 chains (31). The mutation was shown to be a deletion of four base pairs which changed the reading frame of the carboxyl-propeptide of the proα2 chain (29, 30). As a result, the last 33 amino acids had an abnormal sequence. The patient, who had a moderate to severe and progressive form of osteogenesis imperfecta, was homozygous for the defect. All of the type I procollagen synthesized and secreted by his fibroblasts consisted of trimers of proα1(I) chains. The trimers of proα1 chains were normally cleaved by procollagen N-proteinase, but apparently the absence of α2 chains in the type I collagen found in the patient's tissues accounted for his bone defect. The data on this patient and his family make it possible to develop a DNA test for prenatal diagnosis, but the patient's parents, who are third cousins and heterozygous for the same gene defect, have decided not to have any further children.

Conclusions

As indicated above, the number of mutations in procollagen genes that have been definitely identified is still small. Also, there are a number of technical problems in defining mutations in these genes, and we do not know the precise molecular defect in most patients with osteogenesis imperfecta, Ehlers-Danlos syndrome, or related diseases. Therefore, any generalizations about these diseases must be tentative. However, several general features of the diseases are beginning to emerge. One, stressed above, is that similar mutations which alter the

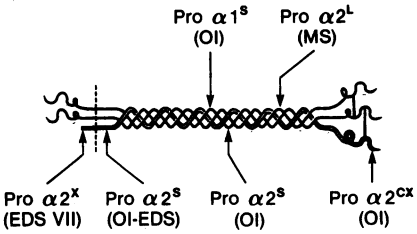

Figure 3. Approximate locations of mutations in the structure of type I procollagen. EDS, Ehlers-Danlos syndrome; MS, Marfan syndrome; and OI, osteogenesis imperfecta. Modified from reference 1 and reproduced with permission.

structure of different regions of the procollagen molecule produce different clinical syndromes. Such data begin to explain why many variants of diseases of osteogenesis imperfecta and Ehlers-Danlos syndrome are highly pleiotropic in terms of the tissues involved and the extent of involvement of each of these tissues (2–5). The same observations begin to establish a "functional topology" (Fig. 3) of the type I procollagen molecule in terms of which features of its structure are critical for the integrity of specific tissues such as ligaments, tendons, skin, and bones. A second, but more tentative conclusion is that the type I procollagen genes appear to be unusually prone to mutations that are new or "sporadic," and that these sporadic mutations tend to be mutations that produce a shortened proα-chain. The procollagen genes may be unusually prone to such mutations because of special features of the genes, features such as their highly repetitive coding sequences or their unusual intron-exon structure. However, the data on this point may be biased by the fact that the analytical techniques currently being used to study mutations in procollagen genes are particularly sensitive to mutations that generate shortened proα-chains and not to other kinds of mutations. A further, and still more tentative, conclusion is that some mutations in procollagen genes, particularly in the heterozygous state, may well provide a basis for understanding diseases that are milder but more common than osteogenesis imperfecta, Ehlers-Danlos syndrome, and related disorders. As a structural protein, collagen is remarkably stable, and the collagen synthesized in many tissues during the growth spurt of adolescence can persist for most of an individual's life. One can therefore readily imagine mutations in procollagen genes that are not consequential enough to produce symptoms early in life, but which may well predispose an individual to osteoporosis or some similar disease of connective tissues late in life. Preliminary evidence for this possibility was obtained from the parents of one of the children with osteogenesis imperfecta described above. The parents are heterozygous carriers for the mutation and asymptomatic, but have early X-ray evidence of osteoporosis (29–31; F. M. Pope, personal communication).

Acknowledgments

Much of the experimental work summarized in this review was generated by a number of investigators within the Department of Biochemistry of this institution. Studies on type I procollagen genes were made possible by the isolation of normal human genes by Drs. Mon-Li Chu, Jeanne C. Myers, and Francesco Ramirez. These three investigators have continued to contribute to major parts of the work on mutations in procollagen genes in patients with osteogenesis imperfecta or Ehlers-Danlos syndrome. Experiments on restriction fragment length polymorphism associated with the human type I procollagen genes were primarily performed by Dr. Petros Tsipouras and Dr. Francesco Ramirez. Major contributions to the work on genetic defects have also been made by Drs. Susan Deak, Wouter de Wet, Taina Pihlajaniemi, Catherine Stolle, and Charlene Williams, and by Maria Sippola.

The research work itself was supported in part by National Institutes of Health (NIH) grant AM-16516 and a grant from the March of Dimes/Birth Defects Foundation.

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