Anti-fibrotic actions of relaxin

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Abstract

Fibrosis refers to the hardening or scarring of tissues that usually results from aberrant wound healing in response to organ injury, and its manifestations in various organs have collectively been estimated to contribute to around 45-50% of deaths in the Western world. Despite this, there is currently no effective cure for the tissue structural and functional damage induced by fibrosis-related disorders. Relaxin meets several criteria of an effective anti-fibrotic based on its specific ability to inhibit pro-fibrotic cytokine and/or growth factor-mediated, but not normal/unstimulated fibroblast proliferation, differentiation and matrix production. Furthermore, relaxin augments matrix degradation through its ability to up-regulate the release and activation of various matrix-degrading matrix metalloproteinases and/or being able to down-regulate tissue inhibitor of metalloproteinase activity. Relaxin can also indirectly suppress fibrosis through its other well-known (anti-inflammatory, anti-oxidant, anti-hypertrophic, anti-apoptotic, angiogenic, wound-healing and vasodilatory) properties. This review will outline the organ-specific and general anti-fibrotic significance of exogenously-administered relaxin, and its mechanisms of action, that have been documented in various non-reproductive organs such as the cardiovascular system, kidney, lung, liver, skin and tendons. In addition, it will outline the influence of sex on relaxin’s anti-fibrotic actions, highlighting its potential as an emerging anti-fibrotic therapeutic.
Abbreviations

Akt, protein kinase B; α-SMA, α-smooth muscle actin; Ang II, angiotensin II; ACE, angiotensin converting enzyme; AT₂R, angiotensin type 2 receptor; bFGF, basic fibroblast growth factor; cAMP, cyclic adenosine monophosphate; CCL₄, carbon tetrachloride; cGMP, cyclic guanosine monophosphate; COPD, chronic obstructive pulmonary disease; ET-1, endothelin-1; ECM, extracellular matrix; FEV₁, forced expiratory volume in one second; FVC, forced vital capacity; GPCR, G protein-coupled receptor; HSC, hepatic stellate cell; INSL, insulin-like; KO, knockout; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; NO, nitric oxide; NOS, nitric oxide synthase; PDGF, platelet-derived growth factor; PGC₁α, PPARγ coactivator protein 1α; PKA, protein kinase A; PMA, phorbol 12-myristate 13-acetate; PPAR, peroxisome proliferator-activated receptor; RLN, relaxin gene; rhRLX, synthetically- or recombinantly-produced drug form of relaxin; RXFP1, relaxin family peptide receptor 1; TGF-β₁, transforming growth factor-β₁; TIMP, tissue inhibitor of metalloproteinase; UUO, unilateral ureteric obstruction; VEGF, vascular endothelial growth factor; WHO, World Health Organization.

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Introduction

The tissue response to stress depends on a number of intrinsic and extrinsic factors including the length and extent of injury. When injury or disease is mild or transient, then the tissue response leads to remodelling or regeneration of the organ parenchyma. However, if the injury is either severe or prolonged, the process is characterised by ongoing inflammation and extracellular matrix (ECM) production. Fibrosis is therefore a failure of the wound healing process, where ECM synthesis is ongoing (Hewitson, 2009; Wynn, 2008). This maladaptive response is particularly dependent on paracrine and autocrine production of transforming growth factor (TGF)-β1, and the consequent recruitment of activated fibroblasts (myofibroblasts). Overabundant ECM production and failure to resolve leads to significant organ damage and dysfunction, which depends not only on the quantity of matrix produced (fibrogenesis), but also the degree of its cross-linking and its reorganisation, or density.

Despite the almost universal significance of fibrosis (Hewitson, 2009; Wynn, 2008), there is a lack of effective anti-fibrotic therapeutic strategies. A growing body of evidence
suggest that relaxin may fulfil this need. This review will focus on its anti-fibrotic effects and mechanism of action in tissues of the circulatory, renal, hepatic and integumentary systems. Moreover, the influence of sex on fibrosis progression will be discussed.

Relaxin

Relaxin is a member of a family of peptide hormones that is structurally similar to insulin, but which diverged from insulin early in vertebrate evolution to form a distinct peptide family based on their two-chain structures, receptor binding motif and ability to bind and activate G protein-coupled receptors (GPCRs) (refer to pp 5846-7 in Alexander et al., 2015b; Bathgate et al., 2006), as opposed to the tyrosine kinase receptors that are activated by insulin and the insulin-like growth factors. The relaxin peptide family is encoded by seven genes in humans, which include the relaxin genes $RLN1$, $RLN2$ and $RLN3$, as well as the insulin-like peptide genes $INSL3$, $INSL4$, $INSL5$ and $INSL6$ (Bathgate et al., 2006). In most other species though, only 5 of these genes exist, including $RLN1$ and $RLN3$, the species equivalents of human $RLN2$ and $RLN3$, respectively. The product of the human $RLN2$ gene (H2 relaxin) and its species equivalent gene (relaxin) represent the major stored and circulating forms of relaxin in their respective species, and will be the forms of relaxin primarily discussed in this review. Recombinant human relaxin (rhRLX; now also referred to as serelaxin) is the drug-based form of H2 relaxin, which will also be discussed throughout the review.

Anti-fibrotic effects of relaxin in the cardiovascular system
Fibrosis is a hallmark of almost all forms of cardiovascular disease and a key contributor to atrial, ventricular and vascular stiffness, impaired cardiac contractility and heart failure (Frieler et al., 2015; Ling et al., 2015; Mandavia et al., 2013). A common fibrotic process develops from a number of cardiac pathologies including ischemic injury and myocardial infarction; hypertrophic, dilated and restrictive cardiomyopathies; valvular heart diseases; hypertension; diabetes; metabolic disorders; and cardiac arrhythmia amongst others.

Continuous exogenous administration of rhRLX, human gene-3 relaxin (H3 RLX) or mouse relaxin (mRLX) to pro-fibrotic factor-stimulated cardiac fibroblasts in vitro (at 100ng/ml over 1-5 days) or various experimental models of cardiovascular disease in vivo (at 0.1mg/kg/day-0.5mg/kg/day over 2-4 weeks; where 0.5mg/kg/day produces circulating levels of ∼20-40ng/ml) has effectively reduced the cardiac fibrosis associated with each model investigated (Table 1).

