The Relaxin Gene Knockout Mouse: A Model of Progressive Scleroderma

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Relaxin is a peptide hormone with anti-fibrotic properties. To investigate the long-term effects of relaxin deficiency on the ageing skin, we compared structural changes in the skin of ageing relaxin-deficient (RLX−/−) and normal (RLX+/+) mice, by biochemical, histological, and magnetic resonance imaging analyses. Skin biopsies from RLX+/+ and RLX−/− mice were obtained at different ages and analyzed for changes in collagen expression and distribution. We demonstrated an age-related progression of dermal fibrosis and thickening in male and female RLX−/− mice, associated with marked increases in types I and III collagen. The increased collagen was observed primarily in the dermis of RLX−/− mice by 1 mo of age, and eventually superseded the hypodermal layer. Additionally, fibroblasts from the dermis of RLX−/− mice were shown to produce increased collagen *in vitro*. Recombinant human gene-2 (H2) relaxin treatment of RLX−/− mice resulted in the complete reversal of dermal fibrosis, when applied to the early onset of disease, but was ineffective when applied to more established stages of dermal scarring. These combined findings demonstrate that relaxin provides a means to regulate excessive collagen deposition in disease states characterized by dermal fibrosis and with our previously published work demonstrate the relaxin-null mouse as a model of progressive scleroderma.

Key words: collagen/dermal fibrosis/relaxin-deficient mice/scleroderma
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Scleroderma or systemic sclerosis is a complex, chronic connective tissue disease, which primarily causes skin thickening and hardening in addition to interstitial fibrosis of various internal organs (lung, heart, kidney, gastrointestinal tract, blood vessels, etc) (Denton and Black, 2000; Simms and Korn, 2002; Kissin and Korn, 2003). The associated fibrosis is potentially driven by several independent factors involving immunological disorders, enhanced workload, hypertrophy, injury-repair mechanisms or metabolic defects, which eventually leads to the proliferation and differentiation of matrix-producing fibroblasts. Stimulated by a number of cytokines and growth factors, these cells differentiate into myofibroblasts and synthesize prodigious amounts of matrix proteins, mainly collagen, leading to tissue scarring and thickening (Korn, 2002). Although the symptoms of scleroderma vary between individuals, it can develop in every age group and if not properly treated, can lead to irreversible tissue damage.

Given that scleroderma offers many targets for therapy, several agents have been used to treat various aspects of the disease (Denton and Black, 2000; Mouthon and Agard, 2001; Korn, 2002; Simms and Korn, 2002), including vasodilators, anti-inflammatory drugs and inhibitors of growth factors, intercellular signalling, gene transcription, collagen production and matrix synthesis pathways; however, many have been limited in their application, whereas others inhibit several mechanisms and are non-specific.

Relaxin is a small dimeric peptide hormone with known anti-fibrotic and vasodilatory properties (Samuel *et al*, 2003a; Sherwood, 2004). Although generally associated with female reproductive tract physiology (Sherwood, 2004), relaxin has also been shown to inhibit fibroblast function (Samuel *et al*, 2004a), decrease collagen secretion (Unemori and Amento, 1990; Unemori *et al*, 1993, 1996; Garber *et al*, 2001; Williams *et al*, 2001; Samuel *et al*, 2004a), increase matrix metalloproteinase expression and inhibit the influence of several pro-fibrotic factors to induce a matrix-degrading phenotype in several non-reproductive organs and cells (reviewed in Gavino and Furst, 2001; Sherwood, 2004). Relaxin acts directly on transforming growth factor (TGF)-β-stimulated human dermal fibroblasts (Unemori and Amento, 1990; Samuel *et al*, 2003b) and on human scleroderma fibroblasts (Unemori *et al*, 1992) to decrease collagen, fibronectin, and fibrillin-2 overexpression. Relaxin was also shown to decrease collagen accumulation in several rodent models of fibrosis (Unemori *et al*, 1996; Garber *et al*, 2001; Williams *et al*, 2001; Samuel *et al*, 2004a), including two rodents models of dermal fibrosis (Unemori *et al*, 1993). These relaxin-induced effects on matrix turnover are species independent, but are most likely influenced by organ-specific factors.

Abbreviations: H2, human gene-2; HDF, human dermal fibroblasts; MRI, magnetic resonance imaging; RLX−/−, relaxin deficient; RLX+/+, relaxin normal

We have used gene-targeting means to establish a relaxin gene-knockout (RLX−/−) mouse that lacks the relaxin protein, the predominant form of relaxin in mice (Zhao *et al*, 1999), which has been found to be expressed in the skin (Bathgate *et al*, 2002). We have since demonstrated that RLX−/− mice undergo an age-related and pregnancy-related progression of fibrosis in several reproductive organs (Zhao *et al*, 2000; Samuel *et al*, 2003c), the lung (Samuel *et al*, 2003d), heart (Du *et al*, 2003; Samuel *et al*, 2004a) and kidneys (Samuel *et al*, 2004b), leading to altered tissue structure and function.

In this study, we used RLX−/− mice to examine the long-term effects of relaxin deprivation on the structure of the skin with the specific aim of determining whether the lack of relaxin affects dermal collagen deposition, types and integrity. We also investigated the use of magnetic resonance imaging as a diagnostic tool to detect the onset of fibrosis in these animals, in addition to the effects of relaxin treatment in RLX-KO (knockout) mice with early and established forms of dermal fibrosis. Our findings, combined with our previously published work, demonstrate that the relaxin-deficient mouse is a model of progressive scleroderma.

Results

The effects of relaxin deficiency on dermal collagen concentration and types A progressive increase in dermal collagen concentration (determined by hydroxyproline analysis) was observed in biopsies from male RLX−/− mice, which was significantly higher than that measured in age-matched RLX+/+ mice at 6 mo (by 40%, $p<0.05$) and 12 mo (by 56%, $p<0.02$) of age (Fig 1*A*). A similar but delayed trend was also observed in the dermis of female RLX−/− mice, resulting in a 30% increase ($p<0.02$) in dermal collagen concentration by 12 mo of age (Fig 1*A*), compared with measurements from age-matched RLX+/+ animals.

