Human relaxin-2: historical perspectives and role in cancer biology.

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Abstract:

One of the most recognised and studied family of peptide hormones is the insulin superfamily. Within this family is the relaxin subfamily which comprises seven members: relaxin-1, -2 and -3 and insulin-like (INSL) peptides 3, 4, 5 and 6. Besides exhibiting sequence similarities, each member exists as an active A-B heterodimer linked by three disulfide bonds. This mini-review is divided into three broad themes: an overview of all insulin superfamily members (including structural similarities); roles of each superfamily member and finally, a focus on the pleiotropic peptide hormone, human relaxin-2. In addition to promoting vasodilatory effects leading to evaluation in Phase III clinical trials for the treatment of acute heart failure, relaxin has recently been shown to be highly expressed by cancer cells, aiding in their proliferation, invasiveness and metastasis. These contrary effects of relaxin are discussed together with current efforts in the development of relaxin antagonists that may possess future therapeutic potential for the treatment of certain cancers.

INTRODUCTION

Evolution – insulin and relaxin family of peptides

In the early 1920s, Frederick Hisaw alongside his then PhD student, Alexander Albert, and other co-workers observed pelvic ligament softening and broadening in pregnant female guinea pigs, which aided in offspring delivery (Hisaw 1926). Albert sectioned sow corpora lutea and extracted and partially purified the hormone involved in the guinea pigs' interpubic ligament relaxation. Injection of this extracted hormone into virgin guinea pigs induced similar effects as seen in the pregnant guinea pigs. Thus, the hormone was termed "relaxin" (Hisaw 1926; Ziel 2000).

In 1945, Robert Kroc, another of Hisaw's former PhD students, took on the pioneering role of developing bioassays to enable the structural study of relaxin. Due to obstacles associated with protein-isolating techniques and the difficulty of preparing pure relaxin, the study the physiology and chemistry of relaxin was a challenge (Friedman 2003). It was not until the mid-1970s that improved techniques to isolate and produce large quantities of purified relaxin enabled determination of the first relaxin primary structure (Bathgate et al. 2006a). Relaxin was shown to share remarkable structural conservation with insulin (approximately 25% structural similarity) in that it consisted of 2 chains held together by three disulfide bonds (Bathgate et al 2006a; Friedman 2003). It was subsequently shown that like insulin, relaxin was also synthesised initially as a single chain pre-prohormone (Sherwood and O'Byrne 1974). Today, with the advancement of DNA sequencing and genomic database searching technologies, primary structures of relaxin from more than twenty different species have been determined (Bathgate et al. 2006).

Further studies have shown that there are several other insulin-like peptides in the genome. These make up what is now known as the insulin superfamily. Within this family, the relaxin subfamily consists of two main groups, the insulin-like peptides (INSL3, 4, 5 and 6) and relaxins (relaxin-1,-2 and -3) (Park et al. 2005) as shown in Figure 1. While the evolutionary pattern and partial sequence homology between the relaxin-1 (RLN1) and -2 (RLN2) genes suggest some similarities between them (Kong 2010), subsequent studies have shown that the RLN1 gene is in fact a pseudo-gene that does not get translated into a functional protein (Bathgate et al. 2006a); while the RLN2 gene encodes the major stored and circulating form of relaxin in humans. Interestingly, lower primates and rodents lack an orthologue of the RLN1 gene, but contain the RLX and RLX3 genes, which encode for relaxin and relaxin-3; the species-equivalent of human relaxin-2 and relaxin-3 (Wilkinson 2005), respectively. Like human relaxin-2, the equivalent relaxin peptide in other species represents the major stored and detectable form. For the purposes of this review, genes expressed in the human are systematically named as RLN and those expressed in other species termed RLX (Bathgate et al. 2006a; Wilkinson 2005).

Overview – relaxin family structures

The principal reason members of relaxin family were classified within the insulin superfamily is because of the structural similarities between the two (Figure 2). Furthermore, there is clear evidence towards the evolution of relaxin from early vertebrates from an ancestral insulin gene (Bathgate et al. 2006).

