REGULATION OF HEAT-SHOCK-PROTEIN 47(HSP47) AND PROCOLLAGEN BY

TRANSFORMING GROWTH FACTOR-β IN AVIAN TENDON CELLS

by

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ABSTRACT

The rupture of chicken gastrocnemius tendon poses a serious problem for the poultry industry. The cause of tendon rupture is unclear, virus infections, extreme environmental conditions or inappropriate growth factor expression have been considered to play a role in pathogenesis. Histological examination in most cases reveals fibrosis and rupture of collagen fibers. I investigated effects of heat-shock, growth factors and mechanical stress on procollagen and heat shock protein 47 (Hsp47) expression in chicken tendon fibroblasts. Hsp47 expression and synthesis rapidly increased in response to heat shock, and its response was reversible after removal of heat shock. Type I procollagen expression transiently increased and then decreased with heat shock. Both Hsp47 and procollagen expression was enhanced by human TGF-β1 treatment. Mechanical stress increased Hsp47 expression and protein production, but had no effect on type I procollagen expression. Because transforming growth factor-β (TGF-β) is a major regulator of collagen I have investigated the effects of TGF-β on collagen expression. As a part of my effort I generated the chicken TGF-β4 cDNA and expressed the corresponding protein and partially characterized it.

Chicken TGF-β4 has 82% amino acid sequence identity to human TGF-β1. I expressed this protein and characterize it *in vitro*. I failed to recover the active TGF-β4 after purification and refolding when the chicken mature TGF-β4 protein was expressed in *E. coli* using a pET-28 vector. I generated the 5' end of TGF-β4 cDNA using the modified 5'RACE. cDNA was produced from mRNA purified from the embryonic chicken tendon fibroblasts using the thermal stable reverse transcriptase and random hexamers at 70°C. Both the first and nested PCR was performed using GC-rich PCR kit. The alignment of obtained sequences showed that the 5' end contained 271 more oligonucleotides with 70% of GC-bases (Genbank accession No: AF395834) than the original partial sequence recovered from the chicken TGF-β4 (Genbank accession No: M31160). I in-frame cloned cDNA expressing the TGF-β4 precursor into pcDNA3.1/V5-His-TOPO plasmid and expressed it in CHO-K1 cell line. The recombinant protein TGF-β4 was purified with Probond Resin under native conditions, and then activated by strong acidification. Protein bioassay showed that recombinant chicken TGF-β4 shares the TGF-β superfamily-related activities. Recombinant chicken TGF-β4 increased Hsp47 expression at both the protein and mRNA levels. I also found that human TGF-β1, chicken TGF-β4, and mechanical stress increased Hsp47 protein synthesis through activation and translocation of HSF1 into the nucleus as heat stress does. Therefore I conclude that chicken TGF-β4 coregulates type I procollagen and Hsp47 protein production in chicken tendon cells as human TGF-β1 does in mammalian species.

INDEX WORDS: Human TGF-β1, Chicken TGF-β4, Procollagen, Heat shock protein 47, Mechanical stress, pET-28, pcDNA3.1, CHO-K1, tenocytes, 5'RACE, HSF1.

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TGF-β IN AVIAN TENDON CELLS

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DEDICATION

To my parents, my son Yang Pan and my wife Sigui Li for their care and love ……

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CHAPTER 1

INTRODUCTION

Failure of the gastrocnemius tendon in broiler chickens has been estimated to cause an annual loss of at least $30 million, due to field losses and waste that is associated with processing. Tendon failure is responsible for a high percentage of lameness and loss due to debilitating musculoskeletal problems in chickens (Riddle, 1997; Agri Stats Bulletin, 1998). What causes tendon rupture is unclear. Virus infections, growth factors malfunction or extreme environment conditions such as heat and humidity have been considered to play a role in tendon pathogenesis (Sullivan, 1994). Histological examination in most cases reveals fibrosis and rupture of collagen fibers (Riddell, 1997). The poor understanding of the pathogenesis of ruptured tendon disease is magnified by our incomplete knowledge of tendon repair, and of normal tendon physiology. Tendon growth and development is a complicated process and is affected by a variety of growth factors and environmental factors, some of which are also involved in tendon injury and repair.

Tendons function as mechanical links, transmitting the force generated by a muscle to a distant bone and bringing about the movement of a segment of a limb. The major component in mature tendons and their sheaths is type I collagen. Type III and V collagens are also present as minor collagens (Tsuzaki et al., 1993; Fan et al., 1997). Collagen is a rigid, inextensible fibrous protein that is a principal constituent of tendons. The high tensile strength of collagen fibers in tendons make possible the various animal activities such as running and jumping that put severe stresses on joints and the skeleton.

I hypothesized that both intrinsic and extrinsic factors play a role in regulation of collagen expression (and thus in modulation of tendon function) in the avian gastrocnemius tendon. To test my hypothesis I examined the effect of transforming growth factor β (TGF-β), an intrinsic factor, and the effect of increased temperature and mechanical stress, two extrinsic factors, on collagen expression.

TGF-βs are major regulators of the production of connective tissues including procollagen and fibronectin (Ignotz et al., 1986; Postlethwaite et al., 1987). TGF-β induces fibrosis in most tissue repair processes (O'Kane et al., 1997). There are three isoforms, TGF-β1-3 in mammalian animals and four isoforms, TGF-β1-4 in chickens and other avian species. These isoforms share 60-80% homology in the amino acid sequences of the mature proteins. Therefore, their biological properties are very similar. They are involved in many aspects of growth, differentiation, and the fate of metazoan cells through SMAD or other signal transduction pathways (Massague et al., 2000; Attisano and Wrana, 2002). Heat-shock protein 47(Hsp47), one of the endoplasmic reticulum (ER)-resident proteins, is characterized by its substrate specificity for collagen. It plays a major role in collagen processing and quality control under stress conditions by preventing the secretion of procollagen with abnormal conformations (Nagata, 1996; Tasab et al., 2000; Hosokawa et al., 2000). The expression of Hsp47 is always closely correlated with the expression of the various types of collagens (Nagata et al., 1986; Clarke et al., 1993). Human TGF-β1 rapidly induced Hsp70 and Hsp90 molecular chaperones in cultured chicken embryo cells through posttranscriptional regulation (Takenaka et al., 1992 and 1993). The relationship between TGF-βs and Hsp47 in avian tendon fibroblasts has not been studied in detail. However, TGF-β1 increased Hsp47

mRNA and protein levels in human normal skin cultured fibroblasts (Kuroda et al., 1998) and human diploid fibroblasts (Sasaki et al., 2002). Treatment of mouse osteoblast MC3T3-E1 cells with 5 ng/ml TGF-β1 for 24 hours increased the level of Hsp47 mRNA three-fold. A dose-dependent induction by TGF-β1 was observed for both Hsp47 mRNA and collagen α1(I) mRNA (Yamamura et al., 1998). TGF-β4 has been characterized in chicken embryo chondrocytes and has only been detected in the chicken (Jakowlew et al., 1988a; Jakowlew et al., 1992). The mature chicken TGF-β1 shows 100% identity with human TGF-β1 (Jakowlew et al., 1988b). However, the authors could not exclude a possibility of contamination of their chicken cDNA library with porcine cDNA, and thus the sequences of chicken TGF-β1 and 4 may have been contaminated with porcine TGF-β1 (Genbank Accession NO: x12373). TGF-β4 and human TGF-β1 proteins share 82% amino acid sequence identity in the mature region and 47% homology in the pro-region. The functions of chicken TGF-β4 are unknown. In my preliminary study the human TGF-β1 enhanced both Hsp47 and pro-collagen protein syntheses in the avian tendon. I hypothesized that TGF-β co-regulates Hsp47 and procollagen syntheses in avian tendon fibroblasts and that chicken TGF-β4 may be involved in this process. I also investigated an effect of mechanical stress on Hsp47 and procollagen in avian tendon cells because it is known that mechanical stress induced heat-shock protein 70 expressions in vascular smooth muscle cell (Xu et al., 1997 and 2000).

My results show that both intrinsic and extrinsic factors have a direct or at least an indirect effect on collagen expression and synthesis. I propose that such data enhance understanding of the failure of gastrocnemius tendon in broiler chickens

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CHAPTER 2

LITERATURE REVIEW

The rupture of chicken gastrocnemius tendon poses a serious problem for poultry

industry. What causes tendon rupture is unclear. Virus infections or extreme

environmental conditions such as heat and humidity have been considered to play a role

in tendon pathogenesis. Histological examination of the tendon in most cases reveals

fibrosis and rupture of collagen fibers. Because the major component in tendon is type I

collagen, I hypothesized that factors regulating collagen expression or synthesis may play

a role in the pathogenesis that leads to tendon rupture. In this work I have been studying

the effects of several of these factors (transforming growth factor-β, increased

environmental temperature, and mechanical stress) on collagen and Hsp47 expression

and synthesis in chicken gastrocnemius tendon cells. My expectations are that my results

will contribute to our understanding of tendon physiology and pathogenesis.

Transforming growth factor β (TGF-β) is the prototype of a family of

multifunctional cytokines that regulate many aspects of cell physiology, including cell

growth, differentiation, motility, and apoptosis. TGF-β plays important roles in many

developmental and pathological processes, such as the deposition of extracellular matrix

(ECM) and wound healing. Collagen, an important component in connective tissues such

as tendons, is regulated by a variety of factors including cytokines or growth factors and by physical factors. Heat shock protein 47 (Hsp47) is a procollagen/collagen-specific molecular chaperone protein derived from the serpin family of proteins and essential for the early stages of collagen biosynthesis. Co-expression of procollagen and Hsp47has been widely documented. However, the relationship between Hsp47 and TGFβ is rarely reported. The following review focuses on recent research on these proteins and their interaction, and their importance in tendon physiology and repair.

A. Tendon

Tendons transduce skeletal muscle shortening into movement of one bone relative to another, resist tension with little to no elongation, and transmit forces generated during skeletal muscle contraction to the skeleton. They are structurally attached to skeletal muscle at the myotendinous junction and to bone at the teno-osseous junction. Research studies have shown that tendon structure is malleable and responds to changes in stress levels (Michna and Hartmann, 1989; Wren et al., 1998). Alterations in tendon structure due to intrinsic (e.g., adaptation to a change in activity level), as well as extrinsic factors (e.g., compression caused by structural impingement) can lead to losses in a tendon's ability to resist tensile forces. Maladaptations can weaken the ability of a tendon to resist additional loads and lead to structural failure. Excessive acute and chronic stress loads on a tendon may result in partial muscle tears that further compromise tendon function and eventually result in tendon rupture (Gerriets et al., 1993).

Tendons are histologically characterized as a dense connective tissue. Like most connective tissues, tendons are relatively acellular. The acellular component of

connective tissues is referred to as ground substance or extracellular matrix. The major components of the extracellular matrix of tendons are collagen (primarily collagen type I) and proteoglycan. Collagen is an inextensible extracellular matrix protein that plays a major role in maintaining the structural integrity of tissues and organs throughout the body. In human tendons, hierarchical organization of the tendon can be observed (Jozsa et al., 1997). Collagen molecules assemble together and become ordered polymers, which contribute to collagen fibrils. Collagen fibrils visualized by electron microscopy make up the collagen fiber. Collagen fibers get assemble together and make a primary fiber bundle (15-400 um in diameter) that is also called a subfascicle. Several primary fiber bundles make up secondary fiber bundle (150-1000 μm in diameter) that is called fascicle. Secondary fiber bundles make up tertiary fiber bundle (1-3 mm in diameter). Finally, tertiary fiber bundles make up tendon (Scott, 1984). Collagen fibrils are oriented parallel to each other in the direction of the tensile force. Interspersed among the collagen fibers are differentiated fibroblasts called tenocytes. Tenocytes synthesize and secrete the extracellular matrix components including collagen, glycoproteins and proteoglycan (Alberts et al., 1994). The primary, secondary, and tertiary fiber bundles are surrounded by the endotenon, while whole tendon is surrounded by the epitenon (Reynolds et al., 1991). Collagen synthesis, secretion and regulation are described later in the Literature Review. The blood supply to most tendons is supplied by branches of the blood vessels that supply the attached muscle with arterioles projecting into the body of the tendon from the myotendinous junction, and from blood vessels entering the tendon at the osseous attachment sites. Both perfusion and diffusion through the extracellular matrix are important in providing tenocytes, and other resident and migrating cell population,

with adequate nutrients and oxygen supplies, as well as providing conduits for removing

metabolic waste products. The colloid-like properties of the proteoglycans help create the

diffusion "pathway" that allows nutrients and oxygen to reach the resident and migratory

cells present in tendon and metabolic wastes to exit the tissue (Robinson e t al., 1983).

Proteoglycans consist of a polypeptide 'core', to which is attached one or more glycan

chains, which is also called glycosaminoglycan (GAG). The glycan chains are of four

types, 1) heparan (D-glucuronic and L-iduronic acid and glucosamine, linked $1 \rightarrow 4$), 2)

keratan (N-acetylglucosamine and D-galactose, linked $1 \rightarrow 3$ and $1 \rightarrow 4$ respectively) 3)

chondroitin-dermatan (N-acetylgalactosamine, with D-glucuronic and /or L-iduronic acid

linked $1 \rightarrow 4$ and $1 \rightarrow 3$, respectively) and 4) hyaluronan (glucuronic acid and N-

acetylglucosamine, linked $1 \rightarrow 3$). They consist of repeating disaccharide units, one

residue of which is always hexosamine, usually with sulphate ester groups attached at the

4 or 6 positions (Scott, 1988). GAG chains form porous hydrated gels that fill most of

the extracellular space. They provide mechanical support against compression to tissues

and are mediators of rapid diffusion of water molecules and migration of cells (Alberts et

al., 1994). Proteoglycans are thought to play a major part in chemical signaling between

cells. They bind to various secreted signaling molecules. Decorin binds to TGF-β and

regulates TGF-β activity (Schonherr et al., 1998; Kresse et al., 1993). Betaglycan binds to

TGF-β and presents it to TGF-β receptors (Lopez-Casillas et al., 1994). Proteoglycans of

the extracellular matrix may be divided into several families. The lecticans are

multidomain proteoglycans containing an N-terminal globular domain, which can interact

with hyaluronan, a C-terminal selectin-like domain and chondroitin sulfate side chains.

Family members are aggrecan, versican, neurocan, and brevican, the latter two being

characteristic components of neural tissue (Kresse et al., 2001). The second family comprises the so-called small proteoglycans/glycoproteins, whose core proteins are composed of a leucine-rich repeat structure (SLRPs), which presumably allow the molecules to adopt a horseshoe-like structure well suited for protein-protein interactions. Well-known family members are decorin, biglycan, fibromodulin, lumican, keratocan, and asporin (Kresse et al., 2001; Lorenzo et al., 2001). Their core proteins most often have either chondroitin/dermatan sulfate or keratan sulfate chains. The other families include testicans and syndecan (Kress et al., 2001). I focus on the second family in the review because of their role in collagen fibrillogenesis. Decorin limits collagen fibril diameters by inhibiting the lateral fusion of fibrils (Scott and Parry, 1992) and inhibits the mineralization of fibrillar collagen matrices (Scott and Haigh, 1985). Decorin will be discussed latter. Mice lacking a functional fibromodulin gene exhibit an altered morphological phenotype in tail tendon with fewer and abnormal collagen fiber bundles. In fibromodulin-null animals, virtually all collagen fiber bundles are disorganized and have an abnormal morphology. Morphometric analysis shows that fibromodulin-null mice have, on the average, thinner fibrils than wild type animals. Protein and RNA analysis showed an approximately 4-fold increase in the content of lumican in fibromodulin-null mice tail tendon as compared with wild type tail tendon. These results demonstrate a role for fibromodulin in collagen fibrillogenesis (Svensson et al., 1999).

Collagen and proteoglycans are the major components of tendons. Therefore all factors affecting collagen and proteoglycan can influence tendon integrity.

B. Collagen

Collagen composition and Structure

The major component in mature tendons is type I collagen. Type III and V collagens are present as minor collagens (Tsuzaki et al., 1993; Fan et al., 1997). There is more type III collagen in the insertion zones and sheaths than in the tendon proper (Jarvinen M., 1991; Fan et al., 1997). An inverse relationship between type III collagen function and fibril diameter exists in the developing tendon (Birk et al., 1997). The characteristic feature of a typical collagen molecule is its long, stiff, triple-stranded helical structure, in which three collagen polypeptide chains, called α chains, are wounded around one another in a rope-like superhelix. An α chain is composed of a series of triple Gly-X-Y sequences in which X and Y can be any amino acid (but X is usually proline and Y is usuallly hydroxyproline). Type I collagen is composed of two $\alpha 1$ and one $\alpha 2$ chains, and type III collagen of three $\alpha 1$ chains. The individual collagen polypeptide chains are synthesized on membrane-bound ribosomes and inserted into the lumen of the endoplasmic reticulum (ER) as larger precursor pro-α chains, containing a central triple helix-forming domain in which the Gly-X-Y repeat motif is continuous for approximately 1000 amino acids, flanked by N- and C-propeptide globular domains (Figure 2.1) (Lamande et al., 1999). In the ER lumen, the selected proline and lysine residues are hydroxylated to form hydroxylproline and hydroxylysine, respectively, and some of hydroxylysine residues are glycosylated. The hydroxylation is catalyzed by two enzymes: prolyl hydroxylase and lysyl hydroxylase, which require molecular oxygen, α-ketoglutarate, ascorbic acid and Fe^{2+}. Scurvy results from a dietary vitamin C deficiency and it is caused by the inability of prolyl hydroxylase and lysyl hydroxylase to form

collagen fibrils properly. Each pro-α chain then combines with two other pro-α chains to form a hydrogen-bonded, triple-stranded helical molecule known as procollagen. The imaging of procollagen transport reveals that the COPI-dependent cargo sorting takes place during ER-to-Golgi transport in mammalian cell using the GFP-procollagen fusion protein in the living cells (Stephens et al., 2002). COPI are protein-coated vesicles, which move materials from the Golgi complex back to the ER. They may also play a role in trafficking from the ER to the Golgi complex, through the Golgi complex from cis to trans face, and between compartments of endocytic pathway. The secreted forms of fibrillar collagens are converted to collagen molecules in the extracellular space by the removal of the propeptides. Both N- and C- propeptide are cleaved by procollagen N-proteinase and C-proteinase, respectively; both of which belong to the zinc-binding metalloproteinase superfamily (Fukui et al., 2002). The C-proteinase is identical to bone morphogenetic protein-1 (BMP-1)(Prockop et al., 1998; Unsold et al., 2002). Interestingly, TGF-β1 elevates the levels of BMP-1 mRNA approximately 7- fold in MG-63 osteosacrcoma cells. Upregulation of BMP-1 mRNA is dose- and time-dependent, and can be inhibited by cycloheximide. TGF-β1 treatment also induced procollagen N-proteinase activity in fibroblast cultures (Lee et al., 1997). Adult mice, after the removal of the NH_2 –termimal propeptide (N-propeptide) through targeted mutagenesis, were normal in appearance and were fertile. Procollagen synthesis, secretion, and proteolytic processing were also normal in these mice. These findings suggest that N-propeptide is not essential for collagen biogenesis (Bornstein et al., 2002). Dermatosparaxis is a recessive disorder of animals (including man) which is caused by mutations in the gene for the enzyme procollagen N-proteinase and is characterized by extreme skin fragility.

Partial loss of enzyme activity results in accumulation of pN-collagen (collagen with N-propeptides) and abnormal collagen fibrils in the fragile skin (Watson et al., 1998).

After being secreted into the extracellullar space, normal collagen molecules assemble into ordered polymers, called collagen fibrils, which are thin (10-300 nm in diameter) structures, many hundreds of micrometers long in mature tissues and clearly visible in electron micrographs. The collagen fibrils often aggregate into larger, cable-like bundles, which can be seen in the light microscope as collagen fibers several micrometers in diameter (Alberts et al., 1994). Collagen stability mainly results from the requisite *trans* conformation of the hydroxyprolyl peptide bond, rather than water bridges (hydrogen bond) (Holmgren et al., 1998). The collagen fibrils are further strengthened and stabilized by the formation of both intramolecular and intermolecular cross-links. Intramolecular cross-links are formed between lysine residues in the (nonhelical) N-terminal region of collagen molecules. The enzyme lysyl oxidase catalyzes the formation of aldehyde group at the lysine side chains in a copper-dependent reaction. The aldehyde groups of two such side chains then link covalently in a spontaneous nonenzymatic aldol condensation. β-Aminopropionitrile (present in sweet pea) covalently inactivates lysyl oxidase, preventing intramolecular cross-linking of collagen and causing abnormalities in joints, bones, and blood vessels. This condition is called Lathyrism. The intermolecular cross-linking of collagen molecules involves the formation of a unique hydroxypyridinium structure from one lysine and two hydroxylysine residues. These cross-links are formed between the N-terminal region of one collagen molecule and the C-terminal region of an adjacent collagen in the fibril (Garrett et al., 2nd). Osteogenesis imperfecta (OI) is characterized by brittle bones and caused by mutations in type I

collagen genes, COL1A1 and COL1A2. A single amino acid substitution (D1441Y) in the carboxyl-terminal propeptide of the proα1 (I) chain of type I collagen results in a lethal variant of osteogenesis imperfecta with features of dense bone disease. Abnormal proα1 (I) chains were slow to assemble into dimers and trimers; abnormal molecules were retained intracellularly for an extended period (Pace et al., 2002). Procollagen synthesis and folding require a number of chaperones or folding enzymes.

Collagen folding and processing

Biosynthesis, folding, and assembly of procollagen are complex processes and involve post-translational modifications by at least nine ER-resident enzymes (Bateman et al., 1996). While some steps in procollagen biosynthesis, including binding of BiP, and GRP94, N-linked glycosylation, and PDI-catalyzed disulfide bonding, are common in other secreted proteins, hydroxylation of proline residues and interaction with the molecular chaperone Hsp47 are unique to collagen biosynthetic events. The initial interactions in collagen subunit assembly occur between the C-propeptides. The triple helix then folds in a zipper-like fashion from the C- to the N-terminus (Figure 2.1). When folded into their native conformation, fibrillar collagen C-propeptides contain two PDI-catalyzed intrachain disulfide bonds (Doege et al., 1986), and the correct formation of these disulfides is critical for C-propeptide folding and interactions between the C-propeptides that lead to subunit assembly. The molecular chaperones BiP, calnexin and GPR94 may also assist C-propeptide folding (Figure 2.1) (Lamande et al., 1995; Chessler et al., 1993). Protein disulfide isomerase (PDI) is a protein-thiol oxidoreductase that catalyzes the oxidation, reduction and isomerization of protein disulfides. In the

endoplasmic reticulum, PDI catalyzes both the oxidation and isomerization of disulfides on nascent polypeptides. Under the reducing condition of the cytoplasm, endosomes and cell surface, PDI catalyzes the reduction of protein disulfides. In those locations, PDI has been demonstrated to participate in the regulation of receptor function, cell-cell interaction, gene expression, and actin filament polymerization (Noiva, 1999). In addition to its activity as a protein-thiol oxidoreductase, PDI facilitates protein folding as a molecular chaperone (Cai et al., 1994). Prolyl 4-hydroxylase (P4H) plays a critical role in collagen biosynthesis by catalyzing the hydroxylation of proline residues in Gly-Pro-X triplets (Kivirikko et al., 1998). Vertebrate prolyl 4-hydroxylase is an $\alpha_2\beta_2$ tetramer, in which the β subunit is PDI (Kivirikko et al., 1989). The P4H heterotetramer can self-assemble when the α- and β-subunits are coexpressed in insect cells or in a cell-free system with dog pancreas microsomes. Although the redox active site of the PDI β-substrate is not directly involved in P4H function, the peptide binding site of PDI does bind P4H substrate, such as proline and procollagen. In fact, the peptide-binding site of the β-subunit (PDI) is essential in the assembly of the P4H heterotetramer, as a deletion in that region (residues 452-454) totally abolishes P4H $\alpha_2\beta_2$ heterotetramer formation. The affinity of P4H substrates for the peptide-binding site is enhanced when PDI is a subunit of P4H. The PDI homodimer also participates in the folding and assembly of procollagen. Experiments utilizing semi-permeabilized cells suggest that P4H, Hsp47 and PDI all participate (probably sequentially) in the folding and assembly of collagen. Another function of the β-subunits (PDI) of the P4H complex is in the subcellular localization of the P4H enzyme to the lumen of the rough ER. The presence of the carboxyl terminal –KDEL sequence on the β-subunit (PDI) localizes P4H to the ER

(Noiva, 1999). Almost complete hydroxylation of appropriate proline residues is necessary for stability of the fibrillar collagen triple helix at 37°C (Berg et al., 1973), and when hydroxylation is inhibited by incubation of cells in the absence of ascorbate, an essential enzyme cofactor, or by the addition of the iron cheletor α,α'-dipyridyl, unfolded chains accumulate within the ER. There is also evidence that prolyl 4-hydroxylase may help prevent secretion of type I collagen molecules with abnormal triple helical domains. In fibroblasts from an OI patient with a 180 amino acid deletion within the triple helix, there is almost complete intracellular retention of the mutant-containing molecules (Chessler et al., 1992). Therefore the stable interaction of procollagen with prolyl 4-hydroxylase (PDI) may be another of the quality control mechanisms acting to prevent secretion (Bottomley et al., 2001). In the cornea, procollagen I is synthesized and intracellularly degraded in corneal endothelial cells (CEC), whereas type IV and VIII collagens are secreted into Descemet's membrane. CECs produce more PDI, P4Hα, and Grp78/Bip than do CSFs (corneal stromal fibroblasts). On the other hand, CECs produce less Hsp47 than do CSFs. The excess amount of PDI in CEC may be required for the ER retention of procollagen I, whereas the higher level of Grp78/Bip in CEC than in CSF may be caused by the presence of unfolded procollagen I (Ko et al., 2002). These data support the hypothesis that Hsp47 interacts with, and stabilizes correctly folded procollagen (Tasab et al., 2000).

