



# Human Recombinant Relaxin (Serelaxin) as Anti-fibrotic Agent: Pharmacology, Limitations and Actual Perspectives

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**Abstract:** Relaxin (recombinant human relaxin-2 hormone; RLX-2; serelaxin) had raised expectations as a new medication for fibrotic diseases. A plethora of *in vitro* and *in vivo* studies have offered convincing demonstrations that relaxin promotes remodeling of connective tissue extracellular matrix mediated by inhibition of multiple fibrogenic pathways, especially the downstream signaling of transforming growth factor (TGF)- $\beta$ 1, a major pro-fibrotic cytokine, and the recruitment and activation of myofibroblasts, the main fibrosis-generating cells. However, all clinical trials with relaxin in patients with fibrotic diseases gave inconclusive results. In this review, we have summarized the molecular mechanisms of fibrosis, highlighting those which can be effectively targeted by relaxin. Then, we have performed a critical reappraisal of the clinical trials performed to date with relaxin as an anti-fibrotic drug, in order to highlight their key points of strength and weakness and to identify some future opportunities for the therapeutic use of relaxin, or its analogues, in fibrotic diseases and pathologic scarring which, in our opinion, deserve to be investigated.

**Keywords:** Connective tissue, extracellular matrix (ECM), fibrosis, myofibroblasts, relaxin (RLX), RXFP1, serelaxin, TGF- $\beta$ .

## 1. RELAXIN AND CONNECTIVE TISSUE

Relaxin (RLX) is a peptide hormone (m.w. 6,000 Da) whose close relationship with the connective tissue dates back to its discovery, when it was found to elongate the pelvic ligaments and soften the organs of the birth canal in preparation for delivery [1]. It is structurally similar to insulin and insulin-like growth factors (IGFs), being formed by two peptide chains, A and B, linked by disulphide bonds. In spite of their biochemical similarity, the hormone families of insulin and RLX are kept distinct based on substantial differences in receptors, signal transduction pathways and biological effects. In humans, the RLX peptide family is composed of seven members, which include 3 RLXs, termed H1-3, and 4 insulin-like peptides INSL3-6. These peptides bind to and activate G-protein coupled receptors (GPCRs) [2, 3]. Although RLXs and INSLs have diverse sources and biological functions [3], the classical effects on the connective tissue are mostly specific to H2 RLX, or RLX-2 (and of its recombinant equivalent suitable for pharmaceutical use, named serelaxin), which is also the main hormone released by the corpus luteum and circulating in the blood in measurable amounts [4].

Therefore, from now on, the abbreviation RLX is intended to designate RLX-2 and serelaxin.

Most effects of RLX are mediated via the Relaxin Family Peptide Receptor 1 (RXFP1), a leucine-rich-repeat (LGR)-containing GPCR, originally discovered as LGR7 [5]. In particular, through this receptor, RLX has been demonstrated to induce its remodeling effects on connective tissue extracellular matrix (ECM) in several target organs. A common hallmark of this ECM-remodeling action is the ability of RLX to inhibit the signal transduction mechanisms of pro-fibrotic cytokines, primarily transforming growth factor (TGF)- $\beta$ 1, and hence their ability to promote differentiation of myofibroblasts, the key cellular players of fibrosis, and subsequent over-production and accumulation of ECM [6, 7]. Additionally, RLX stimulates a net increase in ECM degradation by up-regulation of matrix metalloproteases (MMPs) and down-regulation of their specific tissue inhibitors TIMPs [8, 9]. The major role played by RLX in promoting ECM remodeling suggests that the RLX/RXFP1 pathway can be a natural suppressor of age-related fibrosis in many organs, including the skin, lung, kidney, and heart. This view is further confirmed by the observation that RLX-null mice, in which the *RLN2* gene was knocked out, undergo age-dependent multi-organ fibrosis [10]. Furthermore, exogenously administered RLX has shown its efficacy in the prevention and treatment of experimentally induced fibrosis in animal models [11, 12]. These combined actions of RLX, together with the

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progress in technologies for bulk production of clinical-grade recombinant and synthetic RLX, have collectively contributed to its development as a potential anti-fibrotic drug.

This review gives an updated description of the main cellular and molecular mechanisms triggering fibrosis, highlighting those which can be effectively targeted by serelaxin/RLX. The final part of this review will be devoted to a critical reappraisal of the clinical trials performed to date with serelaxin as an anti-fibrotic drug.