Like insulin, relaxin is initially synthesised on the ribosome as an immature pre-prohormone, prorelaxin, as a single chain structure attached to an N-terminal signal sequence (Bathgate et al. 2006) (Figure 3). The signal sequence directs the transport of the peptide from the ribosomal ubiquitins. Following the loss of signal peptide, the pre-prohormone is converted to a prohormone via co-translational modifications. Proteolytic cleavage of C-chain then yields the A- and B- chain combination, forming mature, active 2-chain heterodimeric peptide. In this two-chain structure, the A- and B-chains are cross-linked by three disulfide bonds with two linking both A- and B-chains and an intra-chain disulfide bond within the A-chain (Bathgate et al. 2006; Liu et al. 2003). With the exception of INSL3, these post-translational modifications remain to be confirmed for members of the INSL family (Büllesbach and Schwabe 2002).

The amino acid sequences of all relaxin family members have been determined by DNA recombinant technologies. Little homology is observed amongst the members with the only invariant residues being the

cysteines across both A- and B- chains making up the disulfide bonds and a single glycine within the B-chains. These non-conserved regions have been implicated in the various roles and receptor binding abilities of each relaxin family member. For human relaxin-2, despite its established role as a multi-functioning hormone, the first crystal structure of this pleiotropic hormone was determined and published only in the early 1990's (Eigenbrot et al. 1991).

Receptors – Relaxin Family Peptide Receptors (RXFPs)

Peptides within the relaxin family interact with G-protein coupled receptors (GPCRs), the biggest class of receptors found in humans. Relaxins-1 (chemically synthesised from its annotated sequence) and -2 can bind and activate leucine-rich repeats containing LGR7 while INSL3 binds and activates LGR8 (Hsu et al. 2002; Kumagai et al. 2002). Following characterization, these two receptors were later termed Relaxin Family Peptides Receptor 1 (RXFP1) and 2 (RXFP2), respectively, by the International Union of Pharmacology (Bathgate 2006a). Structurally, both receptors are more than 50% similar sequentially and contain a seven-transmembrane spanning domain and ectodomains comprising low density lipoproteins Class A (LDL-A) at the N-terminus. These structures contain leucine rich repeats (LRR), which act as the primary binding site for relaxin and connect the seven transmembrane spanning domain to the ectodomain. Relaxin-3 and INSL-5 bind to GPCR135 (now referred to as RXFP3) and GPCR 142 (RXFP4), respectively (Liu and Lovenberg 2008; Zhu 2008). These latter two receptors lack the ectodomain region, LRR, and LDL-A modules. Activation of RXFP1 and RXFP2 results in intracellular cAMP production, via receptor coupling to G_{α_s} and G_{α_o} . In contrast, RXFP3 and RXFP4 activation leads to inhibition of cAMP production due to downward actions of inhibitory G proteins (Lu et al. 2006; Lin 2004).

Due to the structural similarity of peptides within the relaxin superfamily, cross activation of non-native receptors does exist. For example, relaxin-3 has been shown to bind and activate RXFP1 (Bathgate et al. 2006b; Hossain et al. 2011; Zhang et al. 2012) and RXFP4 as well as its native receptor, RXFP3. Likewise, relaxin-2 binds to and activates RXFP2, the native INSL3 receptor (Sherwood 2004).

RELAXIN PEPTIDE SUBFAMILY MEMBERS – ROLES AND THERAPEUTIC APPLICATIONS

Insulin-Like Peptides (INSLs)

INSL3 has been implicated as having reproductive and non-reproductive roles. In male rats, INSL3 has been shown to prevent male germ cells from entering the apoptosis stage of the cell cycle (Kawamura et al. 2004). Additionally, the previously termed Leydig cell insulin-like peptides (Adham et al. 1993) are produced by foetal Leydig cells and are involved in testicular descent via the growth of gubernacular ligament (Ivell et al. 2005). In females, INSL3 is also implicated in a similar anti-apoptotic role, especially in ovarian follicles and thecal cells, during the follicle selection processes (Kawamura et al. 2004; Ferlin et al. 2009). INSL3 antagonists are thus expected to be potential male and female contraceptive agents (Shabanpoor et al. 2010).

The fetal perichondrium and cytotrophoblasts have been shown to express INSL4, and accounts for it also being known as Early Placenta Insulin-Like Peptide (Bellet et al. 1997). Similar to the other members of this superfamily, INSL4 is expressed as a 15kDa precursor, which then undergoes cleavage to form its tertiary structure (either two or three chain structures) (Büllesbach and Schwabe 2001). INSL4 has been implicated in being involved in trophoblastic development, including early cell proliferation and development (Laurent et al. 1998). Despite being exclusive to higher primates, the exact signal transduction and physiological role of INSL4 remain unknown.