Hsp47 is a collagen-binding, heat-inducible protein, resident in the vertebrate endoplasmic reticulum. The human, rat, mouse, and chicken cDNAs have been cloned, and many studies have demonstrated Hsp47 expression in collagen producing cells and tissues, and its ability to bind a wide range of collagens, both in *vitro* and in cells. The

intracellular location, binding, and expression characteristics have led to the proposal that Hsp47 is a collagen-specific molecular chaperone.
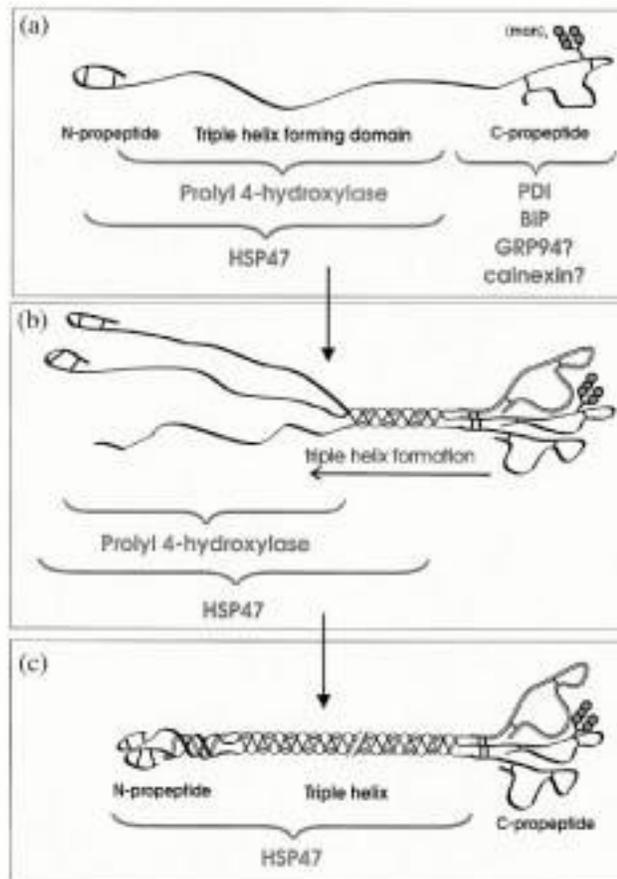

Fig. 2.1. Molecular chaperones involved in procollagen folding and assembly. Three stages of type I procollagen folding and assembly are shown. (a) Synthesis of proα1 (I) chain and folding of the C-propeptide domain. (b) Trimerisation of two proα1 (I) and one proα2 (I) chain and folding of the triple helix. (c) A fully folded procollagen molecule. Enzymes and molecular chaperones that interact with particular domains during each stage are indicated below the protein chains in each panel. For simplicity, a high-

mannose oligosaccharide is shown on only one of the proα1 (I) chains, although the C-propeptides of all three chains are substituted with an N-linked carbohydrate (Lamande, 1999).

Another highly prevalent macromolecule in connective tissues is decorin. Decorin is the prototype of a group of small proteoglycans characterized by a core protein of ~40 kDa, which consists mainly of leucine-rich, repeats of 20-24 amino acid (Patthy, 1987). So far, a number of proteoglycans have been cloned; in addition to decorin, these are biglycan, asporin, fibromodulin, keratocan, and lumican (Lorenzo et al., 2001). Decorin limits collagen fibril diameters by inhibiting the lateral fusion of fibrils (Scott and Parry, 1992) and inhibits the mineralization of fibrillar collagen matrices (Scott and Haigh, 1985). Decorin inhibits fibrillogenesis *in vitro* (Vogel et al., 1984 and 1987). Mice harboring a targeted disruption of the decorin gene display uncontrolled lateral fusion of collagen fibrils and skin fragility (Danielson et al., 1997). In addition, decorin interacts with a variety of extracellular matrix proteins, e.g., fibronectin and thrombospondin, as well as with TGF-β (Schonherr et al., 1998; Kresse et al., 1993). In human mesangial cells, decorin can disrupt the TGF-β/Smad signaling pathway through a mechanism that involves an increase in Ca^{2+} signaling, the subsequent activation of Cam kinase II, and the phosphorylation of Smad2 at Ser-240, an important negative regulatory site (Wicks et al., 2000). Decorin also induces Ser-240 phospho-Smad2 hetero-oligomerization with Smad4 and the nuclear localization of this complex independently of TGF-β receptor activation, and the sequestration of Smad4 in the nucleus (Abdel-Wahab et al., 2002). To some degree, decorin downregulates collagen depostion through the disruption of TGF-β/Smad signaling pathway. On the other hand, decorin binds to TGF-β, and thereby

down-regulates all of its biological activities and displays anti-fibrosis activity in the bleomycin hamster model of lung fibrosis (Giri et al., 1997). In CHO cells, recombinant expression of decorin was accompanied by an inhibition of cell proliferation. The effect was considered to result from the capability of decorin to inhibit the activity of TGF-β. In A431 squamous carcinoma cells, decorin suppresses the malignant phenotype by activating the EGF receptor through its dimerization and increased phosphorylation (Kress et al., 2001). Therefore, one of decorin functions is involvement in growth control.

Some physical factors affecting hydroxylation of lysine or proline, fibril cross-linking or proteoglycan synthesis may induce collagen instability, and therefore can increase or decrease collagen tensile strength. Environmental factors such as exercise, heat or humidity may also affect collagen stability. After one week of physical training, mice demonstrated an increase in mean diameter, in number, and in cross-sectional area of collagen fibril in mouse tendon. In the long-term, there was an increase in fibril number and a decrease in mean diameter (Michna and Hartmann, 1989). While tendon fibroblasts were significantly more resistant to hyperthermia than dermal fibroblasts, repeated hyperthermic insults may compromise cell metabolism of matrix components, resulting in tendon central core degeneration (Birch et al., 1997). Patellar tendon shrinkage by laser-induced heat was precise and dose related. A sharp increase in shrinkage to approximately 70% of resting length was noted around $70^{o}C$ (Vangsness et al., 1997). Therefore, tendon collagen is relatively heat-resistant. During normal developmental tissue growth and in a number of diseases of the cardiopulmonary system, adventitial and interstitial fibroblasts are subjected to increased mechanical strain. This leads to fibroblast activation and enhanced collagen synthesis. To identify mechanical

strain-responsive elements in the rat procollagen alpha 1(I) (COL1A1) gene, COL1A1 promoter constructs were established. The activity of the COL1A1 promoter constructs, transiently transfected into cardiac fibroblasts, was increased between 2- and 4- fold by continuous cyclic mechanic strain. This was accompanied by an approximately 3-fold increase in the levels of total active transforming growth factor–beta (TGF-β) released into the medium. Inclusion of a pan-specific TGF-β neutralizing antibody inhibited strain-induced COL1A1 promoter activation. Deletion analysis revealed the presence of two potential strain response regions within the proximal promoter, one of which contains an inverted CCAAT-box overlapping a GC-rich element. Both mechanical strain and exogenously added TGF-β1 enhanced the binding activity of CCAAT–binding factor, CBF/NF-Y, at this site (Lindahl et al., 2002). In addition to physical factors, growth factors or cytokines play pivotal roles in the regulation of collagen production in physiological and pathological conditions.

Growth factors or cytokines affecting collagen production

A variety of cytokines or growth factors regulate collagen production under normal or pathological conditions. TGF-βs strongly stimulate collagen production (Roberts et al., 1986) and powerfully enhance expression of the plasminogen activator inhibitor type-1 (PAI-1) gene (Nordt et al., 2001). The various functions of TGF-β are discussed in more detail later in this chapter. Interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) decrease collagen synthesis and activate matrix metalloproteinases (MMPs) that degrade collagen when they were added into neonatal and adult rat cardiac fibroblasts cultures *in vitro* (Siwik et al., 2000). The platelet-derived growth factor

(PDGF) has three isoforms, PDGF-AA, PDGF-BB and PDGF-AB. In cultured fibroblasts from normal human wounds, PDGF-AA and PDGF-BB down-regulated both the steady-state levels of pro $\alpha 1$ (I) and pro α (III) collagen chain mRNAs and the production of collagen in a dose-dependent manner. But low concentrations (1ng/ml) of PDGF-AB up-regulated the expression of type I and III procollagen mRNAs. The proliferation rate of wound fibroblasts was stimulated by PDGF-BB, which elicited a dose-dependent (1-3ng/ml) stimulation of cell proliferation, whereas PDGF-AB and AA were less effective in this respect (Lepisto et al., 1995). The expression of IL-1α was constitutively observed in systemic sclerosis (SSc) while no such effect was detected in normal fibroblasts. An antisense oligodeoxynucleotide complementary to IL-1α mRNA was used to suppress endogenous IL-1α. Inhibition of endogenous IL-1α led to decreased levels of the IL-6 and PDGF-A expression in SSc fibroblasts. Moreover, the blocking of the IL-6 response using anti-IL-6 antibody resulted in a significant reduction of procollagen type I in SSc fibroblasts. These results suggest that endogenous IL-1α expressed by SSc fibroblasts may play a key role in the abnormal function of SSc fibroblasts through the expression of IL-6 and PDGF-A (Kawaguchi et al., 1999).

Connective tissue growth factor (CTGF) is a cysteine-rich peptide synthesized and secreted by fibroblastic cells after activation with TGF-β. CTGF acts as a downstream mediator of TGF-β-induced fibroblast proliferation. *In vitro* studies on normal rat kidney (NRK) fibroblasts demonstrated that CTGF potently induced collagen synthesis, and transfection with an antisense CTGF gene blocked TGFβ stimulated collagen synthesis. Moreover, TGF-β-induced collagen synthesis in both NRK and human foreskin fibroblasts was effectively blocked with specific anti-CTGF antibodies

and by suppressing TGF β-induced CTGF gene expression by elevating intracellular cAMP levels with either membrane-permeable 8-Br-cAMP or an adenylyl cyclase activator, cholera toxin (Duncan et al., 1999). In the rat remnant kidney model, proximity of CTGF, TGF-β, and PDGF mRNA expression to regenerative epithelial cells, and those transdifferentiating in myofibroblasts, suggested that growth factors may modulate renal tubular epithelial differentiation (Frazier et al., 2000). The overexpression of CTGF also promotes vascular smooth muscle cells to express more extracellular matrix proteins such as collagen I and fibronectin (Fan et al., 2000). The above described growth factors or cytokines have other activities in addition to regulating collagen production. Some of many cytokines involved in wound healing or growth development are usually able to regulate collagen production.

The regulated turnover of extracellular matrix macromolecules is critical to a variety of important biological processes such as the uterus involution following childbirth, white blood cells migration in response to infection or injury, and cancer cell metastasis. In each of these cases, matrix components are degraded by extracellular proteolytic enzymes that are secreted locally by cells. Most of these proteases belong to one of two general classes: many are MMPs, which depend on bound Ca^{2+} or Zn^{2+} for activity, while the others are serine proteases, which have a highly reactive serine residue in their active sites. After supraspinatus tendon rupture, there was increased activity of MMP-1, reduced activity of MMP-2 and MMP-3, increased denatured collagen and further deterioration in the quality of the collagen network. Tendon degeneration is shown to be an active, cell-mediated process that may result from a failure to regulate specific MMP activities in response to repeated injury or mechanical strain (Riley et al.,

2002). MMPs, particularly MMP-2, produced by cancer cells or, more typically, induced in the normal stroma adjacent to cancer cells are necessary for the degradation of extracellular matrix. That is an essential step in cancer metastasis. The bone and bone marrow are the most common sites of metastasis in breast cancer. MMP-2 is stored in an inactive conformation in association with the cell surface or extracellular matrix of bone marrow fibroblasts (BMF). Cocultures of BMFs and human breast cancer cells induced the release of MMP-2 into the culture medium without up-regulation of MMP-2 synthesis in either cells (Saad et al., 2002). Adenoviral delivery of p53 gene, which is translated into the tumor suppressor protein, suppressed the expression of collagenase-3 (MMP-13) in squamous carcinoma cells (Ala-aho et al., 2002a). On the other hand, expression of collagenase-3 (MMP-13) enhanced invasion of human fibrosarcoma HT-1080 cells (Ala-aho et al., 2002b). The MMPs are regulated mainly by tissue inhibitors of MMPs (TIMP). TIMPs non-covalently bind to the active site of MMPs, but also bind to other domains of MMP-2 and MMP-9. Currently, four TIMPs have been characterized: TIMP-1, TIMP-2 and TIMP-4 are present in soluble form while TIMP-3 is bound to the ECM (Gomez et al., 1997). TGF-β1 induced TIMP production to facilitate collagen deposition (Hui et al., 2001). An important serine protease involved in matrix degradation is urokinase-type plasminogen activator (U-PA). U-PA cleaves a single bond in plasminogen to yield the active protease plasmin, which cleaves fibrin, fibronectin, and laminin (`Alberts et al., 1994`). Collagen production and degradation in tissues are highly regulated by two opposite group of enzymes modulated by a variety of cytokines and growth factor to maintain the tissue equilibrium.

In summary, procollagen biosynthesis, folding, secretion and degeneration are highly controlled by a group of chaperones and enzymes. Any factor affecting those processes is able to alter collagen production and deposition. In normal tissues those processes are precisely modulated to maintain tissue or organ equilibrium. Otherwise, pathological progresses could occur.

C. Heat shock protein 47 (Hsp47)

Hsp47 function

Newly synthesized membrane and secretory proteins are translocated across the endoplasmic reticulum (ER) membrane and transported to the Golgi apparatus for subsequent distribution to the cell surface, lysosomes, and secretory vesicles. Many of these proteins are not exported from the ER until they are folded and assembled correctly. The ER contains several proteins that are termed either molecular chaperones or folding enzymes. The molecular chaperones are involved in the processing and maturation of secretory and membrane proteins. They are thought to associate with folding intermediates or misfolded proteins to prevent nonproductive side reactions, such as irreversible aggregation. They may accelerate the slower steps in the folding process (Bergerron et al., 1994 and De Silva et al., 1990). Of these proteins, certain stress proteins, such as glucose-regulated protein-78 and GRP94, are thought to facilitate translocation into the ER and then to assist in folding, oligomeric assembly, and sorting in the ER. The folding enzymes, which include prolyl cis-trans-isomerase and protein disulfide isomerase (PDI), are involved in the posttranslational modification of proteins such as procollagen.

Heat shock protein 47 (Hsp47) is one such ER-resident protein, first identified as the major collagen-binding heat-inducible glycoprotein in fibroblasts (Nagata et al., 1986). It is characterized by its substrate specificity for collagen, and plays a major role in collagen processing and quality control under stress conditions by preventing the secretion of procollagen with abnormal conformations (Nagata 1996). It is normally located on fibroblasts in connective tissue in various organs, chondrocytes in cartilage, smooth muscle cells in gastrointestinal tract and blood vessels, vitamin A storage cells in sinusoidal area of liver, endothelial cells in blood vessels, and epithelial cells in renal glomeruli, tubules, and basal layer of epidermis (Miyaishi et al., 1992), where Hsp47 functions as a collagen-binding glycoprotein. The same type of cells also co-express one or more types of collagen molecules. Satoh et al., 1996 investigated the intracellular interaction of collagen-specific stress protein Hsp47 with newly synthesized procollagen using biochemical co-precipitation with anti-Hsp47 and anti-collagen antibodies, combined with pulse-label and chase experiments in the presence or absence of various inhibitors for protein secretion. They found that Hsp47 associates with both nascent single procollagen polypeptide chains and mature triple-helix procollagen. When the secretion of procollagen was inhibited by the presence of α, α'-dipyridyl, an iron chelator that inhibits procollagen triple-helix formation, or by the presence of brefeldin A, which inhibits protein transport between the ER and Golgi apparatus, procollagen was found to be bound to Hsp47 during the chase period in the intermediate compartment. In contrast, the dissociation of procollagen chains from Hsp47 was not inhibited by monensin or bafilomycin A1, both of which are known to be inhibitors of post-cis-Golgi transport. These findings suggest that Hsp47 and procollagen dissociate between the post-ER and

cis-Golgi compartments. Hsp47 with the RDEL sequence deleted was secreted out of the cells, which suggests that RDEL sequence actually acts as an ER-retention signal, as the KDEL sequence does. *In vitro* studies (Dafforn et al., 2001) showed that the mature recombinant mouse Hsp47 is able to bind to a monomeric and partially folded conformation collagen mimic peptide. Upon peptide binding Hsp47 has the capacity to induce the peptide backbone to fold into a polyproline type II conformation. Induction of this conformation results in peptides associating into higher order assemblies with increased stability compared with the monomeric peptide alone. Besides, Hsp47 has been reported to have an inhibitory action against the degradation of procollagens in the ER (Jain et al., 1994). This is more conceivable considering the similarity in the primary structures of Hsp47 and the serpin (serine protease inhibitor) super family (Hirayoshi et al., 1991). One characteristic of procollagen molecules that separates them from most proteins synthesized by cells is that their triple-helical domain is not thermostable, being denatured at temperatures a few degrees above physiological temperature. Thermal stability depends on the content of hydroxylproline residues within the triple-helical domain (Kivirikko et al., 1992). Thus, a triple-helical domain containing unhydroxylated proline residues unfolds at temperatures above 25°C. Hsp47 interacts preferentially with triple-helical procollagen molecules and stabilizes correctly-folded procollagen (Tasab et al., 2000). Conflicting results were reported by Macdonald et al., 2001. They found that chicken recombinant Hsp47 bound equally well to native type II and type III procollagen without the carboxyl-terminal propeptide, but binding to triple helical collagen-like peptides was much weaker. The weak binding occurred to both hydroxylated and nonhydroxylated collagen-like peptide. Binding of Hsp47 to bovine pN type III collagen

(collagen with N-propeptides) has only minor effects on the thermal stability of the triple helix and does not influence the refolding kinetics of the triple helix. To better understand the substrate recognition by Hsp47, Koide et al., 2002 synthesized various collagen model peptides and examined their interaction with Hsp47 *in vitro*. They found that the Pro-Arg-Gly triplet forms a Hsp47-binding site. The Hsp47 binding was observed only when Arg residues were incorporated in the Yaa positions of the Xaa-Yaa-Gly triplets. Amino acids in the Xaa position did not largely affect the interaction. The recognition of the Arg residue by Hsp47 was specific because replacement of the Arg residue by other basic amino acids decreased the affinity to Hsp47. The significance of Arg residues for binding of collagen to Hsp47 was further confirmed by using residue-specific chemical modification of types III collagen and I. Therefore, Hsp47 most likely binds to both monomeric and triple helical collagen to ensure the proper folding of triple helical collagen, and to prevent the unfolded procollagen from being secreted and to stabilize correctly folded procollagen.

Co-expression of Hsp47 and procollagen

As a molecular chaperone, Hsp47 is closely associated with collagen processing and secretion, as well as with the inhibition of procollagen degradation in the ER. In addition to these functions, the expression of Hsp47 is always closely correlated with the expression of the various types of collagens. In fibroblasts, the synthesis of both Hsp47 and type I collagen decreases after malignant transformation by Rous sarcoma virus (Nagata et al., 1986; Clarke et al., 1993), and simian virus 40 (Nakai et al., 1990). Phosphorothioate antisense oligodeoxynucleotides to Hsp47 mRNA inhibit firstly Hsp47

production and consequently diminish the production of type I procollagen α1(I) chains (Sauk et al., 1994). In contrast, both Hsp47 and type IV collagen synthesis increased markedly during the differentiation of the mouse teratocarcinoma cell line F9 after treatment with retinoic acid alone or with retinoic acid plus dibutyryl cAMP (Kurkinen et al., 1984; Takechi et al., 1992). Interestingly, Hsp47 synthesis is not observed in cells where collagen synthesis is not detected, such as mouse myeloid leukemic M1 cells and mouse pheochromocytoma PC12 cells (Nagata et al., 1991). Such a correlation between the expression of Hsp47 and several types of collagen has also been reported in rat cells (Clark et al., 1993). The strong correlation of Hsp47 with collagen synthesis under pathological conditions has been observed *in vivo*: the synthesis of Hsp47, as well as collagens, are dramatically increased during the progression of rat liver fibrosis induced by carbon tetrachloride (Masuda et al., 1994), in oral submucous fibrosis (Kaur et al., 2001), the dermal fibrotic disease (Naitoh et al., 2001) and the ballon-injured rat carotid artery (Murakami et al., 2001). Thus, Hsp47 is a stress protein that has substrate specificity, and whose regulation is also closely correlated with the substrate, collagen.

The correct folding and assembly of proteins within the endoplasmic reticulum (ER) are prerequisites for subsequent transport from this organelle to the Golgi apparatus. The mechanisms underlying the ability of the cell to recognize and retain unassembled or malfolded proteins generally requires binding to molecular chaperones within the ER. Vitamin C (ascorbic acid) is an essential co-factor of the enzymes prolyl 4-hydroxylase and lysyl hydroxylase (Garrett et al., 1995). The accumulation of partially folded procollagen occurs during vitamin C deficiency due to the incomplete proline hydroxylation. By using a combination of cross-linking and sucrose gradient analyses,

Walmsley et al., 1999 found that the major protein binding to procollagen during its biosynthesis is prolyl 4-hydroxylase (P4H), and no binding to other ER resident proteins including Hsp47 was detected. This result demonstrates that this enzyme can also recognize and retain unfolded procollagen chains and can release these chains for further transport once they have folded correctly. HoIver, Hosokawa et al., 2000 showed that procollagen bound to both prolyl 4-hydroxylase/protein disulfide isomerase and Hsp47 within the endoplasmic reticulum in the absence of ascobate. Probably Hsp47 and PDI compete for binding to procollagen since the binding of PDI to procollagen decreased when Hsp47 was co-transfected. Thus P4H, PDI and Hsp47 bind cooperatively and timely to procollagen during biosyntheses to facilitate its folding and secretion.

Like other heat shock protein genes, cloned Hsp47 genes reveal a well-conserved heat-shock element (Hse) consisting of one or more tandem repeats of five base-pair units (nGAAn, where n is any nucleotide) at the promoter region (about −80 from the transcription start site) (Hosokawa et al., 1993). Under stressful conditions, various heat shock proteins are induced by Heat shock factors (HSF) binding to Hse (Sarge et al., 1991; Pirkkala et al., 2001). Heat shock factor (HSF) is a sequence-specific DNA binding protein that binds tightly to multiple copies of a highly conserved sequence motif (nGAA). In vertebrate cells, members of the HSF gene family, which includes HSF1, HSF2, HSF3, and HSF4, are thought to respond differently to various forms of stresses (Nakai et al., 1993; Tanabe et al., 1997). HSFs are present constitutively in the cell in a non-DNA binding state, and are activated to a DNA binding form in response to various stresses. The activation process appears to involve HSF oligomerization from monomeric to a trimeric state, and is associated with its hyperphosphorylation (Sorger, 1991). Nitric

oxide (NO) and mechanical stress induces heat-shock protein 70 expression in vascular smooth muscle cells via activation of heat shock factor1 (Xu et al., 1997 and 2000). Induction of Hsp47 synthesis by TGFβ and IL-1β has been reported via enhancement of the heat shock element binding activity of heat shock transcription factor1 in human diploid fibroblasts (Sasaki et al., 2002). Avian cells also express three HSF genes encoding a unique factor, HSF3, as well as homologues of mammalian HSF1 and HSF2 (Tanabe et al., 1997).

D. Transforming growth factor beta (TGF-β)

TGFβ superfamily structure and activation

The transforming growth factor β superfamily (TGF-β) is one of the most complex groups of cytokines with widespread effects on many aspects of growth, differentiation, and final fate of metazoan cells (Massague et al., 2000). The TGF-β superfamily currently consists of more than 25 molecules, isolated from many species, e.g. man, mice, chickens, Drosophila melanogaster and Xenopus laevis and encompasses a wide range of functions (Kingsley, 1994a; Kingsley, 1994b; Meno et al., 1996). The TGF-β superfamily is composed of numerous growth and differentiation factors, including transforming growth factor βs 1-5; growth/differentiation factors (GDFs); Mullerian inhibiting substance (MIS); bone morphogenic proteins (BMPs); glial cell line-derived neurotrophic factor (GDNF); inhibins or activins, Lefty and Nodal (Massague et al., 1994). Members of the TGF-β superfamily are involved in embryonic development and adult tissue homeostasis (Meno et al., 1996; Schmid et al., 1991). The superfamily is characterized by a conserved carboxyl terminal feature consisting of seven cysteine

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residues, six of which form a rigid cysteine 'knot'. The seventh cysteine (Cys77 in TGF-β1) on the respective monomers forms a single disulfide bridge betIen the two subunits (dimer), stabilized by two paired complementary hydrophobic interfaces between them (Amatayakul-Chantler et al., 1994).   The TGF-β family of proteins are synthesized and secreted as large pro-peptide molecules consisting of three regions: an amino terminal (5') signaling sequence, a pro-domain and a mature protein carboxyl (3') domain. The precursor protein domains form homo- or occasionally heterodimers (Ogawa et al., 1992) of two 390 amino acid chains. The associated pro-protein region is called the Latency Associated Peptide (LAP). It has three side chains, two of which are asparagines-linked-mannose-6-phosphate (M-6-P) oligosaccharides (Purchio et al., 1988). In addition, the latent TGF-β (LTGF-β) can contain a protein of variable size called the Latent TGF-β Binding Protein (LTBP), which facilitates the secretion of LTGF-β (Saharinen et al., 1996). Both the LTBP and LAP must be removed before the mature protein can function; therefore activation of TGF-β is a crucial target for biological control of the molecule. Activation of TGF-β1 has been reported by various *in vitro* methods and considerably fewer *in vivo*. *In vitro* methods include extremes of pH, heat, plasmin (Lyons et al., 1990), deglycosylation, binding to the 450 kD platelet protein thrombospondin (Schultz-Cherry et al., 1993), and proteolytic processing by convertase furin (DuBois et al., 1995). *In vivo* the process of activation has not yet been fully established but evidence suggests that plasmin may also activate TGF-β1 under the control of tissue plasminogen activator (tPA), urokinase plasmingen activator (U-PA) and the plasminogen activator inhibitors (PAI). TGF-β can bind to a variety of matrix proteins including biglycan, decorin, fibronectin, collagen, α-2-macroglobulin and vitronectin (Schoppet et al., 2002).

TGF-β is a multifunctional regulator of cell proliferation (Moses et al., 1985; Sporn et al., 1987). TGF-β can act as both a positive and negative regulator of cell division. Studies in several laboratories have demonstrated that TGF-β stimulated the proliferation of mesenchymal derived cells, such as NRK cells (Roberts et al., 1980; Tucker et al., 1984) and AKR-2B cells (Childs et al., 1982), but inhibited the proliferation of epithelial or endothelial cells (Heimark et al., 1986; Takehara et al., 1987). The treatment of mouse BALB/MK keratinocyte cells with either antisense c-myc oligonucleotides or TGF-β1 inhibited cell entry into S phase. TGF-β inhibition of c-myc expression may be essential for growth inhibition by TGF-β1. The block in c-myc expression by TGF-β1 occurred at the level of transcription level (Pietenpol et al., 1990). In addition, TGF-β also inhibits the growth of T and B lymphocytes (Kehrl et al., 1986a,b). The most abundant smyce of this growth factor appears to be platelets (Childs et al., 1982) although other inflammatory cells, including macrophages, neutrophils and astrocytes, produce the TGF-β peptide. TGF-β rapidly induced fibrosis and angiogenesis *in vivo* and stimulated collagen formation *in vitro* when injected subcutaneously in newborn mice (Roberts et al., 1986). Similarly the cellular distribution of TGF-β and procollagen types I, III, and IV transcripts in carbon tetrachloride-induced rat liver fibrosis (Nakatsukasa et al., 1988) suggested that TGF-β1 may play an important role in the production of fibrosis. Mustoe et al. (1987) investigated the effect of exogenous platelet purified TGF-β1 in the healing of incisional wounds in the backs of male rats and reported an increase in breaking strength of 220% after only 5 days, with healing appearing to be accelerated by approx. 3 days. Non-healing human chronic wounds, whatever the cause, whether diabetic, decubitus or venous are a significant clinical

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problem. A clinical trial using bovine derived TGF-β2 in the treatment of venous stasis ulcers provided initially encouraging data (Robson et al., 1995). The total ulcer area was reduced and showed that TGF-β2 was not detrimental to healing, and may have accelerated healing of other chronic wounds. TGF-β2 antibody attenuates fibrosis in experimental diabetic rat kidney (Hill et al., 2001). Low oxygen tension stimulates collagen synthesis and COL1A1 transcription through the action of TGF-β1. Human dermal fibroblasts exposed to low oxygen tension (hypoxia) upregulate the mRNA levels of the human proα1(I) and TGF-β1 genes. Upregulation of COL1A1 mRNA levels in hypoxia was blocked by a TGFβ1 anti-sense oligonucleotide, and failed to occur in fibroblasts from TGF-β1 knockout mice (Falanga et al., 2002). The cultured 3T3-L1 fibroblasts under low partial oxygen pressure enhanced secretion of type IV collagen 10-fold and accelerated adipose conversion of the cells (Tajima et al., 2001). TGF-β is capable of stimulating fibroblast chemotaxis and the production of collagen and fibronectin (Ignotz et al., 1986; Postlethwaite et al., 1987). Raghow et al. (1987) reported that TGF-β increases steady state levels of type I procollagen and fibronectin mRNAs postranscriptionally in cultured human dermal fibroblasts. However, Ignotz et al. (1987) demonstrated that the levels of mRNA for type I collagen and fibronectin proteins were increased several-fold when NRK-49 rat fibroblasts and L_6E_9 rat myoblasts were treated with TGF-β1. Morever, actinomycin D, but not cycloheximide, inhibited the increase in fibronectin and α2(I) procollagen mRNA levels induced by TGF-β1. TGF-β causes transcriptional activation of the α2(I) collagen promoter through an NF1 binding site (Rossi et al., 1988). In contrast, Chung et al. (1996) reported that the AP-1 binding sequence, other than NF-1 or NF-κB, is essential for regulation of human α2(I) collagen

(COL1A2) promoter activity by transforming growth factor-β. TGF-β1 also induced

TIMP production (MMPs inhibitor) to favor collagen deposition (Hui et al., 2001). In

addition, TGF-βs are also involved in animal embryogenesis and morphogenesis (Pelton

et al., 1990; Millan et al., 1991; Schmid et al., 1991). TGF-β1 induces its own message in

normal and transformed cells (Obberghen-Schilling et al., 1988). It was suggested that

autoregulation of this transcript by its ligand possibly provided a feedback loop to further

modulate growth regulation.

TGF-βs have been identified in chicken embryo chondrocytes and myocytes

(Jakowlew et al., 1988a; Jakowlew et al., 1988b; Jakowlew et al., 1991). The mature

chicken TGF-β1 shows 100% identity with human TGF-β1(Jakowlew et al., 1988a).

TGF-β1 mRNA was not detected in fibroblast mRNA when TGF-βs 1, 2, 3 and 4 cDNA

probes were hybridized to total RNA extracted from 7- to 10-day-old chicken embryos.

Expression of TGF-βs 3 and 4 mRNA was independent of developmental age, while

expression TGF-β2 mRNA decreased in fibroblasts from 10-day-old embryos that were

cultured for 3 days. TGF-β4 has been characterized in chicken embryo chondrocytes

(Jakowlew et al., 1988c; Jakowlew et al., 1992a). TGF-β4 may also play an important

role in the development of many tissues in the chicken embryos (Jakowlew et al., 1992b).

The comparison of fmy isoforms reveals that the functionality of TGF-β4 is more like

TGF-β1 than any other TGF-β proteins. The TGF-β4 and TGF-β1 proteins share 82%

sequence identity in the mature region and 47% in the pro-region, respectively. TGF-β4

has only been detected in the chicken, and it remains to be seen if this TGF-β isoform is

unique to birds. However, the authors (1996) commented that the chicken cDNA library

was contaminated with porcine cDNA, and that the sequence is in fact porcine TGF-

β1(Genbank accession no. X12373). Therefore, the chicken TGF-β1 sequence is likely
unknown.
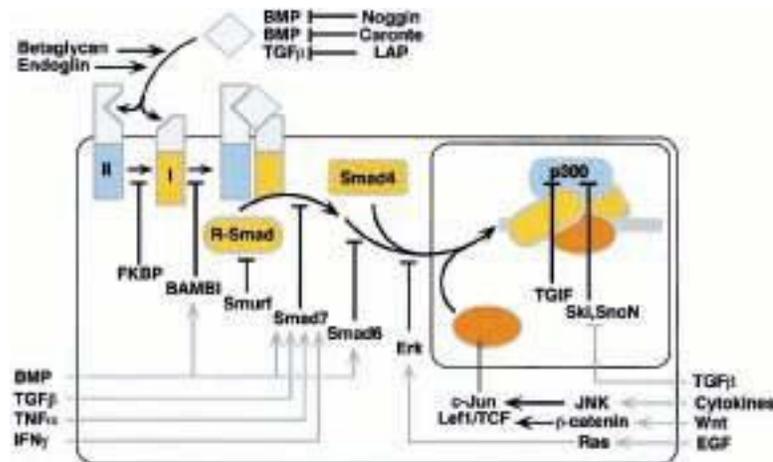
Fig. 2.2. TGF-β signaling. A signaling network controls the activity of the TGF-β/Smad
pathway at multiple levels. Only a few representative examples are shown. Noggin,
Caronte, and LAP are inhibitors of ligand binding to the signal receptors. Betaglycan and
endoglin are enhancers of ligand-access to the signaling receptors. FKBP12 keeps the
type I receptors in the basal state. BAMBI is a truncated receptor-like protein that inhibits
type I receptor activation. Smurf is an E3 ubiquitin ligase that mediates Smad
degradation. Smad7 and Smad6 are decoy Smads that interfere with receptor interaction
with R-Smads or R-Smad interaction with Smad4. Erk MAP kinase phosporylation
attenuates nuclear accumulation of Smads. TGIF, Ski, and SnoN are Smad transcriptional
corepressors. TGIF competes with the coactivator p300 for binding to the Smad complex.
The level or activity of several of these components is controlled by diverse signals as
indicated (Joan Massague, 2000).

TGFβ signaling

TGF-β signaling has been widely studied. Most mammalian cells express three abundant high affinity receptors, which can bind and be cross-linked to TGF-β: the type I (~53kDa), type II (~65kDa), and type III (~100-280 kDa), based upon the molecular mass of the cross-linked products as analyzed by gel electrophoresis (Massague et al., 1992). Tβ-RI and Tβ-RII, the Type I and Type II receptors, are type I transmembrane proteins with cytosolic domains that contain a serine-threonine kinase (Bassing et al., 1994; ten Dijke et al., 1994). Both receptors are essential for intracellular signaling. The TGF-β type III receptor, or betaglycan, is a membrane-bound proteoglycan with a short cytoplasmic tail that has no apparent signaling motif (Wang et al., 1991; Lopez-Casillas et al., 1991). The main role of betaglycan seems to be in binding, and then presenting TGF-β ligand to the signaling receptors Tβ-RI and Tβ-RII (Lopez-Casillas et al., 1994). Overexpression of Tβ-RIII in L6 myoblasts leads to a dramatic increase in TGF-β2 binding to Tβ-RI and Tβ-RII (Wang et al., 1991; Lopez-Casillas et al., 1993). Studies in chemically mutagenized cell lines showed that TGF-β1 binds to Tβ-RII with high affinity in the absence of Tβ-RI and that binding of TGF-β1 to Tβ-RI requires the presence of Tβ-RII (Laiho et al., 1990 and 1991). TGF-β1 binds with relatively high affinity to the soluble secreted exoplasmic domain of Tβ-RII. Tetrameric complexes of the type II and I receptors are found on the surface of many cells after ligand binding, and are important for signal transduction (Lopez-Casillas et al., 1993; Moustakas et al., 1993). Addition of TGF-β1 allows the cytosolic domains of Tβ-RI and Tβ-RII to interact such that the cytoplasmic domain of Tβ-RI is transphosphorylated by the constitutively active Tβ-RII kinase. The activated Tβ-RI then directly signals to downstream intracellular substrate,

Smads (Zhang et al., 1996; Heldin et al., 1997). Adjacent to the kinase domain of Tβ-RI

is a conserved 30 amino acid segment known as the GS region (for a GSGS sequence it

contains). In the basal state, the GS region forms a wedge that presses against the

catalytic center (Huse et al., 1999). The immunophilins FKBP binds to the GS domain

and stabilize this inactive conformation. Activation occurs when the Tβ-RII receptors

phosphorylate the GS domain. Several structurally diverse soluble proteins have been

identified that bind TGF-β factors, preventing their access to membrane receptors (Fig.

2.2). The pro-peptide from the TGF-β precursor (LAP) binds TGF-βs; noggin and

caronte bind BMPs. In contrast, a group of membrane-anchored protein functions as

enhancers of ligand binding to the receptors. Via its protein moiety, Tβ-RIII (betaglycan)

enhances TGF-β binding to its signaling receptors (Lopez-Casillas et al., 1993) and

enables the activin antagonist, inhibin, to bind to activin receptors. A structurally related

protein, endoglin, may have a similar role for TGF-β1. Smads are ubiquitously expressed

through development and in all adult tissues (Flanders et al., 2001; Luukko et al., 2001),

and many of them (Smad2, Smad4, Smad5, Smad6 and Smad8) are produced from

alternatively spliced mRNAs (Gene encyclopaedia, GeneCards). So far, eight vertebrate

Smad proteins in three different functional classes have been identified (Massague et al.,

1998). Smads 1, 2, 3, 5, and 8 make up the receptor-regulated Smad subfamily with a

conserved carboxyl-terminal SSXS motif (R-Smad); Smad4 is a collaborating Smad (or

Co-Smad); and Smads 6 and 7 form an inhibitory Smad subfamily (I-Smad) (Massague et

al., 1998). All Smad proteins share two regions of sequence similarity: Mad homology

MH1 at the NH_2 terminus and MH2 at the COOH terminus. The receptor-regulated

Smads 1, 5, and 8 appeared to mediate specific signaling downstream of Bone

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Morphogenetic Protein (BMP) and its receptors, whereas Smads 2 and 3 function in TGF-β and activin signaling pathways (Heldin et al., 1997; Attisano et al., 1998; Massague et al., 1998, Moustakas et al., 2001). TGF-β-activated Tβ-RI transiently and directly interacts with Smad2 (Macias-Silva et al., 1996; Nakao et al., 1997) and Smad3 (Zhang et al., 1996), resulting in phosphorylaion of the SSXS motif (Abdollah et al., 1997; Liu et al., 1997). Once phosphorylated, Smad 2 and Smad3 associate with Smad4 and translocate to the nucleus. In the nucleus, the Smad complex associates with the forkhead DNA-binding protein FAST2 and binds to DNA, forming a transcriptional active DNA complex (Labbe et al., 1998; Zhou et al., 1998), and in turn activate some gene transcriptions.

Recent findings have demonstrated that accessory/scaffolding proteins interact with the type I and II receptors and/or the Smads. One example is SARA (Smad anchor for receptor activation), a cytoplasmic protein that specifically interacts with non-activated Smad2 and the receptor complex, thus forming a bridge between the receptor and Smad2 and assisting in the specific phosphorylation of Smad2 by the type I receptor (Tsukazaki et al., 1998). The stable interaction of SARA with non-phosphorylated Smad2 also inhibits nuclear import of Smad2 (Xu et al., 2000). As SARA contains a FYVE domain, a motif known to bind phosphatidylinositol 3-phospate, it might anchor Smad2 to the inner leaflet of the plasma membrane or endosomal vesicles. SARA thus provides a first example of how TGF-β signaling centers may be organized at the plasma membrane. A second FYVE-domain-containing protein, Hrs, also facilitates Smad2 signaling and cooperates with SARA-mediated signaling (Miura et al., 2000). Several other proteins with possible roles in Smad anchoring consist of microtubules anchoring inactive Smads

in the cytoplasm (Dong et al., 2000), Filamin positively regulates transduction of Smad signals (Sasaki et al., 2001) and caveolin1 interacting with the type 1 receptor and inhibits Smad2-mediated signaling (Razani et al., 2001).   The interactions betIen TGF-β superfamily receptors and Smads with adaptor/scaffolding proteins are an important regulatory mechanism. Proper receptor localization in plasma membrane or endocytic vesicle microdomains, their proximity to cytoplasmic anchors that hold the Smads, and the ability of such complexes to be mobilized betIen various cytoplasmic compartments are exciting new aspects of the regulation of Smad signaling. Such mechanisms could provide cell-context specificity, allowing differential regulation of the basic Smad pathway. All R-Smads, mammalian Smad4 and Xenopus Smad4α reside in the cytoplasm. In contrast, Xenopus Smad4β and I-Smads localize to the cell nucleus (Itoh et al., 2001; Masuyama et al., 1999). Coprecipitation experiments indicate that phosphorylated R-Smads quickly form complexes with the Co-Smad, prior to nuclear translocation. This notion is enhanced by studies of Smad4 mutants that cannot translocate to nucleus yet oligomerise efficiently with R-Smads (Moren et al., 2000). The MH1 domains of all eight Smad each contain a lysine-rich motif that, in the case of Smad1 and Smad3, has been shown to act as a nuclear localization signal (NLS). In Smad3, C-terminal phosphorylation results in conformational changes that expose the NLS so that importin β1 can bind and mediate Ran-dependent nuclear import. In contrast, Smad2, which has the same lysine-rich sequence in its MH1 domain, is released from the anchoring SARA after C-terminal phosphorylation and then translocates into the nucleus by cytosolic-factor-independent import activity that requires a region of the MH2 domain (Moustakas et al., 2001). All Smads have transcriptional activity (Itoh et al., 2000).

Heteromeric R-Smad-Co-Smad complexes are the transcriptionally relevant entities *in vivo*. Smad3 and Smad4 binds directly, but with low affinity, to Smad-binding elements (5'CAGAC3') through a conserved β-hairpin loop in the MH1 domain, and also associate with GC-rich motifs in promoters of certain genes (Labbe et al., 1998). The transactivation function of Smads maps to the MH2 domain and is mediated by direct association the MH2 domain with co-activators of the p300 and P/CAF (p300- and CBP-associating factor) family (Itoh et al., 2000). Smad4 appears to play a crucial role in regulating the efficiency of transactivation of the Smad complexes in the nucleus. This is thought to involve the unique Smad-activation domain (SAD) of Smad4, which allows stronger association with the p300/CBP-co-activators and confers a unique conformation on the Smad4 MH2 domain (Chacko et al., 2001). Smad signaling can also lead to repression of gene expression. Smad3 has been reported to associate with histone deacetylase (HDAC) activities through its MH1 domain. On the other hand, Smads can interact with co-repressors that recruit HDACs. These co-repressors include the homeodomain DNA-binding protein TGIF and the pro-oncogene products Ski and SnoN (Liberati et al., 2001; Wotton et al., 1999; Liu et al., 2001). Such co-repressors appear to modulate the nuclear activity of Smads, and their levels of expression define the level of Smad transcriptional activity. Protein ubiquitination and subsequent proteasomal degradation is applied to Smads. The Hect-domain ubiquitin ligase, designated Smurf, has been shown to interact with the Smads (Moustakas et al., 2001). Smad function is involved in most actions of the TGFβ family, which is not to say that the TGF-β receptors could not act on other substrates and activate other pathways. Several Smad4-defective cell lines from human or mouse retain some level of responsiveness to TGF-β,

suggesting that, if R-Smads are involved in these responses, they can do so without Smad4 (Dai et al., 1999; Lawrence, 2002). A series of reports indicate that several MAP kinases (JNK, p38, and Erk) can be rapidly activated by TGF-β in a manner that is highly dependent on the cell type and conditions. TGF-β may simultaneously activate Smad and MAP kinase pathway that then physically converge on target genes (Zhang et al., 1998). The Smad signal transduction process itself may be simple but it is under the control of a complex web of regulators (Fig. 2). Several of these molecules, including the truncated receptor-like molecule BAMBI, the ubiquitin ligase Smurf1, and the antagonistic Smads, Smad6 and Smad7, specialize in regulating this pathway. The levels of many of these molecules are controlled by diverse signals, providing feedback and cross-talk links. Additional control and integration are provided by signals that regulate the levels or activity of Smad DNA binding cofactors, including Wnt (another signal pathway) (Bienz et al., 2000; Moon et al., 2002) and diverse cytokines signals. The Smad pathway is therefore well integrated into the signaling networks of the cell at large (Massague et al., 2000; Attisano et al., 2002).

Clearly, important aspects of the cell biology of the Smad pathway are yet to be understood. The mechanism of oligomerisation and the stoichiometry of various Smad complexes are primary goals. The mechanisms of nucleocytoplasmic shuttling also deserve further clarification. Similarly, the *in vivo* mechanisms of gene activation and repression in the context of chromatin need to be addressed, and the ubiquitin-mediated shut-off pathways for the different Smads must be analyzed systematically.

Cell cycle arrest and tumorigensis in response to TGF-β

Inhibition of cell proliferation is central to the TGF-β response in epithelial, endothelial, hematopoietic, neural, and certain types of mesenchymal cells, and escape from this response is a hallmark of many cancer cells. Two classes of antiproliferative gene responses are known to be induced by TGF-β. The first is c-Myc (activation of transcription of growth stimulation genes in leukemia and lung cancer) downregulation, observed in most cell types that are growth inhibited by TGF-β. The second are cdk-inhibitory responses, including the induction of p15 and p21 (both are cyclin-dependent kinase inhibitors. p15 acts on cyclin D/CDK4 or CDK6; p21 inhibits all CDKs) and the downregulation of cdc25A. c-Myc antagonizes TGF-β signaling by acting as a repressor of cdk-inhibitory responses. Downregulation of c-Myc is thus necessary for TGF-β-induced cell cycle arrest. The cdc25 family of tyrosine phosphatases removes inhibitory tyrosine phosphorylation from cdks. Loss of cdc25A and the induction of p21 or p15 lead to the direct inhibition of cyclinD-cdk4. p15 binding to cyclin D-cdk4 leads to the shuttle of p27 from active cyclin D-cdk4-p27 complexes to cyclin E-cdk2 complexes, resulting in their ultimate inhibition as well. p27 is a cdk2 inhibitor that, in the proliferating cells, can stay bound to cdk4 and cdk6 complexes without causing inhibition. When mobilized by TGF-β, p27 binds to and blocks cdk2 (Massague et al., 2000). The overall roles of those proteins induced by TGF-β result in cell cycle arrest. In addition to causing reversible cell cycle arrest in some types, TGF-β can induce programmed cell death in others (Hagimoto et al., 2002). In fact, apoptosis induced by TGF-β family members is an essential component of the proper development of various tissues and organs. TGF-β-

induced apoptosis and the selective elimination of preneoplastic cells may also be involved in the tumor suppression mediated by TGF-β, as a body of largely circumstantial evidence suggests (Gold, 1999). Increased apoptosis with HIV and TGF-β1 was associated with reduced levels of Bcl-2 and increased expression of apoptosis-inducing factor, caspase-3, and cleavage of BID, c-IAP-1, and X-linked inhibitor of apoptosis. These results show that TGF-β1 promotes depletion of CD4$^+$ T cells after R5 HIV-1 infection by inducing apoptosis (Wang et al., 2001). Approximately 25% HIV+ patients produced TGF-β1 in response to stimulation with HIV proteins or peptides. The production of TGF-β1 was sufficient to significantly reduce the IFN-γ response of CD8+ cells to both HIV and vaccinia virus proteins. Antibody to TGF-β1 reversed the suppression. The source of the TGF-β1 predominantly came from CD8+ cells (Garba et al., 2002). Although TGF-β is a potent growth inhibitor in epithelial tissues, it is both a suppressor and a promoter of tumorigenesis. On the one hand, TGF-β has a tumor suppression function that is lost in many tumor-derived cell lines. It has been estimated that nearly all pancreatic cancers (Goggins et al., 1998) and colon cancers (Grady et al., 1999) have mutations disabling a components of the TGF-β signaling pathway. Such mutations occur in TGF-β receptors or Smads. Defective repression of c-myc in breast cancer cells contributes to a loss at the core of the transforming growth factor β arrest program (Chen et al., 2001). Inactivating mutations in TβRII in most human colorectal and gastric carcinomas have been reported with microsatellite instability. Stable transfection of wild-type TβRII into a human colon cancer cell line and a human gastric cancer cell line restored TGF-β-mediated growth arrest and reduced tumorigenicity in

athymic mice, providing further evidence that mutational inactivation of TGF-β receptors is a pathogenic event (Massague et al., 2000). The TGF-β signaling network is also disrupted in cancer by mutations in Smad4 and Smad2. Smad4, initially identified as DPC4 (deleted in pancreatic carcinoma locus 4), suffers biallelic loss in one half of all of pancreatic cancers, one third of metastatic colon tumors and other smaller subsets of other carcinomas (Massague et al., 2000). On the other hand, TGF-β exacerbates the malignant phenotype of transformed and tumor-derived cells in experimental systems. High levels of TGF-β expression are correlated with advanced clinical stage of the tumor (Gold, 1999). Tumor-derived TGF-β could contribute to tumor growth indirectly by suppressing immune surveillance or stimulating production of angiogenic factor. However, TGF-β can also act directly on cancer cells to foster tumorigenesis. Tumor cells that have selectively lost their growth-inhibitory responsiveness to TGF-β but retain an otherwise functional TGF-β signaling pathway may exhibit enhanced migration and invasive behavior in response to TGF-β stimulation (Cui et al., 1996). Some experiments showed that TGF-β1 acts as a tumor suppressor of human malignant keratinocytes independently of Smad4 expression and ligand-induced G (1) arrest (Paterson et al., 2002). TGF-β/Smad signaling pathway remains functional in human ovarian cancer cells, suggesting that if abnormalities exist in the cellular response of TGFβ signals, they must lie down-stream of the Smad proteins (Lesley et al., 2002).

In summary, TGF-β is involved in a variety of physiological activities in tissues and organs including development and differentiation, and wound healing where its activities are highly regulated. Loss of those activities could induce cell transformation or tumorigenesis. Some mechanisms behind TGF-β signaling are still unknown. Elucidation

of the signaling effectors that link the receptors to these alternative pathways and their

functional crosstalk with Smads is of importance. The recent advent of functional

genomics and the ability to globally monitor gene expression at the RNA and protein

levels provides an important approach for the future. A major quest is to identify co-

regulated groups of genes that respond to TGF-β superfamily signals and classify them

on the basis of their mode and kinetics of regulation, the functions of the encoded

proteins and the cell type and developmental context. The new technologies also hold

promise for a better understanding of the contribution of Smads to various disease

conditions and thus may provide novel drug targets.

E. Relationship between Hsp47 and TGF-β

Heat-shock-protein 47 is a 47-kDa collagen-binding heat shock protein, the

expression of which is always correlated with that of collagens in various cell lines

((Nagata et al., 1986; Clarke et al., 1993; Nakai et al., 1990; Sauk et al., 1994; Kurkinen

et al., 1984; Takechi et al., 1992; Nagata et al., 1991). Multifunctional transforming

growth factor (TGF-β) was originally identified in neoplastic cells and subsequently

reported to be present in most cells exerting a variety of effects on cell proliferation, cell

differentiation and embryogenesis (Roberts et al., 1980; Tucker et al., 1984; Pelton et al.,

1990; Millan et al., 1991; Schmid et al., 1991). TGF-β has also been shown to stimulate

the production of extracellular matrix components including collagens and fibronectin

(Ignotz et al., 1986; Raghow et al., 1987; Ignotz et al., 1987; Postlethwaite et al., 1987).

CCl_4-administered rats exhibited increased expression of TGF-β1 and its receptors as Ill

as collagen in the liver (Nakatsukasa et al., 1988). The relationship betIen Hsp47 and

TGF-β has been rarely reported in the literatures. HoIver, TGF-β1 rapidly induces Hsp70 and Hsp90 molecular chaperones in cultured chicken embryo cells (CEC) while PDGF, FGF, and EGF were not effective and they did not enhance the stimulatory effect of TGF-β on the Hsp's (Takenaka et al., 1992). TGF-β did not increase rates of Hsp70 and Hsp90 gene transcription as measured by run-on transcription assays of isolated nuclei. This result suggests that Hsp70 and Hsp90 gene expression is regulated posttranscriptionally in TGF-β-treated CEC (Takenaka et al., 1993). TGF-β1 increased Hsp47 mRNA and protein levels in normal skin cultured fibroblasts (Kuroda et al., 1998) and human diploid fibroblasts (Sasaki et al., 2002). Treatment of mouse osteoblast MC3T3-E1 cells with 5 ng/ml TGF-β1 for 24h increased the level of Hsp47 mRNA three-fold. Dose-dependent induction by TGF-β1 was observed for both Hsp47 mRNA and collagen α1(I) mRNA, and actinomycin D inhibited this increase of Hsp47 mRNA. By generating a series of 5'-deletion promoters fused to luciferase reporter constructs, transient transfection assays shoId that TGF-β1 induced 4-6 fold the promoter activity of a region approximately –5.5 kbp upstream of the Hsp47 gene (Yamamura et al., 1998). In conclusion, TGF-β1 enhances both Hsp47 and pro-collagen synthesis in human cell lines. I hypothesize that the chicken TGF-β4 also increases both Hsp47 and pro-collagen synthesis in avian tendon.

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CHAPTER 3

REGULATION OF HEAT SHOCK PROTEIN 47 AND TYPE I PROCOLLAGEN EXPRESSION IN AVIAN TENDON CELLS

Heat shock protein 47(Hsp47) is a collagen binding stress protein that acts as a collagen-specific molecular chaperone during the biosynthesis and secretion of procollagen. Type I collagen is a major component of tendons. Co-expression of genes for both proteins was reported in various tissues, where many growth factors likely regulate their expressions in different ways. In this study I described the effects of increased temperature, mechanical stress and growth factors on Hsp47 and type I procollagen expression in fibroblasts isolated from embryonic chicken tendons. My results show that elevated temperature affected significantly the expression of Hsp47. The expression of Hsp47 mRNA at 45°C increased within 30 minutes and returned to baseline in 4 hours after the temperature decreased to 37°C. My data also show that human TGF-β1 is another regulator of Hsp47 expression as the addition of TGF-β1 led to a moderate increase in the expression of Hsp47 mRNA. TGF-β2 exerted only a small effect, and TGF-β3, epidermal growth factor (EGF) and tumor necrosis factor α (TNF-α) had no impact on it. TGF-β1 increased type I procollagen mRNA expression and TNF-α reduced this expression. TGF-β1 delays the degradation of Hsp47 mRNA after heat shock so that TGF-β1 likely regulated Hsp47 gene posttranscriptionally in my experiments. In this study I also report that mechanical stress increased Hsp47 mRNA

expression and Hsp47 protein synthesis. I show that induction of Hsp47 protein

expression by heat shock, mechanic stress and TGF-β1 is likely achieved through

activation and translocation of Heat shock transcription factor 1 (HSF1) into the nucleus.

My data indicate that TGF-β1 is a major regulator of both procollagen and Hsp47 genes.

INTRODUCTION

Heat-shock protein 47 (Hsp47) is a 47-kD heat-inducible stress protein found in

collagen-producing cells, where it functions as a collagen-binding glycoprotein (Nagata

et al.1986). It is one of the ER-resident proteins that are termed "molecular chaperones"

or "folding enzymes". It has an RDEL retention signal at the C terminus, which is a

variant of the KDEL retention signal for retention of proteins in the endoplasmic

reticulum. Northern blot analyses and nuclear run-on assays revealed that the induction of

Hsp47 by heat shock and its suppression after transformation of chicken embryo

fibroblasts by Rous sarcoma virus were regulated at the transcriptional level (Hirayoshi et

al. 1991). Hsp47 was shown to bind to single polypeptide chains of newly synthesized

procollagen and to the mature triple-helix form of procollagen, therefore helping them be

folded and secreted properly (Satoh et al. 1996). Co-expression of Hsp47 and collagen

genes has been widely investigated (Clarke et al. 1993; Nakai et al. 1990). Anti-sense

oligonucleotides of Hsp47 mRNA suppressed collagen accumulation in experimental

glomerulonephritis (Sunamoto et al. 1998). Interestingly, Hsp47 synthesis was not

observed in cells where collagen synthesis was not detected, such as mouse myeloid

leukemia M1 cells and mouse pheochromocytoma PC12 cells (Nagata et al. 1991). The

mechanism of Hsp protein expression in heat stress involves a heat shock transcription

factor (HSF) interacting with a highly conserved sequence (nGAAn) in heat shock

protein genes termed the heat shock element (HSE) (Sarge et al., 1993). By heat

treatment, the HSF monomer is activated by conversion to a trimer that translocates into

the nucleus and is capable of binding to the HSE. Binding of HSF to the HSE induces

transcription of heat shock genes (Baler et al., 1993). Chicken HSF1, HSF2 and HSF3

have been reported, and HSF3 is unique to avian species (Nakai et al., 1993). Xu et al.,

1995 have shown that acute hypertension induces a rapid expression of Hsp70 mRNA

folloId by elevated Hsp70 protein in rat aorta. Later their *in vitro*- experiments proved

that this induction was mediated by HSF1 activation (Xu et al., 2000).

The transforming growth factor β (TGF-β) family consists of related proteins

involved in the regulation of growth and differentiation of most cell types. This group

includes TGF-β1(Roberts et al. 1980), TGF-β2 (Lioubin et al. 1992), TGF-β3 (Derynck

et al. 1988), TGF-β4 (Jakowlew et al. 1988), TGF-β5 (Kondaiah et al. 1990), Mullerian

inhibitory substance, and the inhibins among others. Sequence analysis of cDNA

encoding TGF-βs 1 through 5 indicates that these proteins are synthesized as large

precursor molecules, the carboxy terminus of which is cleaved to yield the 112-amino-

acid monomer or a 114 amino acid monomer in the case of TGF-β4 (Bmydrel et al. 1993;

Lioubin et al. 1992; Jakowlew et al. 1988). TGF-βs are called multifunctional regulators

of cell proliferation (Moses et al. 1985; Sporn et al. 1987). TGF-β1 stimulates the

proliferation of mesenchyme-derived cells, such as NRK cells (Roberts et al. 1980;

Tucker et al. 1984) and AKR-2B cells (Childs et al. 1982), but inhibits the proliferation

of epithelial or endothelial cells (Heimark et al. 1986; Takehara et al. 1987) and the

growth of B and T lymphocytes (Kehrl et al. 1986 a, b). TGF-β1 rapidly induces fibrosis

and angiogenesis *in vivo* and stimulates collagen formation *in vivo* when injected subcutaneously in newborn mice (Roberts et al. 1986).

TGF-β1 stimulates the deposition of the extracellular matrix (ECM) that is mostly composed of collagen, fibronectin, elastin, laminin, proteoglycans, and related molecules (Alberts et al.1994). Hsp47 protein binds to collagen chains and guides them through proper folding and secretion processes (Satoh et al. 1996). Interestingly, treatment of mouse osteoblast MC3T3-E1 cells with 5 ng/ml TGF-β1 for 24 hmys increased both type I collagen α1(I) and Hsp47 mRNA expression levels. Transient transfection assays showed that TGF-β1 induced 4-6 fold the promoter activity of a region approximately 5.5 kb upstream of Hsp47 gene (Yamamura et al. 1998). TGF-β1 increased Hsp47 mRNA and protein levels in normal human skin cultured fibroblasts (Kuroda et al. 1998) and human diploid fibroblasts (Sasaki et al., 2002). The relationship betIen TGF-β and Hsp47 has not been studied in tendons, tissues rich in collagen and fibroblasts. I hypothesized that TGF-β regulates both type I procollagen and Hsp47 protein to facilitate the proper folding and secretion of triple helical collagen molecules. In the same time, I investigated the effects of other cytokines or mechanical stresses on the expression of Hsp47 protein and procollagen. In this experiment I used tendon cell cultures established from 18-day old chicken embryonic gastrocnemius tendons.

MATERIALS AND METHODS

Cell culture

Chicken embryonic tendon fibroblasts Ire isolated from gastrocnemius tendons removed from 18-day old chicken embryos and grown in Dulbecco's modified Eagle's

medium (DMEM) with 0.37g of sodium bicarbonate/100 ml and 10% fetal bovine serum

(FBS). The cells were maintained in an incubator with humidified 5% CO_2, 95% air

atmosphere at 37°C. The cell cultures were grown to confluence in 60-mm dishes. After

confluence was reached, the cells were placed in the quiescent state by the serum-free

DMEM for another 24 hours prior to various treatments. Human recombinant TGF-β1,

2, 3, EGF and TNF-α were obtained from R&D system (Minneapolis, MN). Mechanical

stress was induced by placing quiescent cells on a platform shaking at a speed of 50-60

rpm/min in a 37°C incubator.

Probe preparation

The complete chicken Hsp47 (Accession No: X57157) and partial type I procollagen

α1 (Accession No: J00836) and β-actin (Acession No: L08165) cDNA sequences were

obtained from Genbank. The primers were designed using Generunner program (Hastings

Software, Inc): Hsp47, forward primer 5'-ATCCAACGTCTTCCATGCC-3' (1062-1080)

and reverse 5'-AATCCCCCCCTAAAAAACAC-3' (1304-1323); type I procollagen

α1(I), forward 5'-AACGAGATCGAGATCAGG (1207-1226) and reverse 5'-

TTACTCTCTCCTGTCACGC (1477-1496); Chicken β-actin, forward 5'-

GCTACGTCGACATGGATTTCG (718-738) and reverse 5'-

TAGAAGCATTTGCGGAC (1176-1195). We constructed a 512 bp long RNA probe

complementary to a segment of 2745 bp long human TGF-β1 mRNA between 1486 and

1998 bases (Halper et al., submitted). This probe has 92% homology to the

corresponding chicken TGF-β4 cDNA sequence and 73% homology to chicken TGFβ3

cDNA sequence (Jakowlew et al. 1988). We used the forward primer: 5'-

TGAGGGCTTTCGCCTTAG-3' and the reverse primer: 5'-

CGCACGCACGATCATGTTGGAC-3'. T7 (ATTTAGGTGACACTATA) and Sp6

(TAATACGACTCACTATAGGG) promoter sequences were added to the 5' end of the

forward and reverse primer, respectively. Total RNA was isolated from the above cells

using TRIzol reagent (Life Technologies, Inc., Grand Island, NY). Each cDNA was

generated by a Titan™ One Tube RT-PCR system (Roche Boehringer Mannheim,

Indianapolis, IN). RT-PCR fidelity was confirmed by sequencing the cDNA. Dig-labeled

mRNA probes were produced by Digoxigenin-RNA Labeling Kit (Roche Boehringer

Mannheim). Labeling efficiency was confirmed by a dot-blot test.

Northern blot analysis

For Northern blot analysis, an aliquot of the total RNA (3-5 μg per lane) was size

fractionated in a 1.5% agarose gel and transferred to a positively charged nylon

membrane (Roche Boehringer Mannheim). Equal loading in all lanes was confirmed by

ethidium bromide (EB) staining. Blots were prehybridized in Dig Easy Hyb solution

(Roche Boehringer Mannheim) for 1 hour at 68°C, subsequently hybridized overnight at

68°C in Dig Easy Hyb containing indicated probes. The membranes were washed in the

following order: one time in 2 X SSC – 0.1% SDS buffer for 15 min at room temperature

(RT), one time in 1 X SSC – 0.1% SDS buffer for 15 min at RT, two times in 1 X SSC –

0.1% SDS buffer for 10 min at 68°C. The colorimetric detection of the signal was

performed using a NBT/BCIP or CSPD system (both from Roche Boehringer

Mannheim). Bands were quantified using AlphaImager™ 2200 System (Alpha Innotech

Corp., San Leandro, CA). Relative transcription was normalized with 18S or 28S rRNA,

or chicken β-actin. In preliminary experiments, both normalization methods (18S or 28S rRNA or ethidium bromide staining and hybridization for chicken β-actin) gave closely corresponding results. Each experiment was performed at least twice.

Western blotting

The cells were harvested by centrifugation for 15 min at 2,000 rpm/min and lyzed in cell lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40 pH 7.8, 1 mM PMSF, 1 μg/ml pepstatin and 1 μg/ml leupeptin) on ice for 15 min. The lysates were clarified at 4°C for 30 min at 14,000 rpm/min. For nuclear protein extraction the procedure used was similar to that described by Xu et al., 2000. The human HSF1 polyclonal antibody was obtain from Santa Cruz (Santa Cruz Biotechnology, Inc, Santa Cruz, CA.). The protein concentration was measured using BIO-RAD Assay DC kit. Ten μg protein aliquots of collected supernatants were separated under reducing conditions on a 12% SDS-PAGE, and transferred by electroblotting to nitrocellulose membranes. The nitrocellulose membranes were incubated overnight at 4°C in 5% non-fat milk in PBS containing the mouse monoclonal anti-Hsp47 antibody (StressGen Biotechnologies Corp, Vancouver, BC, Canada), diluted 1:500. Excess antibody was washed away with at least 3-4 changes of TTBS buffer (100 mM Tris, 0.9% NaCl, 0.1%(v/v) TIen 20, pH 7.5) over 15 min. The nitrocellulose membranes were incubated for 1 hour at room temperature in TTBS buffer containing the biotinylated secondary antibody (1:1,000 dilution in TTBS), followed by washing three times in TTBS. The detection was performed using ABC kit (Vector Laboratories, Burlingame, CA). 3,3'-diaminobenzidine tetrachloride (DAB) was used as a chromogen.

RESULTS

Heat shock leads to a rapid elevation of Hsp47 expression and synthesis in tendon cells

Hsp47 protein is a major collagen-binding heat-inducible glycoprotein in collagen-producing cells. To determine the effect of temperature elevation on Hsp47 mRNA expression in chicken tendon cells in culture, total RNA was isolated from cell cultures exposed to $37^{o}C$ or higher temperatures for 2 hours. Northern blot analyses showed that Hsp47 mRNA expression increased proportionately to the temperature increase (Fig. 3.1A and B). Hsp47 mRNA level was five-fold higher at 45°C than 37°C after a 2 hours exposure. Hsp47 protein level increased after exposure to 45°C as determined by Western blotting. This increase was time dependent (Fig. 3.1C). This means that Hsp47 protein synthesis corresponded to an increase in Hsp47 mRNA. To detect whether heat shock induced transcription of other genes, Northern blotting was performed on the total RNA extracted from tendon cells exposed to increased temperatures using type I procollagen and TGF-β probes. The elevated temperature increased Hsp47 mRNA expression (Fig. 3.2A and a), and only transiently procollagen mRNA expression (Fig. 3.2C and c), but not expression of TGF-β mRNA (Fig. 3.2B and b). The increase in Hsp47 mRNA expression was rapid: Hsp47 mRNA increased within 30 minutes of exposure. The level of Hsp47 mRNA increased more than 5-fold at 45°C than at 37°C at the end of this 6 hours experiment (Fig. 3.2A and a). Heat shock reduced TGF-β1 expression after 4 hours exposure to $45^{o}C$ (Fig. 3.2B and b).   To test whether the Hsp47 mRNA response to heat shock was reversible, the cells were placed at 37°C for indicated time periods after a 2-hour exposure to 45°C. The increase in Hsp47

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mRNA expression after 2 hours exposure to 45°C, began to decrease after subsequent 2 hours incubation at 37°C, and returned to baseline after more than 4 hours incubation at 37°C (Fig. 3.3A and a). This result indicates that Hsp47 response to heat shock is reversible.

TGF-β1 stimulates Hsp47 and type I procollagen α1(I) mRNA expression

TGF-β has a highly conserved interspecies amino acid sequence. It plays an important role in collagen formation. It exists as several isoforms, which have very similar functions. To test whether isoforms other than TGF-β1 also induce Hsp47 mRNA expression, I added human recombinant TGF-β1, 2, or 3 to embryonic chicken tendon cell cultures to see which isoform regulates Hsp47 expression. EGF and culture medium only were used as controls. TGF-β1 induced a moderate increase in Hsp47 mRNA expression. TGF-β2 had only a small effect, and TGF-β3, EGF and medium alone had no effect on Hsp47 expression (Fig. 3.4A and a). The increase in Hsp47 mRNA expression induced by TGF-β1 was time and dose dependent (Fig. 3.4B and b and Fig. 3.5B and b, respectively) and was smaller than the increase induced by increased temperature. Hsp47 mRNA levels started to increase 12 hours after the addition of exogenous TGF-β1 and reached a peak after 24 hours (Fig. 3.4B and b). This increase in the level of Hsp47 mRNA, though reproducible, was only moderate.

Comparison of TGF-β1 and TNF-α effects on Hsp47 and type I procollagen α1(I) mRNA expression

TNF-α is a pro-inflammatory cytokine, which contributes to the accumulation of leukocytes and fibroblasts at local sites of inflammation. To test if this cytokine stimulates Hsp47 and procollagen gene expression, I added increasing concentrations of this cytokine to quiescent cells to compare its effects to those of TGF-β1. Northern blotting showed that TNF-α had no effect on Hsp47 mRNA level even at concentrations as high as 100 ng/ml (Fig. 3.5A and a) while 10 ng/ml or less TGF-β1 increased the level of Hsp47 mRNA over the control level (Fig. 3.5B and b). The increased Hsp47 mRNA expression corresponded to an increase in Hsp47 protein levels as determined by Western blotting (Fig. 3.5C). Higher concentration of TNF-α reduced type I procollagen α1(I) mRNA expression (Fig. 3.6A or a) while TGF-β1 increased procollagen α mRNA expression (Fig. 3.6B or b). These data further confirmed that TGF-β1 stimulated both Hsp47 mRNA and type I procollagen α1(I) gene expression and that this action was specific for TGF-β.

TGF-β1 delays the degradation of Hsp47 mRNA after the heat shock

Heat shock rapidly induced Hsp47 mRNA expression (Fig. 3.1A or a). This induction was reversible (Fig. 3.3 A or a). Previously, Yamamura et al (1998) shoId that the induction of Hsp47 mRNA expression by TGF-β1 was transcriptional. To test whether TGF-β1 has an effect on Hsp47 mRNA transcription in my system, tendon cells Ire exposed first for 2 hours to 45°C, and then incubated at 37°C with or without the presence of 5 ng/ml TGF-β1 and/or 100 ng/ml Actinomycin D. Incubation at 37°C (after a 2 hours exposure to 45°C) led to decrease in the level of Hsp47 mRNA in control cells (Fig. 3.3). The addition of either 5 ng/ml TGF-β1 or 100 ng/ml Actinomycin D

maintained the heat shock-induced increase in Hsp47 mRNA expression even after 6 hours of exposure to 37°C (Fig. 3.7, lanes 3 and 4 and a). When TGF-β1 and Actinomycin D were added together under the same temperature conditions, an additional increase in Hsp47 mRNA level was noted (Fig. 3.7, lane 5). The continuous presence of TGF-β1, and/or Actinomycin D protected Hsp47 mRNA from degradation. On the other hand Actinomycin D also inhibits a new mRNA production. Therefore, TGF-β1 probably regulates Hsp47 mRNA posttranscriptionally, preserving the steady state of Hsp47 mRNA under varying stressful conditions.

Effect of mechanical stress on Hsp47 protein

Xu et al., 2000 previously reported that mechanical forces evoked rapid activation of heat shock protein transcription factor (HSF) and Hsp70 accumulation (Xu et al., 2000). I determined whether mechanical stress stimulates Hsp47 protein production. The cells were placed on a shaker at a speed of 50 rpm/min at 37°C. Hsp47 mRNA expression increased between 6 and 8 hmys (Fig. 3.8A). Western blot analysis showed that Hsp47 protein also elevated with time (Fig. 3.8B). The level of procollagen α1(I) mRNA was unchanged after shaking for 8 hours. These findings demonstrate that mechanical stress induces Hsp47 in both protein and mRNA levels.

Translocation of HSF1 into the nucleus following mechanical stress and TGF-β1 treatment

To determine whether the induction of Hsp47 protein synthesis by mechanical stress and TGF-β1 was regulated through activation of HSF1, nuclear proteins from

differently treated cells were extracted and subjected to Western blot analysis. The results showed that HSF1 was translocated after the application of mechanical stress and treatment with TGF-β1 (Fig. 3.9, lane 3 and 4) as well as the application of heat shock (Fig. 3.9, lane 2) when compared with control, i.e., untreated cells (Fig. 3.9, lane 1).

DISCUSSION

Hsp47 protein is closely associated with collagen processing and secretion as well as with the inhibition of procollagen degradation in the ER (Jain et al. 1994). Hsp47 expression rapidly increases and decreases in response to changing heat shock and stress conditions. These physiological responses protect procollagen from damage due to various external stresses. In my experiments I found that heat shock led to a rapid induction of Hsp47 both at the transcriptional and protein synthesis levels, and that this effect is specific because heat shock induction of type I procollagen α1 expression was only transient, and TGF-β1 gene expression was decreased as a result of heat shock application.

My data show TGF-β1 induces Hsp47 expression, though to a lesser degree than heat, and that this effect is specific, as TGF-β2 has a much smaller effect and TGF-β3 and EGF have no effect. This action of TGF-β1 on Hsp47 protein is likely to be important during later stages of wound healing and tissue repair as both TGF-β1 and Hsp47 regulate collagen synthesis, a crucial event during the later stages of wound healing. This action can either be a direct induction of collagen expression as in the case of TGF-β1, or an indirect effect in the case of Hsp47, i.e., its role as a chaperone. The persistent effect of TGF-β1 on Hsp47 mRNA expression, even after the cessation of heat

shock, is a further evidence of sustained and persistent effect of TGF-β on collagen processing.

My data correlate well with reports from other laboratories. A strong correlation between the regulation of Hsp47 synthesis and collagen synthesis under pathological conditions has been observed *in vivo*: the synthesis of Hsp47, as well as type I and III collagens, are dramatically increased during the progression of rat liver fibrosis induced by carbon tetrachloride (Masuda et al. 1994). This parallel regulation of type I procollagen α1(I) and Hsp47 mRNA by TGF-β was also reported in cultured human smooth muscle cells (Rocnik et al. 2000; Murakami et al. 2001). The mechanism of this coexpression or coregulation is unknown. My finding that TGF-β1 stimulated both Hsp47 and type I procollagen α1 gene expression in avian tenocytes confirms the finding of Kuroda et al. (1998). They found that TGF-β1 increased both Hsp47 and procollagen α1 gene expression in normal fibroblasts (Kuroda et al. 1998). Those data indicate that TGF-β1 "supervises" more than directs the synthesis of collagen.

TGF-β rapidly induced fibrosis (excessive collagen formation) and angiogenesis *in vivo* when injected subcutaneously in newborn mice (Roberts et al.1986). The proper manipulation of TGF-β levels in wounds may result in an acceleration of healing, and in anti-scarring and anti-fibrotic therapies (O'kane et al. 1997). Neutralization of TGF-β1 and 2 by anti- TGF-β antibodies reduced the inflammatory and angiogenic responses and altered the deposition of ECM, resulting in scarless repair (Shah et al. 1995). Whether neutralization of TGF-β1 and 2 reduces Hsp47 protein remains to be elucidated.

Actinomycin D inhibits the proliferation of cells in a nonspecific way by forming a stable complex with double-stranded DNA, thus inhibiting DNA-primed RNA

synthesis. Therefore, this compound blocks cellular mRNA expression; on the other hand, it also protects the accumulated mRNA stimulated by other factors from the degradation (Chong et al. 2000; Kehlen et al. 2000). The regulation of Hsp47 mRNA expression by TGF-β1 is likely to be transcriptional because the increase in Hsp47 mRNA was blocked when the cells were treated with TGF-β1 in the presence of Actinomycin D (Yamamura et al.1998). To determine whether TGF-β1 protects the Hsp47mRNA accumulated during heat shock, I took advantage of Hsp47 mRNA reversible expression in response to the heat shock. The addition of TGF-β1, similar to the addition of Actinomycin D, led to an accumulation of Hsp47 mRNA in response to a reversal of temperature following heat shock. The regulation of Hsp47 mRNA by TGF-β1 likely involves both transcriptional and posttranscriptional mechanisms.

The effect of TGF-β1 on collagen expression in embryonic chicken tendon cells in culture was moderate when compared with the effect of TGF-β1 on collagen induction in mammalian systems (Alberts et al. 1994). I hypothesize that TGF-β4, an avian isoform of TGF-β, fills the role of TGF-β1 in chicken cells. TGF-β4 gene has been characterized in chicken embryos (Jakowlew et al. 1988). The amino sequence of the chicken TGF-β4 shares 82% homology with human TGF-β1 in the mature region. Successful cloning and recombinant expression of chicken TGF-β4, currently underway in my laboratory, will help to determine whether that is really the case.

The cyclic strain stress induces Hsp70-protein production and Hsp70-mRNA expression in cultured vascular smooth muscle cells, mediated by HSF1 activation (Xu et al., 2000). I subjected cultured chicken tendon fibroblasts to mechanical stress with gentle shaking instead of the Cyclic Stress Unit. I found that mechanical stress also

increased Hsp47 expression at both the protein and mRNA levels. Procollagen α1(I) mRNA expression did not change under this type of stress (Fig. 3.8A and B). Hsp47 protein elevation likely protects tendons or tissues from damages under mechanical stress. The induction of Hsp47 protein production by mechanical stress and TGF-β1 is regulated through activation and translocation of HSF1 into the nucleus in chicken embryonic tendon fibroblasts. These results are in line with previous findings reported by Xu et al. (2000) and Sasaki et al. (2002). Wren et al (1998) showed that tendon structure is malleable and responds to changes in certain stress levels. Thus, Hsp47 might influence the process of tendon remodeling via its effects on tenocytes apoptosis and proliferation in response to heat or mechanical stress and may exert a role in maintaining cellular homeostasis of the tendon.

ACKNOWLEDGEMENTS

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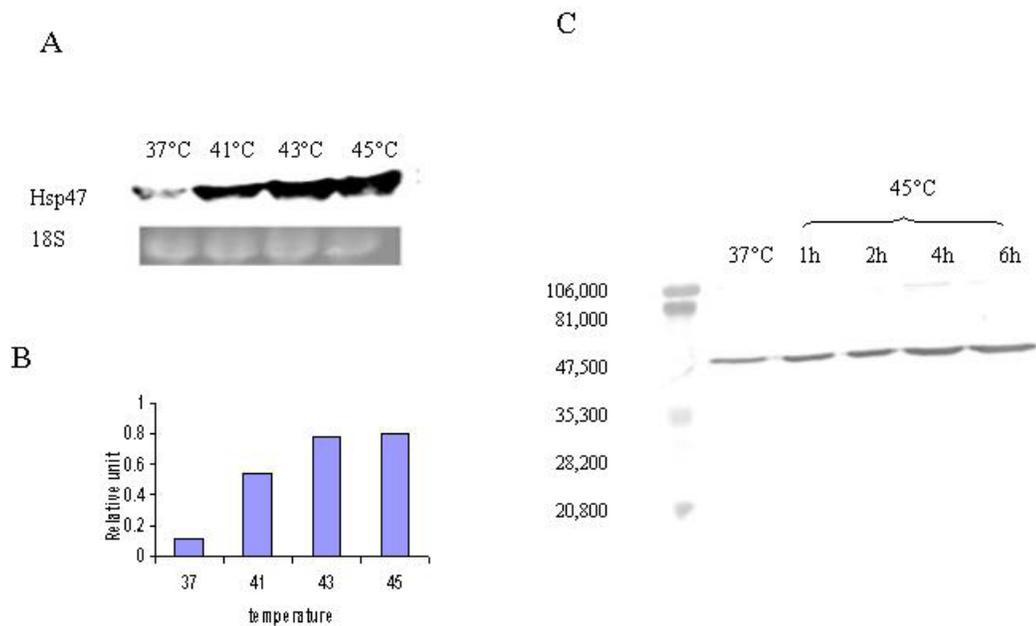
Fig. 3.1. Effect of heat-shock on Hsp47 mRNA and protein levels. Embryonic chicken tendon fibroblasts were cultured in 10% FBS DMEM to reach confluence, and then cultured for another 24 h in the serum-free DMEM. The quiescent cells were exposed to the heat-shock at the indicated temperatures and time periods. The mRNA expression of given genes or protein levels was determined by Northern and Western blotting, respectively. (A) Hsp47 mRNA expression increased with temperatures, determined by Northern blot. (B) Northern blot from (A) converted into a graph by densitometry. The cells were exposed to 37°C, 41°C, 43°C and 45°C for 2 h. (C) Hsp47 protein level increased with time at 45°C, determined by Western blot. The cells were exposed to 45°C for indicated periods. Hsp47: Heat shock protein 47 mRNA; 18S: 18S rRNA.
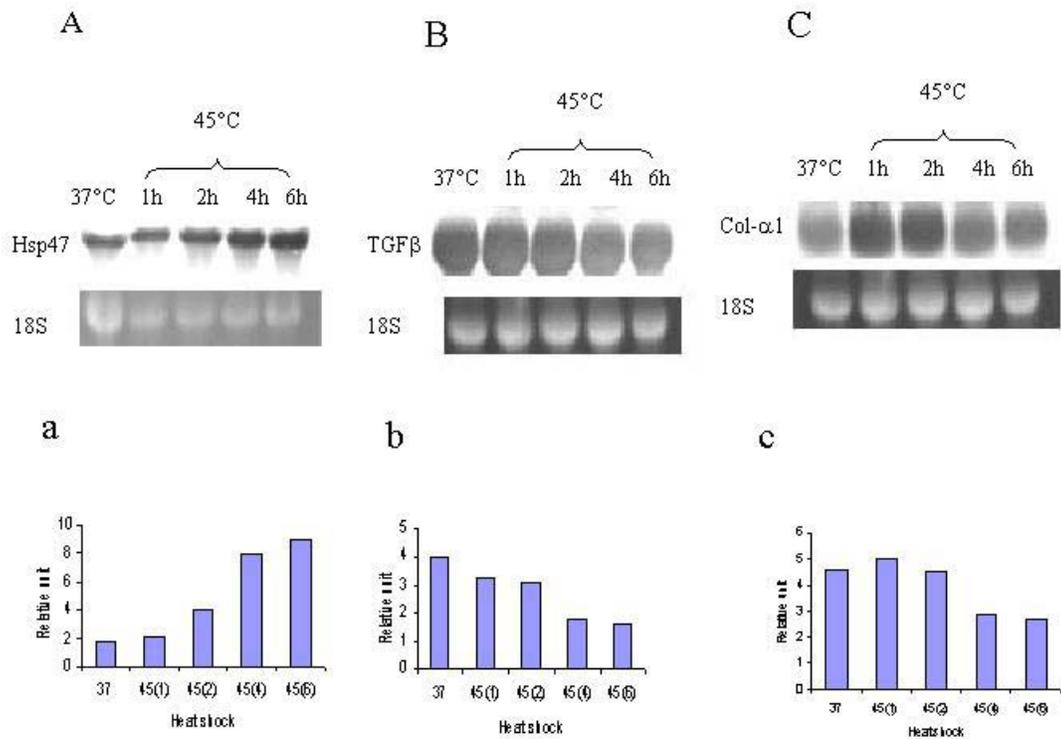
Fig. 3.2. Heat shock increased Hsp47 mRNA expression (A) and, only transiently, pro-
collagen mRNA expression (C), not TGF-β (B). (a), (b), (c) Northern blots converted in
to corresponding graphs by densitometry. Embryonic chicken tendon cells were cultured
in 10% FBS DMEM to reach confluence and continued to culture for another 24 h in
serum-free DMEM. The cells were subjected to heat shock for 1-6 h. The expression
level of indicated genes was determined by Northern blotting. Hsp47: Heat shock protein
47 mRNA; 18S: 18S rRNA; Col-α: Type I procollagen α1 chain mRNA.

A

a
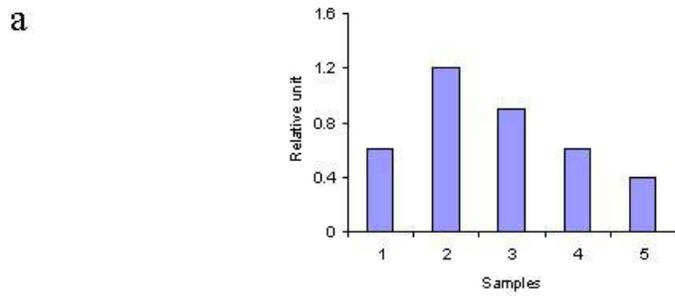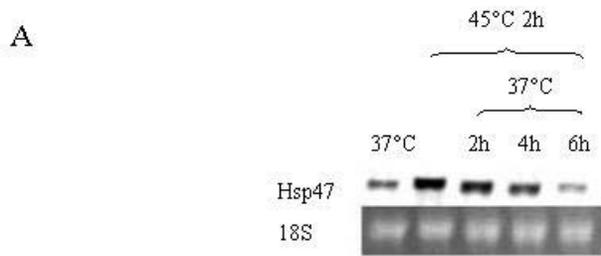
Fig. 3.3. Hsp47 mRNA expression is reversible in response to heat stress, determined by Northern blot. After they reached confluence, chicken tendon fibroblasts were incubated at 45°C for 2 h and then returned to 37°C for indicated intervals. Hsp47: Heat shock protein 47 mRNA; 18S: 18S rRNA.
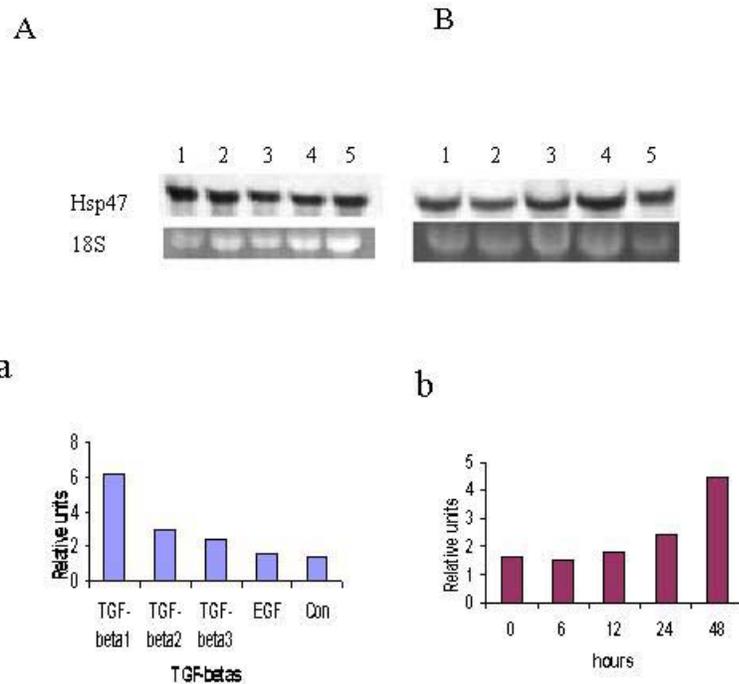
Fig. 3.4. TGF-β1 regulates Hsp47 mRNA expression, as determined by Northern blotting. The cell preparation was described above. (A) Confluent tendon fibroblasts were treated with TGF-β1, 2, 3 at 2.5ng/ml and EGF at 10ng/ml for 24 hours prior to harvesting cells. Lane 1: TGF-β1; Lane 2: TGF-β2; Lane 3: TGF-β3; Lane 4: EGF; Lane 5: medium only. (B) The time course of TGF- β1 treatment. (a) and (b) Northern blots converted into graph form by densitometry. Lane 1-5: 0, 6, 12, 24 and 48 h, respectively.
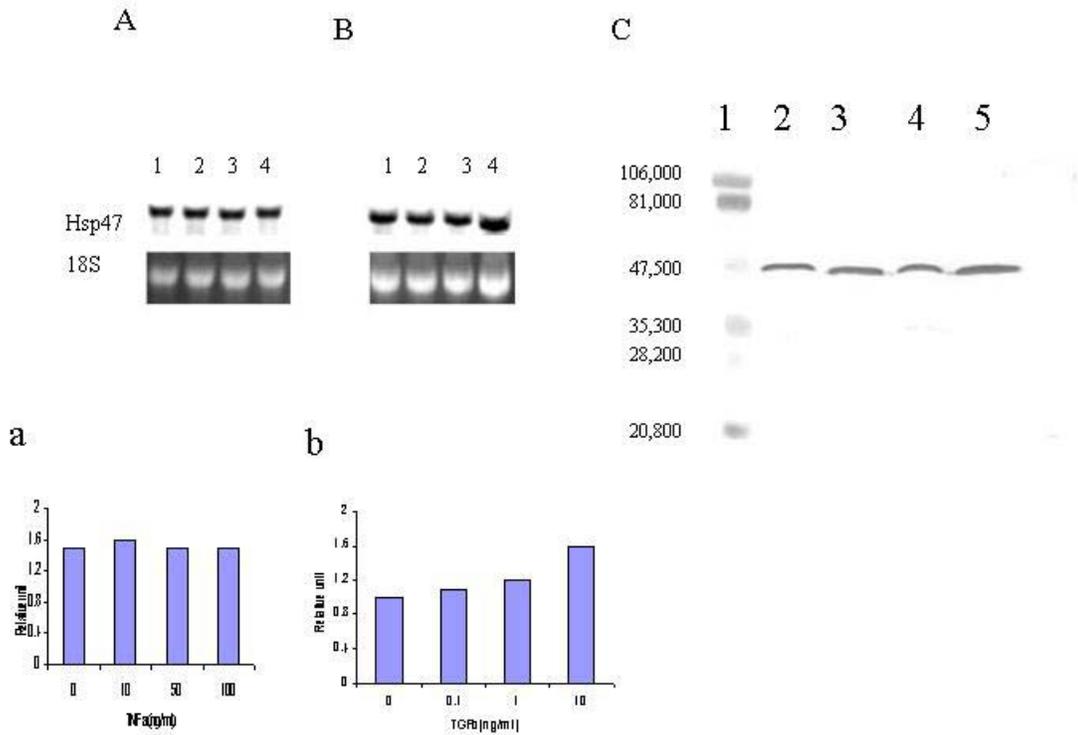
Fig. 3.5. TNF-α does not induce Hsp47 mRNA expression. The cell preparation was described above. (A) Quiescent cells were cultured for 24 h with the addition of TNF-α. Lane 1, 2, 3 and 4: TNF-α at 0, 20, 50, 100ng/ml, respectively. (B) Quiescent cells weIre cultured for 24 h with the addition of TGF-β1. Lane 1, 2, 3 and 4: TGF-β1 at 0, 0.1, 1, 10 ng/ml, respectively. (a) and (b) Northern blots converted into graph form by densitometry. (C) Hsp47 protein increased with increasing TGF-β1 concentration, as determined by Western blotting. Lane 1: protein MW markers; Lane 2, 3, 4 and 5: TGF-β1 at 0, 0.1, 1, 10ng/ml, respectively.
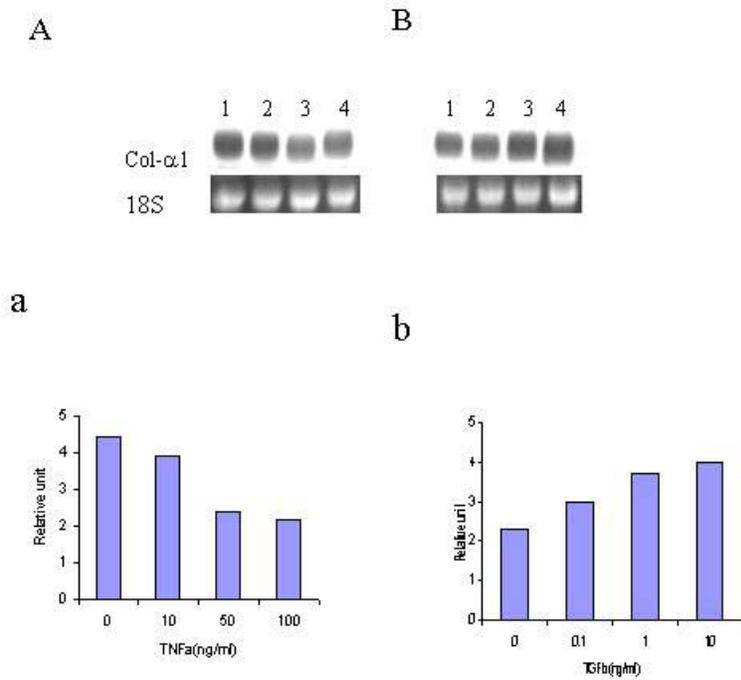
Fig. 3.6. TGF-β1 increases pro-collagen α1 mRNA expression and TNF-α reduces that, determined by Northern blot. After they reached confluence, chicken fibroblasts were incubated for 24 h with TGF-β1 and TNF-α. (A) Lane 1, 2, 3 and 4: TNF-α at 0, 20, 50 and 100 ng/ml, respectively; (B) Lane 1, 2, 3 and 4: TGF-β1 at 0, 0.1, 1, 10 ng/ml, respectively. (a) and (b) Northern blots converted into graph form by densitometry.

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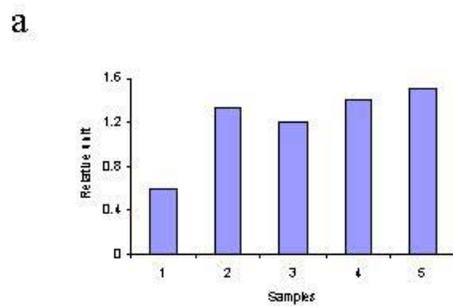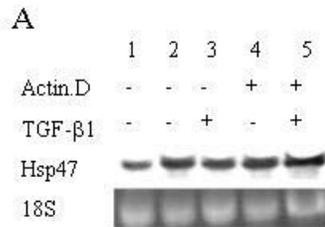
Fig. 3.7. TGF-β1 delays the degradation of Hsp47 mRNA, as does actinomycin D,

determined by Northern blot (A), or a graph obtained by densitometric conversion of the

Northern blot (a). The cell preparation was described in Materials and Method.

Quiescent cells were incubated at 45°C for 2 h, and then returned to 37°C, and cultured

for indicated periods with the addition of TGF-β1 or actinomycin D or both. Lane 1,

37°C as a control; Lane 2, 45°C for 2 h; Lane 3, TGF-1β (5 ng/ml) for 6 h at 37°C after 2

h heat shock at 45°C. Lane 4, actinomycin D (100 ng/ml) for 6 h at 37°C after 2 h heat

shock at 45°C; Lane 5, TGFF-β1 (5 ng/ml) and actinomycin D (100 ng/ml) for 6 h at

37°C after 2 h heat shock at 45°C.
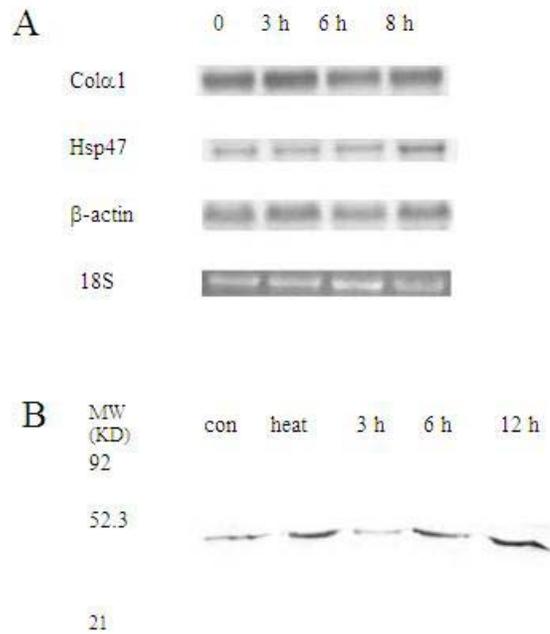
Fig. 3.8. Mechanical stress enhances Hsp47 both mRNA (A) and protein expression (B). The cell preparation is described as Materials and Method. (A) Hsp47 mRNA expression increased over time after shaking, determined by Northern blot analysis. β-actin and 18S rRNA act as a internal control. Colα1: procollagen α1(I) mRNA; Hsp47: heat shock protein 47 mRNA. (B) Hsp47 protein synthesis elevated over time after mechanical stress, determined by Western blot analysis. MW: protein standard marker; con: control (no shaking); heat: cells heated for 3 h at 43°C; cells shake at 50 rpm/min at 37°C for 3h, 6h, 12h, respectively.
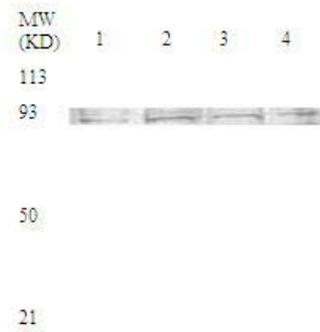
Fig. 3.9. Translocation of heat shock transcription factor 1 (HSF1) into the nucleus by heat–shock, mechanical stress and human TGF-β1 as determined by Western blot analysis. The nuclear protein preparation is as described in Materials and Methods. The samples (10 μg) were separated on 10% SDS-polyacrylamide gel, transferred onto membranes and probed using human HSF1 polyclonal antibody. MW: protein standard marker; Lane 1: control; Lane 2, heat shock at 43°C for 3 h; Lane 3, mechanical stress for 10 h; Lane 4; human TGF-β1 treatment for 24 h.

CHAPTER 4

GENERATION OF THE GC-RICH 5'END OF THE CHICKEN TGF-ß4 CDNA

USING THE 5' RACE METHOD

I have sequenced the GC-rich 5'end of chicken transforming growth factor β4

(TGF-β4) cDNA using a modified 5' RACE methodology. cDNA was produced from

mRNA purified from the 19-day embryonic chicken tendon fibroblast culture using the

thermal stable reverse transcriptase and random hexamers at 70°C. The cDNA was then

tailed with dATP. The first PCR was performed using the dA-tailed cDNA as a template.

The second (nested) PCR was done using the first PCR products as templates. A GC-rich

PCR kit was employed in both PCR amplifications. The second PCR products were

cloned into the pGEM-T vector. The identity of inserts was verified with dot-blotting.

Positive samples were subjected to sequencing. The alignment of sequenced data showed

that the 5' end contained 271 more nucleotides (Genbank accession No: AF395834) than

the original sequence recovered from the chicken TGF-β4 cDNA. The additional

sequence consisted of 70% GC bases. The analysis of the mRNA sequence of this region

by the MFOLD predicted that it likely contains the multiple stem-loop secondary

structures. The cDNA and amino acid sequence analyses show that this region makes up

the complete open reading frame of the chicken TGF-β4 cDNA, in conjunction with a

partial chicken TGF-β4 cDNA in Genbank (Genbank accession No: M31160). My results

indicate that the 5' end of GC-rich cDNA templates could be recovered by the 5' RACE

method combined with the thermostable reverse transcriptase and GC-rich PCR reagents.

INTRODUCTION

Rapid amplification of cDNA ends (RACE) is a polymerase chain reaction-based technique which uses one or two gene-specific oligonucleotide primers (1) to facilitate the cloning of full-length cDNA sequences when only a partial cDNA sequence is available. Traditionally, cDNA sequence is obtained from clones isolated from plasmid or phage libraries. Frequently these clones lack sequences corresponding to the 5' ends of the mRNA transcripts (2). The missing sequence information is typically sought by repeatedly screening the cDNA library in an effort to obtain clones that are extended further towards the 5' end of the message. The nature of the enzymatic reactions employed to produce cDNA libraries limits the probability of retrieving the extreme 5' sequence even from libraries of very high quality. The RACE is the best solution to such problems.

The correct RACE product largely depends on the integrity of the cDNA generated by reverse transcriptase from mRNA. A stable secondary structure of mRNA, especially that which is GC-rich in its 5' end, commonly induces the early termination of the synthesis of full-length cDNA (3,4). Under such extreme circumstances, a thermostable RT could be employed to overcome this problem. Use of this enzyme allows the reaction to proceed at 65°C-70°C (e.g., THERMOSCRIPT™ reverse transcriptase, GIBCO BRL). The GC-rich cDNA represents another obstacle to DNA amplification by polymerase chain reaction (PCR). Several reagents have been used to disrupt base pairing or isostabilize DNA, such as DMSO, formamide, glycerol, Betaine, deoxyinosine and

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low molecular-Iight sulfones (5,6,7,8,9). GC-rich PCR kits in which concentrations of various reagents are optimized are now available from several smyces (e.g., GC-RICH PCR system, Roche Applied Science). The partial sequence of chicken transforming growth factor-β4 (TGF-β4) mRNA is available in Genbank (2) (Genbank accession no. M31160). To characterize the biological activity of this protein, it was necessary to obtain its full-length cDNA. In combination with the thermostable reverse transcriptase, I successfully recovered the 5'-flanking region of the chicken TGF-β4 cDNA using the GC-rich PCR kit.

MATERIALS AND METHODS

cDNA Synthesis and dATP Tailing

The strategy to generate the 5'end of the chicken TGF-β4 cDNA by the 5'RACE is shown in Fig. 4.1. The total RNA was isolated from cultured chicken embryonic fibroblasts using TRIzol® reagent (GIBCO-BRL, Grand Island, NY, USA). mRNA was purified from the total RNA using mRNA isolation kit (Roche Applied Science, Indianapolis, IN). The amount of mRNA was determined spectrophotometrically. The integrity of mRNA was determined by performing PCR with chicken β-actin primers. The purified mRNA was stored at -70°C for later use. To generate full-length cDNA transcripts of the targeted gene that had secondary structure of the mRNA during reverse transcription, THERMOSCRIPT™ reverse transcriptase (GIBCO-BRL) with random-hexamer primers was used. THERMOSCRIPT™ reverse transcriptase, an avian RNase H-minus reverse transcriptase, has higher thermal stability than other reverse transcriptases. Briefly, 50 ng of random primers, 100 ng mRNA, and 1mM dNTP were mixed in a 0.5

ml tube and their volume was adjusted to 12 μl with DEPC-treated water. mRNA and primers were denatured by incubating at 70°C for 5 min and the reaction was placed on ice. The following reagents were added: 4 μl of 5 X cDNA synthesis buffer, 1 μl of 0.1 M DTT, 20 U of RNaseOUT and 15 U of THERMOSCRIPT™ reverse transcriptase, and volume was adjusted to 20 μl with DEPC-treated water. The sample was incubated in a thermal cycler at 25°C for 10 min, followed by at 70°C for 40 min. The reaction was terminated by incubation at 85°C for 5 min. cDNA was purified with the High Pure PCR Product Purification Kit (Roche Applied Science). For the tailing reaction, the purified cDNA was mixed with reaction buffer and 0.2 mM dATP adjusted to a final volume of 24 μl and incubated for 3 min at 94°C and chilled on ice. After the addition of 1 μl of terminal transferase (10 U/μl), the sample was incubated at 37°C for 30 min, and then at 70°C for 10 min. The dA-tailed cDNA was stored at –20°C until used.

PCR Reaction

The design of two reverse primers [primer 1 and primer 2 (Fig. 4.1)] was based on the cDNA sequence of chicken TGF-β4 (Genbank accession number: M31160). The sequences of both primers are 5'CGGTGCTTCTTGGCAATGCTCTGCATGTC3' (primer 1: between positions 675 and 703) and 5'CGGTCAGACGCAGTTTGCTGAGGATTTG 3' (primer 2: between positions 72 and 99). Two forward primers were provided in 5'/3' RACE kit; oligo dT-anchor primer and PCR anchor primer (Roche Applied Science). The GC-rich PCR system (Roche Applied Science) was used in both the first and second PCR reactions. The GC-RICH resolution solution (5 M) containing various organic solvents was tested at 0.5, 1.0 and 1.5 M. My PCR strategy is shown in Fig. 4.1. Briefly, primer 1 and oligo dT-anchor primer primed

the first PCR reaction with the dA-tailed cDNA as templates; primer 2 and PCR anchor primer were then used in the nested PCR with the first PCR products as templates. Both PCR reaction profiles were 2 min at 94°C, 35 cycles of 1 min at 94°C, 30 s at 55°C, 1 min at 72°C, 7 min at 72°C. The first and second PCR products were analyzed on 1% ethidium bromide (EB)-stained agarose gels.

Cloning of RACE-PCR Products and Transformation

The gel-purified RACE-PCR product was ligated into pGEM$^®$-T vector (Promega Corp., Madison, WI) as described in the protocol provided by the manufacturer. Ten white positive colonies were chosen, inoculated into 3 ml of LB medium containing 100 μg/ml ampicillin, and cultured overnight at 37°C. The purification of plasmid DNA from transformed bacteria was performed using QIAprep Spin Miniprep kit (QIAGEN, Inc., Valencia, CA). The insert sizes were determined by cleaving the plasmids with Apa*I* and Pst*I* restriction enzymes.

Dot Blotting

Probes composed of 35 oligonucleotides based on the sequence of the 5' end of chicken TGF-ß4 cDNA (Genbank accession number: M31160) were synthesized by the Molecular Genetics Instrumentation Facility at The University of Georgia. The probes were tailed with digoxigenin-dUTP using Dig-oligonucleotide tailing kit (Roche Applied Science). One μl of purified plasmids from each colony was dropped on nylon membrane (Roche Applied Science), soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 min and neutralization solution (1.5 M NaCl, 1.0 M Tris-HCl, pH 7.4) for another 15 min, and briefly rinsed with 2 X SSC solution. The denatured plasmid DNA was fixed on

the positively charged nylon membrane by an autocross-link device (Strategene, La Jolla, CA). Prehybridization was performed in Dig-Easy-Hyb (Roche Applied Science) containing 0.1 mg/ml poly (A)-solution for 1 hour at 68°C, followed by hybridization in the same solution containing Dig-labeled oligonucleotide probes for 3 hours at 54°C. The blots were washed once in 2 X SSC-0.1% SDS at room temperature, twice in 1 X SSC-0.1%SDS for 10 min at room temperature and twice in 1 X SSC-0.1% SDS for 10 min at 54°C. Positive plasmids detected with the NBT/BCIP system were sequenced. To confirm the sequence of the recovered 5' flanking region, a forward primer was designed from the middle of the 5' flank region, and a reverse primer from the known region in the downstream region of the sequence of the chicken TGF-β4 cDNA. PCR was performed using both primers.

DNA Sequencing and Analysis

The recombinant plasmid DNA containing the RACE-PCR product was first sequenced using the BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City CA). The kit was replaced by the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ) in later experiments. The sequencing was done by the Molecular Genetics Instrumentation Facility at The University of Georgia on an ABI PRISM 373-S Sequencer (Applied Biosystems). The sequences were assembled with a Sequencher program (Gene Codes Corp, Ann Arbor, MI). The corresponding mRNA structures were analyzed by the MFOLD program (Version 3.1; http://bioinfo.math.rpi.edu/~mfold/rna/form1.cgi).

RESULTS

The mRNA (a total of 25 μg) was purified from total RNA isolated from cultured embryonic chicken tendon cells. The chicken β-actin specific band was obtained from the purified mRNA by RT-PCR using chicken β-actin primers (data not shown). This result indicated that the purified mRNA was of a good quality. To generate full-length cDNA transcripts of the targeted TGF-β4 gene that likely contains a very stable secondary structure of mRNA, the reverse transcription was performed at 70°C using THERMOSCRIPT™ reverse transcriptase and random-hexamer primers. The synthesized first strand of cDNA was purified by using High Pure PCR Purification Kit. The purified cDNA was then tailed with dATP using the terminal transferase. The tailing product was the so-called dA-cDNA, which was used as the template for the later PCR reaction.

Both the first and second (nested) PCRs were performed using the GC-RICH PCR System. The first PCR did not produce any products in several GC-RICH resolution solutions differing in molarity, though, the second PCR bands were noted when 1 M solution was used in the second PCR. The second PCR, in which the first PCR products were used as templates, showed a strong band of 250 bp in size and several weak bands of about 300 bp or 400 bp in size (Fig. 4.2). The strong and weak bands in each lane were excised together. cDNA was purified from this pooled material and cloned into the pGEM-T vector. Plasmid cDNA was extracted from 10 positive colonies and digested with Apa*I* and Pst*I*. The electrophoretic analysis showed that all bands were recovered (Fig.4.3A). To confirm that plasmid cDNA indeed contained the targeted gene, one μl of each plasmid was loaded on a nylon membrane and hybridized with Dig-labeled

oligonucleotides. Southern blotting showed that all 10 plasmids were positive (Fig. 4.3B). The alignment of the sequenced data showed that three plasmids of different size (obtained from lanes 1, 4 and 5 in Fig. 4.3A) contained the targeted gene. The sequence analysis revealed the presence of an additional, previously not sequenced region of 271 nucleotides in the 5'end of cDNA of chicken TGF-β4 (Fig. 4.4A). The composition of the newly recovered region was about 70% GC bases. The complete open reading frame (ORF) of the chicken TGF-β4 is shown in Fig. 4.4A, in conjunction with the partial chicken TGF-β4 (Genbank Accession No. M31160). The three inserts overlap in their 3'end (indicated by arrows in Fig. 4.4A). Analysis by the MFOLD program predicted the formation of multiple stem-loop secondary structures in the mRNA in this region (Fig. 4. 4B). To verify if this sequence was an integral part of the TGF-β4 cDNA, a forward primer was designed from the middle of the newly sequenced region. The specific band was obtained using this forward primer and a reverse primer derived from the downstream region (Fig. 4.5).

DISCUSSION

GC-rich regions in mRNAs and the corresponding cDNAs pose an obstacle to obtaining full-length cDNAs during reverse transcription and subsequent PCR (3, 10). I have successfully generated and sequenced the 5'end cDNA of the chicken TGF-β4 by using thermostable THERMOSCRIPT™ reverse transcriptase for reverse transcription and GC-RICH resolution solution during PCR. When I used the AMV reverse transcriptase provided in a 5'/3' RACE kit for reverse transcription at 55°C, as recommended by the manufacturer, I did not obtain any products even though PCR was done in GC-RICH

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resolution solution. THERMOSCRIPT™ reverse transcriptase, an avian RNase H-minus

reverse transcriptase, is engineered for higher thermal stability. It produces higher yields

and more full-length cDNA transcripts than AMV reverse transcriptase. The first strand

cDNA synthesis using THERMOSCRIPT™ reverse transcriptase can be performed at 65°C-

70°C. In the preliminary assay I tried to perform reverse transcription at 60°C, 65°C and

70°C using this reverse transcriptase. I obtained the best results with the reaction

performed at 70°C. In most cases gene-specific primers would be preferred to prime the

first strand cDNA synthesis. In GC-rich templates random hexamers may be more

efficient primers to produce more 5' ends of cDNA, compared with gene-specific primers

by which the first strand cDNA synthesis would pre-terminate when it encounters stable

secondary structures of mRNA.

The addition of 5% -10% DMSO into the PCR buffer did not improve yields in

my experiment. I, therefore, turned to a GC-RICH PCR system, which contains other

organic solvents besides DMSO (5, 6, 7, 8). Both the first and second PCR were

performed using this system. Though no bands were visualized on the gel after the

separation of material obtained in the first PCR, several bands were visible as a result of

the second, nested PCR. It is likely that the first PCR produces amounts of cDNA too

small to be identified in an EB-stained agarose gel. Because the concentration of products

from the second PCR did not increase significantly at annealing temperatures 55°C to

65°C, I used the annealing temperature of 55°C. The GC-RICH resolution solution at 1M

gave the best results in the second PCR (Fig. 4.2). This may change with different

templates. PCR reaction sometimes gives a false positive result (The 5' and 3' end in

cDNA contain forward and reverse primers, but the sequence inside was nonsense) even

though one or two unique bands are identified in an EB-stained agarose gel. The verification of positive bands or plasmids by Southern blotting facilitates the definitive identification of positive bands or plasmids. The sequence of a 35 oligonucleotide long probe was based on the sequence of the known 5' ends of TGF-β4 and was not included in the reverse primer of the second PCR.

I sequenced 3 positive plasmids that contained inserts differing in size. One plasmid containing a shorter (~250 bp) insert was first read through using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit. Two other plasmids containing longer inserts (~400 bp) were not read through using the same kit. In later experiments I used the DYEnamic ET Terminator Cycle Sequencing Kit for the same plasmids. The sequence of the three inserts was finally obtained in more than ten sequence cycles done with concomitant sequencing of 5' and 3' end. The sequence alignment showed that the three inserts overlapped in their 3' end in addition to the previously sequenced region (2). The longer inserts consist of several poly-G and -C or GC stretches (Fig. 4.4A). In the newly sequenced region the total GC-content was about 70% of the bases. Analysis of the corresponding mRNA by the MFOLD program predicted that this sequence forms multiple stem-loop secondary structures. This phenomenon would explain why the reverse transcription using regular reverse transcription enzymes failed to read through this region and why the normal PCR protocol also did not work on the corresponding cDNA.

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mRNA 5' ————————————————————— (A)$_n$-3' Synthesis of first stand cDNA at 70°C
Probe   ← random hexamers

cDNA 3' ————————————————————— 5'

3'-(A)$_n$AAA ————————————————————— 5' Tailing of the purified cDNA with dATP

Oligo dT-Anchor primer
→ (T)$_n$TTTV
(A)$_n$AAAA ————————————————————— 5' The first PCR reaction
                           ← primer1

PCR Anchor primer
→
————————————————————— 5' The second PCR reaction
        ← primer2

PCR products
↓
Cloning into pGEM-T vector
↓
Southern blotting and sequencing and assembly
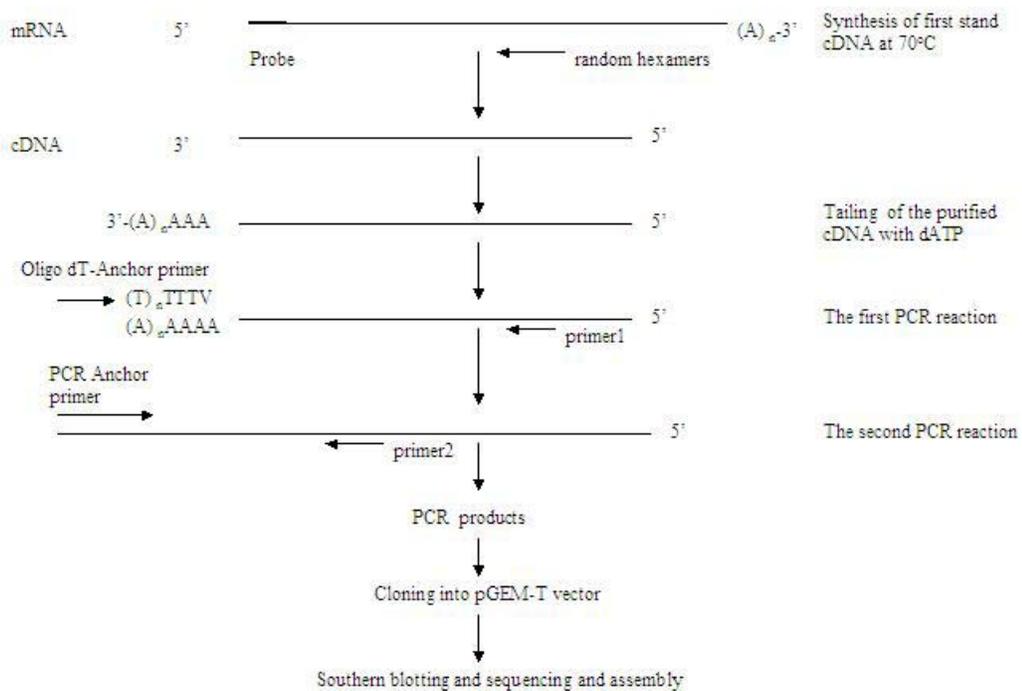
Fig. 4.1. Strategy to generate a 5'end of cDNA with the 5' RACE. The synthesis of the first strand cDNA was carried out at 70°C with the THERMOSCRIPT™ reverse transcriptase and random hexamers. The purified cDNA was tailed with dATP using the terminal transferase (its products called dA-cDNA). The first PCR with primer 1 and oligo dT-Anchor primer was performed using the dA-cDNA as templates. The second PCR with primer 2 and PCR anchor primer was done using the 1st PCR products as templates. Both PCRs were performed in the GC-RICH PCR system. The 2nd PCR products were cloned into a pGEM-T vector. The specificity of the inserts in plasmids was verified by Southern Blotting and sequencing. The first 35 bases of the 5' end of the known region were synthesized as probes (in Bold).

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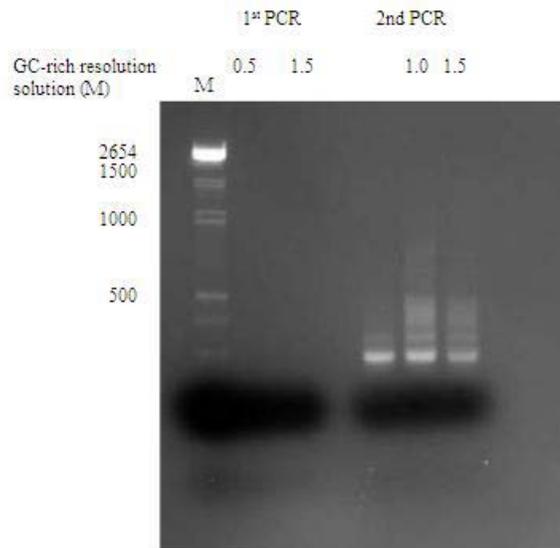
Fig. 4.2. The First and second (nested) PCR products. Both the first PCR and nested

PCR were performed in the GC-RICH system as described in **MATERIALS AND**

METHODS. The reaction products were analyzed by electrophoresis and EB staining.

DNA size markers (System XIV, Roche Applied Science) are indicated in the first lane

on the left. 0.5, 1 and 1.5 M indicate the molarity of GC-rich resolution solution.
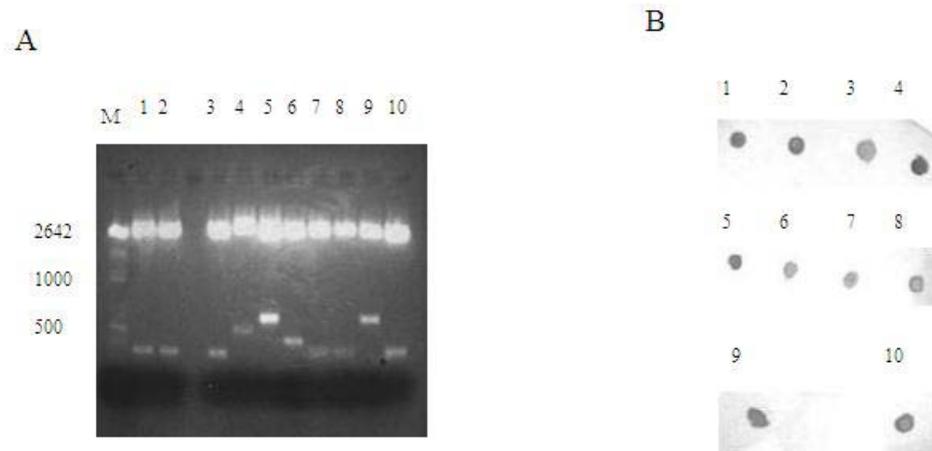
Fig. 4.3 A. Analysis of insert sizes by cleaving pGEM-T-PCR products with Apa*I* **and Pst*I*.** The excised PCR products from the second PCR were purified and ligated into a pGEM-T vector (pGEM-T-PCR) as described in **MATERIALS AND METHODS**. Ten positive colonies Ire selected and transformed into JM109. The purified plasmids were digested with ApaI and PstI and analyzed by electrophoresis and EB staining. DNA size markers (XIV) are indicated in the first left lane. **B. Screening of positive plasmids by Southern blotting.** One μl of the purified plasmids was loaded onto a positively charged nylon membrane and hybridized with Dig-labelled oligonucleotides as described in **MATERIALS AND METHODS**. The detection was done with the NBT/BCIP system. The label 1-10 corresponds to one in Figure 3A.

A
1   CTGGGGGTGGTGGGGGTGTCCCATACCATTTAGAGAAGACCCCCCCCGCCCC
52   CCCTCACACTTAATGGGGAACCCCGTTGGATTGAAGGGGGAATCCCTCTGC
103 GTTAAAGGGGGAATCCCGCAGCCGCGCCTACGGGGGATCCCGGCCCCTTTG
154 CGGGGGGGGGAGGGGTCCCGGTGCCCCATCGGAGTTATTTGGGGGGGGGGGTC
206 CCTCCGCCCCCCCCCATGGATCCGTCGCCGCTGCTGGCGCTGCTGCTGCTGC
257 TGCTGGGGGCCGCC

B
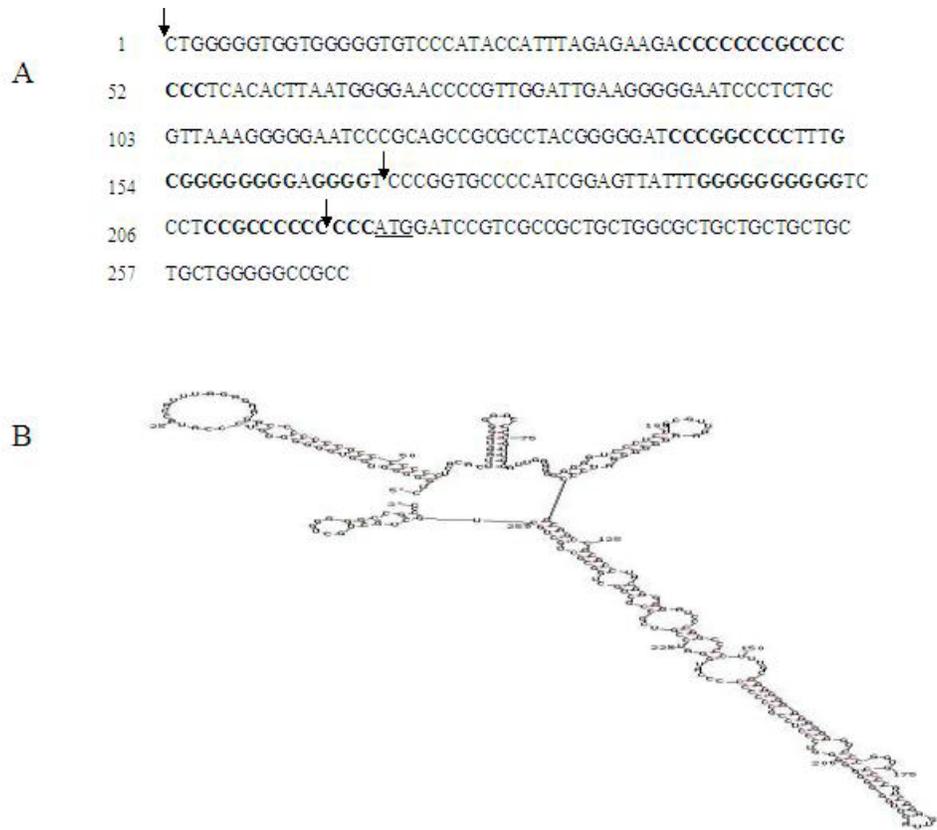
Fig. 4.4. Analysis of the sequence obtained by RACE. A. 5' RACE product. The newly identified stretch of 271 oligonucleotides contained about 70% of GC. It has several poly-G or C stretches (in bold) and ATG initiation site (underlined). Arrows indicate overlapping sequences of the 3 inserts. B. The presence of multiple stem-loop secondary structures of the 5' end of TGF-β4 cDNA (5' RACE product) were predicted with MFOLD.
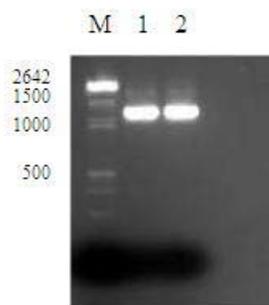
Fig. 4.5. Verification of the 5' flank region generated by the 5' RACE. Both primers were designed as described in **MATERIALS AND METHODS**. The PCR results were analyzed by electrophoresis and EB staining. DNA size markers (XIV) are indicated in the first left lane. Lanes 2 and 3 are replicates.

CHAPTER 5

CLONING, EXPRESSION AND CHARACTERIZATION OF CHICKEN

TRANSFORMING GROWTH FACTOR β4

Chicken transforming growth factor β4 (TGF-β4) is unique to avian species; it shares 82% amino acid sequence identity with human TGF-β1. My previous studies showed that human TGF-β1 enhances both procollagen and Hsp47 protein production in chicken embryonic tendon fibroblasts. In current studies I expressed and partially characterized chicken TGF-β4. I failed to recover active TGF-β4 after purification and refolding when the chicken mature TGF-β4 protein was expressed in *E. coli* using a pET-28 vector. I, therefore, generated the 5' end of cDNA encoding the precursor of this protein using the modified 5'RACE. cDNA encoding this precursor protein was in-frame cloned into pcDNA3.1/V5-His-TOPO vector and transfected into Chinese hamster ovary (CHO-K1) cell line. Individual colonies were grown in F10 nutrient mixture medium and selected with G418. A cell line expressing the TGF-β4 precursor protein was established and expanded. After purification with Ni-NTA beads and activation by acid solution the mature TGF-β4 exhibited strong inhibition of mink lung (Mv1Lu) epithelial cell growth and stimulation of procollagen production.

Further studies showed that TGF-β4 enhanced Hsp47 expression. Induction of

Hsp47 expression by chicken TGF-β4 is regulated by activation and translocation of

chicken HSF1 into the nucleus. My data show that chicken TGF-β4 might play a role in

avian species similar to the role human TGF-β1 plays in mammalian species.

INTRODUCTION

The transforming growth factor-β (TGF-β) family is a group of multifunctional

cytokines that control growth, differentiation and apoptosis of cells, and have important

functions during embryonic development (Moustakas et al., 2001; Derynck et al., 2001).

Members of the TGF-β superfamily are synthesized as large inactive precursor molecules

containing a proteolytical cleavage site R-X-X-R, that can be cleaved by extreme pH

(Lyons et al., 1988), increased temperature, furin (Leitlein et al., 2001; Dubois et al.,

2001) or plasmin to release a carboxyl terminal peptide of 110-140 amino acids. This

region contains 7-9 cysteine residues that are engaged in intermolecular disulfide bonds

necessary for the assembly of biologically active dimers and a "cysteine knot" (Kingsley

1994; O'kane et al., 1997). All members of the TGF-β family share an identical carboxyl

terminus, Cys-X-Cys-X-COOH. The associated pro-protein region is called the Latency

Associated Peptide (LAP). This region has three side chains; two of them are asparagine

linked mannose-6-phosphate (M-6-P) oligosaccharides (Purchio et al., 1988). In addition,

latent TGF-β can contain a protein of variable size called the Latent TGF-β Binding

Protein (LTBP) (Yin et al., 1995). Both the LTBP and LAP must be removed before the

mature protein can function. There are three major TGF-β isoforms in mammalian cells,

designated TGF-β1, TGF-β2, and TGF-β3. The sequence homology among these

isoforms is greater than 65% and their biology activity is similar in many *in vitro* assays (Graycar et al., 1989). TGF-β1effects can be both inhibitory and stimulatory. TGF-βs initiate signaling by assembling type I and type II receptors complexes that activate Smad transcription factors that increase or decrease the expression of certain genes (Massague et al., 2000). Functionally, Smads fall into three subfamilies: receptor-activated Smads (R-Smads: Smad1, 2, 3, 5, 8), which become phosphorylated by the type I receptors; common mediator Smads (Co-Smads: Smad4), which oligomerise with activated R-Smads; and inhibitory Smads (I-Smads: Smad 6 and 7) (Moustakas et al., 2001). Major sources of TGF-β include platelets, leukocytes, bone and placental tissue. TGF-β is a potent chemoattractant for monocytes, macrophages, lymphocytes, neutrophils and fibroblasts. It stimulates the release of cytokines (e.g., IL-1, IL-6, TNFα, bFGF) from these cells (Roberts et al., 1990). TGF-β1 is an important major regulator of the extracellular matrix (ECM), stimulating fibroplasia and collagen deposition, inhibiting ECM degrading proteases and upregulating the synthesis of protease inhibitors (O'kane et al., 1997). Since all these processes are integral to wound healing, the role of TGF-βs in wound healing and tissue repair, and the regulation of their activity are of major clinical significance.

The presence of four TGF-β isoforms (1-4) has been reported in avian species; TGF-β4 is unique to avian species (Jakowlew et al., 1991). Chicken TGF-β1 and TGF-β4 share 100% and 82% amino acid identity to human TGF-β1, respectively (Jakowlew et al., 1988a; 1988b). However, the authors could not exclude a possibility of contamination of their chicken cDNA library with porcine cDNA, and, as a consequence, their sequences of chicken TGF-β1 and 4 might have contained at least a portion of porcine

TGF-β1 sequence (Genbank Accession NO: x12373). My previous data have shown that human TGF-β1 exhibited only moderate stimulation of collagen synthesis in chicken tendon cells. I, therefore, hypothesized that chicken TGFβ-4 plays the role of mammalian TGF-β1 in avian species. To characterize the biological function of TGF-β4, it was necessary to obtain its complete cDNA sequence and express this protein *in vitro.* Chicken TGF-β4 has 114 amino acids in its predicted mature protein after the proteolytical cleavage of the cleavage site RRRR in its precursor. I generated the 5'end of chicken TGF-β4 mRNA by 5'RACE (Chapter 4, GenBank accession no: AF395834) and successfully expressed this protein in CHO-K1. *In* vitro assays confirmed its biological activities.

Heat-shock protein 47 is a 47-kD collagen-binding heat shock protein, expression of which is always correlated with that of collagens in various cell lines (Nagata et al., 1986; Nakai et al., 1990; Clarke et al., 1993). Like other heat shock protein genes, cloned Hsp47 genes reveal a well-conserved heat-shock element (HSE) consisting of one or more tandem repeats of five base-pair units (nGAAn, where n is any nucleotide) at the promoter region (about −80 from the transcription start site) (Hosokawa et al., 1993). Under stressful conditions, various heat shock proteins are induced by the binding of Heat shock factors (HSF) to HSE (Sarge et al., 1991). Heat shock factor (HSF) is a sequence-specific DNA binding protein that binds tightly to multiple copies of a highly conserved sequence motif (nGAAn). In vertebrate cells, members of the HSF gene family, which includes HSF1, HSF2, HSF3 and HSF4, are thought to respond differently to various forms of stress (Tanabe et al., 1997). HSFs are present constitutively in the cell in a non-DNA binding state, and are activated to a DNA binding form in response to

various stresses. The activation process appears to involve HSF oligomerization from a monomeric to a trimeric state, and it is associated with hyperphosphorylation of HSF (Sorger, 1991). I tried to determine if recombinant chicken TGFβ4 play similar roles in chicken tendon fibroblasts.

MATERIALS AND METHODS

Plasmid Construction and Expression in Bacteria

The total RNA extracted from chicken tendon fibroblast cells was reverse transcribed and PCR-amplified into cDNA encoding the mature TGF-β4 protein by using two primers. The sequence of the sense primer was GCATATGGACCTCGACACCGACTACTGC and that of the anti-sense primer was GCTCGAGTCAGCTGCACTTGCAGGCACGG. The underlined sequences represent *Nde*I and *Xho*I restriction sites. Amplified cDNA was resolved on agarose gels, purified by a gel extraction kit (Qiagen Inc., Valencia, CA), and ligated into the recipient TA cloning vector pGEM-T (Promega Corp., Madison, WI). The excised inserts were ligated in-frame into a pET28 expression vector (Novagen Inc., Madison, WI) which has a 6 x histidine (His-tag) attached to the amino terminus of fusion protein. cDNA fidelity was confirmed by sequencing. For recombinant protein expression, pET28-β4 constructs were transformed into the BL21 (DE3) strain of *E.coli*. Primary cultures were grown from single plated colonies and flasks containing 50-ml cultures were inoculated with primary cultures at a 1:50 dilution. Transformed bacteria were cultured in LB containing kanamycin (100 μg/ml) until OD_{600} reading reached 0.6-1, after which protein expression was induced in the presence of 1.0 mM IPTG for 3 h at 37 °C. Bacteria pellets were

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collected by centrifugation at 5000 x g for 10 min and stored at –70°C. For expression of

TGF-β4 in bacterial periplasmic space, pBAD/gIIIA expression vector (Invitrogen Corp.,

Carlsbad, CA) and bacterial strain TOP10 were utilized under the induction of L-

arabinose. The periplasmic protein preparation under osmotic shock is described in the

protocol supplied by the manufacturer.

Isolation of inclusion bodies

The bacteria pellets were washed with a lysis buffer (20mM Tris-HCl, 250 mM

NaCl, 0.1% Triton X-100, pH 8.0), and lyzed by the addition of 0.4 mg/ml lysozyme, 5

ng/ml DNase and 0.8 mM PMSF in 1/10 of the original volume of cell suspension. Lysis

was facilitated by sonication, followed by centrifugation at 10,000 x g for 20 min at 4°C

to pellet the inclusion bodies.

Solubilization and purification of TGF-β4 fusion protein from inclusion bodies

Inclusion bodies were solubilized in 8 M urea, 20 mM Tris-HCl, 500 mM NaCl

lysis buffer, pH 8.0, at RT for 5 min. This process was facilitated by sonication.

Solubilized fusion proteins were separated from insoluble debris by centrifugation at

10,000 x g for 20 min. Purification of solubilized TGF-β4 fusion protein was

accomplished by binding to a Ni-NTA column, followed by elution with an imidazole

gradient (Invitrogen). Ni-NTA beads were first equilibrated with the lysis buffer and

mixed with the protein solution. The mixture was shaken for 1 h and loaded into the

column. The column was washed with 5-10 volumes of the lysis buffer, and the bound

material eluted with a step-wise imidazole gradient (50 mM, 100 mM and 400 mM). The

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presence of protein in individual fractions was determined by a 15% SDS-PAGE gel. For

N-terminal sequencing, fusion proteins were separated by electrophoresis in 15% SDS

polyacrylamide gel and transferred onto a PVDF membrane which was sent for amino aci

sequencing by Edman degradation to the Molecular Genetics Instrumentation Facility at

The University of Georgia.

TGF-β4 refolding

After Ni-NTA affinity purification samples were diluted to an appropriate

concentration (<100 μg/ml) in the lysis buffer. Samples were first dialyzed against a

dialysis buffer (20 mM Tris-HCl, 100 mM NaCl, pH 8.0) containing 0.1 mM DTT for 3

h, and then against the dialysis buffer without DTT for an additional 3 h. To promote

disulfide bond formation, the above samples were again dialyzed against a dialysis buffer

containing 1 mM reduced glutathione and 0.2 mM oxidized glutathione. After prolonged

oxidative refolding, the sample was dialyzed against 500 mM NaCl, 20 mM Tris-HCl,

10% glycerol, pH 8.0. The sample was either frozen directly, or lyophilized and

reconstituted into 1% BSA/0.04 M HCl prior to freezing. Dimerization was visualized in

a non-reduced SDS-polyacrylamide gel.

TGF-β4 bioassay

TGF-β4 biological activities *in vitro* were determined using the mink lung cell

(Mv1Lu) growth inhibition (Kelley et al., 1992) and procollagen stimulation assays.

Mv1Lu cells were plated at a density of 1×10^4 cells/Ill (24-well plate) in 0.5 ml of

Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum

(FBS), and allowed to grow overnight at 37°. The samples to be assayed were added in 0.5 ml in serum-free DMEM. The cells were incubated with the sample for 48 h at 37°, then trypsinized and counted with a hemacytometer. The procollagen stimulation was determined by Northern blotting.

Northern blot analysis

Chicken embryonic tendon fibroblasts were isolated from gastrocnemius tendons removed from 18-day old chicken embryos and grown in DMEM supplemented with 0.37 g of sodium bicarbonate/100ml and 10% FBS. The cells were maintained in humidified 5% CO_2, 95% air incubator at 37°C. After confluence was reached in 60-mm dishes, the cells were kept in the quiescent state by placing them into serum-free DMEM for another 24 hours prior to various treatments. Total cellular RNA was extracted using TRIzol™ reagent. An aliquot of total RNA (5 or 10 μg per lane) was size fractionated in a 1.5% agarose gel and transferred to a positively charged nylon membrane (Roche). Equal loading in all lanes was confirmed by ethidium bromide (EB) staining. Blots were prehybridized in Dig Easy Hyb solution (Roche) for 1 hour at 68°C, subsequently hybridized overnight at 68°C in Dig Easy Hyb containing indicated probes. The membranes were washed in the following order: 2 x SSC – 0.1% SDS for 15 min at room temperature, 1 x SSC – 0.1% SDS for 15 min at room temperature, two times 1 x SSC – 0.1% SDS for 10 min at 68°C. Signal detection was performed in NBT/BCIP or CSPD (Roche) system. Bands were quantified by AlphaImager™ 2200 System (Alpha Innotech Corp., San Leandro, CA). Relative transcription was normalized with 18S or 28S rRNA, or hybridization with chicken β-actin RNA probe. In preliminary

experiments, normalization methods, 18S or 28S rRNA EB staining and hybridization for chicken β-actin mRNA, gave closely corresponding results. Each experiment was performed at least twice.

Western blotting

Mouse monoclonal anti-Hsp47 antibody was purchased from StressGen Biotechnologies Corp. (Vancouver, BC, Canada). The cells were cultured as described above and were harvested by centrifugation for 15 min at 2,000 x rmp/min and then lyzed in cell lysis buffer (50 mM Tris, 150 mM NaCl 1% Nonidet P-40 pH 7.8, 1 mM PMSF, 1 ug/ml pepstatin and 1 μg/ml leupeptin) on ice for 15 min. The lysates were centrifugated at 4°C for 30 min at 14,000 rpm/min. The supernatants were transferred to clean tubes and measured for protein concentration using the Bio-Rad DC protein assay kit (BIO-RAD). For nuclear protein extraction the procedure used was similar to that described by Xu et al., 2000. The human HSF1 polyclonal antibody was obtain from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Equal amounts of protein (15 μg) from each sample were separated under reducing conditions on a 12% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C in 5% non-fat milk PBS containing the primary antibody (dilution: 1: 500). Excess antibody was washed away with at least 3-4 changes of TTBS buffer (100 mM Tris, 0.9% NaCl, 0.1%(v/v) TIen 20, pH 7.5) over 15 min. The membranes were incubated in 10 ml TTBS buffer containing the biotinylated secondary antibody (1: 1000) for 1 hour at room temperature, followed by washing three times in TTBS. The detection of antigen-antibody complexes was performed using the ABC kit (Vector Laboratories, Inc., Burlingame, CA).

Transfection and establishment of the TGFβ-4 precursor-producing CHO-K1 cell line

I generated the 5'end of chicken TGFβ4 cDNA (Genbank accession no: AF395834) using the 5'RACE (Chapter 4). The complete open reading frame (ORF) cDNAs was produced by RT-PCR, one including the plasmid-tag DNA sequence in the 3'end and the other only 6 x His tag. The primers were: forward CCCATGGATCCGTCGCCGCTGCTG; downstream GCTGCACTTGCAGGCACGGACC and CTCGAGTCAATGATGGTGATGGTGATGGCTGCACTTGCAGGCACGGAC. cDNAs were in-frame cloned into a expression vector pcDNA3.1/V5-His-TOPO. The orientation was checked by digestion with *Kpn*I. Plasmid DNA (5 μg) was introduced into CHO-K1 in 25 cm^2 by 30 μg of liposome (NovaFECTOR, VennNova, LLC, Pompano Beach, FL). Next day, the cells were split into two 100-mm plates containing 10% FBS F10-nutrient mixture (Ham) medium (GIBCO BRL) with 600 μg/ml G418 (Geneticin$^®$). After 10 to 14 days, 24 clones were individually picked. Clones were grown separately in 10% FBS F10 medium to reach confluence, and then grown in serum-free medium overnight for recombinant protein testing. Conditioned media from individual clones were harvested and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting and immunostaining using a goat polyclonal IgG specific for human TGF-β1 (Santa Cruz Biotechnology). A single highest-producing clone was selected and utilized for later use. For moderate recombinant protein production, the CHO-K1 cell line expressing chicken TGF-β4 was amplified in ten 175 cm^2-flasks, grown to confluency, and maintained in serum-free medium for 48 h. Conditioned medium was harvested and concentrated using Centricon

plus-80 (Amicon Bioseparations). The recombinant protein was purified using ProBond Resin (Invitrogen) under native conditions. Recombinant TGF-β4 activation was achieved by dialyzing the protein preparation for 24 to 48 h against 0.04 M HCl. The sample was either frozen directly, or lyophilized and reconstituted into 1% BSA/0.04 M HCl prior to freezing.

RESULTS

Plasmid construction and expression of chicken TGF-β4 in bacteria

Human TGF-β2 fusion protein activities, expressed in *E. coli,* were recovered after solubilization and refolding (Han et al., 1997). Because this protocol is less time- and labor-consuming I used it in my first attempt to express chicken TGF-β4 in a bacterial system. I utilized RT-PCR to generate cDNA encoding the mature TGF-β4 protein with *Nde*I and *Xho*I restriction sites and ligated it into the TA cloning vector. The excised cDNA was ligated into the pET-28 vector digested with the two above-mentioned restriction enzymes. The pET28-TGF-β4 plasmid was transformed into the BL21 (DE3) strain of *E. coli*. In the presence of 1 mM IPTG, the level of recombinant TGF-β4 expression approached 40% of cellular protein, as determined by SDS-PAGE and protein staining with Coomassie blue (Fig. 5.1A, Lane 3). The reducing SDS-polyacrylamide gel shows the 15 kDa TGF-β4 monomer. The expression of TGF-β4 was further confirmed by Western blotting using the human TGF-β1 polyclonal antibody (Fig. 5.1B). Since this recombinant protein contains 6 x Histidine tag, I used Ni-NTA resin to purify this protein. The expressed protein was solubilized in 8 M urea, mixed with Ni-NTA resin and loaded into a Probond column (Invitrogen). The recombinant protein was eluted

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using the stepwise gradient elution with imidazole. Fractions containing the targeted

protein were determined by SDS-PAGE and protein staining (Fig. 5.2A).

The proper folding of recombinant protein is critical to its biological activities. To

prevent the concentrated protein from precipitation, the solubilized protein was diluted (<

100μg/ml), and then dialyzed against dialysis buffer (20 mM Tris-HCl, 100 mM NaCl,

pH 8.0) containing 0.1 mM DTT for 3 hours, and further dialyze without DTT for an

additional 3 hours. For the refolding or disulfide bond formation, the sample was then

dialyzed against the redox system containing 1 mM reduced glutathione and 0.2 mM

oxidized glutathione overnight at room temperature. The dimerization was confirmed by

SDS-PAGE using the non-reduced condition (Fig. 5.2B, Lane 2). For the amino acid

sequencing, the fusion protein was digested with thrombin to remove His-Tag. Using the

Edman degradatin process the N-terminal sequencing revealed H, M, D, L, D, T, D, Y, C,

F, G, P, X, T, D, E, K, N, X, X ("X" stands for the Weak signal). The first two amino

acid H, M come from the plasmid and the remaining sequence corresponds exactly to the

predicted amino acid sequence of chicken TGF-β4 (Jakowlew et al., 1988; Burt et al.,

1992).

TGF-β bioassay

A typical activity of TGF-β superfamily is to inhibit the proliferation of epithelial

cells, e.g., mink lung epithelial cells (Mv1Lu). Another is the stimulation of procollagen

production. The recombinant TGF-β4 expressed in bacteria displayed almost no

inhibition of proliferation of Mv1Lu (Fig. 5.3A), as compared to 95% inhibitory activity

achieved with human TGF-β1. The recombinant refolded chicken TGF-β4 did not exhibit

stimulation of the procollagen $\alpha1(I)$ mRNA expression as determined by Northern

blotting (Fig. 5.3B). I hypothesized that the chicken TGF-β4 synthesized by bacteria did

not form the proper native structure after refolding.

Expression of chicken TGF-β4 in bacterial periplasmic space

The formation of disulfide bonds occurs during folding of proteins in the

endoplasmic reticulum of eukaryotes and the periplasmic space of prokaryotes. The

pBAD/gIII plasmids are pUC-derived expression vectors designed for the expression of

regulated, secreted recombinant proteins and their purification from *E.coli*. The gene III

signal sequence is utilized to facilitate the secretion of the recombinant protein into the

periplasmic space. cDNA encoding the mature chicken TGF-β4 was in-frame cloned into

pBAD/gIIIa plasmid and expressed in the bacterial strain TOP10. The chicken TGF-β4

was expressed in bacterial cytoplasm as confirmed by SDS-PAGE analysis, and by

comparison with the positive (pBAD/gIII-Calmodulin) and negative controls (pBAD/gIII

only) (Fig. 5.4). Under osmotic shock conditions calmodulin was secreted into the

bacterial periplasmic space while TGF-β4 was not (data not shown).

Establishment of the TGF-β4 precursor-producing CHO-K1 cell line

Several active human recombinant TGF-β proteins have been expressed in Chinese

hamster ovary cell line (CHO) (Lioubin et al., 1991; Bmydrel et al., 1993). cDNA

encoding the chicken TGF-β4 precursor protein was in-frame cloned into

pcDNA3.1/V5-His-TOPO (pcDNA3.1-β4). The presence of the precursor of TGF-β4

cDNA sequence in constructed plasmids was confirmed by sequencing. pcDNA3.1-β4

was transfected into CHO-K1 cells with liposomes. The transfected CHO-K1 cells were grown in F10 nutrient mixture medium supplemented with 10% FBS, together with G418 for two weeks. Twenty-four individual colonies were transferred into two 12-well plates and monitored for their expression status. Sixteen colonies of transfected CHO-K1 cells grew very well and all sixteen expressed the chicken precursor TGF-β4 molecule (about 55 kD) in the serum-free medium. The protein expression was verified by Coomassie Blue staining (Fig. 5.5 A, Lane 3-8) and Western blot analysis (Fig. 5.5B, Lane 3-8), compared with untransfected CHO-K1 cells (Fig. 5.5 A and B, Lane 1-2). Because the recombinant TGF-β4 was attached to 6 x His-tag, the protein was purified with ProBond Resin, and eluted with a stepwise gradient of imidazole (Fig. 5.5C). The recombinant TGF-β4 precursor activation was achieved by dialysis against 0.04 M HCl (pH 1.5) for 48 hours or by furin treatment. After activation the monomer of TGF-β4 showed a weak band of 15 kD (data not shown). The recombinant TGF-β4 bioassay demonstrated that this protein strongly inhibited Mv1Lu cells growth after 48-hours treatment, almost equivalent to the human TGFβ-1 inhibition (Fig. 5.6A). Procollagen stimulation assay confirmed that the recombinant chicken TGFβ-4 expressed in CHO-K1 had the TGF-β superfamily related activities (Fig. 5.6B). When comparing the effect of human TGF-β1 (chapter 3) and chicken TGF-β4 on procollagen mRNA expression in chicken fibroblasts, recombinant chicken TGF-β4 showed 60% stronger stimulation than human TGF-β1.

Recombinant chicken TGF-β4 stimulates Hsp47 expression and activation of HSF1

I have shown that the human TGF-β1 stimulated chicken Hsp47 expression at both mRNA and protein levels, and activation of HSF1 (Chapter 3). I tried to determine if

recombinant chicken TGF-β4 plays a similar role in chicken fibroblasts. The addition of recombinant TGF-β4 to the quiescent cultures of chicken fibroblasts increased Hsp47 mRNA expression and Hsp47 protein synthesis in a dose-dependent manner (Fig. 5.7A and 5.7B). Therefore, chicken TGF-β4 not only increases procollagen production, but also enhances Hsp47 expression. Western blot analysis also showed that chicken TGF-β induced translocation of HSF1 into the cell nucleus in a dose-dependent manner (Fig. 5.8. Lane 4 and 5), compared with no treatment (Lane 1), human TGF-β1 treatment (Lane 2) and heat shock (Lane 3).

DISCUSSION

In this study I partially characterized chicken TGF-β4. Because this TGF-β isoform is not only commercially unavailable, but also its cDNA sequence had not been fully elucidated (Jakowlew et al., 1991), it was necessary to express it first *in vitro*. Human TGF-β2 fusion protein activities, expressed in *E. coli,* were recovered after solubilization and refolding (Han et al., 1997). Because work with *E. coli* expression systems is less time- and labor-consuming than work with eukaryotic systems I first tried to express mature chicken TGF-β4 in *E. coli* using the pET-28 vector expression. After purification with Probond Resin under denatured conditions, and refolding using several different conditions I did not recover TGF-β biological activities (Fig. 5.3) even though the recombinant protein exhibited dimeric structure (Fig. 5.2B). N-terminal sequencing of the recombinant protein showed that the first 15 amino acids correspond to the predicted sequence from its cDNA. I hypothesize that the dimeric protein was misfolded and did not posses the native structure of TGF-β. Formation of disulfide bridges does not occur in

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the reducing cytosol of bacterial cells while the bacterial periplasm is a compartment that allows the formation of disulfide bonds (Foti et al., 1998). Alternatively, I tried to express this mature protein in bacterial strain (TOP10) with pBAD/gIIIA vector, which contains the gene III signal sequence for secretion of a recombinant protein into the periplasmic space. After induction with L-arabinose, mature TGF-β4 was expressed in the bacterial cytoplasm (Fig. 5.4), but not secreted into the periplasmic space after osmotic shock. It is likely that the presence of the precursor protein of TGF-β4 and/or Latent TGFβ Binding Protein is necessary to facilitate secretion of this protein (Miyazono et al., 1991; Saharinen et al., 1996).

Biologically active human TGF-βs have been successfully expressed in CHO cells or insect cells (Bmydrel et al., 1993; Lioubin et al., 1991). A partial chicken TGF-β4 cDNA gene sequence (Genbank: M31160) was available, lacking the 5'end in its open reading frame. I successfully generated the 5'end of this gene using the modified 5'RACE (Genbank: AF395834) (Chapter 4). In order to express this protein in CHO-K1 cells, I in-frame cloned cDNA encoding the chicken TGF-β4 protein precursor into pcDNA3.1/V5-His-TOPO. CHO-K1 cell lines expressing this precursor protein were established through G418 selection. The recombinant protein was detected with Coomassie Blue and its identity was confirmed by Western blot analysis. The monomer of this fusion protein has a molecular mass about 55 kD (plus tag). This is in line with the human TGF-β2 precursor protein (52.5 kD molecular mass), which was expressed in CHO by Lioubin et al. (1991). The recombinant precursor protein of TGF-β4 was purified using Probond Resin under the native conditions. The precursor protein was activated by dialyzing against strong acid or by furin digestion. The active protein

exhibited strong activities in TGF-β bioassays (Fig. 5.6A and B). Comparison of effects

of the recombinant human TGF-β1 and chicken TGF-β4 on procollagen and Hsp47

protein production in chicken tendon fibroblasts, indicated that chicken TGF-β4 protein

activity was 60% stronger than human TGF-β1 in its effect on collagen expression. Both

proteins show 82% identity in their amino acid sequence and contain the active motif

WXXD in their mature region (human TGF-β WSLD versus chicken TGF-β4 WSAD)

(Huang et al., 1999). The attached His-tag did not hinder activities of this fusion protein

in my experiment. In summary, my data thus indicate that chicken TGF-β4 plays roles in

avian species similar to roles of human TGF-β1 in mammalian species.

Hsp47 is a heat-shock protein that interacts with procollagen during its folding,

assembly and transport from the ER of mammalian cells. It has been suggested to carry

out a diverse range of functions, such as acting as a molecular chaperone that facilitates

the folding and assembly of procollagen molecules, retains unfolded molecules within the

ER, and assists the transport of correctly folded molecules from the ER to Golgi

apparatus (Tasab et al., 2000). Induction of Hsp47 by TGF-β has been previously

reported in rat skeletal myoblasts (Clarke et al., 1993), mouse osteoblasts (Yamamura et

al., 1998) and human diploid fibroblasts (Sasaki et al., 2002). TGF-β induces trimer

formation of HSF1, which in turn bind to HSE of Hsp47, resulting in the enhancement of

Hsp47 expression (Sasaki et al., 2002). In my experiments I demonstrated that

recombinant TGF-β4 enhanced Hsp47 expression at both protein and mRNA levels in

chicken embryo tendon fibroblast (Fig. 5.7A and B). Induction of Hsp47 protein

expression by chicken TGF-β4 is also regulated by activation of HSF1. These results

suggested that chicken TGF-β4 in avian species has roles similar to those of TGF-β1 in mammalian species.

In conclusion TGF-β4 co-regulates chicken procollagen and Hsp47 protein production in chicken tendon fibroblasts. This means that TGF-β4 overexpression resulting from virus infections or other inflammation would induce collagen deposition, which would lead to tissue fibrosis. Tissue fibrosis, as the final result of various chronic immunological and inflammatory processes, modifies the outcome of many disorders (Masuda et al., 1994; O'kane et al., 1999). Therefore, the development of the modalities to inhibit fibrosis has been in progress for long time. In this regard, the present results suggest that Hsp47 may be an appropriate candidate target to be inhibited. Because Hsp47 acts as a universal molecular chaperone for all types of collagen (Nagata, 1996), inhibition of Hsp47 is thought to interfere with the secretion various collagens simultaneously. In fact anti-sense oligonucleotides against collagen-binding stress protein Hsp47 mRNA suppressed collagen accumulation in experimental glomerulonephritis (Sunamoto et al., 1998). Therefore, a modality to attenuate the expression of Hsp47 could be a promising approach to prevent fibrosis.

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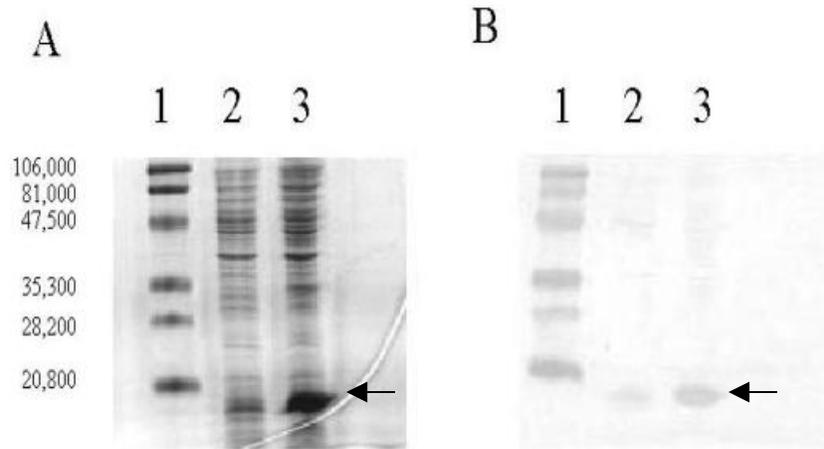
Fig. 5.1. Expression of the recombinant fusion protein TGF-β4 in *E. coli*. The plasmid and bacteria preparation is described in the Materials and Methods. Protein expression is visualized on reducing SDS-polyacrylamide gel. A) TGF-β4 fusion protein accumulated in inclusion bodies after induction by 1 mM IPTG. Lane 1, molecular weight markers; Lane 2, non-induced bacteria; Lane 3, induction for 4 h by 1 mM IPTG. B) Recombinant fusion protein TGF-β4 expression was confirmed by Western blot using human TGF-β1 polyclonal antibody. Lane 1, Molecular weight markers; Lane 2, non-inducted bacteria; lane 3 induction for 4 h by 1 mM IPTG. Arrow indicates positions of mature TGF-β4.
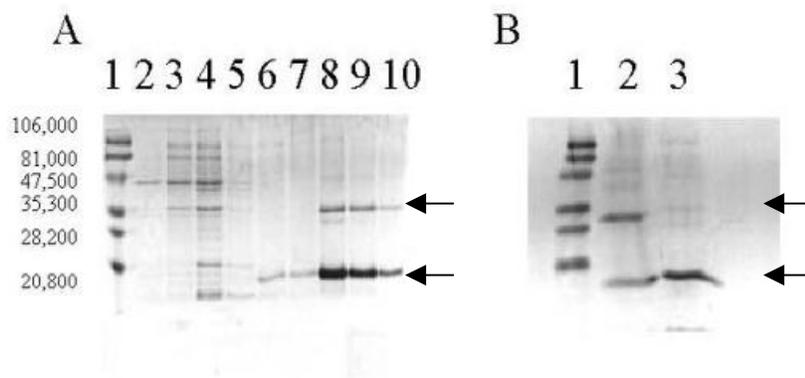
Fig. 5.2. Purification and refolding of recombinant fusion protein TGF-β4 by Ni-NTA chromatography. The recombinant protein purification and refolding procedures are described in the Materials and Methods. A) The solubilized fusion protein was eluted by a stepwise imidazole gradient under denatured conditions. Lane 1, molecular weight markers; Lane 2, 3, 4, elution of TGF-β4 by 50 mM imidazole; Lane 5, 6, 7, elution by 100 mM imidazole; Lane 8, 9, 10, elution by 400 mM imidazole. The top arrow: nonspecific protein; The lower arrow: recombinant mature TGF-β4. B) The dimerized TGF-β4 (30 kD) was formed under the control of redox conditions. Lane 1, Molecular weight markers; Lane 2, under non-reducing conditions; Lane 3; under reducing conditions. The top arrow: dimer protein; the lower arrow: monomer protein.
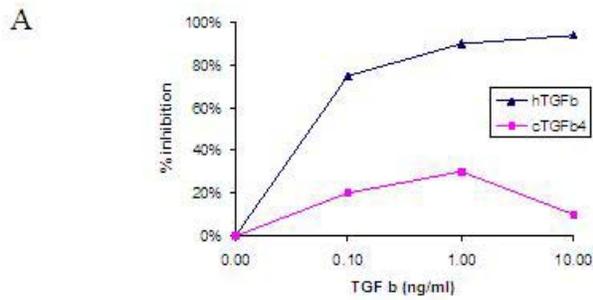
Fig. 5.3. The recombinant chicken TGF-β4 expressed in *E.coli* did not inhibit Mv1Lu
growth (A) and did not stimulate production of procollagen α1(I) and Hsp47 mRNA (B),
determined by Northern blot. Mv1Lu cells and chicken embryonic tendon fibroblasts
preparations are as described in Materials and Methods. The total RNA was isolated from
those cells. The 5 μg of total RNA from specified treatments was loaded into each lane.
The β-actin was used as an internal control.
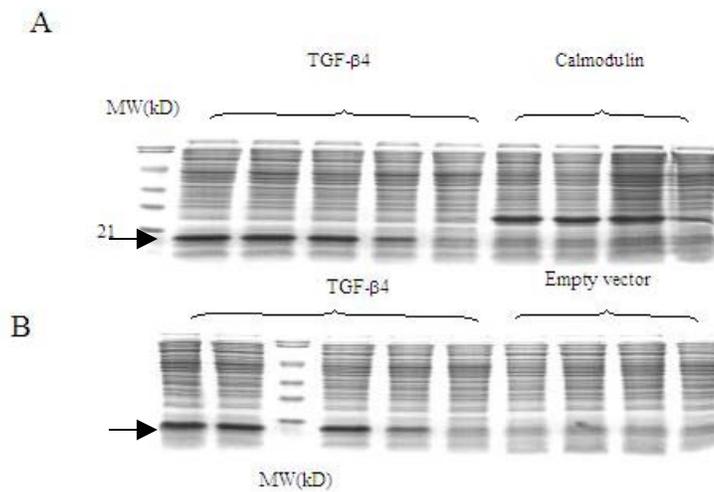
Fig. 5.4. Chicken TGF-β4 was expressed in bacterial strain Top10 with pBAD/gIIIA

vector (A and B), but it was not secreted into the bacterial periplasmic space. A. Chicken

TGF-β4 protein expression decreased with reduction of L-arabinose, 0.2%, 0.02%,

0.002%, 0.0002% and 0.0002%, respectively. Calmodulin was used as a positive control.

B. Empty vector was used a negative control. Chicken TGF-β4 was not secreted into

bacterial periplasmic space under osmotic shock conditions, but calmodulin was (data not

shown). The arrow: recombinant mature monomer TGF-β4.
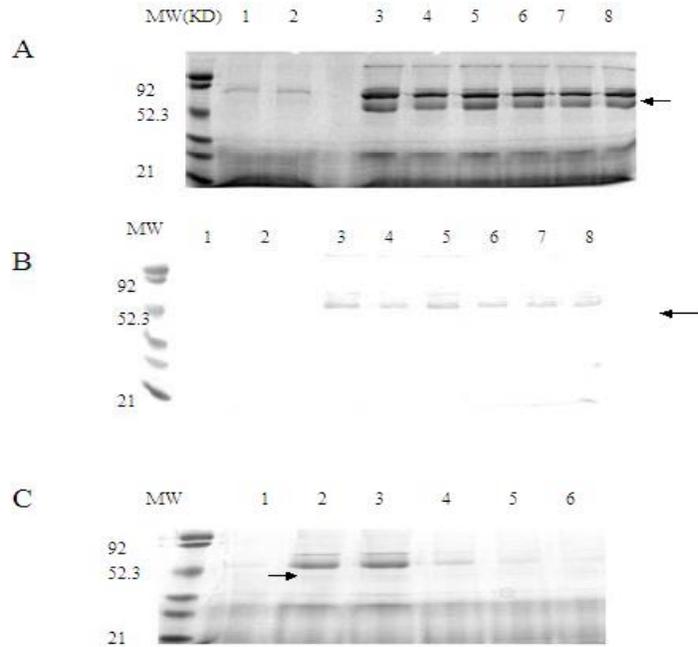
Fig. 5.5. Establishment of Chinese hamster ovary cell line (CHO-K1) expressing chicken TGF-β4 precursor. Plasmid construction and preparation is described in the Materials and Methods. cDNA encoding the chicken TGF-β4 precursor was in-frame cloned into pcDNA3.1/V5-His-TOPO, and then transfected into CHO-K1 cells with liposome. G418 was used to suppress untransfected cells. After 2 weeks individual colonies were selected for the further culture. A. Coomassie Blue staining showed that selected individual colonies have extra bands (Lane 3-8), compared with the untransfected cells (Lane 1-2). B. Western blotting using TGF-β1 antibody confirmed that TGF-β4 was expressed in those colonies (Lane 3-8), compared with the untransfected cells (Lane 1-2). C. The expressed recombinant chicken TGF-β4 was purified using Probond Resin under native conditions. The protein was eluted with a stepwise imidazole gradient: 50 mM (Lane 1), 100 mM (Lane 2 and 3) and 300 mM (Lane 4-6).
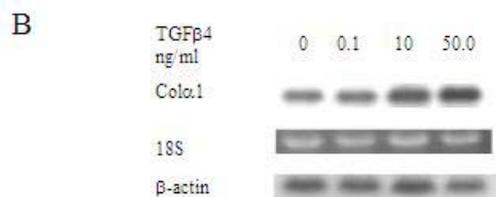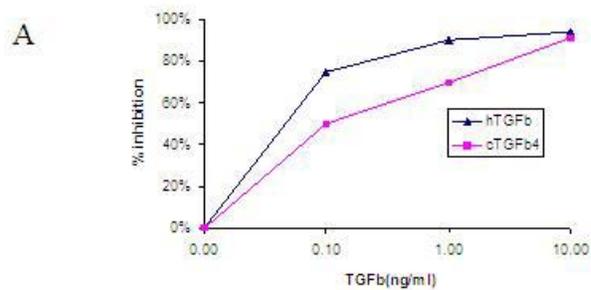
Fig. 5.6. Recombinant chicken TGF-β4 exhibits TGF-β superfamily main activities. A. Recombinant chicken TGF-β4 inhibited growth of mink lung epithelial cells (Mv1Lu) in an assay described in Materials and Methods. Human TGF-β1 was used as a positive control. B. Recombinant chicken TGF-β4 stimulated procollagen mRNA expression in a dose-dependent manner as determined by Northern blotting. 18S tRNA and β-actin were used as an internal control. CTGF-β4: chicken transforming growth factor β4; hTGF-β1: human transformation growth factor β1; Colα1: procollagen α1(I) mRNA;
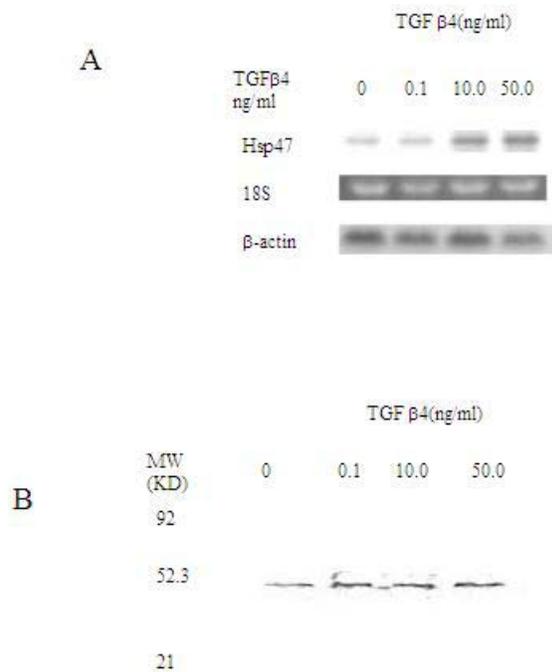
Fig. 5.7. Recombinant chicken TGF-β4 increases Hsp47 expression in both protein and mRNA levels. A. Recombinant TGF-β4 stimulated Hsp47 mRNA expression in a dose-dependent manner as determined by Northern blotting. The total RNA preparation is described in Materials and Methods. 18S tRNA and β-actin were used as an internal control. B. Recombinant TGF-β4 increased Hsp47 protein expression in a dose-dependent manner as determined by Western blotting.
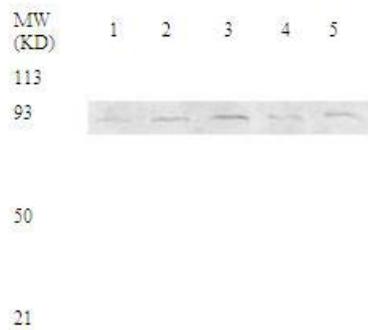
Fig. 5.8. Recombinant chicken TGF-β4 induces activation and translocation of HFS1 into

the cell nucleus. The nuclear protein preparation is as described in the Materials and

Method. Ten μg of samples from each treatment were loaded onto 10% SDS-

polyacrymide gels and transferred onto a nitro-cellulose membrane and probed with

human HSF1 polyclonal antibody. Lane 1, no treatment; Lane 2, human TGF-β1; Lane 3,

heat at 43°C for 3 h; Lane 4 and 5, chicken TGF-β4 treatment at 1 ng/ml, 20ng/ml for 24

h, respectively.

CHAPTER 6

CONCLUSIONS

In the first study I described the effects of increased temperature, mechanical stress and growth factors on Hsp47 and type I procollagen expression in fibroblasts isolated from embryonic chicken tendons. My results show that elevated temperature had a significant effect on the expression of Hsp47. The expression of Hsp47 mRNA at 45°C increased within 30 minutes and returned to baseline within 4 hours after the temperature decreased to 37°C. My data also show that human TGF-β1 is another regulator of Hsp47 expression as the addition of TGF-β1 led to a moderate increase in the expression of Hsp47 mRNA. TGF-β2 exerted only a small effect, and TGF-β3, epidermal growth factor (EGF) and tumor necrosis factor α (TNF-α) had no impact on Hsp47 expression. TGF-β1 also increased type I procollagen mRNA expression and TNF-α reduced this expression. TGF-β1 delays the degradation of Hsp47 mRNA after heat shock, suggesting that TGF-β1 regulates Hsp47 gene posttranscriptionally. I also reported that mechanical stress increased Hsp47 mRNA expression and Hsp47 protein synthesis. I show that induction of Hsp47 protein expression by heat shock, mechanical stress and TGF-β1 is likely achieved through activation and translocation of Heat shock transcription factor 1 (HSF1) into the nucleus. My data indicate that TGF-β1 is a major regulator of both procollagen and Hsp47 genes.

Next I determined whether chicken TGF-β has similar roles in the same cell cultures. The cDNA sequences of chicken TGF-β 1-4 have been reported, but the chicken

TGF-β1 gene sequence entered in GenBank is dubious. Chicken TGF-β4 has 82% amino acid sequence identity with human TGF-β1. I was interested in this protein because I suspected that TGF-β4 plays a role in avian species similar to the role TGF-β1 plays in mammalian species.

I failed to recover active TGF-β4 after purification and refolding of the chicken mature TGF-β4 protein expressed in *E. coli* using a pET-28 vector. I, therefore, generated the 5' end of the cDNA encoding the precursor of this protein using the modified 5'RACE. cDNA encoding this precursor protein was in-frame cloned into pcDNA3.1/V5-His-TOPO vector and transfected into Chinese hamster ovary (CHO-K1) cell line. Individual colonies were grown in F10 nutrient mixture medium and selected with G418. A cell line expressing the TGF-β4 precursor protein was established and expanded. After purification with Ni-NTA beads and activation by acid solution, the mature TGF-β4 exhibited strong inhibition of mink lung (Mv1Lu) epithelial cell growth and stimulation of procollagen production. Further studies showed that TGF-β4 enhanced Hsp47 expression. Induction of Hsp47 expression by chicken TGF-β4 is regulated by activation and translocation of chicken HSF1 into the nucleus. My data show that chicken TGF-β4 might play a role in avian species similar to the role human TGF-β1 plays in mammalian species.

In conclusion, TGF-β4 co-regulates chicken procollagen and Hsp47 protein production in chicken tendon fibroblasts. This means that TGF-β4 overexpression resulting from virus infections or other inflammatory would induce collagen deposition, which would lead to tissue fibrosis. Tissue fibrosis, as the final result of various chronic

immunological and inflammatory processes, modifies the outcome of many disorders, which would cause tendon failure or rupture.

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