The anti-fibrotic effects of rhRLX have consistently been demonstrated in various pre-clinical models of cardiovascular disease and heart failure in vivo, regardless of etiology, including models of ageing (Samuel et al., 2007; Samuel et al., 2004), fibrotic cardiomyopathy (Chan et al., 2012; Samuel et al., 2014), myocardial infarction (Bonacchi et al., 2009; Formigli et al., 2007; Samuel et al., 2011), hypertension (Gu et al., 2012; Lekgabe et al., 2005), type 1 diabetes (Samuel et al., 2008) and atrial fibrillation (Henry et al., 2015; Parikh et al., 2013) (Table 1). Furthermore, rhRLX (0.5mg/kg/day) was found to have improved anti-fibrotic efficacy over the clinically used ACE inhibitor, enalapril (48mg/kg/day) in an experimental model of fibrotic cardiomyopathy, but also augmented the anti-fibrotic efficacy of enalapril when both treatments were administered 10 days after the
onset of injury (Samuel et al., 2014). Likewise, both H3 RLX (Hossain et al., 2011a) and mRLX (Bathgate et al., 2008; Samuel et al., 2007) have also demonstrated similar anti-fibrotic efficacy to that of hRLX in murine models of cardiomyopathy induced by transgenic over-expression of β2-adrenoreceptors. All forms of relaxin studied to date mediate their anti-fibrotic actions by inhibiting the effects of various pro-fibrotic factors (discussed below) rather than by directly regulating collagen or other ECM proteins per se, and rhRLX demonstrated greater anti-fibrotic efficacy over enalapril due to its greater ability to inhibit both TGF-β1 expression and its signal transduction at the level of intracellular Smad2 phosphorylation (Samuel et al., 2014). On the other hand, rhRLX did not affect chronic pressure overload-induced cardiac hypertrophy or fibrosis that was associated with biochemical wall stress rather than elevated TGF-β1 levels (Xu et al., 2008).

Relevant mechanisms of the anti-fibrotic actions of relaxin

Several studies conducted at the in vitro level have offered more in-depth insights into the mechanisms and signal transduction pathways associated with relaxin’s anti-fibrotic actions in the diseased myocardium, primarily involving rhRLX. rhRLX (and H3 RLX (Hossain et al., 2011a)) acting via RXFP1 specially inhibits cardiac fibroblast proliferation and differentiation into activated myofibroblasts and hence, myofibroblast-mediated aberrant collagen deposition (the basis of fibrosis) when cells are stimulated with either angiotensin II (Ang II) (Gu et al., 2012; Samuel et al., 2004), TGF-β1 (Samuel et al., 2004; Sassoli et al., 2013; Squecco et al., 2015), phorbol 12-myristate 13-acetate (PMA) (Wang et al., 2014) or
high glucose (Su et al., 2014; Wang et al., 2009). The ability of rhRLX to inhibit TGF-β1-mediated cardiac fibrosis progression was shown to involve activation of the Notch-1 and nitric oxide (NO) pathways, and down-regulation of Smad2 (Samuel et al., 2014) and/or Smad3 (Sassoli et al., 2013) phosphorylation. This collectively results in the rhRLX-induced inhibition of the pro-fibrotic actions of TGF-β1 on myofibroblast differentiation and aberrant ECM/collagen deposition.

Furthermore, by suppressing the TGF-β1/Smad2 and/or TGF-β1/Smad3 axes, which themselves promote fibrosis by inhibiting the actions of matrix-degrading matrix metalloproteinases (MMPs) and promoting tissue inhibitor of metalloproteinase (TIMP) activity (which are natural inhibitors of MMPs) (Spinale et al., 2014), rhRLX releases and promotes various MMPs (including MMP-1 and its rodent orthologue MMP-13, MMP-2 and MMP-9) (Formigli et al., 2007; Lekgabe et al., 2005; Parikh et al., 2013; Samuel et al., 2014; Samuel et al., 2011; Samuel et al., 2008; Samuel et al., 2004; Sarwar et al., 2015), and/or decreases both cardiac TIMP-1 (Samuel et al., 2008) and TIMP-2 (Sassoli et al., 2013) activity; which would likely result in the degradation of existing collagen fibers. Hence, relaxin mediates its anti-fibrotic effects in the heart by suppressing aberrant pro-fibrotic cytokine-induced ECM/collagen deposition, while also being able to promote MMP-induced ECM/collagen breakdown (Figure 1).

rhRLX may also indirectly inhibit cardiac fibrosis through its anti-inflammatory (Nistri et al., 2003; Nistri et al., 2008; Perna et al., 2005), anti-oxidant (Perna et al., 2005), anti-hypertrophic (Dschietzig et al., 2005; Moore et al., 2007; Parikh et al., 2013) and anti-apoptotic (Bonacchi et al., 2009; Moore et al., 2007; Samuel et al., 2011; Zhang et al., 2015)
actions, while being able to promote tissue repair via its angiogenic (Formigli et al., 2007; Samuel et al., 2011; Segal et al., 2012), vasodilatory (Conrad et al., 2011; McGuane et al., 2011a; McGuane et al., 2011b) and wound-healing (Mu et al., 2010) properties. However the effects of rhRLX have largely been shown to be independent of blood pressure regulation (Du et al., 2010; Samuel et al., 2014; Samuel et al., 2008). These combined effects of rhRLX, along with its direct anti-fibrotic actions, have resulted in its ability to improve cardiac output and ventricular performance (Perna et al., 2005; Teichman et al., 2008), while being able to reduce cardiac contractility and ventricular stiffness (Du et al., 2010; Samuel et al., 2008) as well as atrial (Parikh et al., 2013) and ventricular (Nistri et al., 2008) arrhythmias.

**Anti-fibrotic actions of relaxin in the kidney**

The close association between cardiovascular pathology and renal dysfunction are well documented and significant. Patients with conventional risk factors for cardiovascular disease also suffer renal dysfunction. The pathology of the heart and kidney are therefore inexorably linked (Hewitson et al., 2015).