Examination of the expressed sequence tags databases resulted in the discovery of a novel insulin-like sequence, termed INSL5. The primary physiological function of INSL5 remains unknown but recent studies imply it may play a role in appetite control and gut motility (Belgi et al. 2011). The peptide has been produced by both chemical synthesis and recombinant DNA expression (Hossain et al. 2008, Luo et al 2010) which, in turn, is aiding the identification of its functional roles.

Another member of this ancient superfamily of functionally diverse peptide hormones is INSL6. It is predominantly expressed in the primary reproductive male organ, the testis. However, published results do not concur regarding the specific cell type expressing INSL6. For example, studies by Hsu (Hsu 1999) concluded INSL6 is expressed in Leydig cells but studies by Lok *et al.* (Lok *et al.* 2000) established expression in spermatids but none in Leydig cells. Despite this discordance, INSL6 has been shown to be heavily involved in progression of spermatogenesis (Lu 2006). Further research is required to identify the specific receptor for INSL6 and its exact physiological chemistry.

Relaxin

Despite having two peptide-coding genes, relaxin gene 1 (RLN1) and RLN2, the major stored and circulatory form of relaxin in humans is relaxin-2. Relaxin-2 is produced in the prostate by males (Feng and Agoulrik 2007) and corpus lutea in females (Shabanpoor *et al.* 2009). Since relaxin-1 is a pseudogene, which does not translate into a functional peptide in rodents, humans and other non-human species, both relaxin-1 and -2 will be referred to as relaxin here forth.

As mentioned previously, relaxin is the most comprehensively studied member of the relaxin subfamily. Structure-activity studies of relaxin have revealed the receptor “binding cassette” of this multi-functional peptide hormone lies within its B-chain (Arg₁₃-X-X-X-Arg₁₇-X-X-Ile₂₀) and interacts with the binding pocket in native receptor, RXFP1. Büllesbach *et al.* have shown the importance of the binding cassette by replacing the arginine at position 13 and 17 with citrulline, lysine and alanine, rendering inactive the relaxin native receptor (no interaction with RXFP1) (Büllesbach and Schwabe 2000). Arg₁₃ and Arg₁₇ residues on the B-chain of relaxin interact with a network of two aspartic acid/glutamic acid pairs in the LRR within the ectodomain of its native receptor, RXFP1. In addition, the Ile₂₀ residue on the relaxin B-chain interacts with tryptophan-isoleucine-leucine region of the LRR. Non-specific binding is observed on deletion of any of the aforementioned three residues on the ligand (Büllesbach and Schwabe 2000; Hossain and Wade 2010).

The effect of relaxin, particularly during pregnancy, is well established in rodents. Levels of relaxin change with the different stages of pregnancy and these patterns are dissimilar across species. In rodents, sows and dogs, relaxin is untraceable early in the gestation but increases and reaches a maximum before labour (Hwang 1989; Johnson 1991). Conversely, maximum circulating relaxin is observed in humans within the first trimester of pregnancy. Plasma relaxin then reduces and somewhat plateaued for the remaining period of pregnancy, almost in a contrary pattern to rodents, sows and dogs (Burger *et al.* 1998; Eppel *et al.* 1999). As mentioned earlier, the main source of circulating relaxin is from the corpus luteum in females (Shabanpoor *et al.* 2009) and has been shown to aid in embryo implantation via uterine vascularisation and differentiation of endometrial cells (Eppel *et al.* 1999). Besides aiding in pelvic ligament and cervical softening of birth canals, relaxin has been shown to be involved in remodelling and development of mammary glands and nipples of mice (O'Day *et al.* 1989).

Furthermore, relaxin has been shown to increase oocytes fertility (Brener *et al.* 1984). Besides exerting its effect in female reproduction, relaxin is also involved in maintaining sperm motility in the male reproductive system (Weiss 1989). This conclusion was drawn when increased penetration was observed in human cervical mucous for human sperm incubated with porcine relaxin compared to buffer mixture (Weiss *et al.* 1989; Pupula *et al.* 1986). These exciting findings point to the therapeutic potential of relaxin towards assisting with infertility in humans.

