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Epiphyseal growth plate architecture is unaffected by early postnatal activation of the expression of R992C collagen II mutant

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ABSTRACT

Spondyloepiphyseal dysplasia (SED) exemplifies a group of heritable diseases caused by mutations in collagenous proteins of the skeletal system. Its main feature is altered skeletal growth. Pathomechanisms of SED include: changes in the stability of collagen II molecules, inability to form proper collagen fibrils, excessive intracellular retention of mutant molecules, and endoplasmic reticulum stress. The complexity of this pathomechanism presents a challenge for designing therapies for SED. Our earlier research tested whether such therapies only succeed when applied during a limited window of development. Here, employing an inducible mouse model of SED caused by the R992C mutation in collagen II, we corroborate our earlier observations that a therapy must be applied at the prenatal or early postnatal stages of skeletal growth in order to be successful. Moreover, we demonstrate that blocking the expression of the R992C collagen II mutant at the early prenatal stages leads to long-term positive effects. Although, we could not precisely mark the start of the expression of the mutant, these effects are not significantly changed by switching on the mutant production at the early postnatal stages. By demonstrating the need for early therapeutic interventions, our study provides, for the first time, empirically-based directions for designing effective therapies for SED and, quite likely, for other skeletal dysplasias caused by mutations in key macromolecules of the skeletal system.

1. Introduction

Skeletal aberrations in patients harboring mutations in *COL2A1* emphasize the fundamental role of collagen II in the development of the skeleton [1]. Collagen II mutations cause diverse chondrodysplasia phenotypes classified as spondyloepiphyseal dysplasia (SED; OMIM 183900) [1–3]. The SED phenotypes range in severity from achondrogenesis type II, which is lethal at or before birth, to late-onset SED with early-onset osteoarthritis [1–3].

When mutant collagen II molecules are present during skeletal development, cartilage and bone are affected at the molecular, cellular, and tissue levels [1,3]. In brief, mutations in collagen II can lower the thermostability of mutant molecules, change their shape, and alter the formation of collagen II fibrils. Cartilaginous matrix that harbors collagen II mutants has aberrant architecture that may reduce the ability of growth plate chondrocytes to maintain their proper columnar arrangements, thereby limiting the growth of endochondral bones [4]. In addition, mutations that prevent proper folding of collagen chains into triple helices may increase intracellular accumulation of aberrant collagen II molecules and cause endoplasmic reticulum (ER) stress [1,5–7].

Despite significant progress in understanding the pathomechanisms of heritable skeletal dysplasias, no therapies effectively target the molecular basis of these diseases. Experimental approaches to reduce the consequences of collagen mutations have included cell therapies, gene therapies, and therapies to reduce the effects of ER stress [1,8,9]. The efficacies of growth hormone and statins to improve the growth of bones harboring mutant molecules have been also tested [10,11]. Thus far, these approaches have not been effective in preventing skeletal dysplasias.

Researchers have also tested the clinical utility of cells transplanted from healthy donors to patients with severe forms of osteogenesis imperfecta (OI), a bone dysplasia caused predominantly by mutations in collagen I. Infusions of therapeutic cells at the prenatal and the postnatal stages of skeletal growth have resulted in only minimal, transient effects [12–17]. It remains unclear whether these limited outcomes were due to the poor engraftment of donors' cells into bones or due to the ill-defined timing of cell transplantations [1,15]. To advance the

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efforts to prevent skeletal dysplasias, researchers need to develop an effective drug-, gene-, or cell-based therapy to block the expression of the mutant allele or to attenuate the effects imposed by mutant collagen molecules. It then needs to be determined whether this therapy must be applied during a specific time window in order to be effective. Furthermore, it must be shown whether any therapeutic effects are stable or transient.

While the first problem is beyond the realm of this study, we focused instead on determining the optimal time frame for the application of a model therapy and on studying the persistence of any effects. Specifically, to address the problem of the timing of therapy application, we utilized an inducible mouse model of mild SED caused by the R992C substitution in collagen II [4,18]. Because the mutant transgene may be switched off or on at any developmental stage in this model, we were able to study the consequences of stopping the expression of the mutant collagen II chains at the beginning of embryonic development and at various postnatal stages [4,18]. Our earlier studies thoroughly validated this model by demonstrating that growth plate's structure is altered when the mutant R992C collagen II is constantly expressed. Earlier studies further demonstrated that switching off the expression of the mutant collagen II chains at the beginning of embryonic growth blocks the SED phenotype [4]. Subsequently, we studied the consequences of switching off the R992C collagen II at birth and at later postnatal stages. Results of these studies indicated that the structure of cartilage and bone improved significantly only when the mutant molecules were blocked at birth, but not when blocked at later postnatal stages [18].