## 2. KEY CELLULAR AND MOLECULAR MECHANISMS OF FIBROSIS

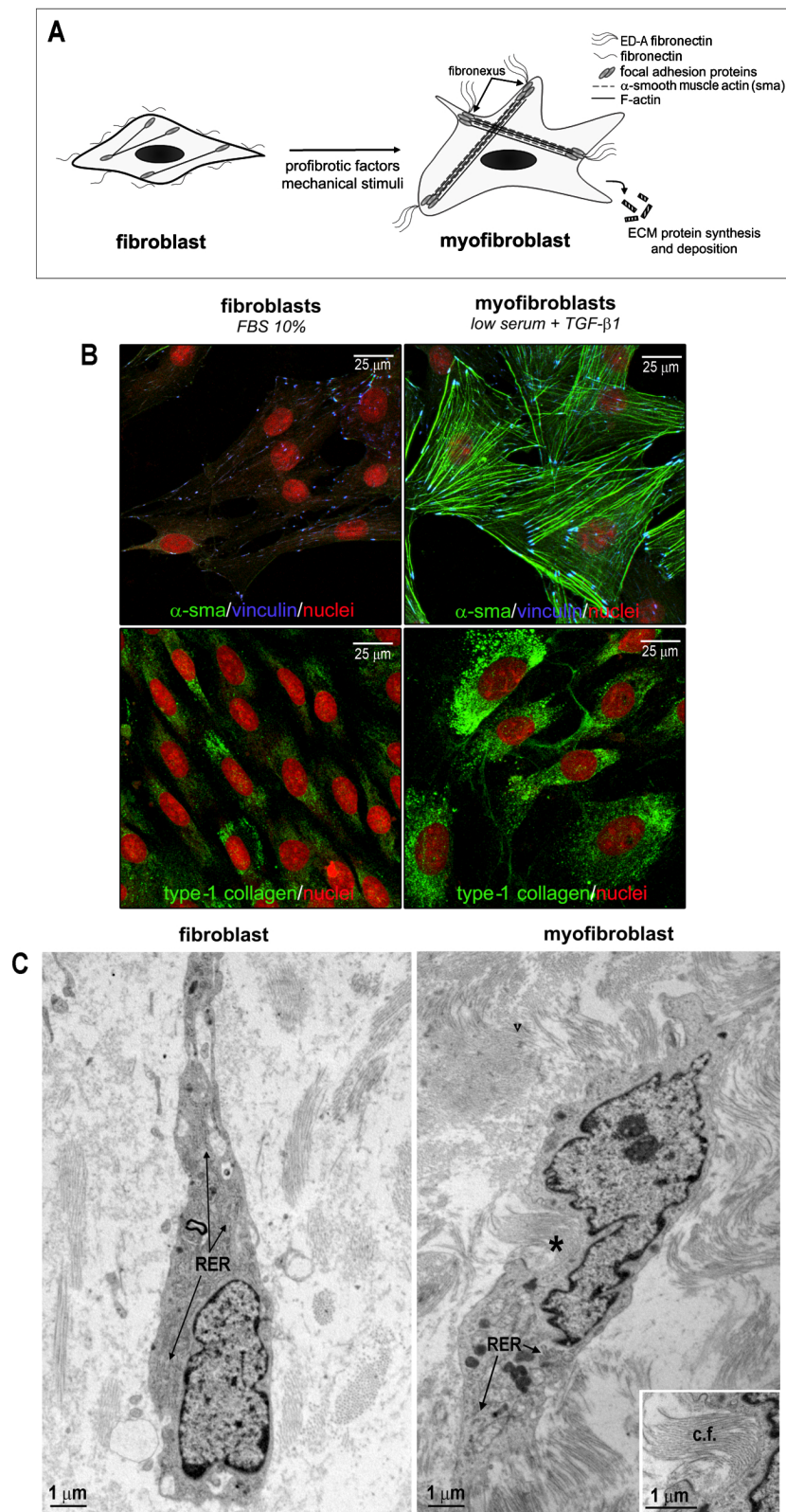
Fibrosis is generally defined as excess ECM production and deposition, often associated with detrimental stromal tissue shrinkage. In tissues endowed with endogenous regenerative potential, fibrosis can also compromise the function of resident progenitor/stem cells, hampering regeneration and ultimately provoking, at worst, disruption of the physiological organ architecture and function [13]. Activation of fibrogenic pathways represents a physiological adaptive response of organs to acute injury. It aims at wound contraction and scar formation, required to rapidly fix the damage, close the wound and preserve residual organ function. In addition to providing a passive mechanical support, scar tissue can also function as a scaffold to facilitate the migration of different cells involved in organ repair, such as vascular cells, inflammatory cells, and, if available, parenchymal stem/progenitor cells, providing instructive signals for their correct positioning and differentiation [14-16].

### 2.1. Myofibroblasts

In post-developmental organs of upper vertebrates, myofibroblasts are the key players of both physiological scarring and pathological fibrosis. Upon acute damage, myofibroblasts are rapidly recruited and activated to give rise to provisional contractile scar matrix, enabling wound size reduction and eventually closure in order to preserve organ function at best. However, myofibroblasts can act as 'good' pro-healing and as 'bad' pro-fibrotic cells depending on their interactions with other cells in the same micro-environment, especially inflammatory cells that represent the first responders to tissue injury, as well as with ECM components. In this way, dysregulated cell-cell or cell-matrix crosstalk may shift myofibroblast behavior from beneficial repair to detrimental fibrosis [17]. To properly achieve a reparative response of the injured tissue, recruitment and function of myofibroblasts must be temporally and spatially limited. Usually, over the course of physiological repair, the scar is degraded and replaced by normal tissue by means of regeneration mechanisms [18, 19]. The finely balanced activity of MMPs selectively digesting ECM components and of their specific tissue inhibitors, TIMPs, released by local cells, including myofibroblasts and inflammatory cells, is usually responsible for scar removal. In turn,

myofibroblasts should progressively disappear by apoptosis and/or senescence or, possibly, revert to a quiescent state [18-20]. By contrast, persistence and accumulation of activated myofibroblasts is a hallmark of pathological fibrosis. In chronic or severe insults, sustained activation of myofibroblasts has been observed in association with unresolved and/or dysregulated inflammatory response: under these conditions, myofibroblasts secrete a progressive excess of ECM proteins, which accumulate and replace the normal tissue [20-22]. Thus, fibrosis can be considered an aberrant/maladaptive tissue reparative response to damage. In such view, activated myofibroblasts have been detected in primarily fibrotic disorders including systemic sclerosis (SSc), sclerodermatous graft vs. host disease, nephrogenic systemic fibrosis, and idiopathic cardiac, pulmonary, liver, and kidney fibrotic syndromes, whose causative mechanisms are heterogeneous and, in part, elusive [23, 24].

