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Relaxin and Fibrosis: Emerging Targets, Challenges, and Future Directions

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Abstract

The peptide hormone relaxin is well-known for its anti-fibrotic actions in several organs, particularly from numerous studies conducted in animals. Acting through its cognate G protein-coupled receptor, relaxin family peptide receptor 1 (RXFP1), serelaxin (recombinant human relaxin) has been shown to consistently inhibit the excessive extracellular matrix production (fibrosis) that results from the aberrant wound-healing response to tissue injury and/or chronic inflammation, and at multiple levels. Furthermore, it can reduce established scarring by promoting the degradation of aberrant extracellular matrix components. Following on from the review that describes the mechanisms and signaling pathways associated with the extracellular matrix remodeling effects of serelaxin (Ng et al., 2019), this review focuses on newly identified tissue targets of serelaxin therapy in fibrosis, and the limitations associated with (se)relaxin research.

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1. Introduction

The earliest functions attributed to relaxin largely related to pregnancy, as evidence showed that it acted to remodel the extracellular matrix (ECM) in the pubic ligament, uterus and vagina (Graham and Dracy, 1953; Hall, 1947; Hisaw, 1926; Krantz et al., 1950). It was also shown to be responsible for the vasodilation and increased cardiac output in pregnancy (Debrah et al., 2006; Novak et al., 2001). However, the finding of relaxin gene expression in the human prostate gland (Gunnensen et al., 1996), relaxin immunoreactive material in porcine seminal vesicles (Kohsaka et al., 1992), and relaxin protein in the seminal fluid of several species, including humans (Weiss et al., 1986) suggested that it had important functions outside of pregnancy. The nonreproductive importance of relaxin was supported by studies using the relaxin-null mouse. These mice spontaneously developed fibrosis in a number of non-reproductive organs, including the kidney (Samuel et al., 2004b), skin (Samuel et al., 2005), lung (Samuel et al., 2003), and heart (Du et al., 2003; Samuel et al., 2004a), and in all cases, the fibrosis was resolved by treatment with recombinant human relaxin-2 (Samuel et al., 2017). Similar (but not identical) effects were observed in mice lacking the relaxin receptor RXFP1 (Kamat et al., 2004). Importantly, these effects were observed in both male and female mice, suggesting that a source of relaxin outside the corpus luteum was responsible for many of these effects.

Relaxin has been studied as a potential treatment for fibrotic diseases since the middle of the 20th century, with the earliest studies focused on the use of partially purified preparations of relaxin from porcine ovary to treat scleroderma, a fibrotic condition of the connective tissue and dermis (Casten and Boucek, 1958; Evans, 1959). While these studies, in general, reported favorable effects on the disease, further studies were hampered by increasingly stringent standards on the quality of therapeutic agents used in clinical trials. With the development of highly purified porcine relaxin and recombinant human relaxin-2 (known as serelaxin), it became possible to conduct more widespread preclinical and clinical studies (Dschiertzig et al., 2009; Khanna et al., 2009; Seibold et al., 2000; Teerlink et al., 2013; Teerlink et al., 2017).

Clinical trials using serelaxin have mostly focused on cardiac dysfunction, particularly acute heart failure. In a phase III trial, serelaxin showed some effectiveness in the reduction of dyspnea, and of all-cause and cardiovascular mortality (Teerlink et al., 2013). However, a second phase III trial did not reproduce these findings. The use of serelaxin in fibrotic diseases has thus far been limited. A phase III trial of serelaxin to treat scleroderma was largely unsuccessful, possibly due to the severity of the disease in the patient population studied (Khanna et al., 2009). Another trial, which explored short-term infusion of serelaxin in cirrhosis, reported improvements in portal hypertension, but did not examine effects on fibrosis (Snowdon et al., 2017). Therefore, while much preclinical data supports the use of (se)relaxin for fibrosis treatment, challenges remain to successful treatment of human fibrotic disease.

In summary, new potential therapeutic targets for (se)relaxin continue to be identified. However, their clinical relevance for the successful treatment of disease continues to present challenges. The purpose of this minireview is to provide an overview of these two extremes

– i) the rapidly increasing targets of serelaxin therapy in fibrosis, and ii) the limitations and difficulties associated with relaxin-related research. In the first section, two emerging potential targets of relaxin (urinary tract and ligaments) are discussed. This is followed by a discussion of the current state of challenges and the potential future directions related to relaxin research.

2. Emerging Targets for Serelaxin in Fibrosis

2.1 Urinary Bladder

The urinary bladder is composed of three distinct layers; the mucosa, muscular detrusor and serosa. The mucosa is comprised of a specialized luminal epithelium referred to as the urothelium and the underlying lamina propria with an ECM composed of collagen-I and III fibers and elastin (Aitken and Bagli, 2009). The lamina propria ECM plays a key role in providing structural support to facilitate force generation during detrusor contractions, and a high degree of elasticity which allows for urine storage under low pressure and increasing bladder capacity (Aitken and Bagli, 2009). Under physiological conditions extracellular collagen is continuously synthesized and degraded. However, disruption of this homeostasis by a variety of pro-inflammatory factors can lead to excessive ECM deposition/fibrosis, driven mainly by the conversion of fibroblasts to myofibroblasts (Pardali et al., 2017; Weiskirchen et al., 2018).

Ionizing radiation is an important therapeutic option for pelvic organ malignancies. However, the risk for developing secondary conditions such as radiation cystitis limits the radiation dose and requires that it be given in fractions over days to weeks (Lobo et al., 2018; Smit and Heyns, 2010). Acutely following radiation exposure, bladder irritation and urinary frequency are the major symptoms of radiation cystitis which resolve within weeks of completing radiotherapy. However, up to 10% of patients can develop chronic radiation cystitis over 6–12 month following treatment with symptomology including vascular endothelial cell damage, inflammation, ischemia, collagen deposition and decreased bladder compliance (Browne et al., 2015; Lobo et al., 2018; Smit and Heyns, 2010). In animal models of radiation cystitis, selective irradiation of the bladder externalized through a laparotomy (Fig. 1A) has been shown to result in chronic bladder fibrosis within 6 weeks, exhibiting decreased bladder compliance, contractility and overflow incontinence (Ikeda et al., 2018).

A recent study has shown that a continuous infusion of serelaxin (400 µg/kg/day/14 days) using subcutaneous osmotic pumps decreased collagen content which improved bladder compliance, and increased detrusor smooth muscle L-type calcium channel α -subunit (Cav1.2) expression which enhanced contractility in mice with chronic radiation cystitis (Ikeda et al., 2018). The therapeutic effects of serelaxin could potentially be mediated by activation of RXFP1 and RXFP2 receptors. The expression of these receptors on the detrusor were demonstrated using *in situ* immunofluorescence, Western blot and quantitative real-time PCR (Ikeda et al., 2018).

The administration of serelaxin did not significantly alter the voiding function in nonirradiated mice (Fig. 1C vs Fig. 1B). Conversely, mice that received bladder irradiation

exhibited loss of the micturition response and overflow incontinence nine weeks post-irradiation (Fig. 1D). External urethral sphincter electromyograms (EUS-EMG, black traces) demonstrated that animals had prolonged guarding reflexes and that normal phasic bursting activity did not occur. The guarding reflex occurs in humans and mice and is an increase in the tonic force of the EUS prior to micturition which continues until a pressure threshold is reached and voiding occurs; this prevents urine leakage and allows for more efficient bladder emptying. In rodents, the tonic activity of the EUS also decreases during voiding but is accompanied by phasic ‘bursting’ activity the function of which is unclear. During voiding in humans, the tonic activity of the EUS simply decreases. When serelaxin was administered (400 µg/kg/day) for 2 weeks in 7-week post-irradiated mice, the CMGs and EUS-EMGs (Fig. 1E) exhibited similar functionality to those observed in non-irradiated mice (Fig. 1B) with a normalized bladder capacity and micturition contraction and in the EUS, the return of tonic relaxation and bursting during voiding.

Ex vivo bladder contractility studies demonstrated a marked increase in the passive tension of irradiated mouse bladders indicative of decreased tissue elasticity/compliance due to fibrosis. The irradiation-induced alteration in tissue elasticity was evident by 4 weeks and peaked by 6–9 weeks (Fig. 2A). The increased passive tension was demonstrated to be due to alterations in ECM rather than the relaxation properties of the detrusor as removal of extracellular Ca^{2+} did not alter passive tension profiles compared to those in physiological solution (Fig. 2B vs. 2C). However, the decreased bladder wall force generation and compliance were reversed by 2 weeks treatment with serelaxin (400 µg/kg/day) where tension measurements became comparable to those of mice with non-irradiated bladders (Fig. 2C). This also correlated with enhanced active force generation evoked by electrical field stimulation (20 Hz) compared to non-irradiated controls (Fig. 2D–E). The increased force generation is in part attributed to the enhanced expression of Cav1.2, which is the key ion channel mediating detrusor contraction. Chronic radiation cystitis appears to decrease Cav1.2 expression in the detrusor layer (Fig. 2F) which was restored following 2-weeks of treatment with serelaxin.

Finally, Van Gieson staining for collagen fibers revealed that long-term radiation cystitis caused urothelial layer disruption, increased collagen content (intense pink staining) and caused significant muscle damage by 9 weeks post-irradiation (Fig. 2H) compared to age matched controls (Fig. 2G). In contrast, mice with chronic radiation cystitis treated with serelaxin exhibited an intact urothelial layer and normal collagen and smooth muscle architecture (Fig. 2I) comparable to non-irradiated controls. Furthermore, the collagen content of irradiated mouse bladders was markedly higher than non-irradiated counterparts and serelaxin therapy restored bladder collagen content to levels comparable to non-irradiated controls (Fig. 2J). These findings, although preclinical, demonstrate the therapeutic potential for serelaxin in treating lower urinary tract pathologies due to radiation cystitis through reversal of fibrosis to improve compliance and enhanced Cav1.2 expression to improve detrusor contractility. It has yet to be determined whether the signaling pathways and the mechanisms of action of serelaxin in the lower urinary tract are like those described in other cell types and tissues. Moreover, the relative contributions of RXFP1 and RXFP2 receptors are unclear as is their role in normal bladder physiology. Accordingly, these are areas that require further investigation.

2.2 Ligaments

Fibrotic diseases, including carpal tunnel syndrome, Dupuytren's disease, adhesive capsulitis, cubital tunnel syndrome, arthrogryposis, and scleroderma, are widespread throughout the populations of many countries. These disease conditions are characterized by increasing areas of fibrosis within tissues and organs, and the conversion of tissue-specific fibroblasts into myofibroblasts (Samuel et al., 2017). As mentioned above, serelaxin has been shown to inhibit fibrosis of the heart, lung, kidneys, and bladder, especially in rodent models of disease [reviewed in (Bathgate et al., 2013; Lam et al., 2018; McVicker and Bennett, 2017; Samuel et al., 2017; Sherwood, 2004)]. Serelaxin achieves this effect by inhibiting the pro-fibrotic actions of cytokines (such as transforming growth factor (TGF)- β 1 (Samuel et al., 2004a; Unemori and Amento, 1990; Unemori et al., 1996), interleukin (IL)-1 β (Unemori and Amento, 1990) and/or angiotensin II (Samuel et al., 2004a)) on fibroblast proliferation and differentiation into myofibroblasts. Additionally, serelaxin has been found to inhibit endothelial-to-mesenchymal cell transition ((Cai et al., 2017; Zheng et al., 2017) to inhibit myofibroblast-induced extracellular matrix (ECM) production (Fig. 3). Furthermore, serelaxin promotes the expression and activation of MMPs while suppressing TIMPs within the ECM to facilitate the breakdown of aberrant ECM components (Samuel et al., 2004a; Unemori and Amento, 1990; Unemori et al., 1996) (as illustrated in Fig. 3).