Here, we employ an inducible SED model to study the consequences of activating the R992C collagen II mutant postnatally in mice in which the expression of this mutant was at first blocked during the entire prenatal development.

2. Materials and methods

2.1. Mutation nomenclature

The R992C (p.R1192C) amino acid substitution is named according to the literature, with amino acid residues numbered from the first glycine residue of the collagen triple helix.

2.2. Transgenic mice

All mice received humane care according to the guidelines in the Guide for the Care and Use of Laboratory Animals. Procedures performed on animals were approved by the Thomas Jefferson University's Institutional Animal Care and Use Committee.

We employed a well-characterized transgenic mouse model of SED based on a DNA construct encoding procollagen II with the R992C substitution. In this model, the transgene is expressed conditionally, while the endogenous *Col2a1* gene is expressed constantly [4,18]. To enable a direct microscopic visualization of the expression patterns of the exogenous procollagen II, its pro-α chains were tagged at the C terminus with green fluorescent protein (GFP). As we demonstrated earlier, the GFP-tag does not interfere with functions of mature collagen II molecules because it is naturally cleaved off together with the C propeptides of the procollagen II-GFP chimeras [4,5,18]. GFP-tagged procollagen II molecules harboring the R992C mutation are referred to as R992C-ProII.

Although we described our model in detail elsewhere, here we present its key characteristics: (i) in addition to the DNA construct for the R992C-ProII mutant, the expression of tetracycline (Tet) transactivator (tTA) is maintained in the mice to enable Tet-dependent regulation of expression of the construct; (ii) the presence of cre recombinase, the expression of which is driven by a chondrocyte-specific promoter (Col2a1-cre), facilitates the chondrocyte-specific expression of the R992C-ProII transgene; (iii) the presence of all three transgenes (*i.e.*, for R992C-ProII; for tTA; and for Col2a1-cre) is needed to express the exogenous R992C-ProII protein; (iv) the expression of the R992C-ProII construct is only possible in the absence of doxycycline (Dox) while the expression is inhibited completely in the presence of Dox supplied in drinking water at 0.2 mg/ml; (v) triple-transgenic mice expressing R992C-ProII, together with tTA and Col2a1-cre, are described as mutant (MT); (vi) because of the genetics, not all littermates are positive for all three transgenes needed to produce R992C-ProII; transgenic mice that lack at least one of the required three transgene DNA constructs (*i.e.*, either for R992C-ProII, for tTA, or for Col2a1-cre) are named wild type (WT); in these mice, the exogenous R992C-ProII protein is not produced but the endogenous normal procollagen II is [4,18].

2.3. Identification of triple transgenes

Offspring generated *via* a breeding strategy described elsewhere were analyzed by PCR for the presence of all three transgenes [4,18].

2.4. Transgene expression and experimental groups

Initially, in the triple-transgene embryos (MT), the R992C-ProII was not produced during development because pregnant mothers were receiving Dox. This mutant was also not produced in WT members of the same developing litter that did not harbor all three transgenes. After birth, the Dox treatment was stopped to initiate the expression of the R992C-ProII in triple-transgene MT littermates. Thus, the triple-transgene mice in which the expression of the R992C-ProII was switched on only postnatally are referred to as MT-pON. The WT and MT-pON littermates were sacrificed at either 7-week or 10-week time points. We selected the 7-week time point to study defined features of the growth plates in growing bones, and the 10-week time point to analyze corresponding features in mature mouse bones [19–22].

We also utilized a control group of mice maintained constantly in the absence of Dox. In this group, the MT littermates harboring all three transgenes produced the R992C-ProII constantly (MT-cON), *i.e.* prenatally and postnatally, while the WT littermates missing at least one transgene did not produce this mutant at all [4,18].

The number of mice from each group available for specific assays is listed in Table 1. Note that the breeding protocol employed here was theoretically predicted to generate 12.5% triple-transgene MT offspring, and our earlier studies confirm this specific percentage [4].

2.5. Tissue harvesting and histology of growth plates

Mice were euthanized by CO_2 overdose. Before processing the mice for whole-skeleton staining with alizarin red and alcian blue, their right hind limbs were collected for histology. Subsequently, the hind paws were separated from the collected limbs. Next, the samples were fixed in a 4% solution of paraformaldehyde and decalcified in a solution of

Table 1
Summary of the growth plate measurements.

Genotype/Age	Number of mice	Average number of individual data points/mouse[a]		
		HZ	AC	BiP
MT-cON/7wks	7	25	10	10
MT-pON/7wks	3	29	3	7
WT/7wks	11	26	8	8
MT-cON/10wks	8	25	10	3
MT-pON/10wks	4	26	3	5
WT/10wks	12	33	8	4

[a] These numbers represent measurements done on various tissue sections and different regions of these sections. Symbols: HZ, hypertrophic zone; AC, articular cartilage; BiP, binding immunoglobulin protein.