Myofibroblasts possess immunophenotypical and ultrastructural features of both collagen-synthesizing cells (like fibroblasts, chondroblasts, osteoblasts), *i.e.* prominent rough endoplasmic reticulum (RER) and Golgi apparatus, and contractile cells (like smooth muscle cells), well-assembled myofilament bundles immunoreactive for  $\alpha$ -smooth muscle actin (sma) and trans-membrane ion currents typical of excitable cells [17, 25]. Typically, myofibroblasts secrete higher amounts of type-1 and -3 collagen, proteoglycans and other ECM components than fibroblasts, and their myofilaments are mostly arranged in stress fibers linked to "supermature" focal surface adhesion complexes, so-called fibronexus, in turn connected with extracellular fibronectin. In particular extracellular domain (ED)-A splice variant of fibronectin is crucial for the differentiated myofibroblastic phenotype. Fibronexus represents a mechano-transduction system by which the force generated by stress fibers is transferred to the surrounding ECM, and *vice versa*, the mechanical stimuli by ECM can be sensed and transduced into intracellular signals [17, 26, 27] (Fig. 1). Myofibroblasts mainly result from activation and differentiation of resident fibroblasts. However, other cells have been demonstrated to contribute to the myofibroblast population: they include mesenchyme-derived cells such as pericytes, hepatic stellate cells and circulating bone marrow-derived mesenchymal progenitor cells, as well as epithelial and endothelial cells exploiting their ability to transdifferentiate into mesenchymal cells owing to unique post-developmental processes termed epithelial-to-mesenchymal (EMT) and endothelial-to-mesenchymal (EndMT) transitions [16, 20, 28-32]. In diseased organs undergoing fibrosis, differentiation of different precursors into myofibroblasts is mainly promoted by the combined action of pro-fibrogenic cytokines and growth factors released by infiltrating inflammatory cells, especially macrophages, and of mechanical stimuli from the surrounding ECM [17, 21, 24, 27, 33]. This interplay activates different complex and sophisticated pro-fibrotic pathways. In turn, the



**Fig. (1).** (A) Schematic drawing of fibroblast-to-myofibroblast transition. (B) Representative confocal immunofluorescence analysis of *in vitro* cultures of normal fibroblasts and TGF-β1-induced myofibroblasts belonging to the NIH/3T3 cell line, labelled for the indicated markers [25]. (C) Representative electron micrographs of a normal fibroblast (left panel), showing an oval euchromatic nucleus and several cisternae of RER (arrows) and surrounded by a loose extracellular matrix, and a myofibroblast (right panel), surrounded by bundles of collagen microfibrils: the asterisk and the higher-magnification inset show a surface groove where the cell appears to build up and align the newly-generated collagen fibres (c.f.). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

contractile activity of myofibroblasts impacts ECM mechanical properties (stiffness) perceived by myofibroblasts themselves, initiating a vicious cycle of continuing myofibroblast activation and macrophage recruitment that maintains a pro-fibrotic state. This is considered one of the mechanisms capable of tipping the balance between beneficial repair and fibrosis, and thus a potential therapeutic target [13, 34]. Other local cells, including the fibroblasts/myofibroblasts themselves or damaged parenchymal cells, contribute to pro-fibrotic stimuli. For example, injured kidney tubular epithelial cells have been shown to release exosomes containing TGF- $\beta$ 1 mRNA [35, 36].

## 2.2. TGF- $\beta$ 1 Pathways

TGF- $\beta$  is regarded as the master cytokine in fibrosis. The TGF- $\beta$  superfamily includes TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3, among which TGF- $\beta$ 1 is the most abundant and prototypical. This is secreted as an inactive complex with latency-associated peptide (LAP), tethered and stored in the ECM. In turn, LAP binds the complex to latent TGF- $\beta$ -binding proteins (LTBP), which form cross-links with ECM proteins. TGF- $\beta$ 1 can be activated by different mechanisms, such as proteolysis, physical-chemical processes, or interaction with thrombospondin and integrins [36]. TGF- $\beta$ 1 signaling on target cells is mediated by a canonical pathway dependent on Smad2/3 or non-canonical Smad-independent pathways involving mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K) and Rho-like GTPases (Rho) [37].

The canonical pathway is activated upon binding of TGF- $\beta$ 1 to its cognate TGF- $\beta$  type II receptor (TGF $\beta$ RII), which in turn activates TGF- $\beta$  type I receptor (TGF $\beta$ RI). Then, TGF $\beta$ RI phosphorylates serine residues of receptor-activated Smad (R-Smad), a complex of Smad2 and Smad3, which binds to the common mediator Smad4. This ternary Smad complex translocates to the nucleus, interacts with specific *cis*-acting elements in the regulatory regions of its target genes, recruits other coactivators and eventually modulates the expression of a variety of genes involved in myofibroblast activation, EMT and EndMT. Such genes include connective tissue growth factor (CTGF),  $\alpha$ -sma, collagen IA2, Wnt, MMP-2, integrin-linked kinase (ILK), and  $\beta$ 1-integrin [37]. Of interest, it has been shown that this TGF- $\beta$ 1-induced canonical Smad2/3 signaling is regulated by ECM stiffness *via* control of Smad2/3 localization. This mechanoregulation appears to depend on the core components of the Hippo pathway, namely Yes-associated protein (YAP) and the transcriptional coactivator with PDZ-binding motif (TAZ). In fact, the Hippo complex has recently emerged as a mechanosensitive pro-fibrotic signaling pathway capable of integrating different biochemical and mechanical stimuli and promoting myofibroblast generation and activation [38-41].

The non-canonical pathways involved in TGF- $\beta$ 1 induced-fibrosis are downstream the three major MAPK subfamilies: extracellular signal-regulated kinases

(ERK) 1/2, p38/MAP kinases ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ), and stress-activated c-Jun N-terminal kinases (Jnk1-3) [37]. Activated MAPKs can exert transcriptional regulation either through direct interaction with the nuclear Smad protein complex or *via* other downstream proteins. A second non-canonical Smad-independent pathway involves PI3K, which activates two downstream pro-fibrotic pathways, protein kinase B(Akt)-mTOR and p21-activated kinase 2 (PAK2)/Abelson kinase (c-Abl), to stimulate collagen gene expression, fibroblast proliferation and differentiation to myofibroblast [37]. A third non-canonical pathway involves Rho GTPases, a subfamily of the Ras small GTP-binding proteins superfamily, whose activity is regulated by Rho guanine nucleotide exchange factors that can interact directly with Rho proteins, allowing the exchange of GDP for GTP. Rho GTPases are involved in cytoskeleton remodeling. In particular GTP-bound active Rho interacts with downstream effector proteins, such as Rho-associated coiled-coil containing protein kinase (ROCK), which promotes polymerization of monomeric G-actin into F-actin to form stress fibers, a hallmark of myofibroblasts. The consequent decrease in G-actin monomers frees G-actin-sequestered transcription factors, such as myocardin-related transcription factor (MRTF), which translocate into the nucleus and cooperate with serum response factor (SRF) to express fibrosis-related genes encoding for CTGF,  $\alpha$ -sma and collagen [42].

Other signaling mechanisms have been reported to interact with TGF- $\beta$ 1 and mediate fibrosis, besides the above canonical and non-canonical pathways. These mechanisms include, for instance, the Wnt/ $\beta$ -catenin pathway and the renin-angiotensin-aldosterone system (RAAS). Wnt proteins interact with Fizzled receptors and co-receptors (members of the LDL receptor-related protein 5/6) and activate a chain of downstream signaling events leading to dephosphorylation and stabilization of  $\beta$ -catenin. Then,  $\beta$ -catenin accumulates in the cytoplasm, translocates into the nucleus, and interacts with T-cell factor (TCF)/lymphocyte-enhancer-binding factor 1 (LEF1) to induce transcription of Wnt target genes. Moreover, by a mutual loop, Wnt/ $\beta$ -catenin can upregulate the expression of TGF- $\beta$ 1, while TGF- $\beta$ 1 can activate  $\beta$ -catenin [43-45]. Several studies have reported the activation of Wnt/ $\beta$ -catenin signaling during fibroblast proliferation, fibroblast-to-myofibroblast transition and EMT [37]. RAAS has been extensively shown to contribute to the development and progression of fibrosis in many tissues and organs [46]. In particular, angiotensin II exerts a pro-fibrotic action through angiotensin II type 1 receptor (AT $_1$ R), known as the classical RAAS pathway. Similar to TGF- $\beta$ 1/TGF $\beta$ RI/II, angiotensin II/AT $_1$ R activates Smad2/3 and MAPKs, even in the presence of TGF- $\beta$  antagonists, and stimulates the synthesis of ECM proteins, especially collagen I, fibronectin, PAI-1/2 and tenascin-C [47, 48]. Moreover, it also promotes the synthesis and release of TGF- $\beta$ , thus eliciting a vicious cycle that amplifies the pro-fibrotic effects of TGF- $\beta$  [46]. On the other hand, recent evidence demonstrates that different processing of angiotensin I, giving rise to



angiotensin-(1–9), angiotensin-(1–7), alamandine (generated by angiotensin-(1–7) decarboxylation), and angiotensin A (generated by angiotensin II decarboxylation and angiotensin-converting enzyme-2 processing), represents an alternative RAAS pathway able to counteract the classical RAAS through AT<sub>2</sub>R and Mas receptor (MasR), thereby ameliorating and preventing fibrosis [46]. Augmentation of the alternative RAAS holds promise for the therapeutic treatment of fibrosis. In this context, the antifibrotic effects of RLX that have been attributed to AT<sub>2</sub>R activation upon heterodimerization with agonist-activated RXFP1 [49] will be discussed in the following section.

Finally, our research group has demonstrated that TGF- $\beta$ 1 endorses fibroblast-to-myofibroblast differentiation by up-regulating the expression and activation of transient receptor potential canonical/stretch-activated (TRPC/SAC) ion channels [50] and of voltage-dependent gap junctions [25] and down-regulating Notch-1 signaling pathway, which is instead potentiated by RLX [51], as discussed in depth in the following section.

Taken together, the many data reported in this section shed light on the complex network of molecular mechanisms regulating differentiation and functional activity of fibrosis-generating stromal cells and allow to focus on the possible molecular targets of new anti-fibrotic therapies.

### 3. RLX AND STROMAL CELLS: EFFECTS AND MECHANISMS OF ACTION

As indicated above, RLX has been found to influence connective tissue cells by inducing an anti-fibrotic phenotype; most of these effects are achieved through activation of its natural receptor, RXFP1. This receptor has a complex molecular structure and an

even more complex mode of activation upon interaction with its RLX ligand [52]. Accordingly, its downstream signaling events are multiple and may differ depending on cell type, leading to a broad range of biological actions that include anti-inflammatory, ECM remodeling, pro-angiogenic and vasodilatory anti-ischemic effects [7, 9, 52–54]. Concerning stromal cells, the main signal transduction mechanisms can be summarized as follows (Fig. 2).

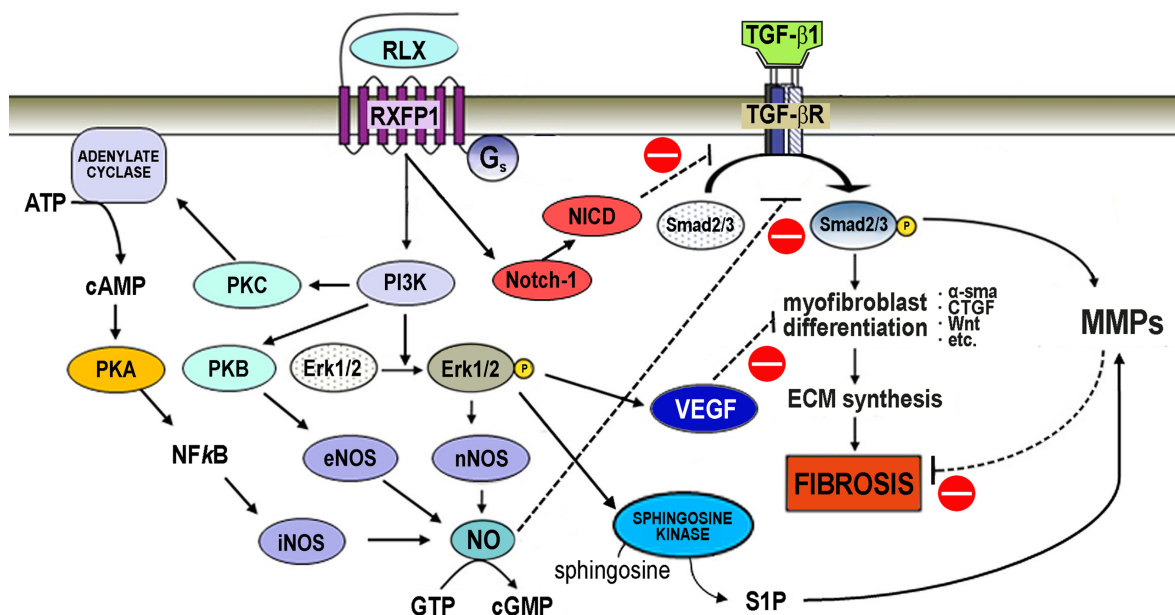
#### 3.1. Adenylate-cyclase/cAMP

Although the G-protein-dependent activation of adenylate cyclase is a classical receptor response mechanism, the pattern of cAMP elevation upon RXFP1 activation is unexpectedly complex. In fact, target cells may exhibit a biphasic cAMP response, an immediate transient one and a delayed sustained one. Peculiarly, many stromal cell types of different tissues and organs, including the uterus, heart, kidney and lung only show a modest or negligible immediate cAMP rise [7, 55, 56], while others, such as decidual stromal cells and monocytoid THP-1 cells, mainly exhibit the delayed one [57–59]. By a cAMP-dependent mechanism, RLX has been shown to mediate some key effects on stromal cells, such as decidualization, reduction of inflammation and accelerated wound healing [53].

#### 3.2. Protein Kinases

These enzymes also represent a key intracellular effector of many signal transduction pathways; those related to RXFP1 signaling are PI3K/protein kinase B (Akt); MAPK and particularly ERK1/2; protein kinase C (PKC).

PI3K/Akt is a canonical G-protein-dependent pathway involved in the regulation of cell survival,



**Fig. (2).** Schematic drawing of the main signaling transduction mechanisms mediated by RLX/RXFP1 in stromal cells, which are involved in the hormone's anti-fibrotic effects. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

apoptosis and proliferation. RXFP1 activation has been shown to activate this pathway in THP-1 monocytic cells [58], endothelial cells [60], primary chondroblasts [61] and NIH/3T3 fibroblasts [62]. By means of PI3K/Akt activation, RLX was shown to exert vasodilatory, anti-apoptotic and anti-fibrotic effects [53].

MAPK-ERK1/2, also activated by G proteins, are ser/thr kinases involved in a broad range of cellular processes, such as survival, proliferation and differentiation. RLX-induced increase in ERK1/2 phosphorylation has been reported in human endometrial stromal cells and THP-1 cells, rat renal myofibroblasts [7, 9] and chondroblasts [61]. By means of ERK1/2 phosphorylation, RLX has been demonstrated to up-regulate the expression of vascular endothelial growth factor (VEGF), thereby inducing angiogenesis [63], and endothelin-B (ET-B) receptor in vascular cells, thereby inducing vasodilatation [64]. This mechanism is also part of the complex intracellular molecular network involved in the anti-fibrotic action of RLX, as specified below.

PKC is another G-protein-dependent ser/thr kinase chiefly involved in cell cycle control; anecdotal evidence exists in the literature that PKC can mediate the response of some target cells to RLX, but not specifically of stromal cells [53].

### 3.3. Nitric Oxide Synthases/NO/cGMP

First evidence that nitric oxide (NO) could mediate the effects of RLX was obtained on mast cells [65] while searching the yet unknown mechanisms of its dilator effect on rat mesocaecal and mouse mammary gland microvessels [66, 67]. Soon after, NO synthases, NO and its second messenger cGMP were demonstrated to be key effectors of the vasodilatory action of RLX, exerted on both vascular endothelial and smooth muscle cells [68, 69]. Besides being a major player in the protection afforded by RLX from ischemia/reperfusion-induced damage as demonstrated in numerous cellular, isolated organs and whole animal models [70], the NO/cGMP system has been shown to be involved in the anti-fibrotic effects of RLX [7, 9, 71, 72]. This pathway is closely interlocked with that of protein kinases. In fact, increased NO generation can result from activation of constitutive NO synthases (eNOS, nNOS) induced by phosphorylation operated by PI3K/Akt, as well as increased expression of inducible NO synthase (iNOS) which follows nuclear translocation of the transcription factor NF- $\kappa$ B upon phosphorylation and inactivation of its inhibitor I $\kappa$ B operated by cAMP/PKA and/or ERK1/2 [73, 74]. RLX may also indirectly activate the NO pathway through the stimulation of ECM remodeling. In fact, RLX-induced MMP activation could convert big endothelin (ET)-1 into bioactive ET<sub>1-32</sub>, which in turn binds to ET-B receptors, thereby inducing eNOS activation [75]. Of note, NO can play an indirect albeit important role in the anti-fibrotic effects of RLX *via* its inhibitory effects on inflammatory cell recruitment and activation, and stimulatory effects on microvascular dilatation [76]; in this way, RLX can reduce the local

levels of pro-fibrotic cytokines driving the conversion of stromal cells into fibrogenic myofibroblasts. Since NO, by binding to Fe-heme, activates guanylate cyclase, an increase in cGMP and downstream activation of protein kinase G (PKG) are expected phenomena upon RLX-induced NO stimulation. In fact, increased cGMP levels upon RLX treatment have been found in lung myofibroblasts [56], and kidney and liver tissue undergoing experimentally induced fibrosis [71, 77]. In an acute experimental setting, the RLX-induced NO/cGMP/PKG signaling has been shown to inhibit myofibroblast contraction by reduced phosphorylation of myosin light chain and Ca<sup>2+</sup> transient [56] and to promote the expression and activity of collagen-degrading MMPs [9, 72].

### 3.4. VEGF

RLX induces the production of VEGF in a variety of cell types, including endometrial stromal cells, THP-1 cells and cardiac fibroblasts [78-81]. By VEGF, RLX can induce angiogenesis, thereby improving tissue perfusion, especially in organs subjected to ischemia and reperfusion, and wound healing. In addition, it can be speculated that the anti-fibrotic action of RLX could be in part VEGF-mediated, given the property of VEGF to inhibit myofibroblast differentiation [82].

### 3.5. Notch-1 & Sphingosine-1-phosphate (S1P)

In recent years, anecdotal evidence has shown that RLX can induce anti-fibrogenic effects on connective tissue by activating the Notch-1 and sphingosine-kinase/S1P pathways [51, 83, 84], which are known as regulators of cell differentiation. By these mechanisms, RLX appears to inhibit differentiation of myofibroblasts, induce activation of MMPs and counteract the pro-fibrotic effects of TGF- $\beta$ 1.

### 3.6. Crosstalk with other Receptors/signaling Pathways

Besides the actions depending directly on RXFP1 and its signal transduction mechanisms, RLX can also induce some of its physiological and pharmacological effects through crosstalk between RXFP1 and other receptors or their signaling pathways, chiefly TGF- $\beta$ R and AT<sub>2</sub>R. Namely, RLX has been demonstrated to inhibit TGF- $\beta$ 1/TGF- $\beta$ R signaling by interfering with the phosphorylation of Smad2/3, the targets of ERK1/2; such inhibition results in decreased myofibroblast activation and ECM deposition [12, 51]. Another newly discovered important crosstalk involves RXFP1 and AT<sub>2</sub>R: starting from the observation that angiotensin metabolites also inhibits TGF- $\beta$ 1 activity through AT<sub>2</sub>R, Dr. Byrna Chow and coworkers demonstrated that RXFP1 and AT<sub>2</sub>R form heterodimer complexes and that these heterodimers are required for RLX's antifibrotic effects [49].

Finally, it is worth mentioning that increased knowledge of RXFP1 structure and function has led to the development of low-molecular-weight oligopeptide analogs, such as CGEN25009 and B7-33 [85, 86] and

non-peptidic agonists, such as ML290 [87], which have been shown to retain many effects of RLX, particularly the ability to reduce fibrosis, being endowed with improved pharmacological and pharmacokinetic features, e.g. extended half-life or reduced immunogenicity [88]. These molecules hold great promise as new therapeutic approaches to fibrotic diseases.

#### 4. SERELAXIN AS ANTI-FIBROTIC DRUG: EVIDENCES FROM CLINICAL TRIALS

Since the early studies of Hisaw [1], evidence has been accumulating that relaxin has marked, specific effects on ECM turnover and remodeling, which have been held responsible for the hormone's effects on cervix ripening and birth canal dilation [4]. On these bases, once available in sufficient amounts for clinical trials, relaxin was investigated as a possible new drug for fibrotic diseases [89].

##### 4.1. Scleroderma/systemic Sclerosis

Anecdotal findings of pioneer studies with partially purified porcine relaxin in patients suffering from systemic sclerosis with cutaneous involvement suggested amelioration of skin stiffness and trophic ulcers [90, 91], whereas subsequent investigations reported little or no improvement of the major fibrosis parameters [92]. Of note, relaxin administration was reported to be associated with accelerated healing of ulcers, putatively because of vasodilatation and improved blood perfusion [92]. A major advancement was the use of recombinant DNA technology to produce human RLX, now termed serelaxin. Using this molecule as a drug, Seibold and co-workers [93] performed an accurate double-blind placebo-controlled clinical trial, carried out to investigate its therapeutic efficacy on scleroderma patients, for whom no effective anti-sclerotic therapy exists. This trial enrolled 64 patients of both sexes: 45 received RLX by continuous s.c. infusion at 25 or 100 µg/kg body weight for up to 24 weeks using microinfusion pumps. Peculiarly, the primary efficacy objective, set as improvement of skin thickness (by modified Rodnan skin score), was only reached with the lower RLX dose of 25 µg/kg body weight. On the other hand, the secondary efficacy objective, set as pulmonary function, was not met at any RLX dosage [93]. The apparent paradox of a lack of efficacy of RLX at high dosage can in fact be explained by later studies demonstrating desensitization and internalization of RXFP1 induced by ligand stimulation [94]. This behavior of RXFP1, first described in primary human decidual cells *in vitro* [94], is likely to consistently occur in other RLX target cells and organs in humans and needs to be taken into account when planning posologic schemes with RLX. Another key issue that can contribute to explaining the unsuccessful outcome of the clinical trial with RLX in scleroderma is the reduced expression of RXFP1 in the skin of such patients, causing a substantial loss of responsiveness of dermal myofibroblasts to RLX, which becomes unable to counteract the pro-fibrotic effects of

TGF-β1 [95, 96]. As a matter of fact, despite the many encouraging theoretical premises, no further clinical studies with RLX in scleroderma or systemic sclerosis have been then undertaken.

##### 4.2. Heart Failure

Similar premises, based on preclinical data from several research groups worldwide, have prompted to investigate the possible clinical efficacy of RLX in acute heart failure (AHF). This disease represents a major challenge in cardiology because of the high incidence in the ageing population, high morbidity and mortality, high financial burden on health care systems, and limited therapeutic options. Clinically, AHF is characterized by symptoms (dyspnoea, orthopnoea, lower limb swelling) and signs (elevated jugular venous pressure, pulmonary congestion and oedema) related to abnormal cardiac stiffness, reduced systolic output and elevated intra-cardiac pressures [97]. Different aetiological causes concur to similar pathological consequences among which fibrosis is a key hallmark, which justifies the RLX hypothesis. A preliminary phase IIb dose-ranging and safety pilot study (Pre-RELAX-AHF, ClinicalTrials.gov n. NCT00520806) on a limited cohort of AHF patients (N=234) suggested that treatment with RLX could be advantageous. The patients were subjected to standard care plus 48-h i.v. infusion of placebo (n=62) or RLX 10 (n=40), 30 (n=43), 100 (n=39), or 250 (n=50) µg/kg b.wt. per day; then primary and secondary endpoints (dyspnoea, days of hospitalization, out-of-hospital days after dismissal, cardiovascular death or re-hospitalization, n. of serious adverse events) were evaluated for up to 60 days. Remarkably, RLX (best dosage 30 µg/kg b.wt) caused a significant reduction of dyspnea as well as of overall cases of death and re-admission [98]. However, a subsequent, extended multi-center study (RELAX-AHF-2, ClinicalTrials.gov n. NCT01870778) was not able to confirm the preliminary data of the Pre-RELAX-AHF study. In this study, placebo (n=580) or RLX (30 µg/kg b.wt., n=581) were administered as continuous i.v. infusion for 48 h in addition to standard care and patients were followed-up to 180 days. Although attention was paid to narrow down the inclusion criteria in order to achieve good standardization of the enrolled patients, RLX only showed modest, albeit significant, advantages over placebo in attenuating dyspnoea in the short term and reducing deaths from cardiovascular causes in the long term [99]. The leading investigators of these clinical trials have recently performed a careful meta-analysis of cumulative results of the previous studies, coming to the conclusion that RLX (n=6105) at the reported administration regimen was associated with a significantly reduced risk of short-term (5-day) worsening of AHF symptoms and slightly better renal function markers in respect with placebo (n=5254), but other key endpoints, such as cardiovascular deaths, occurrence of heart or renal failure, and length of hospitalization or re-hospitalization, were not significantly different from the placebo groups. On a positive note, RLX administration was consistently reported to be well tolerated [100]. These substantially

inconclusive results did not suffice to convince the US and European Medicine Committees for releasing permission to put RLX into the market; despite its favourable safety profile, it was deemed that the limited benefits ascribable to RLX did not outweigh its risks [70].

### 4.3. Cervical Ripening

Poor softening of connective tissues of the birth canal is the main cause of delayed parturition and related maternal-fetal complications. Based on the well-assessed physiological effects of relaxin on reproductive organs in several animal species [101], it seemed logical to exploit its therapeutic efficacy as an inductor of cervical ripening. Despite the favorable results of pioneering studies with local administration of purified swine relaxin [102], later double-blind placebo-controlled clinical trials and meta-analyses on a larger number of parturients failed to confirm significant advantages induced by either porcine relaxin or RLX to ripen the cervix and induce labor [103, 104]. Currently, the place of RLX as a drug for obstetric purposes remains marginal and of little clinical interest [105].

## CONCLUSION AND PERSPECTIVES

The initial enthusiasm towards clinical use of RLX as a new drug for fibrotic diseases, despite a firm theoretical background of its well-known anti-fibrotic actions in cellular and animal models [4, 12, 101], has been tempered by the inconclusive and disappointing results of all the endeavored clinical trials [70, 89, 106]. This ostensible paradox deserves to be carefully analyzed to identify the points of weakness that have hampered a clear demonstration of RLX's clinical efficacy.

**Experimental animal models vs. clinical trials.** A first critical point relies on obvious differences between the highly standardized animal models of disease exploited to demonstrate the anti-fibrotic effects of RLX, commonly using in-bred animals of similar age and sex and the same experimental procedures for disease induction, and the intrinsic heterogeneity of the population of patients suffering for fibrotic diseases enrolled in the clinical trials, who can differ by age, sex, genetic background, etiology, time of disease, previous or concurrent therapies, etc. All these factors cause a broader dispersion of the values of the assayed parameters, which can deeply influence the statistical significance of the final results.

**Interfering therapies.** In most clinical trials with RLX, especially when dealing with severe life-threatening diseases, it was deemed unethical to suspend other previous or concurrent therapies. However, recent experimental studies have demonstrated that AT<sub>1</sub>R blockers, such as sartans, widely used in elderly patients with fibrotic diseases also suffering from hypertension, abrogate the response of myofibroblasts to RLX, thereby reducing or negating its anti-fibrotic effects [107]. Drug interference with the reliability of clinical results has been a major

objection of the US and European Medicine Committees against the commercialization of RLX.

**Pharmacokinetic issues.** Typically, fibrosis is a chronic disease requiring administration of RLX for long times. This is a powerful limiting factor to the clinical use of RLX since it has a short half-life, estimated to be ~2 h [108], and its *in vivo* effects are rapidly lost [8]. Therefore, RLX must be administered parenterally, usually in multiple daily doses, with obvious disadvantages for the patients. Likely, this issue has contributed to the negative outcome of the RELAX-AHF-2 trial because RLX administration was discontinued after 48 h while the clinical data were collected at day 60 and day 180. Indeed, a careful data re-appraisal showed that the beneficial effects of RLX therapy vanished in 14 days after drug cessation [100]. Such issues could be addressed by means of portable infusion devices optimized for the delivery of peptidic drugs. On the other hand, the tempting hypothesis that RLX could be delivered orally by means of appropriate formulations to withstand gastrointestinal digestion and allow absorption in the small bowel [109] has not been validated experimentally yet.

**Immunologic issues.** Since RLX, commonly used for therapeutic purposes, derives from the human *RLN2* gene, its identity with the natural hormone reduces the likelihood of anaphylactic reactions. Nonetheless, upon long-term systemic delivery to scleroderma patients, circulating anti-RLX antibodies were detectable in ~20% of the patients [93]. Although major immunologic adverse effects, such as anaphylaxis, were not reported, the presence of circulating anti-RLX antibodies in these patients could have caused the inactivation of an unpredictable fraction of the administered drug, with obvious negative effects on its actual efficacy.

**Oncologic issues.** Anecdotal reports indicate that H2 RLX is increased in the plasma of patients with osteosarcoma and its levels are positively correlated with the risk for metastases [110]. Although a causative connection was not demonstrated, the possibility that RLX could favor the spread of metastatic cells, possibly by accelerating degradation of basement membranes which surround tumors and limit their invasiveness, dictates extreme caution when planning long-term treatment with RLX because of possible increased metastasis risk from undiagnosed malignancies.

**Future directions and perspectives.** The previous clinical trials with RLX as an anti-fibrotic drug have highlighted some key points of strength and weakness that must be considered when planning future clinical investigations. Since RLX is maximally effective upon acute administration, it could offer actual therapeutic advantages if used to counteract the early pathogenic events of fibrosis, namely inhibition of myofibroblast differentiation and activation. In this view, RLX may hold promise in dermatological and aesthetic surgery: *i*) to reduce the formation of keloids or hypertrophic scars in predisposed subjects or after burns, *ii*) to minimize surgical scars upon aesthetic interventions, *iii*) to reduce sclerotic complications and shrinkage due to



formation of a fibrous capsule around breast prostheses, *iv*) to improve skin compliance upon placement of skin expanders in preparation for plastic surgery (this application was successfully exploited in a swine model) [111]. Another possible area of interest could be idiopathic pulmonary fibrosis, a progressive, often lethal disease for which no effective therapies exist; in the search for novel treatments, molecular targeting of TGF- $\beta$  signalling and myofibroblast activation appears to be the most promising approach [112], thus opening a slot for RLX. A step forward in this direction could be favored by setting up new inhalation delivery modes of RLX. Finally, sound experimental evidence has demonstrated that RLX can promote remodeling of bone, ligaments and muscles and their healing upon induced injury [113] and dramatically improves the recovery of animals subjected to experimental shoulder arthrofibrosis [114]. Although the application of RLX to conditions of musculo-skeletal fibrosis has not yet been the object for clinical trials, this would also represent a further promising therapeutic option.

Finally, as introduced above, low-molecular-weight RLX analogs, such as the oligopeptides CGEN25009 and B7-33 [85, 86], and the non-peptidic RXFP1 agonist ML290 [115] have been developed and demonstrated to retain many of the anti-fibrotic effects of authentic RLX, especially the ability to interfere with TGF- $\beta$  signaling, reduce myofibroblast recruitment and activation, and promote ECM remodeling, while showing improved pharmacological and pharmacokinetic features, such as longer half-life, reduced immunogenicity, and suitability for other delivery routes in addition to classical *i.v.* one [88]. These molecules could represent a valuable alternative to RLX in the treatment of fibrotic diseases.

## CONSENT FOR PUBLICATION

Not applicable.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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