Early studies showed that continuous 2-to-4 week-infusion of rhRLX with osmotic mini-pumps ameliorated progressive fibrosis in several experimental rodent models of fibrosis including bromoethylamine-induced renal papillary necrosis (Garber et al., 2001), experimental anti-glomerular basement membrane nephritis (McDonald et al., 2003), and reduction of renal mass by either surgical ablation or infarction (Garber et al., 2003). The last two models are particularly insightful because they showed a decrease in hypertension in the
infarction model, but no change in blood pressure in the normotensive ablation model, suggesting that structural-functional effects can be independent of changes in blood pressure (Garber et al., 2003). Also key is the observation that systemic administration of rhRLX concurrently limits both cardiac and renal fibrosis in the spontaneously hypertensive rat, indicating that rhRLX may simultaneously ameliorate similar pathologies in multiple organs (Lekgabe et al., 2005). Treatment of acute renal injury with rhRLX also limited loss of structure and function after ischemia-reperfusion (Yoshida et al., 2013) and cisplatin exposure (Yoshida et al., 2014).

However, rhRLX may not be universally renoprotective, as it did not prevent diabetic renal complications in mouse models of type I diabetes (Dschietzig et al., 2015; Wong et al., 2013), when TGF-β1 was not elevated in either model. Importantly in a phase II scleroderma trial (Khanna et al., 2009), cessation of rhRLX after 24 weeks of administration resulted in a significant decline in renal function, as estimated by creatinine clearance (Khanna et al., 2009). It is unclear if this was related to scleroderma-specific factors.

Establishing that pre-treatment with rhRLX can slow progression is an important proof of principle, but the reality is that most renal patients present clinically with established fibrosis. It is those studies in established renal disease that will provide the greatest clinical insight.

Relevant pleiotropic actions of relaxin
The pleiotropic properties of rhRLX can also indirectly limit renal fibrosis and promote wound healing by stimulating NO production (Chow et al., 2012; Chow et al., 2014; Mookerjee et al., 2009; Sasser et al., 2011), improving the local environment through maintaining glomerular filtration rate (Conrad, 2004), preventing parenchymal cell loss through apoptosis (Yoshida et al., 2013), and enhancing cell survival through maintaining vascular supply via angiogenesis (Hewitson et al., 2010; Unemori et al., 2000). Its recognised direct cellular actions in the kidney include a reduction in inflammation (Yoshida et al., 2014), oxidative stress (Sasser et al., 2011) and ECM synthesis (fibrogenesis) and contraction (Masterson et al., 2004). There is also circumstantial evidence to suggest that a rhRLX-mediated increase in activity of MMP-2, MMP-9 or MMP-1 (and its analogue MMP-13 in the rodent) may directly limit renal scarring by increasing collagen degradation (Figure 1). Nevertheless, the spatial and temporal context of these collagenases are important as degradation of collagen IV can limit fibrosis by removing excess collagen, and in other circumstances, exacerbate injury through destruction of basement membranes. An MMP-induced vasodilation occurs through ET1-32 production (Jeyabalan et al., 2003), which again may improve wound repair and the environment in which the kidney functions.

rhRLX may also indirectly reduce fibrosis by augmenting repair with mesenchymal stem cells (MSCs). A combination of rhRLX and bone marrow-derived MSCs abrogated kidney fibrosis after 7 days of UUO (Huuskes et al., 2015). This was accompanied by reduced tissue damage, and enhanced MMP-2 activity compared with either treatment alone, while rhRLX also directly increased MSC proliferation and migration (Huuskes et al., 2015).
**Signal transduction pathways of the anti-fibrotic actions of rhRLX**

Extensive cell culture studies using rat renal myofibroblasts have provided mechanistic insight into rhRLX’s anti-fibrotic actions. rhRLX inhibits myofibroblast differentiation and collagen synthesis, and promotes MMP expression/activity by myofibroblasts through binding to its cognate receptor, RXFP1 (Mookerjee *et al.*, 2009). Recent studies also suggest that heterodimer formation between RXFP1 and other GPCRs, in particular angiotensin receptors, may be necessary (Chow *et al.*, 2014). Regardless, subsequent signal transduction through a nNOS-NO-cGMP pathway appears to be central to the anti-fibrotic actions of rhRLX in renal myofibroblasts. More specifically rhRLX inhibits phosphorylation of Smad2 in both human (Heeg *et al.*, 2005) and rat renal (myo)fibroblasts (Mookerjee *et al.*, 2009). These effects are only partially dose-dependent, with robust effects seen at physiological concentrations (Mookerjee *et al.*, 2009). Importantly, the anti-fibrotic actions of rhRLX may well be contingent on TGF-β1, which may explain the lack of efficacy in models without aberrant TGF-β1 expression (Dschietzig *et al.*, 2015; Wong *et al.*, 2013).

**The influence of sex on the anti-fibrotic actions of relaxin**

There are sex-differences in the incidence, prevalence and progression of cardiovascular and renal disease, with males being at greater risk than females prior to menopause (Silbiger and Neugarten, 2008; Barrett-Connor, 2013). For example, it has been demonstrated that cardiac fibrosis associated with left ventricular hypertrophy due to aortic stenosis is greater in men compared with women (Petrov *et al.*, 2014). This data suggests that there are sex-differences in the adaptation to pressure overload. Indeed, this greater cardiac fibrosis was associated
with higher cardiac tissue levels of TGF-β1, Smad2 phosphorylation, and higher fibrosis related gene expression of collagens I and III, MMP-2 and MMP-9 in men than women (Petrov et al., 2010; Petrov et al., 2014). Similarly, in chronic kidney disease, tissue damage is greater in males than females and this has been associated with enhanced TGF-β1 activation and reduced NO bioavailability (Silbiger and Neugarten, 2008). It is notable that these pathways are amenable to the actions of relaxin. Therefore, relaxin may act as a protective buffer against cardiovascular and renal disease in pre-menopausal women.

The literature is unclear as to whether serum relaxin levels are greater in women than men. In the main, data suggests that relaxin levels are greater in females during the luteal phase of the estrus cycle and pregnancy (Bani, 2008). A recent study (Wolf et al., 2013) detected no difference in serum relaxin levels between men and women. However, given that the subjects were athletes it is possible that ovulation and relaxin secretion was suppressed in these females. Thus, higher circulating levels of relaxin may confer cardio-renal protection during the reproductive years in women.