Relaxin has also been shown to be involved in non-reproductive functions. For example, relaxin plays a crucial role in cardiovascular and renal systems (Conrad *et al.* 2004). Relaxin promotes collagen breakdown in systemic tissues (Unemori 1990), rendering a high possibility for an ability to reduce systemic fibrosis (Unemori *et al.* 1996; Samuel 2005). Fibrosis occurs when collagens, glycoproteins and other extracellular matrix accumulate in organs. When relaxin is introduced to systemic organs such as the heart and lungs, over-expression of collagen has been reduced (Samuel *et al.* 2004). Moreover, relaxin has also been observed to increase vasodilation and renal functions in pregnant females. This, along with its cardioprotective actions (Dschietzig *et al.* 2006; Samuel

et al. 2006) has led to efforts to exploit its haemodynamic roles and consequently relaxin is currently in Phase III clinical trials for the treatment of acute heart failure. The clinical trials to date have demonstrated relaxin provided relief of dyspnoea (symptom of breathlessness) and reduction of heart failure symptoms (Teerlink et al. 2009).

Relaxin-3

Relaxin-3 exerts its actions through its native receptor, RXFP3, which is found in the hypothalamic paraventricular nucleus of the brain. The expression site of this most recently discovered insulin superfamily neuropeptide was found to be in the nucleus incertus within the hypothalamus. These regions of the brain have been extensively associated with regulation of energy homeostasis and appetite regulation (McGowan et al. 2005). Additionally, the paraventricular nucleus plays a reproductive role during reproduction by providing feedback to the hypothalamic gonadotrophin-releasing hormone neurons (Chan et al. 2011). Interestingly, relaxin-3 knockout mice have shown altered sleep patterns during normal active (night) periods with increased sleep episodes compared to their wildtype counterparts (Ma and Gundlach 2007). Although the exact physiological mechanisms of relaxin-3 in humans still remain vague, studies from rodents have suggested that relaxin-3 may coordinate sleep, hunger and food intake regulation, (Chan et al. 2011; Ma and Gundlach 2007) which make the developments of relaxin-3/RXFP3 agonists and antagonists desirable therapeutic candidates.

RELAXIN AND ITS POTENTIAL ROLE IN CANCER BIOLOGY

While well known for its reproductive and antifibrotic roles, most recently relaxin has been associated with cancer biology. A number of putative roles, including the modulation of tumor growth, neovascularization, metastasis and oncogenic progression, have been correlated to relaxin overexpression (Silvertown et al. 2003). The following sections will focus on the effects of downstream intracellular relaxin signaling and its physiological implications.

Intracellular pathways associated with cancer biology known to be activated by relaxin

In addition to its vasodilatory effects, relaxin has been shown to promote nitric oxide (NO) production in renal, cardiac and hepatic systems. In MCF-7 breast cancer cell lines, production of NO was reported to be increased via heightened nitric oxide synthase (iNOS) production (Failli et al. 2002; Bani et al. 1999b). Increased NO production is implicated with oncogenic cell migration and growth (Bani et al. 1995; Jadeski et al. 2003). As highlighted above, relaxin stimulates increased vasodilation in a range of systemic tissues, including the skeletal and cardiac muscles. Relaxin-induced NO production could possibly encourage blood flow and growth of new blood vessels (angiogenesis) in the MCF-7 mammary cancer cell line. NO encourages cell apoptosis by inhibiting DNA synthesis and mitochondrial respiration, decreasing rate of cellular growth and multiplication (Jadeski et al. 2000). However, studies have shown increased expression of iNOS in an adenocarcinomic breast cancer cell line, MCF-7, incubated with porcine relaxin. These observations may correlate with relaxin assisting cancer cells avoid to apoptosis. This could lead to further invasiveness and metastasis potential, particularly with malignant oncogenic cells (Bani et al. 1995; Jadeski et al. 2000). Further studies investigating the relationship of increased NO production and its impact on oncogenic acceleration may be useful in understanding the role of relaxin in cancer.

The influence of relaxin in cell growth, invasion and angiogenesis

Besides its inherent biochemical signalling pathway, other physiological roles of relaxin may also encourage tumour advancement, further aggravating the severity of cancer. Cancer progression involves oncogenic cell replication, development (tumorigenesis) and spread of the tumour mass from one organ or tissue to another (metastasis). All of these physiological actions involve angiogenesis and tissue growth, remodelling and apoptosis (Failli et al. 2002). These oncogenic “hallmarks” are closely related to one another despite being classified as three separate physiological events – cell growth, cell invasion and angiogenesis. These three events can be seen in two gender-specific leaders in oncogenic mortality: prostate cancer and breast cancer. The following sections address these “hallmarks” and their consequential effects.

