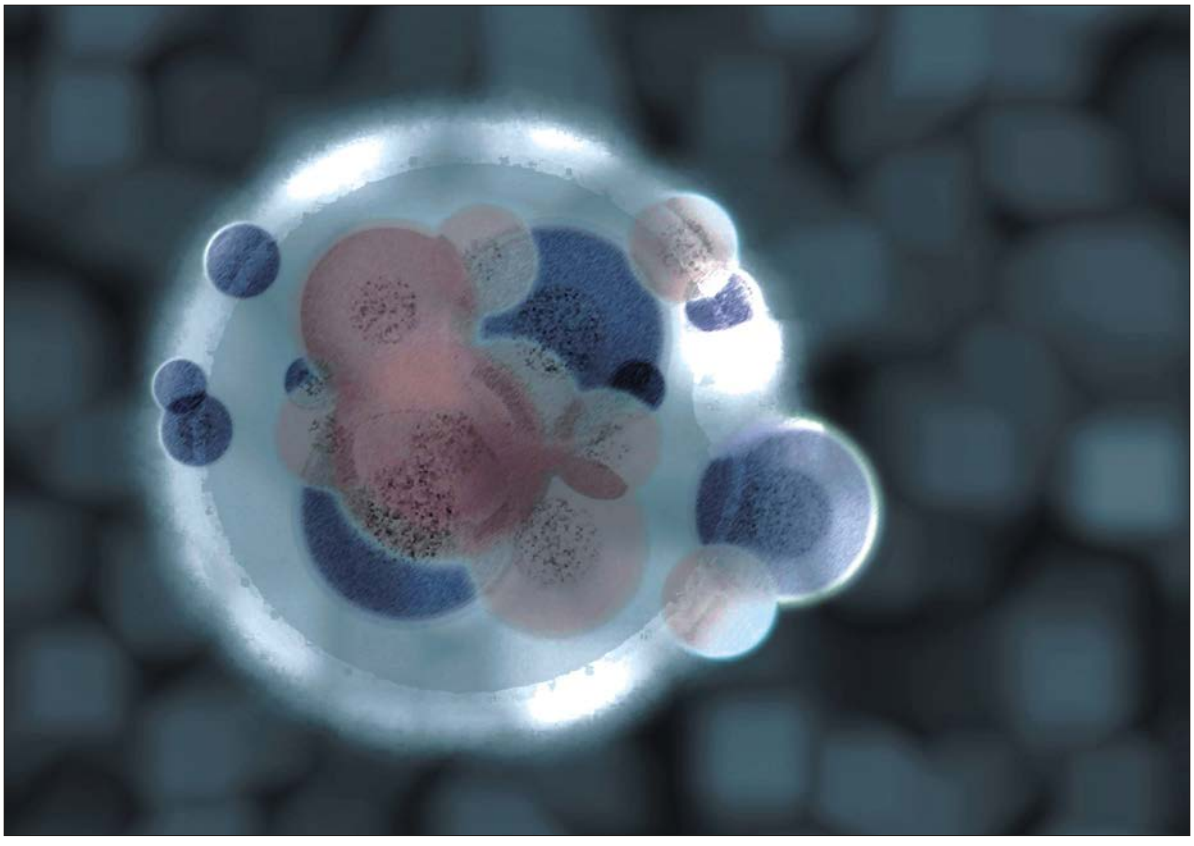

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New Insights into Regulation of Type I Collagen Gene Expression

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Abstract: Elucidation of the regulation of type I collagen synthesis is critical for the understanding of pathophysiology of fibroproliferative disorders, wound healing, tissue remodeling and embryonic development. Transcriptional studies have shown that changes in collagen expression could not be explained solely by changes in the transcription rate of the collagen genes. Recently, it was discovered that stability of collagen α1(I) mRNA changes dramatically by culturing fibroblasts in matrix and by treatment with TGFβ, the most potent profibrotic cytokine. Evidence was provided that the signal transduction pathways leading to activation of phosphatidylinositol 3-kinase (PI3K) and ERK1/2 kinases are involved in this regulation. In hepatic stellate cells (HSCs), which are responsible for excessive collagen synthesis in liver fibrosis, stabilization of collagen α1(I) is primarily responsible for 50-70 fold increase in its steady state level. Two cis-acting sequences in collagen α1(I) mRNA have been identified which are responsible for this effect, 5′ stem-loop and C-rich region in the 3′ untranslated region (UTR). 5′ stem-loop is involved in stabilization and translation of α1(I) mRNA in activated collagen producing, HSCs. This RNA element binds cytosolic and nuclear proteins in sequence specific manner. Sequestration of these proteins by a molecular decoy inhibited synthesis of type I a collagen by more than 50%. No binding to the 5′ stem-loop was detected in collagen nonproducing quiescent HSCs, where collagen α1(I) mRNA has a short half-life. The 3′ C-rich region binds α-CP, a known RNA binding protein involved in stabilization of several long-lived mRNAs. Binding of α-CP can be demonstrated only in extracts of activated HSCs. Differential binding of α-CP probably depends on phosphorylation status of the protein. Cloning of the 5′ stem-loop binding proteins and elucidation of the posttranslational modifications of α-CP are critical for complete understanding of regulation of type I collagen synthesis.

Key words: Collagen type I, posttranscriptional regulation, RNA binding proteins, hepatic stellate cells, translation

INTRODUCTION

Type I collagen is the most abundant protein in human body. It is a heterotrimeric protein composed of two α1(I) polypeptides and one α2(I) polypeptide, which are encoded by separate genes[1]. Type I collagen is expressed at high levels in skin, bone, tendons, placenta, diaphragm, heart, aorta and other tissues requiring high tensile strength, while its expression is low in most other organs and nonstructural tissues. In fibroproliferative disorders uncontrolled expression of type I collagen leads to scarring of tissues (fibrosis) with loss of function[2-4]. The prevalence of fibroproliferative disorders and the lack of treatment has prompted studies on regulation of expression of type I collagen. Since the cloning of α1(I) and α2(I) genes investigators have studied transcription of these genes, as the primary level of regulation[5-13]. Promoter studies have shown that 3.6 kb nt of the promoter of collagen α1(I) gene was sufficient to drive expression of a collagen minigene in stably transfected fibroblasts and expression of a reporter gene in osteoblasts and fibroblasts[14]. Transcription of collagen genes was also studied in HSCs and regulatory promoter elements were identified[11,15]. 1500 nt of the promoter sequence binds several ubiquitous transcription factors like Sp1, NF1, AP1, CBF1, BTEB[9,11,15-19], but the modular nature of α1(I) promoter was discovered by expression studies in transgenic animals. DNA sequences between -2.3 and -1.7 kb were required for α1(I) promoter expression in bone and tooth[6]; sequences that control expression in tendon are distributed between -3.5 and -1.7 kb of the promoter, with sequences downstream of -1.7 kb still capable of directing expression to this tissue[20]. In another study transgenic mice harboring 900 bp of the pro-alpha 1(I) proximal promoter expressed the transgene at relatively low levels almost exclusively in

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skin. In mice containing 2.3 kb of this proximal promoter, the transgene was also expressed at high levels in osteoblasts and odontoblasts, but not in other type I collagen-producing cells[21]. Transgenic mice harboring 3.2 kb of the proximal promoter showed an additional high level expression of the transgene in tendon and fascia fibroblasts[12]. These data strongly suggest a modular arrangement of separate cell-specific cis-acting elements that can activate the mouse α1(I) collagen gene in different type I collagen-producing cells. Distal DNase I hypersensitive sites were also described in the α1(I)[22,23] and α2(I) gene locus[24], as potential binding sites for enhancer-like or locus controlling region factors, but the functional significance of these sites is not clear. From the studies in transgenic animals it also became apparent that to achieve expression similar to the endogenous gene in collagen producing tissue, the transgene had to be present in multiple copies (10 to 12 copies) in these animals[25]. At the same time the mice had overexpression of the transgene in tissues that normally produce low level of collagen, like liver, spleen, thymus and lungs. Clearly, the proximal promoter of α1(I) gene cannot fully account for the regulated expression in transgenic animals.

There is compelling evidence that the steady-state level of many proteins is regulated at multiple steps and when there is a large change in the amount of either mRNA or protein it is likely that multiple steps in the metabolism of the mRNA and protein have been altered[26]. The steady-state level of the mRNA encoding the specific protein is determined not only by the rate of transcription, but also by the percentage of transcripts that are ultimately processed and transported to the cytoplasm and the half-life of the mRNA in the cytoplasm. The amount of protein that accumulates from a particular transcript is influenced not only by the amount of mRNA present in the cytoplasm, but also by the rate of translation of the mRNA and the stability of the protein product. These steps are known as posttranscriptional regulation. Substantial evidence has been accumulated in recent years that collagen α1(I) gene is regulated at post-transcriptional level in various cell types[27-39] and that posttranscriptional regulation is predominant mode of regulation in Hepatic Stellate Cells (HSCs)[33,34,40]. HSCs are responsible for excessive synthesis of type I collagen in liver fibrosis[41], the most common fibroproliferative disorder[42]. This review will focus on some novel finding regarding posttranscriptional regulation of type I collagen gene expression.

Posttranscriptional regulation of collagen α1(I) mRNA in fibroblasts: Previous to studies in HSCs, posttranscriptional regulation of collagen α1(I) mRNA has been studied almost exclusively in fibroblasts[27-31,39,43], as a model of collagen producing cells. There is a wide range of half-lives for different mRNAs, ranging from minutes to hours, despite the fact that most mRNAs decay via a common pathway[44,45]. In fibroblasts collagen mRNA belongs to long-lived mRNAs and its stability depended on culturing conditions. In NIH 3T3 fibroblasts α1(I) mRNA had a half-life between 4h in semi confluent cultures and 9h in confluent cultures[39]. In rat fibroblasts the half-life of collagen α1(I) mRNA was measured to be longer than 16 h[43] and in human fibroblasts it has a half-life of 9 h[46]. Fibroblasts grown within or on the matrix composed of extracellular proteins extracted from Engelbreth-Swarm-Holms tumor (matri-gel) or pure collagen type I gels revert to a more quiescent phenotype. Primary human fibroblasts down regulate collagen α1(I) mRNA steady state levels when cultured within collagen gel matrix by destabilizing the mRNA[27,28,30,31,35]. In mouse Swiss 3T3 fibroblasts grown on plastic there was no decay of collagen α1(I) mRNA 12 h after the inhibition of transcription, while in the cells grown in gel only 50% of the initial level of the mRNA remained, suggesting a decreased stability[35]. Thus, destabilization of collagen α1(I) mRNA is one of the features associated with quiescence of fibroblasts. TGFβ is the major profibrotic cytokine capable of inducing collagen synthesis in various cell types[47-51]. Treatment of fibroblasts with TGFβ increases steady-state level of collagen α1(I) mRNA by increasing the half-life of the transcript several fold[39].

Signal transduction pathways which trigger the changes in stability of collagen α1(I) mRNA are being elucidated. Activation of phosphatidylinositol 3-kinase (PI3K) leads to stabilization of collagen α1(I) mRNA in lung and skin fibroblasts[36,37,52]. For example, basal level of collagen α1(I) mRNA was reduced when the PI3K activity was inhibited by either LY-294002 or wortmannin in lung fibroblasts. These PI3K inhibitors also blocked increases of collagen α1(I) mRNA levels after the addition of TGFβ. The effect of PI3K inhibition was abolished by the removal of the inhibitor or by the addition of cycloheximide. Inhibition of PI3K activity decreased the stability of the α1(I) collagen mRNA with no change in the rate of transcription of the α1(I) collagen gene as assessed by Northern blotting with actinomycin D-treated fibroblasts and nuclear run-off assays. Expression of a truncated α1(I) collagen minigene driven by a constitutive promoter in lung fibroblasts was decreased by LY-294002 treatment, suggesting regulation at the level of mRNA stability[37]. In skin fibroblasts LY294002 also significantly inhibited collagen type I protein and mRNA levels. The

effects of LY294002 were more pronounced on the collagen α(I) gene, which was inhibited at the transcriptional and mRNA stability levels versus collagen α2(I) gene, which was inhibited through a decrease in mRNA stability. Wortmanin did not have any effect in skin fibroblasts[53]. These data indicate that PI3K activation results in increased stabilization of collagen α1(I) mRNA. On the other hand, C(2)-ceramide inhibits expression of type I collagen mRNAs in dermal fibroblasts by reducing the stability of collagen mRNAs. The down-regulatory effect of C(2)-ceramide on type I collagen mRNA levels was abrogated by protein kinase C inhibitors H7, staurosporine and Ro-31-8220 and potently inhibited by a combination of MEK1, 2 inhibitor PD98059 and p38 inhibitor SB203580. Activation of ERK1/2 by adenovirus-mediated expression of constitutively active MEK1 resulted in marked down-regulation of type I collagen mRNA levels and production in fibroblasts. These results identify the ERK1/2 signaling cascade as a potent negative regulatory pathway with respect to type I collagen expression eliciting its effect at the level of mRNA stability[54]. The signaling pathway downstream of ERK1/2 has not been worked out yet.

Posttranscriptional regulation of collagen α1(I) mRNA in HSCs: Cirrhosis is characterized by the accumulation of extracellular matrix proteins in the liver, including type I collagen and is the most common fibroproliferative disorder[55,56]. Hepatic stellate cells (HSCs also named Ito cells, lipocytes, or fat-storing cells) are the major cell type responsible for collagen synthesis in the cirrhotic liver[57,58]. In normal liver, quiescent HSCs store vitamin A[59], but express only trace amounts of type I collagen[33]. Upon a fibrogenic stimulus, HSCs become activated, a process in which they lose retinoid droplets, proliferate, change morphologically into myofibroblasts and increase their synthesis of extracellular matrix proteins[60,61]. Culturing quiescent HSCs on plastic causes activation similar to that seen in liver fibrosis *in vivo*; including the accumulation of collagen α1(I) mRNA and synthesis of type I collagen protein[61,62]. The level of collagen α1(I) mRNA increases 50-70 fold in activated HSCs compared to quiescent HSCs[33,63], therefore HSCs provide an excellent model system to study regulation of type I collagen. We have investigated mechanisms controlling the increase of collagen α1(I) mRNA during culture activation of HSCs. We measured the rate of transcription of α1(I) gene in quiescent HSCs and in activated HSCs by nuclear run-off assay. The transcriptional rate was increased 3 fold in activated HSCs compared to quiescent HSCs, which was insufficient to account for 50-70 fold increase in steady-state level of the mRNA. Therefore, we measured the stability of collagen α1(I) mRNA by blocking transcription with actinomycin D and measuring the rate of disappearance of the transcripts. The half-life of the α1(I) mRNA was estimated to be about 24 h in activated HSCs compared to 1.6 h in quiescent HSCs, an increase of 16 fold. Together with 3 fold increase in transcription rate, this stabilization of the mRNA could fully account for the observed increase in the steady state level[33]. Thus, in HSCs regulation of collagen α1(I) mRNA is predominantly post-transcriptional, at the level of mRNA stability.

Experiments *in vivo* have corroborated this finding. Injection of CCl_4 into experimental animals resulted in approximately a 20-30 fold increase in collagen α1(I) mRNA in total liver RNAs[64]. There was about a two-fold increase in collagen α1(I) gene transcription in total liver nuclei from CCl_4 treated rats compared to normal liver nuclei[65,66]. Taking into account that HSCs are the major liver cells capable of up regulating collagen α1(I) mRNA following a fibrogenic stimulus[67], these results suggested predominantly posttranscriptional regulation of collagen α1(I) mRNA in liver fibrosis.

Mechanism of stabilization of collagen α1(I) mRNA: mRNA competent for translation is circular, formed by an interaction of the cap binding complex assembled at the 5′ end (eIF4) with Poly-A Binding Protein (PABP) bound at the 3′ end[68,69]. The common decay pathway is triggered by shortening of the polyA tail. When the polyA tail reaches a critical length, the loss of PABP disrupts the circular mRNA structure. The mRNA is then decapped and becomes accessible to exonucleases which can degrade the mRNA 5′ to 3′[44,45]. There are also enzymes that can degrade the mRNA 3′ to 5′. Stability of most mRNAs is regulated by sequences in their 3′ untranslated regions (UTRs) and determined by proteins interacting with these sequences. These sequence specific RNA binding proteins divert the mRNA from the degradative pathway, usually by interacting with PABP or the cap binding complex to stabilize the circular conformation[44,45]. Two sequence specific binding activities have been discovered for collagen α1(I) mRNA, they are; α-CP, also known as PCBP or hnRNP-E, which binds to the 3′ UTR[33] and 5′ stem-loop binding proteins targeted to the 5′ UTR[34].

α-CP binding is targeted to the C-rich sequence located 23 nt 3′ to the stop codon of collagen α1(I) mRNA. Binding of α-CP can be demonstrated only in cytoplasmic extracts of activated HSCs, where the collagen mRNA is stable and not in extracts of quiescent HSCs. When α-CP binding site was mutated in the context of a reporter mRNA, expression in activated HSCs was

decreased when compared to the same mRNA with wild type α-CP binding site[33]. α-CP belongs to the KH-domain family of RNA binding proteins and shuttles between the nucleus and cytoplasm[70]. Purified recombinant α-CP binds avidly to the C-rich 30 nt sequence derived from the collagen α1(I) mRNA 3' UTR with a Kd of 2 nM. Change of a single C nucleotide within the binding site dramatically decreases the binding of recombinant protein[71]. Interestingly, α-CP binds a similar C-rich sequence to stabilize the α globin mRNA[72] and binds the C-rich sequences in the 3'UTRs of 15-lipoxygenase and tyrosine hydroxylase mRNA[73]. In extracts of activated HSCs the α globin sequence efficiently competes for α-CP binding to the collagen sequence and collagen sequence competes, although slightly less effectively, for binding to the α globin sequence[33], suggesting interactions with a common factor. Thus, α-CP seems to stabilize several long lived mRNA. The reason why α-CP does not bind collagen α1(I) mRNA in quiescent HSCs is not clear and requires further study. Since both activated and quiescent HSCs express the protein, as judged by Northwestern analysis, it most likely involves a posttranslation modification of α-CP[74]. Phosphorylation of α-CP inhibits its binding to RNA[75] and several kinases have been recognized to phosphorylate the KH domain containing proteins[76-78]. Which kinase may be responsible for phosphorylation of α-CP in quiescent HSCs remains to be discovered. Whether there is a specific phosphatase involved in stimulating of the RNA binding activity of α-CP when HSCs undergo activation, is also unknown.

In the 5′ UTRs of three collagen mRNAs, α1(I), α2(I) and α1(III), there is a stem-loop structure encompassing the translation initiation codon[79]. These three mRNAs are coordinately up regulated in fibrotic processes of various organs[55,67]. The 5′ stem-loop structure is located about 75 nt from the cap and has a stability of ΔG=25-30 kcal mol^{-1} in different collagen mRNAs. Enzymatic probing of a synthetic 5′ stem-loop RNA demonstrated folding into a higher order structure with a bulged A nucleotide[34]. The 5′ stem-loop is well conserved in evolution, differing by only two nucleotides in Xenopus and human collagen mRNAs[80]. The sequence flanking the stem-loop is not conserved. A similar stem-loop structure is also found around the translation start codon of the sea urchin collagen gene[81]. Evolutionary conservation of this sequence suggests an important function. An initial study could not demonstrate a regulatory role for this structure on mRNA steady-state levels or translation, based on transfections of hybrid collagen-human Growth Hormone (hGH) genes into fibroblasts[82]. The subsequent experiments analyzed a regulatory role of the 5′ stem-loop in two experimental systems; quiescent versus activated HSCs[34] and fibroblasts cultured in a three-dimensional matrix[35]. A series of reporter genes driven by the SV40 promoter and containing the 5′ stem-loop was delivered into quiescent and activated HSCs by adenoviral vectors and the mRNA and protein levels were measured. The inability to efficiently introduce genes into quiescent HSCs has previously limited studies of gene expression in this cell type, but adenovirus mediated gene transfer efficiently transduces HSCs with a quiescent phenotype. The 5′ stem-loop prevented expression of the reporter genes in quiescent HSCs, but allowed for expression in activated HSCs. Reporter genes with the mutated stem-loop were constitutively expressed to a high level in both cell types[34]. Because the SV40 promoter is equally active in quiescent and activated HSCs, regulation by the 5′ stem-loop must have been posttranscriptional. Therefore, expression of a reporter mRNA becomes similar to expression of the endogenous collagen α1(I) mRNA if the 5′ stem-loop is included in its sequence.

To provide evidence for a role of the 5′ stem-loop in the accelerated decay of collagen α1(I) mRNA observed in cells grown within three-dimensional gel, full size collagen α1(I) mRNA with or without the 5′ stem-loop was expressed and analyzed in fibroblasts grown in the matrix. When the 5′ stem-loop was mutated, collagen α1(I) mRNA was more stable than the mRNA with the intact 5′ stem-loop[35]. Thus, 5′ stem-loop is required for accelerated decay of collagen α1(I) mRNA in cells which revert to a more quiescent phenotype. This result is in agreement with experiments employing quiescent HSCs.

Multiple protein factors bind the 5′ stem-loop of collagen α1(I) mRNA: A mechanism by which the 5′ stem-loop targets mRNAs for turnover in HSCs and fibroblasts grown in three-dimensional matrix is unknown. Cis-acting RNA elements work through association with their cognate binding proteins. In quiescent HSCs no protein binding to the 5′ stem-loop could be detected *in vitro*, this is probably a reason for accelerated decay of the mRNA. In activated HSCs a cytosolic protein factor(s) of unknown identity binds to the 5′ stem-loop in sequence specific manner and requires 7 mG cap on the RNA for binding[33]. It is not known if the 5′ stem-loop binding factor directly interacts with 7 mG cap or with the cap binding protein, eIF4E[83]. Supershift experiments with anti-eIF4E antibody showed no change in gel mobility of the complex. However, an excess of cap analogue completely prevents formation of this complex *in vitro*. It is likely that protein binding to the 5′ stem-loop increases the steady state level of collagen α1(I) mRNAs by diverting them from the degradative pathway. A protein of 120 kD was crosslinked to the 5′ stem-loop of collagen

α1(I) mRNA in extracts of activated HSCs[33]. Cytosolic 5′ stem-loop binding protein was also detected in fibroblasts (unpublished).

In the nucleus, after or during transcription, numerous mRNAs associate with sequence-specific RNA binding proteins, which have important regulatory roles[84]. Therefore, it is not surprising that a nuclear protein was detected, which specifically binds to the collagen 5′ stem-loop. This binding activity is different from the cytoplasmic binding activity because it can be detected only in nuclear extracts; it does not require the presence of 7 mG cap for binding; and it has a different electrophoretic mobility in native gels. The nuclear binding activity was detected in fibroblasts and was increased when the cells were grown in three-dimensional matrix[35]. The nuclear factor may be required for processing and/or nuclear export of collagen α1(I) mRNA, which takes place along distinctive tracks extending from the gene into the SC-35 domains[85]. The SC-35 domains are 1 μM structures within the nucleus rich in splicing factors, where processing of pre-mRNAs takes place. After leaving the SC-35 domains collagen α1(I) mRNA rapidly exits the nucleus[85]. A nuclear binding activity was also detected in nuclear extracts of activated HSCs (unpublished), quiescent HSCs have not been tested yet.

Human disease Osteogenesis Imperfecta (OI) is characterized by decreased synthesis of type I collagen due to mutations in collagen genes[86]. One patient with OI type I was described who had a mutation in the 5′ stem-loop in the absence of any other mutation of α1(I) collagen gene[87]. This finding emphasizes the importance of the 5′ stem-loop for collagen synthesis *in vivo*.

Molecular decoy with 5′ stem-loop as inhibitor of collagen synthesis: In the absence of the 5′ stem-loop binding proteins in quiescent HSCs the decay of collagen α1(I) mRNA is accelerated. In activated HSCs, where the 5′ stem-loop binding proteins are present, there is a high level of expression of collagen α1(I) mRNA[33]. Therefore it should be possible to inhibit collagen α1(I) expression in activated HSCs by titrating out these proteins. A small chimeric RNA of 108 nt was designed (molecular decoy), which contained collagen 5′ stem-loop fused to the U7 snRNA. When the molecular decoy was expressed in quiescent rat HSCs by adenovirus mediated gene transfer and the cells were subjected to activation *in vitro*, collagen α1(I) mRNA and protein were decreased by 80%. When expressed in fully activated human HSCs molecular decoy was able to reduce collagen protein synthesis by 50%[40]. More than 50% inhibition of collagen synthesis has also been obtained by stably expressing molecular decoy in NIH 3T3 fibroblasts[40]. The effectiveness of the molecular decoy is presumably due to titration of the 5′ stem-loop binding proteins, because similar RNA with the mutated stem-loop was ineffective. Sequestration of the 5′ stem-loop binding proteins results in increased degradation of collagen α1(I) mRNA and decreased protein synthesis. This finding corroborates the importance of 5′stem-loop binding proteins for high collagen expression and suggests that molecules with the ability to inhibit interactions between the 5′ stem-loop and its cognate binding proteins may be effective antifibrotic drugs.

Regulation of translation of collagen α1(I) mRNA: Translation is, in general, controlled at the step of initiation[88]. Translation initiation factors assemble at the mRNA cap, followed by recruitment of the 40S ribosomal subunit. The 40S subunit scans the mRNA from the cap towards the 3′ end until it encounters an AUG codon in the favorable sequence context (initiation codon). The 50S subunit then associates to form a functional ribosome and the translation elongation phase resumes[89]. In the normal cell, there is a competition among the many mRNAs for the translation initiation factors, which clearly limit the translation process[90,91]. Therefore, regulation of translation can then be accomplished by altering the efficiency of assembly of the initiation complex on the mRNA. At least three different types of sequences present in collagen α1(I) mRNA can affect translation initiation: two short upstream open reading frames (uORF), the 5′ stem-loop and the α-CP binding site. The latter two also regulate mRNA stability (see above).

Three collagen mRNAs, α1(I), α2(I) and α1(III) of all vertebrate species have two uORFs preceding the coding region. These ORFs have a potential to encode a peptide of 4-8 amino-acids, but their sequence is not conserved. It is conceivable that these uORFs attenuate scanning of the ribosomes towards the main frame, decreasing the rate of translation initiation at the main coding region[92]. Such a mechanism has been described for several other mRNAs[93-96]. Inefficient translation initiation may be a prerequisite for regulation by the 5′ stem-loop binding proteins and/or α-CP. There was no enhancement of translation of collagen mRNA in reticulocyte lysate when both uORF were abolished (unpublished). However, translation in the lysate is not subjected to regulation, therefore, experiments *in vivo* are needed to elucidate a role of the uORFs. Their evolutionary conservation strongly suggests such function.

As a rule, mRNAs with long and highly structured 5′ UTRs are inefficiently translated[97,98]. Stable stem-loops ($\Delta G > 50$ kcal mol^{-1}) or stem-loops that bind RNA binding proteins can block translation initiation if

placed adjacent to the cap[97]. The 5′ stem-loop of collagen mRNAs has a stability of 25-30 kcal mol^{-1} and is about 75 nt from the cap, therefore it is unlikely that it can block ribosomal scanning by steric hindrance. Since the start codon is part of the 5′ stem-loop, the sequence constraints required to maintain the 5' stem-loop dictate the sequence around translation initiation. Therefore, the start codon in collagen mRNAs is not in the sequence context necessary for optimal translation initiation[99,100], but it is surrounded by the sequence under evolutionary pressure to maintain the 5' stem-loop, as a binding site for regulatory proteins. A reporter mRNA with the collagen 5' stem-loop was translated *in vitro* 3 fold less efficiently than similar mRNA in which the 5' stem-loop was mutated[101]. However, in the presence of a competitor mRNA, reporter mRNA with the 5' stem-loop produced 25-fold less protein then the reporter mRNA without the 5' stem-loop, suggesting that under competitive conditions it is a poor substrate for translation. When the sequence surrounding the initiation codon of α1(I) collagen mRNA was changed to create an optimal translation initiation site the mRNA translation was increased 4-fold under competitive conditions [101]. Thus, it seems that collagen α1(I) mRNA is designed to be inefficiently translated, the structure of the 5' stem-loop together with its suboptimal translation initiation site are both responsible for this effect. Being poor translational substrate, this mRNA may be subjected to regulation by the 5' stem-loop RNA binding proteins.

Structural probing of collagen triple helices by digestion with pepsin[102] provided evidence that 5′ stem-loop also has a critical role in the proper assembly of the collagen triple helix. A synthetic gene encoding the full size collagen α1(I) mRNA containing the wild type 5′ stem-loop synthesized pepsin resistant collagen triple helices. A similar gene, but with the mutated stem-loop synthesized pepsin sensitive collagen[101]. The mutation did not change the coding region of the gene, just the 5′ stem-loop. The pepsin sensitive collagen indicates that, in the absence of the 5′ stem-loop in mRNA, α1(I) polypeptides were not efficiently folded into a triple helix and accumulated as monomers, or alternatively, the monomers were not properly modified and an unstable triple helix was produced. This result implied that the 5' stem-loop couples the translational machinery to the collagen assembly pathway and to our knowledge this is the first example of an RNA element that affects protein folding[101].

α-CP regulates translation of some viral mRNAs[103-105], therefore binding of α-CP to the 3′ UTR of collagen α1(I) mRNAs may also have a potential to regulate translation. mRNAs that are poised for translation are in circular conformation held by an interaction of poly-A binding protein bound to the 3′ end and cap binding complex (eIF4F) bound to the 5′ end[68]. Any event which further stabilizes this circular conformation has a potential to stimulate translation. Collagen α1(I) mRNA has a C-rich sequences in the 3′ UTR, which binds α-CP. α-CP can interact with Poly-A Binding Protein (PABP)[106], therefore, it is possible that α-CP further enhances translation of collagen α1(I) mRNA by stabilizing interaction of poly-A binding protein and eIF4F. Thus, α-CP may enhance translation of collagen α1(I) mRNA, as well as increasing its stability, essentially by the same mechanism.

Coordination of translation of collagen mRNAs: Very few studies have addressed this topic. All collagen α1(I) mRNA is associated with membrane bound polysomes and is not found on free polysomes or in postpolysomal supernatant (our result). Collagens are secreted proteins and their translation is coupled to export of the peptides into the Endoplasmatic Reticulum (ER), with their subsequent posttranslational modifications and folding into triple helix[1]. There is substantial evidence that two α1(I) peptides and one α2(I) peptide initiate folding into the heterotrimer while still associated with polysomes on the membrane of the endoplasmatic reticulum or soon after their release[107-111]. In the human disease osteogenesis imperfecta (OI) certain mutations of α1(I) chain decrease the rate of assembly of collagen type I. Unassembled OI α1(I) chains are hyper modified on proline and lysine residues and degraded[112-114]. This suggests that modification and assembly processes are in a kinetic equilibrium and that translation of α1(I) and α2(I) mRNAs must be coordinated to allow efficient assembly. HSP 47 is a chaperone for folding of collagen type I[115] and it can be coimmunoprecipitated with the polysome associated α1(I) chains[116]. Lysine hydroxylase is also associated with the membrane of ER[117] and we have recently demonstrated coupling of TRAM2 protein, as a component of translation/translocation machinery for type I collagen, with Ca^{++} pump Serca2b[110]. This provides high local concentration of Ca^{++}, which is necessary for the function of molecular chaperones assisting folding of the collagen triple helix. Perhaps, there are specialized sites for collagen synthesis on the membrane of ER where the modifying enzymes, Ca^{++} pumps and chaperones cluster and where collagen mRNAs are targeted. Such coordinated translation would greatly increase the efficiency of collagen synthesis. Because mRNA without the 5′ stem-loop can not synthesize triple helical collagen,

5' stem-loop and its cognate binding proteins may target collagen α1(I) and α2(I) mRNAs to these hypothetical sites of collagen synthesis.

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