Surprisingly few studies have examined whether relaxin has sexually dimorphic effects in ameliorating cardio-renal disease. Studies examining the response to rhRLX infusion observed no difference in the blood pressure, cardiac output, total peripheral resistance or renal function (Danielson et al., 2000; Conrad et al., 2004) between male and female rats. In other studies, the age-related cardiac and renal fibrosis observed in relaxin knockout (RLN1-KO) mice was exacerbated in male but not female mice (Du et al., 2003; Samuel et al., 2004b), which was later found to be associated with testosterone and elevated androgen receptor levels in RLN1-KO mice being causative of the problem (Hewitson et al., 2012).
These data suggest that the effects of relaxin may be of greater importance in males than females. However, an alternate explanation put forward was that females have additional protective mechanisms that compensate and mask the loss of relaxin (Du et al., 2003; Metra et al., 2013). Interestingly though, while neither ovariectomy or estrogen replacement therapy affected the cardiac and renal fibrosis measured in female RLNI-KO mice (Lekgabe et al., 2006), ovariectomised RLNI-KO mice had exacerbated lung myofibroblast burden and subepithelial collagen levels (fibrosis), which were both diminished by estrogen replacement therapy, suggesting that the potential compensation offered by estrogen at least may be organ specific. In the RELAX-AHF trial, subgroup analysis of the response to acute administration of rhRLX did not detect a sex-difference in outcomes (Metra et al., 2013). On the other hand, systemic adenoviral delivery of mRLX abrogated left ventricular fibrosis in male and female β2-adrenoreceptor transgenic mice (Bathgate et al., 2008), suggesting that rhRLX may be equally effective for the treatment of cardiovascular and renal disease in both sexes. However, more detailed studies examining the response to rhRLX administration in models of disease in both males and females are required to validate this. It would also be important to conduct studies in both young and aged animals, as cardiovascular and renal disease occur predominantly in post-menopause women, when loss of ovarian hormones may reduce receptor and or second messenger signalling pathways.

There is also intriguing data to suggest that relaxin may have an enhanced action in females. As detailed above, rhRLX’s NO-promoting and TGF-β1-inhibitory actions were found to involve an interaction with the angiotensin type 2 receptor (AT2R), through heterodimers formed between RXFP1 and the AT2R (Chow et al., 2012; Chow et al., 2014).
Moreover, we have demonstrated that the renal and vascular effects of AT$_2$R stimulation are enhanced in females in association with increased AT$_2$R expression (Sampson et al., 2008; Hilliard et al., 2011; Brown et al., 2012; Hilliard et al., 2013). It is therefore possible that signalling via RXFP1-AT$_2$R heterodimers may contribute to the cardio-renal protection observed in young females and that this mechanism may be down-regulated post-menopause since the enhanced role for the AT$_2$R in females is lost with aging and reproductive senescence (Mirabito et al., 2014a; Mirabito et al., 2014b). This possibility warrants further investigation, as restoration of AT$_2$R expression in older women, or up-regulation in males, may represent a therapeutic target worth pursuing.

In contrast, it is also possible that a deficit in relaxin may play a role in the progression of renal disease in females. In general, females are protected from renal disease except in the case of diabetic nephropathy, in which progression and severity of renal damage is greater in females (Diamond-Stanic et al., 2012; Barrett-Connor, 2013). Evidence suggests that this is due to a reduction in the estrogen to testosterone ratio in females in response to the diabetic environment and a similar change in hormonal balance also occurs post-menopause a time when the risk of cardio-renal disease starts to increase in women (Maric, 2009; Diamond-Stanic et al., 2012). Given the association between relaxin secretion and estrogen status (Lippert et al., 1996; Seeger et al., 2000), relaxin may be altered in diabetic patients. However, rhRLX infusion in type I diabetic models has not been shown to improve renal fibrosis, for the reasons outlined above (Wong et al., 2013; Dschietzig et al., 2015). By contrast, in a high fat diet model of insulin resistance, rhRLX infusion reversed collagen accumulation in the heart (Bonner et al., 2013). As these studies were all conducted in males,
in the future it will be important to examine the effect of rhRLX replacement therapy for diabetic nephropathy in females, in which an aged-related reduction in relaxin might be associated with sex-hormone imbalance. Thus, several lines of evidence suggests that relaxin may explain the protection from cardiovascular and renal disease enjoyed by pre-menopausal women, while its lack may contribute to the exacerbation of disease in females.

**Anti-fibrotic effects of relaxin in the lung**

Fibrosis in the lung is seen histopathologically in asthma, chronic obstructive pulmonary disease, pulmonary fibrosis (including idiopathic pulmonary fibrosis), bronchopulmonary dysplasia and pulmonary hypertension. Its significance in these diseases is that it is associated with progression, severity of disease, resistance to treatment, and contributes to the functional endpoints of dyspnea, airway hyperresponsiveness (AHR) and forced vital capacity (FVC) and forced expiratory volume in one second (FEV1).

In a murine model of chronic allergic airways disease (AAD), which mimics several features of human asthma, systemic treatment with 0.5mg/ml rhRLX via miniosmotic pumps was able to reduce total lung collagen and peribronchial ECM deposition to that seen in control mice (Royce et al., 2009). Furthermore, rhRLX treatment reversed epithelial thickening, and significantly improved AHR, but did not influence airway inflammation or goblet cell metaplasia. rhRLX therapy was also attempted in the most widely used preclinical model of parenchymal pulmonary fibrosis induced by bleomycin administration. In this model, mice are typically given a single or double dose of bleomycin – originally used for the
treatment of cancers where fibrotic side-effects in the lung were identified (Della Latta et al., 2015). rhRLX administration significantly reversed established parenchymal fibrosis as well as myofibroblast contractility (Huang et al., 2011; Pini et al., 2010; Unemori et al., 1996).

Given the fact that relaxin is a hormone and RXFP1 appears to be expressed widely throughout the body it is important to limit off target effects. This is achievable for lung diseases as the organ is available for inhaled delivery. However, there are a number of issues with regard to lung delivery that must be addressed. In a murine AAD model of asthma, similar reversal of established airway fibrosis was achieved with daily intranasal administration of rhRLX over two weeks (Royce et al., 2014a) to earlier studies with continuous systemic administration (Royce et al., 2009). Intranasal administration to mice involves simply allowing the mouse to inhale a bolus of rhRLX micropipetted to the nares. In humans the lung is orders of magnitude larger and hence the challenge is for drugs inhaled at the nose or mouth to reach the small airways (<2mm), which are of particular pathological significance (Thien, 2013) and the alveolar sacs. As such inhaled medications need to be delivered as dry powder or nebulized to achieve small enough particle size to reach the terminal parts of the respiratory tree. rhRLX has yet to meet this but promising new nebulization and delivery technologies are under development that enable a larger range of drugs to be nebulized without conformational change or loss of bioactivity (Cortez-Jugo et al., 2015).

As well-documented, rhRLX has a strong anti-fibrotic effect especially against established fibrosis, particularly with its ability to down-regulate TGF-β1-mediated collagen production (Royce et al., 2014a; Unemori et al., 1996) and up-regulate gelatinases (MMP-2
and MMP-9) to help digest aberrant collagen accumulation in the lung and airway wall (Chow et al., 2012; Royce et al., 2009) (Figure 1). However, if rhRLX is to have potential to treat fibrotic lung diseases, it is important that it is able to complement existing therapies, in particular inhaled and oral corticosteroids. rhRLX has been combined with methylprednisolone to treat established fibrosis in an experimental model of chronic AAD and it was found that combination therapy with the corticosteroid and rhRLX more effectively reduced subepithelial collagen thickness compared to either therapy alone (Royce et al., 2013). However, corticosteroids are not without their limitations such as steroid resistance in some patients, side-effects that limit optimal dosing in very young children, and from the point of view of fibrosis, the fact that blocking inflammation does not eliminate fibrosis; as is clear in the adult asthma population where airway remodelling remains rife despite corticosteroids being in widespread use for decades. This reflects the fact that airway remodelling and fibrosis are often not due to chronic inflammation alone but arise from other aetiologies such as epithelial damage and genetic susceptibility (Holgate et al., 2006).

MSCs are another therapeutic approach used experimentally in combination with rhRLX for lung disease treatment (Royce et al., 2015). Used alone, bone marrow-derived MSCs have been shown to have potential for treating a range of fibrotic lung diseases (Royce et al., 2014b) and have been used successfully to treat AAD (Ogulur et al., 2014) and bleomycin-induced (Moodley et al., 2009; Ortiz et al., 2003) disease models. Stem cell therapies in general are thought to heal either by engraftment into the host tissue or by secretion of exosomes and proteins that have paracrine and immunomodulatory effects in diseased tissue. In many inflammatory lung diseases there are areas of epithelial damage where engraftment
of MSCs would conceivably be of benefit but it seems more plausible that secretions of MSCs explain most of the beneficial effects seen in mouse models (Ge et al., 2013). It was hypothesized that combining rhRLX with MSCs would augment the therapeutic efficacy of the latter, by improving the microenvironment via removal of aberrant ECM accumulation and fibrotic stimuli (Formigli et al., 2007; Huuskes et al., 2015). In a chronic AAD model, this combination treatment further reversed disease-induced airway inflammation and airway/lung fibrosis compared to either treatment alone and further increased MMP-2 and MMP-9 levels (Royce et al., 2015). Another avenue for this method is for the overexpression of relaxin by MSCs, which would provide both a novel delivery method of rhRLX and the likely positive effects of rhRLX in the immediate microenvironment of the MSC. This protein overexpression has already been used with other MSC treatments including overexpression of the CXCR4 receptor to enhance homing (Yang et al., 2015), and with murine C2C12 myoblasts overexpressing relaxin to reduce fibrosis and promote angiogenesis in ischemically-damaged organs (Formigli et al., 2007).

Much progress has been made on rhRLX in the acute heart failure trials towards being a realistic drug treatment for human disease. In addition it has good safety, ability to reverse aberrant collagen (without effecting basal ECM required for normal structure), and other advantages compared to other anti-fibrotic drugs both currently used and under development. Given these factors and the outstanding need for novel therapies to treat lung diseases such as asthma (estimated 235 million sufferers 2004) and COPD (64 million sufferers) (World Health Organization (WHO) Global Burden of Disease), relaxin has great potential as a future therapy for fibrosis associated with various lung diseases.
**Anti-fibrotic effects of relaxin in the liver**

Hepatic fibrosis results from accumulation of fibrillar collagens and other ECM proteins in response to chronic injury from a variety of causes, including alcohol overuse, viral hepatitis, and nonalcoholic steatohepatitis (Trautwein et al., 2015). The liver cells responsible for the production of the majority of the collagen in hepatic fibrosis are the hepatic stellate cells (HSCs), specialized cells that reside in the perisinusoidal regions (Friedman, 2008; Puche et al., 2013). HSCs are unusual in that in the normal (quiescent) state, they function as lipid storing cells, and are not fibroblastic in nature. With liver injury, inflammatory cytokines such as TGF-β1 and platelet-derived growth factor (PDGF) stimulate HSCs, causing their transdifferentiation to an activated myofibroblastic phenotype, characterised by proliferation, contractility, and high expression of α-smooth muscle actin (α-SMA), and fibrillar collagens. At the same time, HSCs secrete TIMP-1 and TIMP-2 (Friedman, 2008; Puche et al., 2013), resulting in a shift in the balance between matrix production and degradation, and a net increase in collagen accumulation. When the cause of liver injury is removed, the number of activated HSCs is decreased, either through apoptosis or reversion to the quiescent phenotype, allowing clearance of the excess collagen and a return to the normal liver architecture and functioning. With chronic injury, however, the collagen accumulation causes disruption of liver architecture, and can eventually lead to cirrhosis. For this reason, HSCs have been a prime target in the development of new treatments for advanced hepatic fibrosis and cirrhosis (Trautwein et al., 2015).
The first study of relaxin’s effects in the liver was in rats treated for 4 days with porcine relaxin (Bani et al., 2001). Relaxin caused dilation of the sinusoids, and changes in contractile, actin-rich cells that were likely either myofibroblasts or activated HSCs. The HSC were confirmed as a target of relaxin in studies using culture-activated rat HSCs, where rhRLX caused a decreased collagen deposition and synthesis, accompanied by decreased secretion of TIMP-1 and TIMP-2, but without a change in the gene expression of MMPs or α-SMA (Williams et al., 2001). In another study, porcine relaxin treatment of culture-activated rat HSCs also caused decreased collagen synthesis and deposition, as well as decreased secretion of type I collagen, TIMP-1 and TIMP-2 (Bennett et al., 2003). Furthermore, relaxin decreased the level of α-SMA protein, increased the degradation of type I collagen, and increased the expression of MMP-13, the major rodent fibrillar collagenase. Similar results were observed using primary human HSCs (Fallowfield et al., 2014). Taken together, these studies provided evidence that human or porcine relaxin can inhibit the profibrotic properties of activated HSC, and promote conditions that favour collagen degradation (Figure 1).

HSCs are the major liver cells that express RXFP1. Early studies using PCR detected RXFP1 expression in human liver (Hsu et al., 2002, Hsu et al., 2003), but not female mouse liver (Scott et al., 2006). It was later revealed that quiescent rat HSCs express very low levels of RXFP1, which increased markedly during transdifferentiation in culture, and RXFP1 was detected in fibrotic mouse liver and cirrhotic human liver (Bennett et al., 2005, Bennett et al., 2007). These findings were later confirmed in primary rat and human HSCs, and in rat and human hepatic fibrosis (Fallowfield et al., 2014). The signalling pathways activated by
relaxin in HSCs are consistent with those in fibroblasts from other tissues, and include cAMP, cGMP, NO and Akt (Bennett et al., 2007, Fallowfield et al., 2014). Furthermore, recent findings suggest that rhRLX activation of RXFP1 can result in activation of the anti-fibrotic transcription factor peroxisome proliferator-activated receptor gamma (PPARγ), through a mechanism involving cAMP, PKA, p38-MAPK, and PPARγ coactivator protein 1α (PGC1α) (Singh and Bennett, 2010, Singh et al., 2015).

Several studies have been conducted to assess the efficacy of relaxin in the treatment of hepatic fibrosis. The first was a prevention study using CCl₄-induced hepatic fibrosis in rats, with concomitant treatment with rhRLX (~0.5 mg/kg/day) for 4 weeks (Williams et al., 2001), in which rhRLX reduced the overall liver collagen content as assessed by hydroxyproline content. Later studies focused on more clinically relevant models of established hepatic fibrosis in mice, using 4 weeks of CCl₄ treatment to induce fibrosis, followed by rhRLX treatment via osmotic pumps with continued CCl₄ administration. In one study using short-term treatment with porcine relaxin (0.5 mg/kg/day), relaxin had a small effect on fibrosis after 1 week, but little effect after 2 weeks (Bennett et al., 2009). A later study of 4 weeks treatment with rhRLX (25 or 75 μg/kg/day) showed significant reductions in the levels of liver collagen and α-SMA, and reduced expression of types I and III collagen, α-SMA, and TIMP-2, increased expression of MMP-3 and MMP-13, and increased collagen degrading activity (Bennett et al., 2014). Finally, another study showed that with short-term (72 hours) administration of 0.5 mg/kg/day rhRLX into rats made fibrotic with 8 weeks of CCl₄, rhRLX reduced the amount of α-SMA protein, but not the amount of liver collagen, and decreased the gene expression of type I collagen, α-SMA, MMP-3, and TGF-β.
(Fallowfield et al., 2014). Furthermore, in both CCl₄ and bile duct ligation models, rhRLX reduced portal pressure and increased the hepatic oxygen supply, consistent with other studies using perfused liver models (Fallowfield et al., 2014, Boehnert et al., 2008).

Taken together, the studies conducted thus far show some positive effects of relaxin on hepatic fibrosis and haemodynamics, at least in part by reducing the activated phenotype of HSCs and/or promoting matrix degradation. However, there are some differences in the various experimental models used thus far, and further studies are needed to determine the optimal conditions necessary for effective relaxin treatment of hepatic fibrosis. Nevertheless, these collective preclinical results led to a phase II trial of recombinant human relaxin on haemodynamics in patients with compensated cirrhosis and portal hypertension, which was completed in early 2015 (clinicaltrials.gov identifier NCT01640964). The results of this trial, when released, may clarify the potential role of relaxin in the treatment of human liver disease.

**Anti-fibrotic effects of relaxin on the integumentary system and connective tissues**

Research involving relaxin’s effect on clinical skin pathology has a storied past, with scleroderma as the most frequently studied pathology. Scleroderma is a spectrum disorder and includes a milder, limited form as well as a ‘diffuse’ form, and involves fibrosis of internal organs. In its limited form, common manifestations in the skin are limited to the extremities, and include skin tightness and ulcerations, thickening, altered vascularity and sensitivity of the hands and digits (Raynaud’s syndrome), difficulty in swallowing
(dysphagia), and localized dilation of small blood vessels (CREST Syndrome). In diffuse scleroderma, major organ fibrosis occurs along with widespread skin involvement. Early reports indicated that estrogen priming followed by 20-40 mg doses of porcine relaxin (then known as ‘Releasin’) partially resolved skin tightness, Raynaud’s symptoms, prompted healing of skin ulcerations that were refractory to corticosteroid treatment, and, in limited cases, relieved dysphagia (Casten & Boucek, 1958; Ismay, 1958; Evans, 1959; Rivelis et al. 1965). No improvements were reported in heart, lung, or kidney function. Therapeutic effectiveness often required weeks of therapy (3-5 weeks, Casten & Boucek, 1958) with therapy continuation for protracted periods (up to 30 months), with discontinuation often exacerbating symptoms. Reports of complications were rare, mainly focused on injection site soreness. Contrary to these reports, others observed treatment failure in four patients treated with 80 mg of Releasin for 3-4 weeks (Jefferis & Dixon, 1962). These reports suggested that treatment efficacy was most often observed with continued therapy and that response variability was present in the outcomes.

More recently, clinical safety trials were conducted to establish tolerability of rhRLX to treat patients with systemic disease of short (< 5 yr.) duration. A 24-week randomized control trial (RCT) showed both safety and preliminary measures of efficacy for 25 µg/kg/day subcutaneous rhRLX infusion; a higher dose, (100 µg/kg/day), however, failed to show an advantage over placebo (Seibold et al., 2000). A 14% improvement over placebo in ratings of standardized scoring of skin thickness was observed in this trial. A subsequent FDA Phase III efficacy randomised clinical trial involving over 200 patients failed to show efficacy over
placebo and evidenced seven instances of serious adverse events involving renal crisis (Khanna et al., 2009).

The failure of this Phase III trial forced a reassessment of relaxin therapeutics for treating skin-associated fibrosis, most especially in the context of targeting tissue competence to respond to relaxin. Certainly, in vitro studies have lent support for therapeutic trials. rhRLX (1-100 ng/ml) dose-dependently reduced type I collagen production from most (but not all) of a small sample of biopsies derived from patients with scleroderma (Unemori et al., 1992), and these effects were synergistically enhanced by co-administration of interferon (IFN)-γ (100 U/ml). Furthermore, rhRLX significantly improved the cosmetic appearance and histology of porcine excisional wounds when administered at 130 µg/kg/day for 6 weeks (Stewart, 2009). Currently, studies are underway to determine the effect of relaxin treatment in animal models of dermal wounding, and the expression of relaxin receptors in biopsy samples from patients with Dupuytren’s Disease (Cooney, unpublished data).

That fibroblasts are likely targets for relaxin therapy is also supported from other clinically-relevant anatomic sites, suggesting a systemic-wide tropism. rhRLX dosing (up to 100 ng/ml) of primary cultures of human fibroblasts obtained from vaginal wall biopsies resulted in reduced TGF-β1 translation (Wen et al., 2008). Fibroblasts comprising knee ligaments and thumb express relaxin receptors (Dragoo et al., 2003; Galey et al., 2003; Lubahn et al., 2006). An in situ study of female lower shank tendons showed an inverse relationship between serum relaxin levels and patellar tendon stiffness (Pearson, et al., 2011). These data strongly suggest that fibroblasts located in connective tissue are competent to respond to rhRLX.
However, the scope of apparent relaxin’s cellular interaction within the integument extends beyond resident fibroblasts. Several studies have shown that keratinocytes derived from normal skin express RXFP1 receptors (Cooney et al., 2009; Giordano et al., 2012). Relaxin effects have also been isolated to a substituent of the ECM, fibrillin-2, in the form of downregulated gene transcription and translation in human dermal fibroblasts in response to rhRLX dosing at 30 ng/ml (Samuel et al., 2003). Cell lines derived from the eye epithelium and surrounding sites (human cornea, conjunctiva, sebaceous, and lacrimal glands) express both RXFP1 and RXFP2 receptors and respond to dosing with INSL3 and rhRLX (Hampel et al., 2012; Hampel et al., 2013). Blood vessel endothelium as well as endothelial precursors have been shown to be rhRLX targets. Subcutaneous dosing of 0.1 mg/kg/day rhRLX in rats enhanced angiogenesis in wound chambers, increased VEGF and bFGF signalling and production, and ultimately determined that effects were likely mediated by monocytes infiltrating hypoxic tissue areas (Unemori et al., 2000). Importantly, effects were confined to the site of wounding and were not seen in alveolar pulmonary macrophages. Others showed increases in the transcription and translation of mediators of angiogenesis and vasculogenesis in genetically diabetic mice dosed with 25 µg/day of porcine relaxin with consequent improvement in wound healing time (Bitto et al., 2013); and that rhRLX signaled through RXFP1 to induce mobilization of bone-marrow-derived endothelial progenitors (Segal et al., 2012). Overall, there are an abundance of histologic sites in the integument that express relaxin receptors and respond to dosing in vitro.

Limitations of using rhRLX as a therapeutic and challenges to consider for future studies

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Whilst these collective findings highlight the therapeutic potential of rhRLX as an anti-fibrotic, there are a number of recognised barriers to clinical translation that are noteworthy. Firstly, the clinical efficacy of various anti-fibrotic agents are difficult to evaluate in general due to the lack of reliable biomarkers that could indicate when fibrosis has developed to the point when these agents should be appropriately administered; the lack of reliable non-invasive end-points that could demonstrate if they have regressed disease progression; and the slow pathogenesis of fibrosis in humans (compared to that in animals), which can take decades to develop. Secondly, rhRLX is a peptide-based drug with a short \textit{in vivo} half-life (~10 minutes (Nair et al., 2015)). Although this has been overcome at the experimental level by administering rhRLX to various animal models via subcutaneously-implanted osmotic mini-pumps, and at the clinical level via microinfusion pumps (Erikson, 2001), weekly injections (McGorray et al., 2012) or intravenous administration (Weiss et al., 2009), the relatively short duration of rhRLX treatment may have contributed to the failed clinical trials to date that have attempted to demonstrate its anti-fibrotic efficacy in patients with varying conditions, particularly given that fibrosis is a chronic condition that likely requires long-term treatment to diminish its progression. Thirdly, the requirement for longer term rhRLX treatment will only add to its expense to produce. While small molecules that activate RXFP1 (Xiao et al., 2013) and single-chain derivatives of rhRLX (Hossain et al., 2016; Hossain et al., 2011b) have recently been developed and will be cheaper to manufacture, further work is required to determine their anti-fibrotic efficacy when chronically administered over long periods. The latter might be difficult to test in rodent models though which have been found to mount antibody responses to rhRLX, which in turn have caused increased and variable circulating levels of rhRLX beyond 10 days post-administration (personal communication; Dr
Elaine Unemori; Corthera Inc and Novartis AG, San Mateo, CA, USA). Consistent with this, mice administered 0.5 mg/kg/day rhRLX undergo a doubling of circulating human relaxin levels after 14 days of administration compared to that after 5 days (Samuel et al., 2003); which may lead to higher concentrations of the drug producing lower physiological responses, given its well-demonstrated bell-shaped dose-response effects (Danielson et al., 2003; Teerlink et al., 2009; Unemori et al., 1996).

Furthermore, the importance of understanding tissue competence to respond to rhRLX along with signalling pathways is paramount to ensuring both efficacy and safety of rhRLX in human clinical trials. Recently, Giadarno et al (2015) showed that skin biopsies from patients with limited systemic scleroderma showed weak or no staining for RXFP1 receptors. In line with this, only 30% of Dupuytren’s nodules stain positive for RXFP1 (Cooney, unpublished observation). These findings beg the question of how receptor expression changes over the course of disease. Restoration of calcium signalling via NO is impaired in the endothelium of spontaneously hypertensive rats and cannot be remediated by rhRLX, unlike that of Wistar Kyoto control rats (Nistri et al., 2015), suggesting differential responsiveness based on phenotype or pathologic state. Indeed, as in the disease state, alterations in the ECM environment of cell culture matters, especially in the context of myofibroblasts. Material properties of the ECM affect mechanical signal transduction in myofibroblasts which, in turn, alters gene transcription (Vi, Gan & O’Gorman, 2010). This data show that culturing myofibroblasts on tissue culture plastic or even type I collagen substrates significantly alters the feedback loop between cells and ECM; and underscores the
importance of recognizing the distinction between the pathologic environment versus one that fosters cell growth and proliferation.

**Conclusions**

rhRLX continues to holds promise as a therapeutic to treat several fibrotic conditions associated with up-regulated TGF-β1 and myofibroblast burden. In addition to directly down-regulating intracellular Smad activity to suppress TGF-β1 signal transduction and the profibrotic actions of TGF-β1 on myofibroblast differentiation and aberrant ECM synthesis, it is able to regulate matrix degradation by altering the MMP-to-TIMP balance (summarised in Figure 1). Additionally, it may also indirectly inhibit fibrosis progression via its other organ-protective actions, including its anti-inflammatory, anti-hypertrophic, anti-apoptotic, angiogenic and vasodilatory effects; and can also promote wound healing and tissue function through these combined actions. Although a number of high-quality randomised trials to date have failed to demonstrate the clinically efficacy of its matrix remodelling actions in human subjects (that have been well characterised in various experimental models), it is possible that the positive effects of rhRLX on blood pressure and renal function that have been observed in various trials may be associated with its ability to inhibit fibrosis progression in affected organs. A significant challenge for future trials is to identify patients who may benefit from rhRLX therapy, identify appropriate endpoints for a peptide-based therapeutic which requires continuous administration, consider the timing and length of treatment as well as appropriate route of administration and ensure tissue penetrability beyond the direct site of
administration. Application of gene sequencing to human biopsy material (to understand whether perturbations involving single nucleotide polymorphisms or deletions/additions that impair RXFP1 functioning affect therapeutic responses to rhRLX), and the development of small molecule mimetics of relaxin that have improved pharmacokinetics and biodistribution properties compared to native H2 relaxin, will undoubtedly aid this effort. Cell culture models that better mimic *in situ* tissue conditions will also provide more relevant information. Overall, this field of relaxin investigation remains vibrant and full of potential.

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References


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Figure Legend

Figure 1: Summary of the mechanisms of relaxin’s anti-fibrotic actions that are mediated through RXFP1 and RXFP1-AT2R heterodimers. Relaxin specifically ameliorates the effects of pro-fibrotic stimuli such as transforming growth factor (TGF)-β1 and angiotensin II (Ang II), the former by inhibiting Smad2 (pSmad2) and/or Smad3 (pSmad3) phosphorylation, which is dependent at least in part through the pERK1/2, nitric oxide and Notch-1 pathways. This causes decreased expression and deposition of interstitial (types I, III, V) and basement membrane (type IV) collagens and reduced activity of TIMP-1 and TIMP-2, accompanied by increased expression and activity of various MMPs, including MMP-1/13, MMP-2 and/or MMP-9. The end result is a decrease in the rate of collagen deposition, and increased collagen degradation, allowing clearance of the fibrotic scar.
**Tables of Links**

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These Tables of Links list key protein targets and ligands in this article that are hyperlinked* to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson et al., 2014), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015a,b,c,d).

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Table 1: Various models of cardiovascular disease that have been used to demonstrate the anti-fibrotic effects of relaxin

<table>
<thead>
<tr>
<th>Model studied</th>
<th>Type of relaxin used</th>
<th>Length of treatment</th>
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<tr>
<td>Ang II-stimulated atrial fibroblasts (rat) (Samuel et al., 2004)</td>
<td>rhRLX</td>
<td>3 days</td>
</tr>
<tr>
<td>Ang II-stimulated cardiac fibroblasts (rat) (Gu et al., 2012)</td>
<td>rhRLX</td>
<td>1 day</td>
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<table>
<thead>
<tr>
<th>Condition</th>
<th>Treatment</th>
<th>Duration</th>
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</thead>
<tbody>
<tr>
<td>TGF-β1-stimulated atrial fibroblasts (rat)</td>
<td>rhRLX</td>
<td>3 days</td>
</tr>
<tr>
<td>TGF-β1-stimulated ventricular fibroblasts (rat)</td>
<td>rhRLX</td>
<td>3 days</td>
</tr>
<tr>
<td>TGF-β1-stimulated cardiac fibroblasts (mouse)</td>
<td>rhRLX</td>
<td>1-5 days</td>
</tr>
<tr>
<td>PMA-stimulated cardiac fibroblasts (rat)</td>
<td>rhRLX</td>
<td>3 days</td>
</tr>
<tr>
<td>High glucose-stimulated cardiac fibroblasts (rat)</td>
<td>rhRLX</td>
<td>2 days</td>
</tr>
<tr>
<td>Age-related fibrosis (mouse)</td>
<td>rhRLX/mRLX*</td>
<td>14 days/ 120 days*</td>
</tr>
<tr>
<td>β2-adrenoreceptor-induced cardiomyopathy (mouse)</td>
<td>Ad-mRLX#/#H3 RLX†/rhRLX</td>
<td>14 days</td>
</tr>
<tr>
<td>Myocardial infarction (pig)</td>
<td>hRLX†/rhRLX</td>
<td>7-30 days</td>
</tr>
<tr>
<td>Hypertension (rat)</td>
<td>rhRLX</td>
<td>14 days</td>
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<tr>
<td>Type 1 diabetes (rat)</td>
<td>rhRLX</td>
<td>14 days</td>
</tr>
<tr>
<td>Atrial fibrillation (rat)</td>
<td>rhRLX</td>
<td>14 days</td>
</tr>
</tbody>
</table>

*Synthetically-produced mouse relaxin (mRLX) or adenovirus-mediated mouse relaxin (Ad-mRLX) or H3 relaxin (H3 RLX) was used; †mouse skeletal myoblasts were engineered to produce relaxin based on the human prepro-relaxin (hRLX) sequence.
Author/s:
Samuel, CS; Royce, SG; Hewitson, TD; Denton, KM; Cooney, TE; Bennett, RG

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