1. Relaxin and prostate cancer

The unresponsive state of uncontrolled cell division and differentiation is a characteristic feature of cancer cells. As previously mentioned, further growths of tumours are aided by vascularisation due to over-expression of NO. The changes observed in matrix metalloproteases contribute to changes in the underlying framework of connective tissues – this increased angiogenesis and changes in connective tissue framework are classic giveaways of relaxin-mediated effects (Hansell et al. 1991; Samuel et al. 2004; Bathgate et al. 2006). Early studies which focus on causatives of neoplastic prostatic cells, particularly abnormal cell increase, conclude peptides may also be involved in hyperplasia (Ivell et al. 1989; Sokol et al. 1989) as well as steroid hormones (Montie and Pienta 1994).

The state of hyperplasia was indicated by angiogenesis and remodelling of the connective tissue framework. Furthermore, marked uncontrolled rate of cell differentiation is another indication of the aforementioned neoplastic prostatic cells observed in men and male dogs (Barrett-Connor et al. 1990; Nomura et al. 1988). Both observations are classic, trademark effects of relaxin (Bathgate et al. 2006). Furthermore, a study has shown a human prostate adenocarcinoma cell line, LNCaP, to express high levels of a relaxin mRNA, FGC. These findings were observed via reverse transcription PCR and Northern blot analysis. This may be reflective *in vivo* as besides being a product of the RLN2 gene, relaxin found in the seminal fluid is expressed by the prostate gland (Gunnarsen 1995). The high levels of mRNA transcripts suggest a link to prostate cancer. Increased neoplastic prostate xenografts have been shown to be due to lentiviral-mediated relaxin delivery into PC-3 prostate cancer cell line (Silvertown 2006) and an R273H p53 mutation directly targets downstream relaxin production in prostatic carcinoma cells (Vinall et al. 2006).

2. Relaxin and breast cancer

Similar to its effects on prostate cancer cell lines, relaxin is associated with impairment in the development of mammary cells, leading to neoplastic mammary tissues (Bani et al. 1984; Binder et al. 2001). Elevated relaxin transcripts in neoplastic mammary tissues compared to non-neoplastic tissues were reported in 1994 (Sacchi et al. 1994). Increased circulating relaxin levels were also observed in women diagnosed with breast cancer (Binder et al. 2004). The canine relaxin precursor, prorelaxin 2, has been shown to increase invasiveness of canine mammary cells (Silvertown et al. 2003). In rodents, relaxin has been shown to encourage replication and differentiation of mammary cells, particularly glands responsible for milk delivery post-gestation (Min and Sherwood 1996; Winn et al. 1994). Similarly, relaxin in human mammary cells carries out comparable roles alongside oestrogen and progesterone (Bani et al. 1984). The presence of relaxin has been demonstrated in all malignant and benign mammary cell samples in comparison to equivalent cells with normal cell proliferation rate, correlating with elevated circulating relaxin levels amongst metastatic breast cancer patients (Binder et al. 2001). Studies which focus on *in vitro* aetiology of mammary adenocarcinogenic mammary cells, show low amounts of relaxin encourage cell metastasis over a short time (Sacchi et al. 1994; Bani et al. 1999a). An independent study determined higher relaxin plasma concentration amongst metastatic breast cancer patients, correlating with the previous observation (Binder et al. 2004).

Despite prostate and breast cancers being the “focus” of most studies, relaxin has been shown to be associated with other types of cancers *in vitro* including gastrointestinal tract, colorectum, thyroid and endometrial cancer cells (Kamat 2006; Hombach-Klonisch et al. 2006). Most of these cancers have been shown to increase matrix metalloprotease activity (Binder et al. 2002). This causes downstream expression of vascular endothelial growth factors which have been previously shown to increase tumour vascularisation and angiogenesis (Liang et al. 2006).

POTENTIAL THERAPEUTICS TOWARDS RELAXIN-INDUCED CANCER

Relaxin expression interference *in vitro* successfully reduced metastasis which led to increased prostate adenocarcinoma cells death (Feng et al. 2007). This particular study was aimed at observing the hallmark characteristics of prostate adenoma: invasion and metastasis rate of prostate cancer cells. Suppression of relaxin levels or reducing the autocrine/paracrine signalling to its native receptor, RXFP1, via short interfering RNAs

(siRNA) significantly reduced prostate cancer cell growth and metastasis. Moreover, prostate cancer cell apoptosis was also increased with the suppression of relaxin. These observations highlight the importance of relaxin signalling and its role in cancer cells development (Feng et al. 2007).