It has been suggested that physiological hormonal imbalance plays a role in the attenuation of the carpometacarpal joint of the thumb, specifically the volar oblique ligament (Lubahn et al., 2006). Before the discovery of the human relaxin receptor, identification of relaxin binding sites was detected by autoradiography using radiolabeled human relaxin (Osheroff et al., 1992; Osheroff and Phillips, 1991) or indirect ligand-antibody staining of tissue sites (Galey et al., 2003). Control tissue (not shown; see (Galey et al., 2003; Lubahn et al., 2006)), incubated without polyclonal antirelaxin antibodies, showed no staining at all. However, antibody staining was found to be detected in surgically removed volar oblique ligaments of perimenopausal female patients (Fig. 4A; (Lubahn et al., 2006)) and in ligaments from an 81 year old woman and a 15 year old girl, (Fig. 4B and Fig. 4C, respectively; (Galey et al., 2003)). With the discovery of the serelaxin receptor (Hsu et al., 2002), more precise localization of the relaxin receptor in tissues and cells was possible. Further studies using antibodies against RXFP1 revealed staining RXFP1 in human fibroblasts (Fig. 4D and Fig. 4E; (Cooney et al., 2009)). In fact, hormones seem to play a role in the stability and biomechanics of the carpometacarpal joint (Komatsu and Lubahn, 2018). Human labial tissue also exhibited RFXP1 binding (Fig. 4F; (Cooney et al., 2009)). More recently, Schoeber and colleagues discussed the need to consider hormonal binding to genital skin during feminizing and masculinizing genital surgery. The role of serelaxin in wound healing needs to be considered during these increasingly common surgeries, along with circulating levels of both male and female hormones (Schoeber et al., 2016).

While fibroblasts do not have a definitive cellular marker, they are characterised by their spindle shape and expression of the cytoskeletal protein vimentin. On the other hand, while myofibroblasts also express vimentin, they are distinguished from fibroblasts by having the additional presence of the cytoskeletal protein, smooth muscle actin. A key feature of fibrotic diseases is the transition of fibroblasts to myofibroblasts (Samuel et al., 2017).

Recently, Wu and colleagues investigated the role of serelaxin in the transition of cardiac fibroblasts to myofibroblasts (Wu et al., 2018). As shown in Fig. 3, the signaling pathway controlling this transition is complex, with TGF- β 1 playing a major role. TGF- β 1 signaling activates activin-like kinase 5, which in turn activates Smad proteins. The Smad complex turns on profibrotic genes, eventually promoting ECM proliferation within damaged cardiac tissue (Wu et al., 2018). This process, found in other organs, also involves MMPs, TIMPs, and collagens (Samuel et al., 2017). Using a rodent cell model, Wu and colleagues examined the effect of serelaxin in mitigating the TGF β 1-induced formation of cardiac myofibroblasts. Cells treated with TGF- β 1 lost the majority of their vimentin staining; however, these same cells expressed higher levels of α -smooth muscle actin (α -SMA) (Fig. 5A). The addition of serelaxin to the cells for a short time reversed these changes (Fig. 5A, Fig. 5B and Fig. 5C). The authors concluded that short-term exposure of cells to serelaxin significantly altered the fibrotic signaling pathways in cardiac tissue and inhibited differentiation of TGF- β 1-induced cardiac myofibroblasts (as characterized by down-regulated expression of α -SMA; (Wu et al., 2018)).

3. Challenges and Future Directions

3.1 Challenges

Several challenges in relaxin-related research have hindered the translation of basic research and preclinical findings into clinical outcomes. In particular, there has been a lack of good assays to detect the relaxin receptor(s) and hence, an inability to predict the effectiveness of serelaxin and serelaxin-based therapies in major disease settings. There are organ- and disease-specific issues concerning delivery and pharmacokinetics. Finally, there have been some challenges in applying serelaxin as a long-term therapy in clinical trials (given its short half-life of ~10 minutes (Chen et al., 1993a; Chen et al., 1993b)) and also in identifying suitable disease settings for which it can be evaluated as a therapeutic in human patients.

Serelaxin is just one example of a drug that has successfully reduced scar tissue accumulation in animal models of fibrotic disease, but has not been so successful in human clinical trials. Rockey has put forward a number of possible explanations for this phenomenon (Rockey, 2008). These include that many animal studies of anti-fibrotic drugs have been conducted in a setting of prevention of development of fibrosis. However, in the case of serelaxin, there are a large number of robust animal studies showing reversal of well-established fibrosis (Bennett, 2009; McVicker and Bennett, 2017; Samuel et al., 2017). Apart from the issue of pharmacokinetics (where it is possible to achieve higher serelaxin concentrations in rodents than is achievable in humans), the duration of injury in rodent models is short and treatable within days. On the other hand, much longer treatment periods (several weeks-to-months) will be required to treat human fibrotic diseases associated with excessive collagen cross-linking and maturation. Currently we do not know the effects of serelaxin treatment over such long periods or its repeated treatment effects over years. However, at least we can predict safety is likely given that relaxin is endogenously present over the human lifespan, and several trials using serelaxin (albeit up to several weeks) have demonstrated its excellent safety profile to date.

The expression of RXFP1 has been reported directly and indirectly *via* the presence of protein detected by antibody-based techniques, mRNA and radioligand binding studies as being almost ubiquitously expressed in human cells to some degree (Bathgate et al., 2013). However some variation in expression has been reported between different organs and between normal versus various fibrotic disease states. While RXFP1 protein expression has been detected in female reproductive tissues (ovary, uterus, cervix, vagina), the nipples and brain, only RXFP1 gene expression has generally been detected in several non-reproductive tissues (Bathgate et al., 2013). This is mainly due to the poor quality of RXFP1 antibodies that have been commercially developed (which have various degrees of validation information available). However, another contributing factor is likely the low levels of receptor expression that may fall beneath the detection limit of currently available methods used to identify its presence. Antibodies developed by Ivell et al. have been used in a number of well-controlled studies (Ivell et al., 2003). In the setting of human disease there have been differing results of RXFP1 protein and mRNA expression depending on the disease and organ site investigated. In heart failure, circulating relaxin concentrations and myocardial gene expression of both H1 and H2 relaxin was found to be increased in correlation with disease severity (Dschietzig et al., 2001), while RXFP1 protein expression was found to be moderately decreased in the failing atrial myocardium compared to that found in the non-failing atria (Dschietzig et al., 2011). In lung diseases characterised by fibrosis (Lam et al., 2018; Tan et al., 2016), RXFP1 gene and protein expression was found to be reduced or unchanged in idiopathic pulmonary fibrosis (Tan et al., 2016), while both RXFP1 and to a greater extent, H2 relaxin immunostaining were found to be lower in biopsies from asthma patients (Lam et al., 2018). In general, RXFP1 is expressed at very low levels and is not secreted from cells. As such the use of RXFP1 as a biomarker and reliable predictor in response to serelaxin-based therapies in individual patients seems unrealistic and it is something that will need to be determined preclinically.

In addition to requiring more sensitive assays that can detect relaxin and RXFP1, attention should be paid to measuring activation of pathways downstream from RXFP1. Some candidates here include extracellular signal-regulated kinase (ERK) $\frac{1}{2}$ phosphorylation (pERK $\frac{1}{2}$), cyclic GMP and/or MMP activity, which are all increased via serelaxin binding to RXFP1 to mediate its anti-fibrotic actions. ERK $\frac{1}{2}$ phosphorylation plays an important role in serelaxin's ability to limit ECM production (Chow et al., 2014; Mookerjee et al., 2009) and upregulate MMPs (Chow et al., 2012). As such, it can be detected at the cellular level via AlphaScreen assays (a bead-based Amplified Luminescent Proximity Homogenous (chemistry) Assay used to study biomolecular interactions and the effects of compounds on these molecular interactions in a microplate format), or at the tissue level via Western blotting and/or by histological staining. Technologies around real-time biosensors for ERK activity (de la Cova et al., 2017) and *in vivo* monitoring of ERK activity at a single cell resolution (Mayr et al., 2018) are also being developed. cGMP sensor mice might be valuable for cGMP measurements and for screening *in vivo* (Paolillo et al., 2018). ELISA kits are also available that allow for the measurement of cGMP from cell lysates, tissue homogenates, plasma or urine. MMPs are already routinely measured in serum usually as a biomarker of acute inflammation intensity. However if we wish to measure increased MMPs (specifically MMP-2 and MMP-9) in patients to determine serelaxin activity in digesting

collagen, these measurements might be affected by acute inflammatory exacerbations of these MMPs seen in chronic disease (Giannandrea and Parks, 2014). Furthermore, whilst we would argue that reducing aberrant collagen accumulation in tissues is the key fundamental aim of any anti-fibrotic therapy, it should always be remembered that there are key clinical endpoints pertaining to each disease including bladder function and lung compliance measurements.

The delivery of serelaxin or related peptides to chronic diseased settings remains a challenge, and not a unique one. Some organs featuring heavily in cardio-vascular fibrotic diseases can only be reached via systemic infusion and this potentially limits long-term serelaxin therapy (Erikson, 2001); which may be associated with potential off-target effects of serelaxin in other RXFP1-expressing tissues. The lung and integumentary system represent good sites for topical delivery of serelaxin, as well as the gastrointestinal tract, although we are not aware of formulations of relaxin with protection against proteolytic digestion.

Given the limited opportunity for serelaxin to be used in large clinical trials and the negative perception it has received based on the various trials that have been conducted in which it did not produce a favourable outcomes (Erikson, 2001; McGorray et al., 2012; Samuel et al., 2007; Weiss et al., 2016), it is important that some strategic planning be made before future trials be attempted. As far as organ fibrosis is concerned, it can be said that many of the completed trials were too short to show outcomes meaningful to long term fibrosis. Attempting to treat chronic diseases that only affect a small percentage of people and in which large cohorts of treatable patients are difficult to find is also likely to lead to low levels of success.

Another complication is finding relevant endpoints that reliably measure the response to serelaxin treatment of fibrotic disease processes, that can be used in human and animal models (with invasive and non-invasive correlates). Serum collagen-breakdown fragments and other soluble biomarkers have not been developed or adopted for indications of fibrosis progression and/or regression and in some cases, serum level of these biomarkers may not necessarily predict the severity of organ disease or the success of therapeutic efficacy. One peculiarity of the mechanisms associated with the anti-fibrotic actions of serelaxin is that it degrades collagen *via* upregulation of MMPs (Unemori and Amento, 1990), so this would preclude the use of MMPs as a fibrotic biomarker; as in many situations, MMPs are often upregulated in response to tissue injury/disease (Caron et al., 2005; Duchossoy et al., 2001). Serum and tissue TGF- β 1 levels have been shown to correlate with fibrotic liver disease progression (Kanzler et al., 2001), but can be influenced by several factors and processes outside fibrosis during disease pathology. Other aspects of fibrosis can only be measured *in situ via* biopsy specimens, including collagen and the presence of α SMA-positive myofibroblasts.

3.2 Future directions

One area that has been identified is the need for new methodologies for measuring responses to anti-fibrotic therapies in experimental systems and patients. Given the limitations and lack of biomarkers to indicate disease progression and the efficacy of serelaxin as a therapy,

measuring responses to therapy will still be reliant on non-invasive imaging techniques, the biopsy and the surgical specimen. Modern imaging techniques used for the detection and monitoring of fibrosis include X-ray imaging, computer-based tomography, ultrasound, magnetic resonance imaging (MRI), positron emission tomography and single-photon emission computed tomography (SPECT) (Baues et al., 2017). MRI has been used to detect alterations in fibrosis associated with atrial fibrillation (Boyle et al., 2018; Luetkens et al., 2018) and hypertrophic cardiomyopathy (Jalanko et al., 2018), renal allograft injury (Kirpalani et al., 2017), liver diseases (Harrison et al., 2018) and systemic diseases affecting multiple organs (Wielputz et al., 2018); and is currently being evaluated as a detection system in various clinical trials (Harrison et al., 2018; Prabhu et al., 2018). At the level of the biopsy or surgical specimen, techniques based on second harmonic generation (SHG) including commercially available instruments such as the HistoIndex platform allow detection of collagen in tissue sections without histochemical staining. This allows visualization of fine collagen deposits as in non-alcoholic steatohepatitis (NASH) liver biopsies. More importantly SHG in conjunction with advances in morphometric software allow evaluation of collagen crosslinking, density and fibre thickness, which may play a crucial role in animal model to human translation.

Another area for future development is methods for delivery of serelaxin. As noted, the lung and integument offer opportunities for topical delivery. Serelaxin has successfully been given intranasally to mice with established airway fibrosis (Royce et al., 2014) but delivering it deep into the human lung presents more challenges. Nanoparticles have been suggested as a vehicle for drug delivery (Hardy et al., 2012). Slow-release hydrogels (Du et al., 2013) and carriers for the targeted delivery of aerosolized macromolecules (Osman et al., 2018) could also be investigated as future modes of serelaxin delivery. Although serelaxin has been proposed to be used as a tablet-ingested therapy for patients with fibromyalgia, it is unclear if it gets absorbed through the gut into the circulation. Another area being explored is the use of stem cells as an adjuvant in relaxin-based therapies or as a vehicle for serelaxin delivery. In the latter scenario, stem cells can be genetically altered to overexpress relaxin (Formigli et al., 2007).

Finally, modified forms of relaxin such as the single chain-derivative, B7–33 (Hossain et al., 2016), and small molecule, ML290 (Kocan et al., 2017), have been developed that maintain the strong anti-fibrotic effects of the hormone whilst reducing some of the possible deleterious and off-target effects of the naturally-occurring hormone. B7–33 contains residues 7–29 of the original B-chain of H2 relaxin in addition to the residues lysine-arginine-serine-leucine (KRSL) at the C-terminus (Hossain et al., 2016). Importantly, B7–33 retains the RXFP1-binding domain of H2 relaxin, and more specifically binds to RXFP1 (at the same site as serelaxin), but with lower affinity than H2 relaxin/serelaxin. ML290 is the first RXFP1-selective small molecule agonist, which has a plasma half-life of 8.56 hours in mice (Xiao et al., 2010). It activates human, monkey and pig, but not mouse RXFP1, and does not compete directly for the orthosteric binding site of human RXFP1, suggesting that it binds RXFP1 at an allosteric site. Characterising the wider organ-protective of these modified relaxin compounds and evaluating their therapeutic benefits in established diseased settings will be key to establishing them as therapies of the future.

4. Conclusion

The interest in relaxin research has been growing steadily in recent years. A contributing factor in this phenomenon was the development of new research tools, such as recombinant human relaxin and the genetic mouse models of relaxin or RXFP1 deficiency. These innovations helped solidify relaxin's role in the protection from fibrosis in a variety of organs. Based on this work, researchers are exploring the use of serelaxin for treatment for a wider range of disease states, as exemplified by the recent work in the ligaments and urinary bladder. However, there are still considerable hurdles to clear in order to reach the ultimate goal of successful treatment of human fibrotic disease. The continued development of validated reagents and stable, potent relaxin analogues will hopefully allow this goal to be reached.

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Highlights

- Fibrosis results from excess tissue extracellular matrix (collagen) deposition.
- Pro-fibrotic cytokines and myofibroblast activation contribute to fibrosis.
- Relaxin is a polypeptide hormone with established antifibrotic properties in several tissues.
- Recent studies have highlighted previously unrecognised targets of relaxin activity.
- There are still several challengers, however, facing clinical evaluation of relaxin as an anti-fibrotic.

Figure 1. Bladder cystometrograms (CMGs) and external urethral sphincter (EUS) electromyograms (EMGs) from irradiated mice with and without serelaxin treatment. A, Method for selective irradiation of the urinary bladder. B-E, CMGs/EUS-EMGs in decerebrated mice. B, Control, nonirradiated mouse (N = 6). C, Nonirradiated mouse treated with serelaxin (400 μ g/kg/day) for 2 weeks (N = 2). D, Irradiated mouse with saline infusion via a subcutaneous osmotic pump for 2 weeks (N = 6). E, Irradiated mouse with serelaxin infusion (400 μ g/kg/day) via a subcutaneous osmotic pump for 2 weeks (N = 4). Treatment in D and E commenced 7 weeks after irradiation. Serelaxin treated mice exhibited more efficient voiding, longer intercontractile intervals, higher bladder compliances, and a normalized EUS activity. Used with permission from (Ikeda et al., 2018).

Figure 2.

Passive properties, bladder wall compliance, detrusor contractility and collagen content changes in chronic radiation cystitis and its reversal by serelaxin treatment. A, The bladders were isolated at 1, 2, 4, 6, and 9 weeks post-irradiation and contractile function was measured in organ bath experiments (N = 3 each time point). Passive tension profiles (an indicator of tissue stiffness) showed significant increases at 6-9 weeks post-irradiation. B, Passive tension recorded in Ca²⁺-free Tyrode's solution demonstrated that serelaxin decreased tension generation, compared to saline treated irradiated mice, suggesting that this effect was due to changes in the elastic properties of the bladder and not smooth muscle relaxation, (N = 5 each). C and D, At 9 weeks post-irradiation, mouse bladders showed increased passive tension and decreased active force generation (red traces) compared to nonirradiated mice (green traces). Two weeks treatment with serelaxin (subcutaneous, 400 μ g/kg/day) commenced 7-week post- irradiation resulted in a passive tension profile similar to nonirradiated controls and increased contractile responses to EFS (blue traces and bars, N = 4 for control and N = 5 each for irradiated + vehicle or serelaxin). E, Force generation in response to EFS were enhanced in serelaxin-treated compared to vehicle treated irradiated mouse bladders (*P < 0.05, Wilcoxon ranked sum test). Responses to muscarinic agonist, oxotremorine-M and 120 mM KCl were not significantly different between the groups (N = 4 for control and N = 5 each for irradiated + vehicle or serelaxin). F, The expression of L-type Ca²⁺ channels (Cav1.2) was decreased in the detrusor layer of mice with chronic radiation cystitis and was increased following serelaxin treatment (N = 3 each). G, Van Gieson staining of control mouse bladder sections. H, Sections from irradiated bladders

showed denuding of the UT and significant collagen staining in the lamina propria (LP) and throughout the detrusor. I, Mice treated with serelaxin showed a decrease in bladder collagen content that was comparable to nonirradiated mice and an intact urothelial layer. J, Collagen:tissue ratio was analyzed using ImageJ (N = 4 each group, Wilcoxon ranked sum test, * indicate $P < 0.01$ vs non-irradiated control and ** $P < 0.01$ vs irradiated + vehicle). Used with permission from (Ikeda et al., 2018).

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Figure 3.

Summary of the mechanisms of relaxin's anti-fibrotic actions that are mediated through RXFP1 receptors and RXFP1–AT₂ receptor heterodimers. Relaxin specifically ameliorates the effects of pro-fibrotic stimuli such as TGF-β1 and Ang II, the former by inhibiting Smad2 (pSmad2) and/or Smad3 (pSmad3) phosphorylation, which is dependent at least in part on the pERK1/2, NO and Notch-1 pathways. This causes decreased expression and deposition of interstitial (types I, III and 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.

Figure 4.

Immunohistological and immunocytochemical staining of tissues and cells for relaxin and hormone receptors. A. Aminoethylcarbazole staining (red) of relaxin binding to the volar oblique ligament, counterstained with hematoxylin (magnification, $\times 100$; see ref 20). B. Ligament staining. Relaxin binding to an ACL specimen from an 81-year-old woman. C. Relaxin staining to an ACL specimen from a 15-year-old girl (for B and C: original magnification, $\times 100$. Positive regions appear red. See ref 21). D. RXFP1 staining in VOL fibroblasts. Cells from a ligament explant culture were fixed and stained with rabbit anti-human RXFP1 coupled to fluorescein (magnification, $\times 1000$; see (Cooney et al., 2009)). E. RXFP1 expression in basement membrane from a labial skin biopsy. The image shows localization of rabbit anti-human RXFP1 antibodies (dark) to cells that rest on the basement membrane in a labial specimen from a pediatric patient (magnification, $\times 400$). Images used with permission from (Cooney et al., 2009; Galey et al., 2003; Lubahn et al., 2006).

Figure 5. Changes of α-SMA expression after treatment with serelaxin. (A) Immunostaining of α-SMA. Bar = 100 μm. (B) Expression of α-SMA mRNA assessed with RT-PCR. (C) The statistical result of α-SMA mRNA expression. (D) Expression of α-SMA assessed with Western blot. (E) The statistical result of α-SMA expression. Serelaxin inhibits α-SMA expression in TGF-β1-induced cells. *p < 0.05 versus control group, #p < 0.05 versus TGF-β1 group. Used with permission from (Wu et al., 2018).