Type I collagen was the predominant form of mature collagen in the skin of immature (1 mo) and adult (12 mo) RLX+/+ and RLX−/− male and female mice (Fig 1*B*), representing over 90% of the total collagen present. Type I collagen was represented by monomers (α1(I) and α2(I) subunits), dimers (β11: dimers of two α1(I) subunits; β12: dimers of α1(I) and α2(I) monomers) and trimers (γ) in the dermis, whereas lower amounts of type III collagen (α1(III) subunits) monomers were also detected. No significant differences in types I or III collagen were detected in the skin of immature RLX+/+ *versus* RLX−/− animals, as determined by densitometric studies (Fig 1*B*). Marked increases, however, in types I ($p<0.01$) and III ($p<0.01$) collagen were observed in 12 mo old RLX−/− mice, compared with that detected from age-matched RLX+/+ animals (Fig 1*B*), consistent with the hydroxyproline data (Fig 1*A*). An identical trend was observed in both male and female mice (data not shown).

The effects of relaxin deficiency on skin histology The dermis of Masson trichrome-stained skin sections from 1 mo (Fig 2*A*), 3 mo (Fig 2*C*), 6 mo (Fig 2*E*), and 14 mo (Fig 2*G*) old RLX+/+ mice consisted of an extracellular matrix of collagen fiber bundles (blue staining) that were packed as parallel arrays of fibrils. The collagen fibers of adult mice became more densely packed with age, contributing to increased skin thickening. Beneath the dermis, the subcutaneous tissue (hypodermis) containing fat and adipose cells

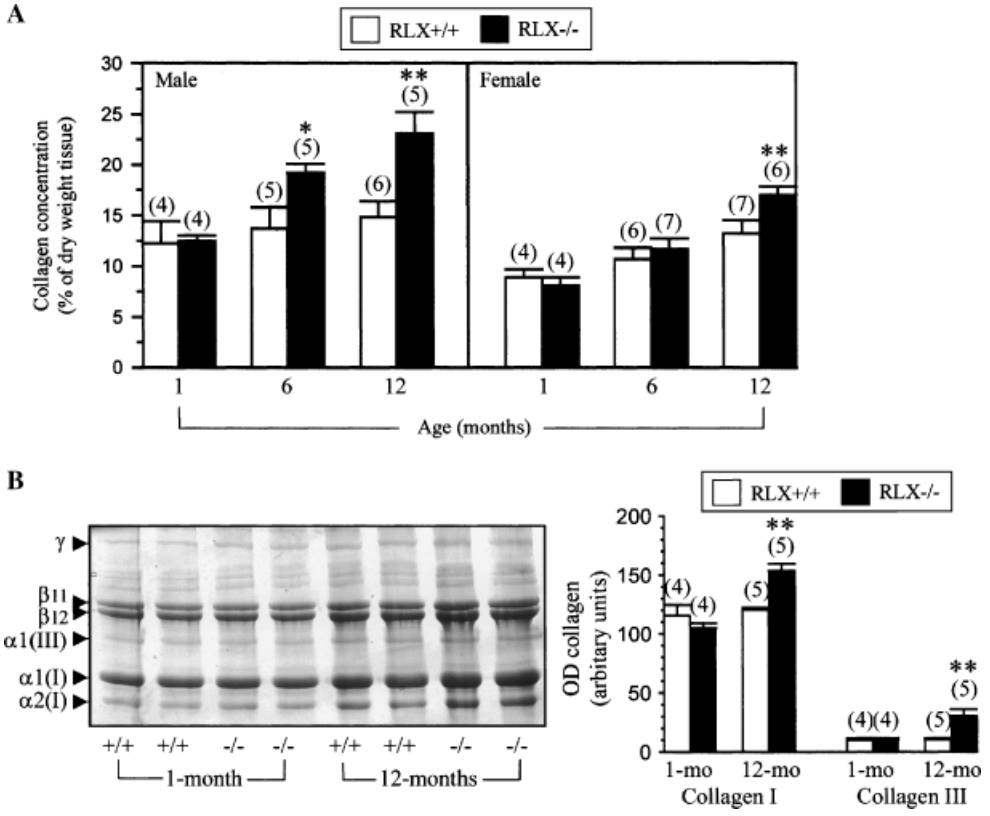

Figure 1
Effect of relaxin deficiency on dermal collagen concentration and types. Collagen concentration (collagen content as a percentage of the dry weight tissue) was determined from 12 mm skin biopsies from ageing male and female relaxin normal (RLX+/+) and deficient (RLX−/−) mice (*A*). The numbers in parentheses represent the number of samples analyzed per group. *$p<0.05$ and **$p<0.02$, when compared with corresponding values from age-matched RLX+/+ mice. The types of maturely cross-linked (pepsin-digested) collagen in the dermis of ageing male RLX+/+ and RLX−/− mice was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (*B*), using delayed reduction of the disulfide bonds with 10% β-mercaptoethanol. Shown is a representative gel of two individual pepsin digests from the skin of 1 and 12 mo old male RLX+/+ and RLX−/− mice. Pepsin digests from four to five individual animals per group and time point were analyzed by SDS-PAGE. Densitometric analysis of the type I (α1(I) and α2(1) chains) and type III (α1(III) chain) collagen chains was also performed on n = 4–5 samples per group and time point. **$p<0.01$, when compared with corresponding values from age-matched RLX+/+ mice. An identical trend was also observed in samples from female mice (data not shown).

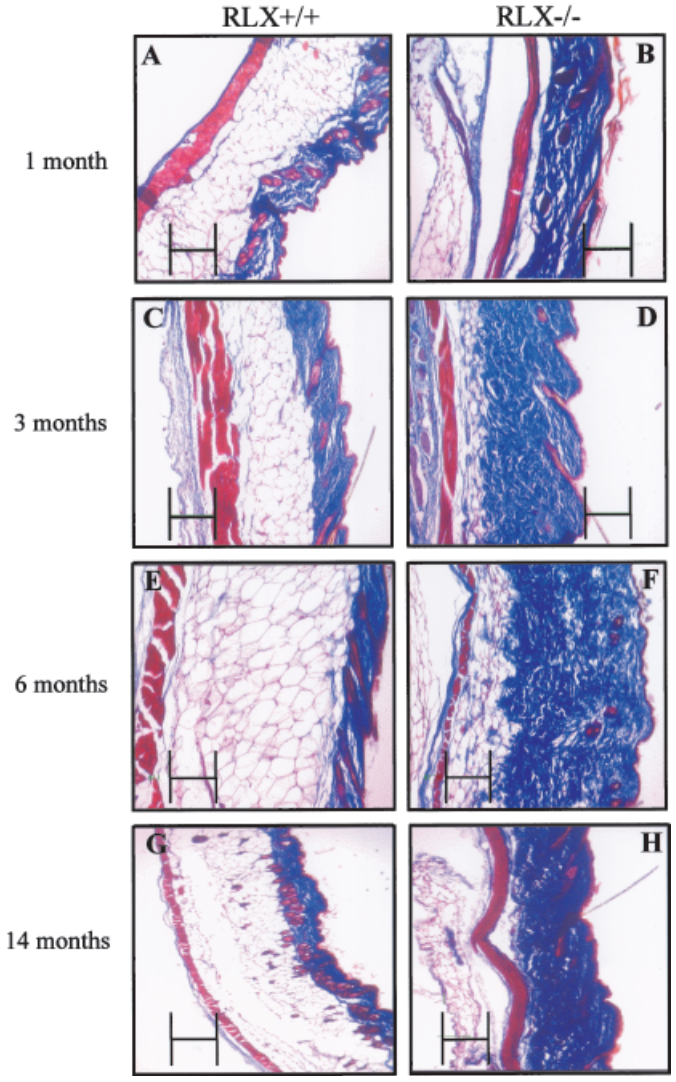

Figure 2
Effects of relaxin deficiency on skin histology. Masson trichrome staining was used to identify changes in collagen (matrix) within the dermis of 1 mo old (*A*, *B*), 3 mo old (*C*, *D*), 6 mo old (*E*, *F*) and 14 mo old (*G*, *H*) relaxin normal (RLX+/+) (*A*, *C*, *E*, *G*) and relaxin-deficient (RLX−/−) (*B*, *D*, *F*, *H*) male mice. Shown in each figure is the epidermis and dermis of the skin, the underlying subcutaneous (hypodermis) tissue and muscle. *Scale bar* = 0.1 mm.

also appeared to increase in thickness by 6 mo of age (Fig 2*E*), but lost much of its integrity by 14 mo of age (Fig 2*G*). The skin of RLX−/− mice was progressively thickened with time, caused by increased fibrosis throughout the dermis, which also appeared to supersede the hypodermis. Dermal fibrosis was evident in RLX−/− mice by 1 mo (Fig 2*B*) of age and the density of collagen fibers contributing to this fibrosis was markedly increased by 3 mo (Fig 2*D*), 6 mo (Fig 2*F*), and 14 mo (Fig 2*H*). The epidermis of RLX−/− mice appeared normal, as compared with that observed in RLX+/+ animals.

The effects of relaxin deficiency on dermal fibroblast-induced collagen secretion and deposition, *in vitro* Dermal fibroblasts from RLX−/− mice were able to deposit 38% more collagen ($p < 0.05$) into the cell matrix, over a 7-d culture period (Fig 3*A*), as compared to that measured from

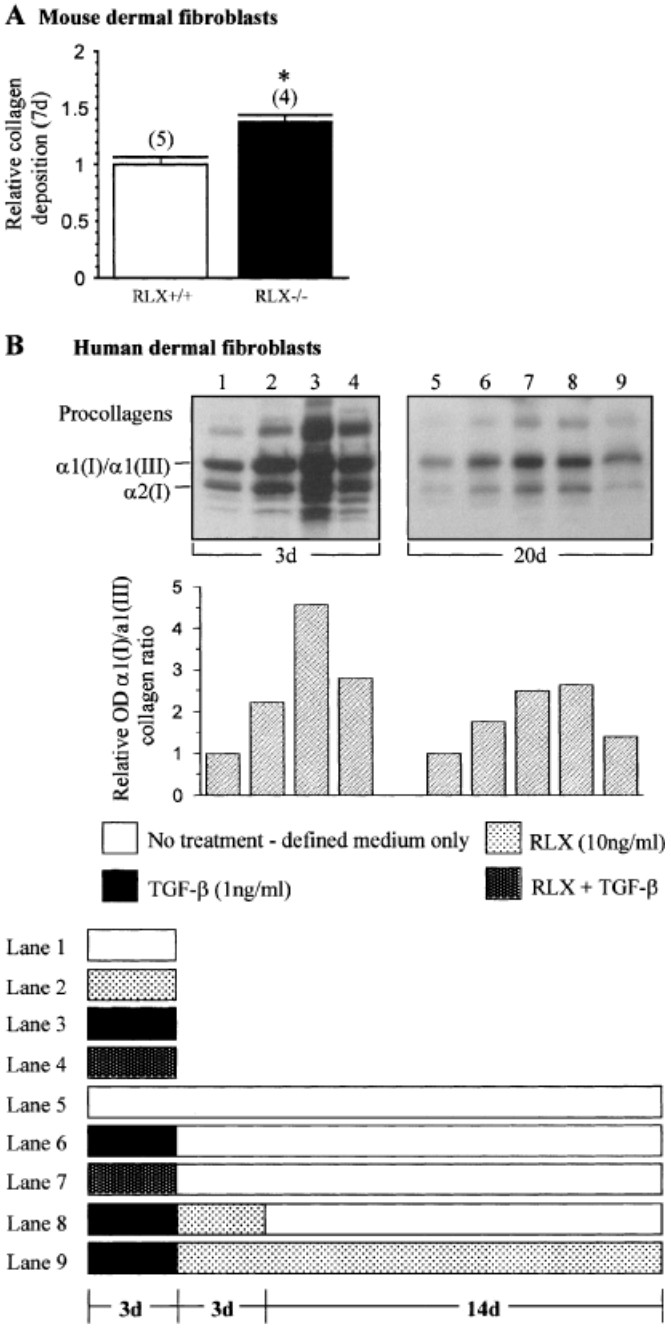

Figure 3
Effects of relaxin deficiency on dermal collagen synthesis and deposition, *in vitro*. Collagen deposition into the cell matrix was determined from dermal fibroblasts, derived from relaxin normal (RLX+/+) and relaxin-deficient (RLX−/−) mice, after 7 d in culture (*A*). Numbers in parentheses represent number of separate cultures grown from the skin of either RLX+/+ or RLX−/− mice. $^{*}p < 0.05$ *versus* corresponding value from RLX+/+ mice. Biosynthetically labelled interstitial collagen synthesis and secretion was also measured from human dermal fibroblasts after short-term or long-term exposure to recombinant human gene-2 (H2) relaxin (*B*). Cells were either treated with media alone (*lane 1*), H2 relaxin alone (10 ng per mL; *lane 2*), transforming growth factor (TGF)-β alone (1 ng per mL; *lane* 3) or TGF-β and H2 relaxin (*lane 4*) for 72-h in culture. Cells were also treated with media alone for 20 d (*lane 5*); TGF-β (1 ng per mL) for 3 d followed by media for 17 d (*lane 6*); TGF-β and H2 relaxin (10 ng per mL) for 3 d followed by media for 17 d (*lane 7*); TGF-β for 3 d, H2 relaxin for 3 d and then media for 14 d (*lane 8*); or TGF-β for 3 d followed by H2 relaxin for 17 d of culture (*lane 9*). Densitometry of the interstitial collagen bands was performed and presented as a ratio of the 3 and 20 d untreated control samples, respectively, which were expressed as 1.0.

skin fibroblasts of age-matched RLX+/+ animals, consistent with the age-related increase in dermal fibrosis measured in RLX−/− mice, *in vivo*.

Collagen secretion was markedly up-regulated by TGF-β treatment of human dermal fibroblasts (HDF), over a 3-d culture period (Fig 3*B*, *lane 3*), by 4–5-fold of that measured in untreated cultures (Fig 3*B*, *lane 1*) over the same time. In comparison, recombinant human gene-2 (H2) relaxin treatment of HDF only had a modest effect on collagen secretion (Fig 3*B*, *lane 2*), but was able to clearly downregulate the TGF-β-induced effects on collagen synthesis by up to 50%, over 3 d in culture (Fig 3*B*, *lane 4*), consistent with previous findings (Unemori and Amento, 1990). Similar findings were also obtained from a separate experiment, using the same culture conditions.

Collagen secretion was still increased by 60%–70%, when HDF were treated with TGF-β for 3 d, then removed for 17 d (Fig 3*B*, *lane 6*), as compared with that measured from HDF treated with media alone over 20 d (Fig 3*B*, *lane 5*). Collagen secretion was further increased when TGF-β-stimulated HDF were either co-incubated with recombinant H2 relaxin for 3 d of culture (Fig 3*B*, *lane 7*) or with H2 relaxin for 3 d after stimulation (Fig 3*B*, *lane 8*), before being withdrawn for a further 7–10 (data not shown) or 14–17 d of culture, respectively. In contrast, collagen synthesis and secretion were markedly inhibited by up to 50%, when HDF were stimulated with TGF-β for 3 d, followed by continuous exposure to recombinant H2 relaxin for 10 (data not shown) or 17 d (Fig 3*B*, *lane 9*). Similar findings were also obtained from a separate experiment, using the same culture conditions. These findings indicated that (i) collagen synthesis, pre-stimulated by a major pro-fibrotic factor, could only be inhibited by relaxin, when continuously exposed to human dermal fibroblasts, and (ii) removal of relaxin from human dermal fibroblast cultures, after short-term exposure, led to an increase in the collagen synthetic rate.

The effects of relaxin treatment on the skin of RLX−/− mice At 6 mo of age (when dermal fibrosis was first apparent by histology and hydroxyproline analysis), administration of H2 relaxin to RLX−/− mice resulted in the significant reversal of dermal collagen deposition back to levels measured in RLX+/+ animals (Fig 4*A*). Histologically, a marked decrease in collagen staining was observed in the dermis of H2 relaxin-treated mice (Fig 4*F*), resulting from less abundant collagen fibers that were not as tightly packed, compared to the level of staining observed in untreated RLX−/− mice (Fig 4*D*). At 12 mo of age (when dermal fibrosis was well established), H2 relaxin treatment of RLX−/− mice had no significant effects on dermal collagen concentration (Fig 4*A*) or structure over a 2 wk period, as compared with that measured in untreated and vehicle-treated RLX−/− mice.

Skin sections from 6 mo old RLX+/+ mice (Fig 4*C*) could be separated into three distinct regions, by magnetic resonance imaging (MRI): a striated lighter gray region (which corresponded to the upper part of the dermis containing hair follicles, surrounded by connective tissue/collagen sheaths (see Fig 4*B*)); a darker gray region (which corresponded to the lower part of the dermis, containing connective tissue/collagen); and a distinct light (white) region (which corresponded to the subcutaneous tissue/hypodermis containing adipose cells and fat). MRI scanning of skin sections from 6 mo old RLX−/− mice (Fig 4*E*) also revealed the two regions of the dermis. These sections, however, lacked the same intensity of the hypodermal region, consistent with Masson trichrome staining of the tissue (Fig 4*D*), which demonstrated increased collagen and decreased adipose tissue in this region of RLX−/− mice. A reduced intensity of the striation pattern of the lighter gray region of these sections (Fig 4*E*) was also consistent with increased collagen accumulation around the hair follicles. H2 relaxin treatment of RLX−/− mice resulted in the increased intensity of the hypodermal layer and the striations in the upper dermal layer (Fig 4*G*), consistent with the decrease in collagen staining that was observed by Masson trichrome staining of sequential sections (Fig 4*F*). Skin sections from 12 mo old RLX+/+ mice (Fig 4*H*) appeared thinner than those from 6 mo old RLX+/+ animals (Fig 4*C*), hence, the dermal and hypodermal layers were detected, but less well resolved, due to limited image resolution. In contrast, skin sections from 12 mo old RLX−/− mice (Fig 4*I*) were much thicker than their RLX+/+ counterparts (Fig 4*H*) and lacked the clarity and intensity of the hypodermal layer, most likely due to the increased collagen that had superseded this layer.

A marked increase ($p < 0.01$) in fibroblast differentiation (detected by α-smooth muscle actin (α-SMA); by 2.5–3-fold) was observed in skin biopsies from 6 mo old untreated RLX−/− mice, compared with that measured in RLX+/+ mice (Fig 4*J*). H2 relaxin treatment of RLX−/− mice was able to normalize α-SMA expression ($p < 0.01$ *vs* levels from untreated RLX−/− mice), to that observed in RLX+/+ mice (Fig 4*J*), consistent with its ability to decrease collagen concentration (Fig 4*A*) and staining (Fig 4*F*).

Discussion

In this study, we demonstrate that mice lacking the relaxin gene over a lifetime, which encodes the major stored and circulating form of relaxin, develop an age-related progression of dermal fibrosis and thickening. The dermis of male and female RLX−/− mice was associated with marked increases in interstitial collagen over time, from as early as 1 mo of age. Dermal fibroblasts from RLX−/− mice were also shown to deposit increased collagen into the cell matrix *in vitro*. Recombinant H2 relaxin treatment of RLX−/− mice resulted in the complete reversal of dermal fibrosis, when applied to early stages of disease, but was ineffective when applied to more established stages of dermal scarring over a 2 wk treatment period. Relaxin was able to markedly inhibit TGF-β-induced collagen synthesis and secretion *in vitro*, only when continuously exposed to human dermal fibroblasts, whereas collagen secretion was again elevated when relaxin was removed from cell cultures, after short-term exposure. Furthermore, MRI scanning of skin biopsies from RLX+/+ and RLX−/− mice treated with or without H2 relaxin demonstrated a number of structural changes within the skin, which correlated to changes observed by histology. These combined findings support our previous observations that relaxin is a naturally occurring inhibitor of

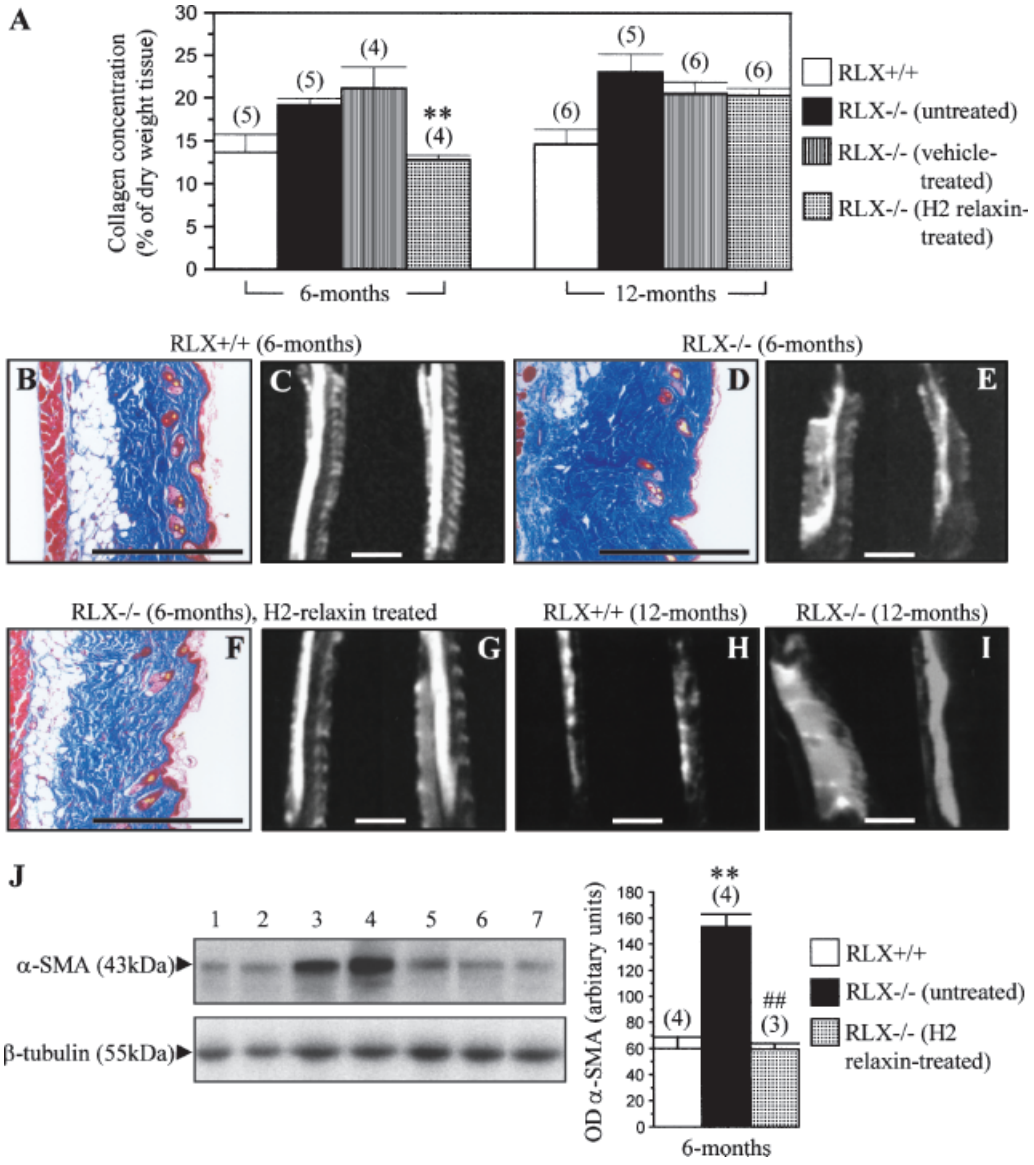

Figure 4

Effects of recombinant human gene-2 (H2) relaxin treatment on the dermis of relaxin deficient mice. Collagen concentration was determined from the skin of relaxin normal (RLX+/+) mice, untreated relaxin-deficient (RLX−/−) mice and RLX−/− mice treated with either vehicle alone or with 0.5 mg per kg per day recombinant H2 relaxin (over 14 d), at 6 and 12 mo of age (*A*). Numbers in parentheses represent the number of samples analyzed per group. $^{**}p<0.01$, when compared with corresponding values from untreated and vehicle-treated RLX−/− mice. Masson trichrome staining of collagen within the dermis and magnetic resonance imaging (MRI) scans of two representative skin sections from separate 6 mo old RLX+/+ (*B* and *C*, respectively), untreated RLX−/− (*D* and *E*, respectively) and recombinant H2 relaxin-treated RLX−/− (*F* and *G*, respectively) mice were used to detect and compare changes in skin architecture, before and after relaxin treatment. MRI scans of two representative skin sections from 12 mo old RLX+/+ (*H*) and RLX−/− (*I*) mice were also used for comparative analysis. *Scale bar* = 0.1 mm. Western blotting of α-smooth muscle actin (α-SMA) (*J*), a marker for myofibroblast accumulation, was conducted on protein extracts (10 μg per sample), from the skin of 6 mo old RLX+/+ (*lanes 1–2*), RLX−/− (*lanes 3–4*) and H2 relaxin-treated RLX−/− (*lanes 5–7*) mice. Shown is a representative blot of n = 3–4 samples per group. Western blots of the housekeeping protein, β-tubulin was also performed to demonstrate equal loading of the protein samples, whereas densitometry scanning of the mean ± SEM optical density values of α-SMA are also shown (*J*). $^{**}p<0.01$ *versus* corresponding values from RLX+/+ mice; $^{\#\#}p<0.01$ *versus* corresponding values from untreated RLX−/− mice.

collagen accumulation (in several organs including the skin) *in vivo* and *in vitro*, by altering key matrix molecules, matrix degrading enzymes and growth factors. Relaxin-null mice offer the first direct evidence that links relaxin to a generalized alteration of collagen turnover in normal skin.

In addition to the findings presented in this study, we had previously demonstrated that RLX−/− mice undergo an age-related progression of interstitial fibrosis in the lung (Samuel *et al*, 2003d), heart (Du *et al*, 2003), and kidneys (Samuel *et al*, 2004b), leading to organ damage and dysfunction. The lungs of RLX−/− mice were associated with marked increases in collagen content and concentration, resulting in altered alveolar structure, lung function and increased bronchiole epithelium thickening (Samuel *et al*, 2003d). The heart of RLX−/− mice underwent an increase in atrial hypertrophy, impeded left ventricular (LV) diastolic filling and venous return, which was attributed to progressive increases in LV collagen content and ventricular chamber stiffness (Du *et al*, 2003). The kidneys of ageing RLX−/− mice were also associated with focal increases in

interstitial fibrosis and a more general diffuse increase in glomerulosclerosis, resulting in increased cortical thickening and reduced renal function (Samuel *et al*, 2004b). Additionally, small renal arteries isolated from RLX−/− animals were less compliant and had significantly reduced myogenic reactivity, as compared with renal arteries from RLX+/+ mice (personal communication: Dr Jacqueline Novak, Dr Laura J. Parry, and Professor Kirk P. Conrad). These combined findings demonstrate that RLX−/− mice undergo certain symptoms of systemic sclerosis, characterized by increased fibrosis of the dermis and internal organs, in addition to altered blood vessel integrity. To date however, the inflammatory and immunological deficiencies that contribute to scleroderma in this model are yet to be demonstrated. Like several other animal models of scleroderma involving induced and/or spontaneous systems (Bocchieri and Jiminez, 1990), the RLX−/− mouse only mimics certain aspects of scleroderma, but along with all other available models, does not mimic the entire spectrum of this complex disorder. The RLX−/− mouse can also be compared to the most extensively used model of scleroderma, the TSK/+ mutant mouse, which also demonstrates fibrosis of the skin and internal organs but is absent of inflammatory, immunological, vascular, gastrointestinal and articular involvement (Bocchieri and Jiminez, 1990).

Although scleroderma affects women more than men, our findings in RLX−/− mice demonstrated that male mice developed a more rapid progression of dermal fibrosis and also consistently developed an age-related progression of pulmonary, cardiac and renal fibrosis. Whereas female RLX−/− mice developed fibrosis in some organs (skin, lung), the severity and onset of disease was less pronounced and delayed, respectively, whereas in other organs (heart, kidney), they did not demonstrate any changes in collagen accumulation, as compared to that measured in RLX+/+ animals. Thus, the phenotype differences observed between male and female RLX−/− mice may be the result of using a genetically modified model, which have been reported to cause some organs to display male gender-restricted or -biased phenotypes (Du, 2004). Alternatively, the presence of androgens in males may have detrimental consequences on the progression of fibrosis or other female-specific hormones or sex steroids may be compensating for the absence of relaxin in female RLX−/− mice. This is consistent with the fact that hormones, such as estrogen, have a profound effect on the ageing skin (Sator *et al*, 2004) and are known to increase collagen biosynthesis by human skin fibroblasts (Surazynski *et al*, 2003).

Remarkably, recombinant H2 relaxin treatment of 6 mo old RLX−/− mice (with an early onset of dermal fibrosis) resulted in the significant reduction and normalization of dermal fibroblast function and collagen concentration to that observed in age-matched RLX+/+ mice. In contrast, H2 relaxin had no significant effects on established dermal fibrosis when administered to 12 mo old RLX−/− mice, despite its ability to significantly inhibit established pulmonary, renal and cardiac fibrosis by 40%–70% at this time point (Samuel *et al*, 2003d, 2004a, 2004b). The increased viscosity of the dermis, compared to the other organs and/or permanent damage to the internal structure of the skin, most likely explains why relaxin may not been responsive at this time point. These findings demonstrate that there is a narrow window within which relaxin can successfully be used as an anti-fibrotic therapy for dermal scarring. These findings are of particular importance, given that clinical trials on recombinant H2 relaxin's effects on systemic sclerosis recently demonstrated that relaxin was biologically active and well tolerated in humans (Erikson and Unemori, 2001). Although relaxin treatment benefited only some individuals, the primary efficacy of relaxin as an antifibrotic agent was not met in phase II/III of these trials, due to the addition of several patients with end-stage scleroderma that appeared to be untreatable. Our studies also demonstrate that the potency and efficacy of relaxin as an antifibrotic therapy is diminished, when applied to more severe stages of dermal fibrosis.

Relaxin has been shown to act at multiple levels to decrease collagen accumulation in several organs, including the skin (Unemori and Amento, 1990; Unemori *et al*, 1993). Although limited studies have demonstrated that relaxin is able to bind to endothelial cells (Quattrone *et al*, 2004) and adipocytes (Bani-Sacchi *et al*, 1987), our studies demonstrate that relaxin acts directly on dermal fibroblasts to inhibit their differentition, which would otherwise lead to the over-production of collagen (and other matrix proteins) in the skin. This is consistent with the finding that H2-relaxin treatment of RLX−/− mice inhibited dermal collagen secretion and deposition to levels observed in RLX+/+ mice. Notably, the re-appearance of the hypodermal layer, post-H2 relaxin treatment, was most likely due to the reduction of myofibroblast and collagen expression, rather than to direct affects of relaxin on adipocytes and/or other cell types.

In this study, we also employed MRI as a potential non-invasive diagnostic tool to detect changes in the skin architecture that were associated with dermal fibrosis. For these studies, we used fixed samples from 6 and 12 mo old mice. MRI scans of skin biopsies from RLX+/+ mice were able to detect distinct regions of the skin, which corresponded to the dermal and hypodermal layers of the tissue. The distinctive feature of the MRI scans was the detection of the hypodermal layer (containing adipose tissue/fat), which appeared as a white/light band with high intensity. In RLX−/− mice, the increased fibrosis was characterized by the loss of intensity of this white band and a progressive increase in dermal thickening with age, which matched what was observed by histology. Importantly, in biopsies from recombinant H2 relaxin-treated (6 mo old) mice, the restoration of this white band (hypodermis) and the striations within the upper dermal layer to that observed in biopsies from RLX+/+ mice was consistent with histological analysis of the tissue. These findings suggested that the MRI may be used a potential diagnostic tool to detect the onset of dermal fibrosis in animals and humans, but requires further optimization. Scanning the progression of disease in live animals is also the subject of future investigation.

In conclusion, we have demonstrated that the removal of the relaxin gene from mice resulted in a progressive build-up of collagen in the skin, resulting in dermal fibrosis and thickening. Furthermore, relaxin may provide an important

means to regulate excessive collagen deposition in skin disorders associated with or characterized by fibrosis.

Materials and Methods

Reagents Recombinant H2 relaxin was generously provided by BAS Medical (San Mateo, California) and is bioactive in mice (Unemori *et al*, 1996; Samuel *et al*, 2003d, 2004a, b).

Animals All male and female relaxin wild-type (RLX+/+) and RLX−/− mice used in this study were age-matched litter-mates, generated from RLX+/− (C57Blk6J × 129SV) parents (Zhao *et al*, 1999). The animals were housed in a controlled environment and maintained as described previously (Samuel *et al*, 2003d; 2004b). These experiments were approved by the Howard Florey Institute and Molecular Medicine Research Institute Animal Experimental Ethics Committees, which adhere to the Australian and NIH Codes of Practice, respectively, for the care and use of laboratory animals for scientific purposes.

Tissue collection Male and female RLX+/+ and RLX−/− mice were obtained at 1, 6, and 12 mo of age, whereas additional mice were obtained at 3 and 14 mo of age (n = 8–12 per genotype, gender, and age-group). All mice were weighed, before being euthanized and shaved for tissue collection. Skin biopsies (12 mm in diameter) were taken from the same location, off the back of each mouse for analysis. Tissues were then either stored at −80°C for hydroxyproline, SDS-PAGE, and Western blot analyses or fixed in 10% formalin for histological and MRI analysis.

Hydroxyproline analysis of skin biopsies The collected skin biopsies from male and female RLX+/+ and RLX−/− mice were lyophilized to dry weight, before being re-hydrated, hydrolyzed with 6 M hydrochloric acid, and treated as described previously (Samuel *et al*, 1996). Hydroxyproline values were then converted to collagen content by multiplying by a factor of 6.94 (Gallop and Paz, 1975), whereas collagen concentration was calculated by dividing the collagen content by the tissue dry weight.

Determination of collagen types in the skin Skin biopsies from male and female 1 and 12 mo old RLX+/+ and RLX−/− mice (n = 4–5 per genotype and gender) were separated and finely diced in the presence of liquid nitrogen. The pepsin-digested collagen was then extracted and analyzed by SDS-PAGE, as described before (Samuel *et al*, 1996). Densitometric analysis of the interstitial collagen chains was then performed for quantitatative studies, as described before (Samuel *et al*, 2003b).

Histology of skin biopsies Fixed skin biopsies from male and female 1, 3, 6, and 14 mo old RLX+/+ and RLX−/− mice (n = 4 per genotype) were washed, processed, paraffin embedded, and cut as described before (Samuel *et al*, 2004b). The sections were cut such that the epidermis, dermis, underlying subcutaneous (adipose) tissue and muscle could be observed. Serial sections from each tissue were stained with hematoxylin and eosin (H&E) and for collagen, with the Masson trichrome stain. The stained slides were viewed and analyzed as described previously (Samuel *et al*, 2004b). Several tissue sections were screened with each stain and a representative slide chosen for figure presentation.

Human recombinant relaxin treatment of RLX−/− mice 5.5 mo (n = 8) and 11.5 mo old (n = 12) male RLX−/− mice, with early and established dermal fibrosis, respectively, were anesthetized and subjected to subcutaneous implantation of osmotic mini-pumps (model 2002; Alza, Cupertino, California) as described previously (Samuel *et al*, 2003d). The osmotic mini-pumps were loaded with either a 20 mM sodium acetate buffer, pH 5.0 (vehicle; n = 10) or 0.5 mg per kg per day (20 μg per day) recombinant H2 relaxin (in sodium acetate buffer; n = 10), which were maintained for 14 d. The dose of recombinant H2 relaxin added was previously shown to produce circulating levels of 20–40 ng per mL after 14 d of treatment and to successfully treat cardiac, pulmonary, and renal fibrosis in 12 mo old, male RLX−/− mice (Samuel *et al*, 2003d; 2004a, b). After 14 d, the animals were euthanized and skin biopsies collected for various analyses.

MRI of mouse skin Fixed skin tissues from male 6 and 12 mo old RLX+/+ and RLX−/− mice and from 5.5 mo old RLX−/− mice treated with recombinant H2 relaxin for 14 d (n = 3–4 per group) were scanned with a 4.7T MR BIOSPEC system (Bruker, Germany). Multiple skin samples were placed on a specially designed sample holder with multi-layer plastic sheets. A total number of 10 samples could be scanned simultaneously using a T_1-weighted spin-echo sequence with TR/TE equal to 800/26.1 ms and scanning time equal to 14 h 34 min for 128 NEX. Sixteen sagittal slices were acquired providing cross-sectional view of all skin samples in each image. A high-resolution imaging matrix of 512 × 512 with a field of view (FOV) of 20 mm along the frequency direction, 13.5 mm along the phase encoding direction and 0.5 mm slice thickness was used. The phase encoding direction was perpendicular to the skin to avoid a chemical-shift effect of the subcutaneous (adipose) layer underneath the skin (dermis). The image resolution was 26 and 39 μm in a perpendicular and parallel direction to the skin layer, respectively.

Western blotting Protein extracts from skin biopsies of 6 mo old male RLX+/+, untreated RLX−/−, and H2 relaxin-treated RLX−/− mice (n = 3–4 per group) were analyzed by Western blotting, for the detection of α-SMA (a marker for fibroblast differentiation), as previously described (Samuel *et al*, 2004a). A monoclonal antibody to α-SMA (M0851, 1:1000 dilution) was commercially purchased (Dako Corporation, Carpinteria, California), whereas a monoclonal antibody to the housekeeping protein, β-tubulin (kindly provided by Dr Zhonglin Chai, Baker Heart Research Institute, Victoria, Australia) was also used to demonstrate equal loading of the protein samples. Densitometric analysis of α-SMA was then performed as described above.

Cell culture

Mouse dermal fibroblast cultures To investigate the effects of relaxin-deficiency on fibroblast-induced collagen deposition *in vitro*, dermal fibroblasts from the skin of adult (4 mo old) RLX+/+ and RLX−/− mice, were isolated using standard collagenase digestion (Unemori and Amento, 1990) and used between passages two and five for these studies. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing Hams F12 medium (1:1 ratio), 15% fetal calf serum and the antibiotics, penicillin (75 U per mL) and streptomycin (75 μg per mL). Five separate fibroblast cultures from RLX+/+ mice and four separate cultures from RLX−/− mice were plated at a density of 2×10^6 cells per 60 mm^2 dish and grown for a 7 d period, before the media discarded and the cell layer collected and analyzed for hydroxyproline content (as a measure of the deposited collagen into the cell layer), as described above.

HDF cultures To investigate the short-term and long-term effects of relaxin on collagen secretion *in vitro*, human dermal fibroblasts (which were generously provided by Dr Elaine Unemori; Connetics Corporation, Palo Alto, California), were maintained in DMEM, as described before (Unemori and Amento, 1990) and used between passages ten and 14. For short-term studies, the fibroblasts (1×10^5 cells per well; 24-well plate) were either incubated with media alone, recombinant H2 relaxin (10 ng per mL) alone, TGF-β (1 ng per mL) alone or recombinant H2 relaxin (10 ng per mL), and TGF-β (1 ng per mL) for a 72 h culture period. For longer-term studies, fibroblasts were incubated with (i) media alone for 20 d, (ii) TGF-β (1 ng per mL) for 3 d, then medium for 17 d, (iii) TGF-β (1 ng per mL), and recombinant H2 relaxin (10 ng per mL) for 3 d, then medium for 17 d, (iv) TGF-β (1 ng per mL) for 3 d followed by recombinant H2 relaxin (10 ng per mL) for 3 d, then media for 14 d,

or (v) TGF-β (1 ng per mL) for 3 d followed by recombinant H2 relaxin (10 ng per mL) treatment for 17 d. Collagen biosynthesis was then determined by labelling cells with [^{3}H]-proline (25 μCi per mL) as described before (Unemori and Amento, 1990), and quantified by densitometry scanning of the α1(I)/α1(III) collagen bands from each sample, using Kodak (Rochester, New York) 1D imaging software. The data from each treatment group was presented as a ratio of the 3 and 20 d untreated control samples, respectively, which were expressed as 1.0.

Statistical analysis The results were analyzed using a one-way ANOVA, using the Newman–Keuls test for multiple comparisons between groups. All data in this paper are presented as the mean ± SEM, with $p < 0.05$ described as statistically significant.

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