Relaxin antagonists

Encouragingly, studies have shown that reducing levels of relaxin and/or its available receptor, RXFP1, in prostate cancer cells reduced metastasis and invasiveness *in vitro* (Feng et al. 2007; Willcox and Summerlee 2010). Silvertown *et al.* first showed that lentivirally-produced B-R13/17K H2 relaxin demonstrated antagonistic properties in *in vitro* studies and successfully impaired prostate cancer xenografts *in vivo* (Silvertown et al. 2007). This relaxin analogue had two arginines (Arg₁₃ and Arg₁₇) in its B-chain substituted by lysines. The antagonist peptide used in the study was from a lentiviral construct designed to produce pro-B-R13/17K H2 relaxin. Despite demonstrating its antagonistic properties, the active, heterodimeric peptide was not chemically characterised and full proteolytic cleavage of the C-chain from the prohormone was not demonstrated. Relaxin peptides with their C-chain intact have previously been shown to retain full activity (Bathgate et al. 2006). Hence, another study soon followed with extensive chemical characterisation of the chemically synthesised and characterised B-R13/17K H2 relaxin (Hossain et al. 2010). The relaxin analogue was synthesised via solid phase peptide synthesis. The latter study confirmed the antagonising ability of the B-R13/17K H2 peptide towards the relaxin native receptor (Hossain et al. 2010). These two initial studies on the progression of prostate cancer *in vivo* and *in vitro* led to the development of molecular agents (as illustrated in Figure 4) that may aid further understanding of the roles of relaxin in prostate cancer cells. Importantly, the studies of Silvertown *et al.* (Silvertown et al. 2007) and Hossain *et al.* have highlighted the need for simpler and efficient relaxin antagonists to effectively reduce metastasis, potentially leading to prospective therapeutic agents (Hossain et al. 2010).

On the other hand, ligand suppression via relaxin antagonists might not be the only approach towards the development of potential therapeutics. An alternative strategy towards an RXFP1 antagonist could be the use of LDL-A module with the aim of relaxin signalling suppression (Feng et al. 2011). This study has successfully shown that the over-expression of the LDL-A module on RXFP1 inhibited downward signalling abilities of the native relaxin receptor on prostate cancer cells, PC3, ultimately providing an unconventional approach towards cancer therapeutics (Feng et al. 2011). However, any RXFP1 antagonist will obviously require careful development to target its anti-tumorigenesis effect without ameliorating the positive actions of relaxin itself. Yet there is little doubt that such compounds represent exciting potential additions to the armoury of clinical treatments towards RXFP1-responsive cancers.

CONCLUSION

The role of relaxin and its involvement in cancer cell proliferation, metastasis and cell invasion have been proven by numerous studies, further reinforcing its function as a pleiotropic peptide hormone in humans. Three independent studies have concluded that reduction of relaxin production significantly reduces oncogenic progression *in vitro* and *in vivo* (Feng et al. 2007), particularly with the aid of a relaxin antagonist (Hossain 2010; Silvertown 2007). These exciting findings illustrate the need for further study of relaxin and its role in cancer biology. In turn, this may improve the afore-mentioned anti-relaxin agent and lead to greater investigation of tumour development and cancers related to relaxin.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table I

Table I: Summary of recognized functions of relaxin peptide subfamily members, performing roles undertaken by specific peptide hormones in humans unless otherwise specified. See text for specific references.

Peptide Hormone	Functions
Relaxin (relaxin-2)	• aids embryo implantation via uterine vascularisation and differentiation of endometrial cells • sperm motility in male reproductive system • promotes collagen breakdown • increased vascularisation and renal functions in pregnant females, lead to haemodynamic roles in acute heart failure patients (Phase III clinical trials) • produced by cancer cells and acts on its receptor (autocrine signalling) to promote cancer growth and metastasis
Relaxin-3	• regulation of energy homeostasis and appetite regulation
INSL3	• involved in testicular descent • regulates germ cell maturation
INSL4	• possibly involved in trophoblastic development
INSL5	• associated with feeding functions
INSL6	• involved in spermatogenesis progression

FIGURES.

Figure legends

Figure 1: Schematic showing the different members within the insulin-relaxin superfamily of peptide hormones and their corresponding receptors. *receptors for INSL4 and INSL6 remain unknown

Figure 2: Sequence homology of members within the human insulin-relaxin superfamily of peptide hormones. Conserved cysteines highlighted in blue denote the intra-molecular disulfide bond within the A-chain; yellow and green inter-disulfide between A- and B-chains, respectively. Glycines highlighted in mauve denote conserved residues unique to the B-chain.

Figure 3: Representation of post-translational modifications undergone by single-chain pre-prohormone to its active heterodimeric stage. Signal peptide in pre-prohormones leads the transport of the peptide from the ribosomal ubiquitins. Following the loss of signal peptide, the pre-prohormone is converted to a prohormone via co-translational modifications. Proteolytic cleavage of C-chain then yields the A- and B- chain combination, forming mature, active 2-chain heterodimeric peptide.

Figure 4: Molecular representations of native human relaxin-2 (**A**) and its analogue H2 B-R-13/17K with Arg₁₃ and Arg₁₇ of the native H2 B-chain mutated to lysines (**B**). The H2 B-R13/17K analogue has demonstrated antagonistic properties towards the relaxin native receptor, reducing the cancer growth and metastasis in both *in vitro* and *in vivo* studies (Silvertown *et al.* 2007; Hossain *et al.* 2009).

FIGURE 1

Insulin						G	I	V	E	Q	C	C	T	S	I	C	S	L	Y	Q	L	E	N	Y	C	N
IGF-I		A	P	Q	T	G	I	V	D	E	C	C	F	R	S	C	D	L	R	R	L	E	M	Y	C	A
IGF-II		R	R	S	R	G	I	V	E	E	C	C	F	R	S	C	D	L	A	L	L	E	T	L	C	A
Relaxin-1		R	P	Y	V	A	L	F	E	K	C	C	L	I	G	C	T	K	R	S	L	A	K	Y	C	
Relaxin-2		Z	L	Y	S	A	L	A	N	K	C	C	H	V	G	C	T	K	R	S	L	A	R	F	C	
Relaxin-3		D	V	L	A	G	L	S	S	S	C	C	K	W	G	C	S	K	S	E	I	S	S	L	C	
INSL3		A	A	A	T	N	P	A	R	Y	C	C	L	S	G	C	T	Q	Q	D	L	L	T	L	C	P
INSL4	R	S	G	R	H	R	F	D	P	F	C	C	E	V	I	C	D	G	T	S	V	K	L	C		
INSL5					Q	D	L	Q	T	L	C	C	T	D	G	C	S	M	T	D	L	S	A	L	C	
INSL6					G	Y	S	E	K	C	C	L	T	G	C	T	K	E	E	L	S	I	A	C		

Insulin		F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A		
IGF-I						G	P	E	T	L	C	G	A	E	L	V	D	A	L	Q	F	V	C	G	D	R	G	F	Y	F	N	K	P
IGF-II						P	S	E	T	L	C	G	G	E	L	V	D	T	L	Q	F	V	C	G	D	R	G	F	Y	F	S	R	P
Relaxin-1		K	W	K	D	D	V	I	K	L	C	G	R	E	L	V	R	A	Q	I	A	I	C	G	M	S	T	W	S				
Relaxin-2	D	S	W	M	E	E	V	I	K	L	C	G	R	E	L	V	R	A	Q	I	A	I	C	G	M	S	T	W	S				
Relaxin-3		R	A	A	P	Y	G	V	R	L	C	G	R	E	F	I	R	A	V	I	F	T	C	G	G	R	W						
INSL 3		P	T	P	E	M	R	E	K	L	C	G	H	H	F	V	R	A	L	V	R	V	C	G	G	P	R	W	S	T	E	A	
INSL 4		Z	S	L	A	A	E	L	R	G	C	G	P	R	F	G	K	H	L	L	S	Y	C	P	M	P	E	K	T	F	T	T	P
INSL 5				S	K	E	S	V	R	L	C	G	L	E	Y	I	R	T	V	I	Y	I	C	A	S	S	R	W					
INSL 6		S	D	I	S	S	A	R	K	L	C	G	R	Y	L	V	K	E	I	E	K	L	C	G	H	A	N	W	S	F	R		

FIGURE 3

FIGURE 4

A:

B: