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RLN2 and its role in cancer

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Abstract

There is clear evidence that relaxin (RLN2 9p24) is involved in tumorigenesis. Relaxin, and a family of related peptides, has significant actions on connective tissue, cell growth and death and vascularization. Originally identified and named for its action on relaxing the ligaments of the pelvic girdle, over the last thirty years a picture has emerged that relaxin is involved in a number of critical tissue and cellular functions which are important attributes of cancer development and growth. This review provides an overview of the relaxin superfamily and focuses attention on evidence that relaxin is involved in different aspects of tumorigenesis.

I. Introduction

In 1926, F.L. Hisaw reported that injection of serum from pregnant guinea pigs or rabbits into virgin guinea pigs resulted in relaxation of the pubic ligament (Hisaw, 1926) and, shortly after in 1930, was able to develop an aqueous extract of this relaxative agent (Fevold et al., 1930). The hormone was named "relaxin" - it was one of earlier peptide hormones to be discovered and its method of discovery and its name have left an indelible impression that it is a hormone of pregnancy. But almost one hundred years on, the hormone is now known as one of a family of related peptides with putative and accepted roles in a variety of tissues and organs throughout the body and across many animal species from paramecium to humans.

Despite its relatively early discovery, relaxin research was hampered by technological barriers - primarily the lack of an ability to isolate pure extracts of relaxin. However, in 1974 techniques were developed to isolate and produce large quantities of purified hormone spawning a renewed interest in relaxin research (Sherwood and O'Byrne, 1974). Relaxin was isolated from a number of species and purified forms were used to determine its primary structure, develop a radioimmunoassay, identify actions in a number of tissues, and develop monoclonal antibodies and knock-out mice to elucidate its action (Bathgate et al., 2006a). But almost all the reports focused on its role in the female (Sherwood, 1994). Although there were reports of its presence in males or in non-reproductive tissue, the predominant focus of relaxin research was in its role as a hormone of pregnancy.

The first substantive observation that relaxin might have actions outside of the reproductive system was published by Summerlee and co-workers in 1984 who showed that relaxin affected the release of other peptide hormones from the brain. Since this discovery, many other actions of relaxin have been identified in tissues ranging from the heart and vascular system (Han et al., 1994), kidney (Novak et al., 2001), and neoplastic tissue (Silvertown et al., 2003). It is now clear that relaxin acts on a multiplicity of tissues in males and females (Bathgate et al., 2006a).

The advent of molecular techniques paved the way to cloning the first relaxin gene: cloning the rat (Hudson et al., 1981) and porcine (Haley et al., 1982) relaxin genes confirmed previous work that relaxin is structurally similar to insulin and is synthesized as a prohormone with three distinct regions or chains designed A, B and C. The A and B chains, with a characteristic signature of disulphide bridges cementing the tertiary structure, form the mature hormone but as relaxin was cloned from different species a remarkable lack of sequence homology between species was confirmed. Two human relaxin genes were cloned - RLN1 (Hudson et al., 1983) and RLN2 (Hudson et al., 1984). We now know that the second of these genes RLN2 is the gene encoding the relaxin peptide

produced in the corpus luteum and released in the circulation in women. It is the ortholog of circulating relaxins in other species and is known as H2 relaxin and has more recently been named systematically as RLX2 (Bathgate et al., 2006b).

The availability of recombinant H2 relaxin and the availability of genome databases rapidly led to the discovery that there were five novel genes with high homology to relaxin: four of these were named insulin-like peptides (INSL) - designated 3-6 (Adham et al., 1993; Chassin et al., 1996; Conklin et al., 1999; Hsu, 1999; Kasik et al., 2000; Lok et al., 2000). The insulin-like peptides do not share the relaxin-binding motif and are unable to mimic the actions of relaxin. Interestingly, in 2002 Bathgate and co-workers reported on a new relaxin gene with almost exclusive expression in the brain; termed RLN3 this discovery also provided researchers with new avenues for study with respect to the central actions of relaxin (Bathgate et al., 2002). Further studies investigating the sequence of RLN3 provide evidence that this peptide is indeed the ancestral form of all relaxins, insulin-like peptides, and insulin itself leading researchers to classify this group of peptides as a family of hormones (Hsu, 2003; Wilkinson et al., 2005; Bathgate et al., 2006b).

Concurrent with the rapid expansion in our knowledge of relaxin genes, there has been a substantial growth in our knowledge of the potentially physiological actions of relaxin; indeed there may be instances where relaxin has pathological actions (e.g. cancer). The hormone acts on a variety of tissues including connective tissue (Unemori and Amento, 1990), blood vessels (Bani, 1997) and neurons (Geddes and Summerlee, 1995) and on a number of organs including the brain (Geddes and Summerlee, 1995), heart (Han et al., 1994), and on the male and female productive reproductive tracts (Sherwood, 2004). And most recently has been implicated in tumour biology (Silvertown et al., 2003a) with a number of putative roles including modulation of tumour growth, neovascularization, migration and tumour progression (Silvertown et al., 2003a; ; Silvertown et al., 2006, Silvertown et al.,2007). The purpose of the current review is to focus on the potential role of relaxin in facilitating and supporting tumour development and metastasis and spread but before highlighting some of the key actions of relaxin in cancer, it is important to highlight one other fascinating feature of this unique, pleiomorphic hormone - the nature of its receptors.

Once again, the story of the discovery of "the"
relaxin receptor is remarkable - remarkable for three reasons: (1) it took almost eighty years from the discovery of the hormone to the first receptor was
identified (Hsu et al., 2002); (2) despite the structural similarities and in some cases sequence homology with insulin, relaxin appears to use a completely different family of receptors (Hsu et al., 2002; Kumagi et al., 2002; Liu et al., 2003a, Liu et al., 2003b; Liu et al., 2005) from insulin; and (3) there are several receptors and specific ligand-receptor pairings and even some specific peptide and species specific interactions between ligands and receptors (Bathgate et al., 2006b) that may complicate our understanding of the way these hormones may bring about their effects at the cellular level.

With all these complexities, it is important to understand and situate the biology of RLN2 9p24 within the framework of the family of peptides and to appreciate that the observations about the potential role of relaxin in cancer biology in one species may not necessarily be extrapolated to another species. There have been a number of critically important reviews of the actions of relaxin published over the years which provide a more detailed account of the history, chemistry and biology of relaxin (Sherwood, 1994; Schwabe and Büllesbach, 1994; Goldsmith et al., 1995; Bani, 1997; Ivell and Einspanier, 2002; Bathgate et al., 2003; Dschietzig and Stangl, 2003; Samuel et al., 2003; Silvertown et al., 2003b) and conference proceedings from meetings in 2000 (Tregear et al., 2001), 2004 (Sherwood et al., 2005) and 2008 (Bryant-Greenwood et al., 2009a). However, the current review is focused on the role of relaxin in cancer. It therefore outlines the isolation and cloning of relaxin and the relationship between the relaxin family of genes using RLN2 as the principal reference point. We then provide information on identification of the binding sites and receptors for relaxin and the actions of relaxin, primarily in non-reproductive tissues, that might underlie roles of relaxin in cancer biology. Finally, we review the evidence that supports the contention that relaxin has a role in the development and maintenance of cancer and in metastasis. We conclude with some remarks about the opportunities and challenges for further work in this field.

II. Isolation and purification of relaxin

The initial work isolating and purifying relaxin was published by Fevold et al., (1930) who reported that relaxin was probably a peptide as it was soluble in water, amphoteric and could be readily digested by trypsin (Fevold et al., 1930; Fevold et al., 1932). However, the early studies were limited by the lack of techniques for isolating and purifying proteins and by the lack of an ability to determine the purity of a substance. A significant break-through was achieved by Sherwood and O'Byrne (1974) who described a procedure for isolating the peptide in high yields from pig ovaries in kilogram quantities. For the first time it was possible to sequence the hormone and show the similarity between relaxin and insulin (Figure 1).

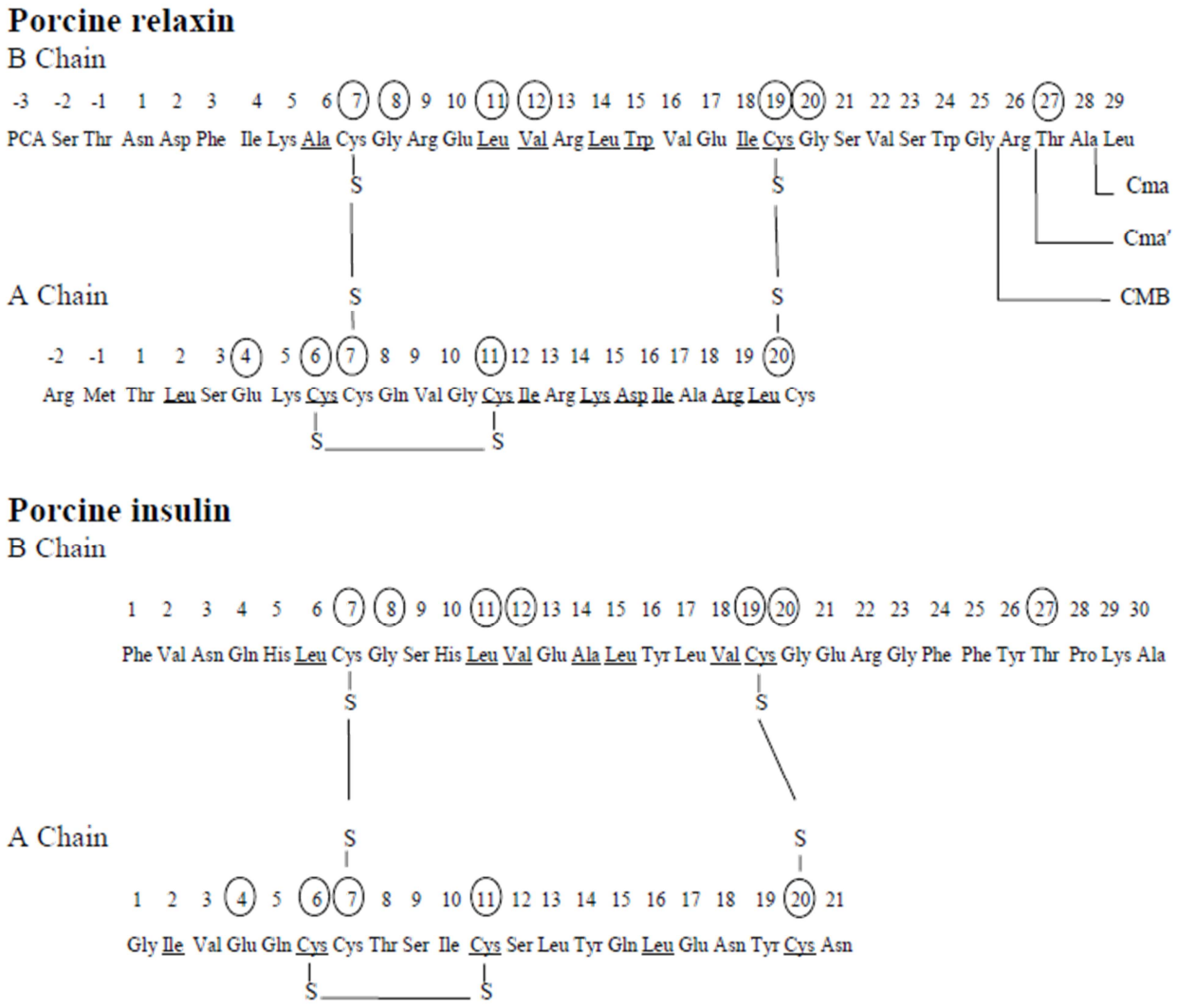

Figure 1: The structure of porcine relaxin (equivalent to H2 relaxin) and porcine insulin to illustrate the similarities and differences between the two peptides. The residues are numbered according to the insulin sequence to facilitate comparison. There are minor differences between three forms of porcine relaxin reported (CMB, Cma and Cma') which are shown on the B Chain of porcine relaxin. There are minor differences in the lengths of the B Chain between residues 25 and 26, 26 and 27 and 28 and 29 respectively. The amino acids which are identical in the two hormones are circled and those which contribute to the hydrophobic core of insulin and the comparable positions in relaxin are underlined.

Much of the work done on the structure of relaxin has been focused on the isolation and purification in three species - the pig (Sherwood and O'Byrne, 1974); the rat (Sherwood, 1979) and the horse (Stewart and Papkoff, 1986). The comparisons between these three types of relaxin underscore that despite the overall framework of two peptide chains held together in a characteristic tertiary conformation with an approximate molecular weight of roughly 6000 Da, there is considerable heterogeneity. Despite the notion that sequence homology is not highly conserved between species, three invariant structural characteristics are highly conserved: (1) the overall two-chain structure designated A and B; (2) the location of the disulfide bridges yielding the tertiary structure of the peptide; and (3) because the tertiary structure is highly conserved, the distinctive binding motif (R-x-x-x-R-x-x-I/V) is exposed and confers biological activity of the peptide.

Isolation of human relaxin did not occur until the late 1980s and early nineties because the hormone is present in lower concentrations in human tissues and initial attempts to isolate the hormone were confounded by lack of purity of the isolate but eventually sufficient hormone was extracted and purified for amino acid sequence analysis from human relaxin corpora lutea (Winslow et al., 1989) and later Winslow et al., (1992) were also able to extract relaxin from seminal plasma and show that the luteal and seminal relaxin were identical.

The heterogeneity of relaxin between species is remarkable with differences in lengths of the chains - particularly the B chain and considerable differences and differences within the chains. In some animals, the B chain is particularly long, for example, the domestic

dog (Canis familiaris) (Stewart et al., 1992) and the skate (Raja erinacea) has the longest B chain (Büllesbach et al., 1987): in some species not only is hormone different but its biological activity is considerably different - for example, shark relaxin shows very poor bioactivity in the mouse interpubic ligament bioassay (Büllesbach et al., 1986, Reinig et al., 1981); whilst in some species there is an astonishing conservation of amino acid sequence - for example, there is virtually no difference between porcine (Sus scrofa) relaxin and relaxin obtained from a mike whale (Blaenoptera acutorostrata) (Schwabe et al., 1989) or the porpoise (Phocaena phcaena) (Woods et al., 1991).

III. Cloning of relaxin

Work began on the cloning of relaxin genes with the activities of Niall and colleagues (Hudson et al., 1981; Haley et al., 1982) who determined the complete amino acid sequences of porcine (Haley et al., 1982) and rat (Hudson et al., 1981) preprorelaxin by cloning of relaxin cDNA. They confirmed that relaxin is synthesized as one single chain peptide with a signal tail connected to the B chain, a connecting peptide and the A chain in that order. Since then, first porcine (Haley et al., 1987) and then rat (Soloff et al., 2003) relaxin genes were cloned from genomic libraries. The sequences are identical in both circumstances with the potential of a single allelic variation in the porcine sequence (Haley et al., 1987) and the structure conforms to the gene structure for all relaxin genes.

There has now been analysis of the genomic DNA from humans, primates, pigs, rats and mice and their general structure is similar. There is a consistent view that an intron interrupts the coding region at the Glu in position 46 of the C peptide (Hudson et al., 1983; Haley et al., 1987; Crawford et al., 1989; Evans et al., 1993; Soloff et al., 2003) and the position of this intron matches that of one of the two introns found in insulin genes (Bell et al., 1980). Although there is no evidence of the second intron seen in insulin (Bell et al., 1980).

IV. Relaxin-family of related peptides

The first hint that there might be other members of the relaxin family came in the early 1990s when two groups independently identified a new cDNA clone that was differentially expressed in porcine (Adham et al., 1993) and mouse (Pusch et al., 1996) testis.

The newly identified clone encoded for a protein that was structurally similar to insulin and relaxin. In both cases the cDNA was highly expressed in the Leydig cells and initially known as Leydig cell insulin-like peptide (Adham et al., 1993) and relaxin-like factor (Büllesbach and Schwabe, 1995) which provides some confusion in the early literature. Subsequently, the gene for this peptide was cloned from human, porcine, mouse and rat (Burkhardt et al., 1994; Koskimies et al., 1997; Zimmerman et al., 1997; Spiess et al., 1999) and showed to be a single-copy gene similar to the relaxin gene - two exons and a single intron in the middle of the coding for the C-peptide - remarkably similar to relaxin. (See Figure 2). The name of this new member of the relaxin family was rationalized to insulin-like peptides and the peptide produce from this particular gene designated INSL3 because it was the third insulin-like gene to be discovered.

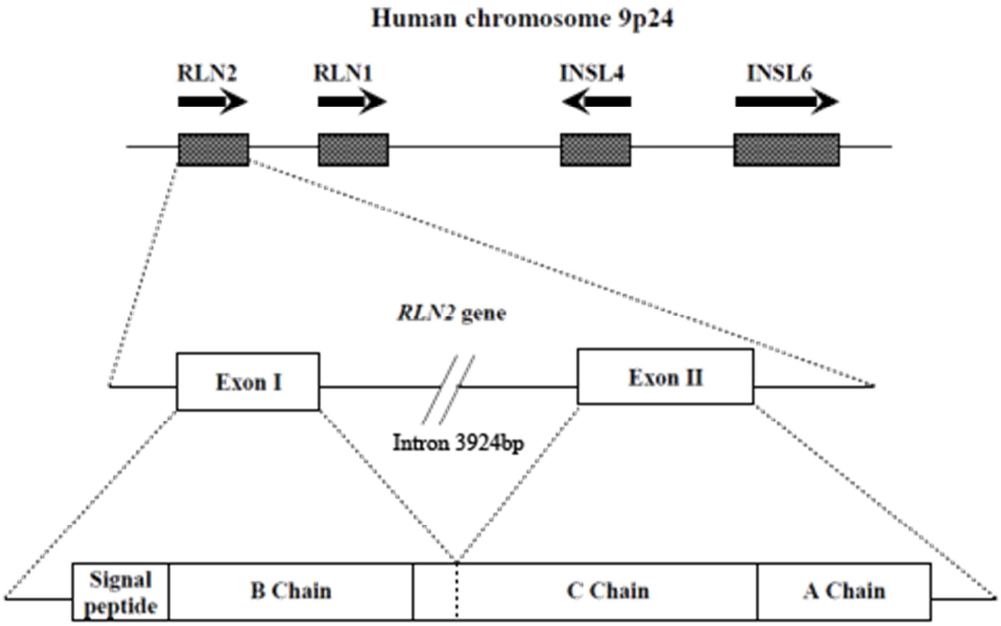

Figure 2: Schematic representation of the transcription of the human RLN2 gene. Adapted from Bathgate et al. 2006a (with permission). The gene is located with the RLN1, INSL4 and INSL6 genes on chromosome 9 at 9p24. The RLN2 gene consists of two exons and is transcribed to give preprorelaxin-2 mRNA. Exon I encodes for the signal peptide, the B Chain and part of the C Chain, and Exon II encodes for the remainder of the C Chain and the A chain of H2 relaxin. The arrows on the diagrams indicate the orientation of the genes. Although insulin and H2 relaxin are similar, there is no report that the insulin gene posses an intron.

Although the gene for INSL3 was discovered in the early 1990s, it was not until 2002 that the structure of INSL3 synthesized in vivo was identified (Büllesbach and Schwabe, 2002). Comparing the predicted and the actual sequence of the peptide revealed another surprise about these relaxin-like peptides: the A chain of bovine INSL3 was exactly as predicted but the B chain is longer by 8-9 amino acids - paradoxically the longer, naturally-produced INLS3 is less bioactive than an artificially synthesized version with a shorter B chain (Büllesbach and Schwabe, 2002). This implies that there may be mechanisms for processing relaxins once it is released or at the target tissue. Although this observation has only been specifically verified for INSL3, there are reports that transfected cells in vitro and in vivo with a cDNA for prorelaxin (H2 relaxin) will produce a peptide that appears to be prorelaxin which is as biologically active as relaxin. Such a possibility raises more questions about the possibility to there could be local control at the site of action for relaxin and members of the relaxin family of peptides that might be critical in cancer or in mitigation of the effects of relaxin in cancer.

V. The Evolving Story of the Relaxin Family of Peptides

In total, six human relaxin-like genes have been discovered. These are shown in Table I along with their specific chromosomal location. The key facet that links these genes and their products is the greater similar to relaxin (H2) than to either insulin or the insulin-like growth factors although there are clearly similarities across these three groups (Hsu, 2003).

Phylogenetic analysis has revealed that there is a common ancestor (Hsu, 2003) and this is most likely to be the third relaxin gene that was identified most recently (RLN3) and is located predominantly in the brain (Bathgate et al., 2002).

The similarities and differences among the relaxins, insulin and insulin-like growth factors are highlighted by their clustering on different chromosomes. These are illustrated in Figure 3. The focus of the remainder of the review will concentrate on RLN2 which is located on chromosome 9p24 closely associated with RLN1, INSL4 and INSL6 on the same chromosome.

VI. Binding sites and receptors for relaxin

Relaxin binding sites were identified in reproductive and non-reproductive tissue before the discovery of the relaxin receptor. The principal challenge was labeling pure hormone in a way that the labeled relaxin retained its biological activity. The early studies used two different techniques to label porcine relaxin: (1) iodination of tyrosine residues added to the N terminus producing a polytyrosyl-relaxin (Sherwood et al., 1975) or (2) incorporation of a ^{125}I group directly into the N terminus of porcine relaxin (McMurtry et al., 1978). Both methods produced labeled hormone that was biologically active and binding sites were demonstrated in reproductive tissues such as uterus (McMurtry et al., 1978; Mercado-Simmen et al., 1980; Mercado-Simmen et al., 1982; Weiss and Bryant-Greenwood, 1982) and placental membranes (Koay et al., 1986). Binding sites were also reported in fibroblasts in human skin (McMurtry et al., 1980). Although neither approach yielded completely pure iodinated forms, binding of the radioactive labeled hormone could not be displaced by insulin, IGF-1 or IGF-2.

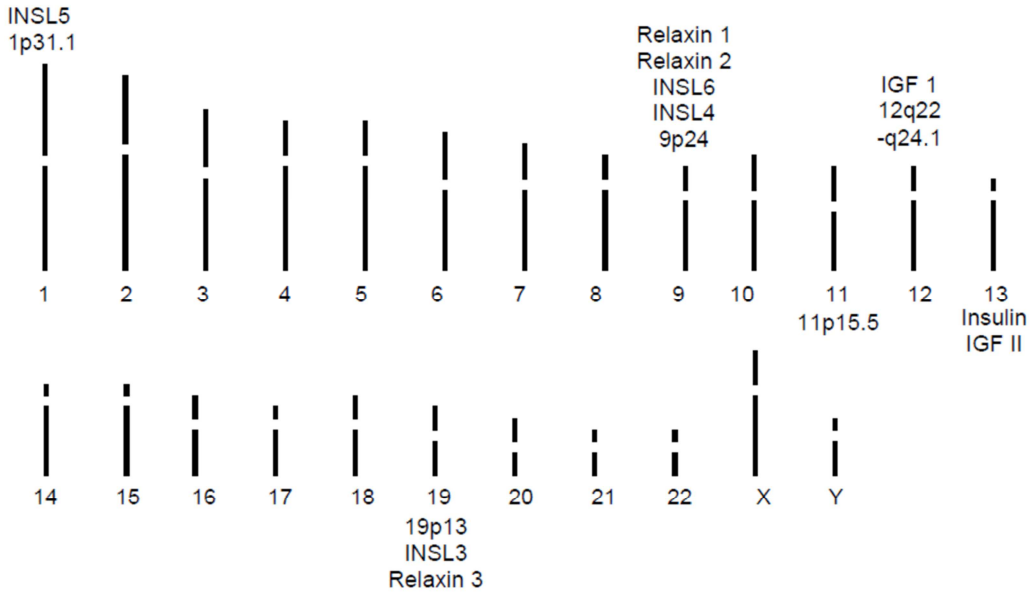

Figure 3: Schema showing the human chromosomal locations of the 10 members of the relaxin and insulin-like family of peptides genes. The relaxin peptide family genes are in different locations from the insulin and IGF-1 genes. The human RLN1 and RLN2 genes map in a tight cluster with INSL4 and INSL6 genes on chromosome 9 at 9p24. The RLN3 gene is located on chromosome 19 at 19p13.3 in close proximity to INSL3 at 19p13.2. In contrast, the INSL5 gene is located in chromosome 1 at 1p31.1 and is not closely associated with the other relaxin-like genes.

Peptide name	Abbreviations	Gene name
Insulin	INS	INS
Insulin-like growth factor-1	IGF-1	IGF1
Insulin-like growth factor-2	IGF-2	IGF2
Relaxin-1	RLX1 (human H1)	RLN1
Relaxin-2	RLX2 (human H2)	RLN2
Relaxin-3	RLX3 or INSL7	RLN3
Insulin-like peptide 3 Leydig-insulin-like peptide Relaxin-like factor	INSL3 Ley-I-L RLF	INSL3
Placentin Early placental insulin-like factor	INSL4 EPIL	INSL4
Insulin-like peptide 5 Relaxin-insulin-like factor 2	INSL5 RIF2	INSL5
Insulin-like peptide 6 Relaxin-insulin-like factor 1	INSL6 RIF1	INSL6

Table I
Members of the relaxin and insulin-like peptide family of genes.

More recently, relaxin binding studies were expanded using a ^{32}P (Osheroff et al., 1990) or ^{33}P (Tan et al., 1989) labeled relaxin. Specific binding with the ^{32}P labeled relaxin was confirmed in the uterus and cervix but also extended to the brain (Osheroff et al., 1990; Osheroff and Phillips, 1991; Osheroff and Ho, 1993). Interestingly, latter studies demonstrated binding in the rat heart atrium (Osheroff et al., 1992) and rat atrial cardiomyocytes (Osheroff and King, 1995).

In 1990, Büllesbach and Schwabe reported that the relaxin molecule could be biotinylated yet preserve its biological activity. Binding sites have subsequently been confirmed in the rat cervix, mammary gland and nipple (Kuenzi and Sherwood, 1995), cervix, mammary gland, nipple, small intestine, skin, ovary and testis of pigs (Min and Sherwood, 1998); the reproductive tract and breast tissue of women (Kohsaka et al., 1998); and prostate (Hornsby et al., 2001).

As a result of the chemical structure of members of the relaxin and insulin families of peptides and the evidence for the coevolution of the two peptide families and probably their receptors, it was assumed that receptors for relaxin and the INSLs 3-7 would be related to the known insulin receptors with tyrosine kinase activity. Indeed some of the initial work suggested that stimulation with relaxin resulted in tyrosine phosphorylation (Büllesbach and Schwabe, 2000). But the relaxin receptor remained illusive and attempts to purify the receptors were confounded by high levels of non-specific binding of tracer and apparently low levels of binding sites in target tissues.

At the turn of the twenty first century, there was a significant break through in relaxin receptor biology. Investigating the phenotypes of mice deficient in INSL3 (Nef and Parada, 1999; Zimmerman et al., 1999) two groups of researchers reported that bilateral cryptorcidism was a consistent feature of the INSL3 knock-out mouse and it was suggested that a leucine-rich glycoprotein receptor might be the receptor for relaxin (Hsu et al., 2002). It was shown that porcine relaxin stimulates both LGR7 and LGR8 receptors and results in increased cAMP (Hsu et al., 2002). Subsequent work has shown that LGR7 transcripts are located in a number of reproductive and non-reproductive tissues throughout the body. Although there is some evidence that relaxin activates both LGR7 and LGR8 there are clearly species differences in both the ability of relaxin to bind to LGR8 and the sensitivity of that binding (Bathgate et al., 2006b).

The complexity of the receptor-ligand story for relaxin was further compounded by the discovery that RLX3 has a relatively low affinity for LGR7 (Bathgate et al., 2002; Sudo et al., 2003). It now appears as if RLX3, which is located specifically within the brain, is a ligand to two orphan receptors GPCR135 (also known as somatostatin and angiotensin-like peptide receptor [SALPR]) and GPCR142 (Liu et al., 2003a; Liu et al., 2003b). There close links between the sites of concentration of these GPCR receptors and binding sites for relaxin and for relaxin-3 message (Osheroff and Phillips, 1991; Bathgate et al., 2006b) but low levels of GPCR142 message have also been reported in a variety of non-neural tissues throughout the body (Liu et al., 2003b).

Identification of receptors for relaxin created the possibility of confirming the intricate signaling cascade in normal and neoplastic tissues (Hsu et al., 2002; Kumagi et al., 2002; Sudo et al., 2003).

VII. Signaling pathways

Relaxin enacts its many physiological actions through a number of distinct signaling pathways that ultimately upregulate cAMP (Braddon, 1978; Sanborn et al., 1980; Sanborn and Sherwood, 1981; Hsu et al., 1985). Interaction of relaxin and its cognate GPCR stimulates cAMP production in a bi-phasic manner through G_S to enhance the activity of adenylate cyclase (Halls et al., 2006). Relaxin has also been reported to act through Gbetagamma thereby activating PI3K and further increasing cAMP production (Nguyen et al., 2003; Nguyen and Dessauer 2005). Downstream signaling of PI3K has also indicated that relaxin stimulates PKCzeta to stimulate cAMP (Nguyen and Dessauer, 2005). PKA has also been implicated in the signaling cascade initiated by relaxin. Inhibition of PKA has been reported to reduce contractility of heart cells (inotropy) (Han et al., 1994) and also has been demonstrated to be involved in affecting contractility of the myometrium by modulating potassium channels (Meera et al., 1995). Taken together it is clear that relaxin stimulates profound changes in cAMP levels in many cell types and tissues in order to bring about diverse physiological actions.

Relaxin has also been demonstrated to affect expression of NOS expression both acutely and chronically (Nistri and Bani, 2003). Modulation of NOS expression has been reported in endothelial cells (Failli et al., 2001) and vascular smooth muscle cells (Bani et al., 1998). It appears that two NOS isoforms are implicated: NOSII (iNOS) is likely affected by chronic administration of relaxin (Quattrone et al., 2004) while shorter term NO production is likely through NOSIII (eNOS) (Willcox et al., 2009).

The intracellular signaling pathways affected by relaxin have a number of implications in cancer and may explain the invasive, growth promoting, and angiogenic phenotypes promoted by relaxin in tumours. Relaxin has been reported to increase cAMP levels in a number of tumour cell lines including MCF-7 breast cancer cells (Bigazzi et al., 1992), PC-3 prostate cancer cells (Silvertown et al., 2007), and MDA-MB-231 human breast cancer cells (Radestock et al., 2008). Liu and colleagues (2008) also reported an involvement of the PI3K/PKB (Akt) pathway in a LNCaP prostate cancer cell model. Taken together these studies indicate that congruent to physiological actions, relaxin retains a diverse signaling profile and an ability to activate multiple signaling pathways in order to promote tumour growth and invasion characteristics. Whether or not these pathways are working in parallel or converge remains to be elucidated and requires further study in order to further understand relaxin's action in these cancers and develop potential therapeutic targets to treat this disease.

Relaxin has also been reported to increase NO production through increased iNOS activity in MCF-7 breast cancer cells (Bani et al., 1995). In spite of this observation, whether or not this is a positive effect of relaxin remains to be determined. It is possible that this phenotype contributes to the inhibition of tumour cell growth by the inhibition of DNA synthesis and mitochondrial respiration (Silvertown et al., 2003) however conversely increased NO may also induce cellular resistance to apoptotic events thereby contributing to cellular growth of the tumour. However other studies investigating the effect of NO on tumour development clearly report on the increased tumour cell migration (Jadeski et al., 2003) and tumour cell growth and angiogenesis (Jadeski et al., 2000). Furthermore, relaxin-induced expression of NO may affect the blood supply of the tumour contributing to the increased blood supply required by tumours to promote their own growth. In a number of vascular beds, relaxin has been noted to increase NO and therefore induce vasodilation in tissues ranging from the heart (Fisher et al., 2002) to skeletal muscle (Willcox et al., 2009). Given that NO is a potent vasodilator and has been reported to increase blood flow (Di Bellow et al., 1995) and angiogenesis in mammary cancer (Jadeski et al., 2000) the fact that relaxin-induced NO signaling may play a role in the development of tumours presents opportunities for further and intruiging studies.

VIII. Biological actions of relaxin that might underlie a role in cancer biology

A number of actions of relaxin at the tissue and cellular level are also important components of tumour growth, development, and metastasis and present the possibility that relaxin is involved the progression of cancer. Its action modulating connective tissue, inducing angiogenesis and affecting cell growth and apoptosis are critical in tumorigenesis and metastasis.

Evidence that relaxin affects tumour growth and development

Relaxin, acting in concert with estrogen and progesterone plays a critical role in mammary gland development (Min and Sherwood, 1996; Winn et al., 1994). In the mouse, the hormone induces mammary growth and differentiation (Bani and Bigazzi, 1984). Conversely, mammary development is retarded and nipple development impaired in the relaxin-deficient mouse (Zhao et al., 1999). Although lactational changes do occur in the mammary tissue in the knock-out mice, the young are unable to suck milk and starve to death which confirms the essential role that relaxin plays in remodeling connective and epithelial tissue and development of the nipples. Similarly, both H1 and H2 relaxin are present in human breast and have been linked to normal development. They have also been implicated in neoplastic growth of the breast (Tashima et al., 1994; Mazoujian and Bryant-Greenwood, 1990; Bryant-Greenwood et al., 1994). Moveover, Tashima et al., (1994) reported the presence of relaxin (H2) transcripts were identified in 100% of neoplastic mammary tissue (benign and malignant) with relatively

low proportions in non-neoplastic tissue. LGR7 receptors are present in both malignant human breast cancer tissues and in human mammary tumour cell lines (Silvertown et al., 2003a) suggesting that the neoplastic tissue is not only producing relaxin but is also a target for the hormone. The possible extracellular roles of relaxin in tumour growth, development and metastasis are discussed later in the review. Low concentrations of relaxin over short periods of time appear to promote the growth of breast adenocarcinoma cells in vitro (Sacchi et al., 1994; Bani et al., 1999) and Binder et al., (2004) reported that there are elevated circulating levels of relaxin in women with breast cancer - particularly those with metastatic disease. Relaxin stimulates invasiveness and migration of breast tissue, thyroid, and endometrial carcinoma cells in vitro and is accompanied by up-regulation of matrix metalloproteinase activity and expression of vascular endothelial growth factors (VEGF) (Binder et al., 2002; Kamat et al., 2006; Hombach-Klonisch et al., 2006). Prorelaxin 2 (the precursor of relaxin) also stimulates the invasiveness of canine mammary carcinoma cells (Silvertown et al., 2003b).

Similar to reports of the presence and action of relaxin in normal development of human breast tissue, relaxin is present in prostatic tissue (Ivell et al., 1989; Sokol et al., 1989; Hansell et al., 1991) and has been implicated in development and maturation of prostatic tissue in rats (Hornsby et al., 2001; Feng et al., 2007). The prostate gland undergoes a number of structural changes during life and prostatic hypertrophy and tumour are condition of men over 45 years of age (Carter and Coffey, 1990) with similar age-related changes reported in other species (Gann et al., 1996). Much of the work on the etiology of both prostatic hyperplasia and carcinoma and adenocarcinoma has focused on the role of steroid hormones (Montie and Pienta, 1994; Barret-Connor et al., 1990; Normura et al., 1988) but the findings are not entirely consistent and there is a persistent view that peptides may also be involved in the disease. There is a clear progression of the disease from hypertrophy to cancer which is characterized by an unresponsive switch to a differentiated state and uncontrollable proliferation of cells (Hanahan and Weinberg, 2000) reported in both men and male dogs (Nomura et al., 1988). The hyperplastic state is associated with a change in the connective tissue framework of the gland and a marked angiogenesis (Lissbrant et al., 1997): changes which are further exaggerated in the neoplastic state - both of these changes are hallmarks of the action of relaxin (Bathgate et al., 2006a; Bathgate et al., 2006b). Gunnerson et al. (1995) reported that the human prostate adenocarcinoma cell line LNCaP. FGC expresses high levels of relaxin transcripts which implies a link with prostatic cancer. Lentiviral-mediated delivery of relaxin into PC-3 prostate cancer cells increases growth of prostate tumour xenografts (Silvertown et al., 2006) and it has been shown that relaxin is a direct downstream target of R273H p53 mutation in prostate carcinoma cells (Vinall et al., 2006). Moreover, relaxin expression appears to be up-regulated by androgen withdrawal both in vivo and in vitro (Thompson et al., 2006). Finally, Feng and colleagues (Feng et al., 2007) reported that there is a strong correlation between significantly higher levels of relaxin message and message for its receptor LGR7 in recurrent prostate cancer samples from human patients and congruent with reports in breast tissue, relaxin stimulates cell proliferation, invasiveness and adhesion in vitro (Feng et al., 2007). Interfering with the production of relaxin and its receptor in vitro on prostate adenocarcinoma cells decreased cell invasiveness and growth and increased cell death in vitro (Feng et al., 2007). Finally, experiments conducted by Feng and colleagues, (2007) in vivo using a transgenic mouse with overexpression of RLN1 demonstrated a shorter survival time for mice with excess relaxin in the presence of prostate adenocarcinoma compared with controls. Further evidence that relaxin modulated tumour growth and progression was provided by Silvertown et al., (2007) when this group reported that an analog of relaxin which appears to be a relaxin anatgonist impairs prostate tumour growth in vivo both reducing the growth of a prostate cell line xenograft and reducing the incidence of metastasis. This was the first study to indicate the possible use of a relaxin antagonist to both investigate the progression and course of tumourigenesis as well as it suggest a possible therapeutic agent for use in the treatment of prostate cancer.

Relaxin-like peptides and INSL3 have been associated with a number of other tumours (Klonisch et al., 2005) including malignancies in the gastrointestinal tract (Stemmermann et al., 1994) thyroid gland (Homach-Klonisch et al., 2006), colorectum (Alfonso et al., 2005), and the male and female reproductive tracts (Silvertown et al., 2003a) in addition to the report above on relaxin and tumour development in breast and prostate. Although the data are not as fulsome for these other cancers, common themes emerge: there are higher levels of expression of transcripts for relaxin and its receptor in malignant cell forms, and in some cases correlations reported between increased relaxin expression, circulating levels of hormone, tendency to malignancy and incidence of metastasis (Homach-Klonisch et al., 2006). Studies in vitro suggest that relaxin promotes proliferation, invasion and metastasis of tumour cells. There is some evidence that levels of circulating hormone can be linked to survival times. Taken together, evidence is accumulating to suggest that relaxin signaling plays a significant role in tumour development and progression.

Relaxin and cell growth

Relaxin affects cancer cell differentiation and growth. Relaxin induced a transient growth followed by a

reduction in growth of mammary tumours induced by estrogen and radiation in rats (Segaloff, 1983). Human breast cancer MCF-7 cells show marked proliferation and differentiation to relatively low levels of relaxin. However at higher doses relaxin seems to suppress proliferation although differentiation is still observed both in coculture (Bani et al., 1994) and in an in vivo preparation in nude mice (Bani et al., 1999). This raises intriguing questions about the possible role of relaxin in cancer suppression that need to be answered but at the same time Zhang and colleagues demonstrated that relaxin caused cellular proliferation by increasing MAPK and MEK protein expression in a variety of cells including normal human endometrial stromal cells, THP-1 myelomonocytic leukemia cells, and coronary and pulmonary artery smooth muscle cells (Zhang et al., 2002). Insulin, IGF-1 and platelet derived growth factor (PDGF) activate proliferative, apoptotic and metabolic signals via both MAPK and P13-Kinase/Akt. Although relaxin appears to stimulate P13-Kinase in blood vessels (Willcox et al., 2009) it appears that its action in human endometrial stromal cells stimulates the transcription factor CREB but does not involve Akt or Jun N-terminal kinase (JNK) (Zhang et al., 2002).

One of the principal intracellular pathways activated by relaxin is the nitric oxide (NO) cascade (see previous section). Activation of NO results in cytoskeletal and organellular changes and, depending on conditions be involved in antiapoptosis or cytostasis (Rivoltini et al., 2002): suppression of NO synthesis in human melanoma results in induction of the intrinsic apoptosis pathway. Cell survival is thereby promoted against chemotherapeutic drugs, mediating hypoxia induced drug resistance in human and murine tumours and assisting neoplastic cells to avoid immune destruction. Nitric oxide also induces a cytostatic state by inhibiting DNA synthesis, mitochondrial respiration and cytochroms P-450 activity (Bani et al., 1995; Bani et al., 1998; Bogdan, 2001). There is either spontaneous or induced expression of NO-synthase (iNOS) in both mouse mammary and melanoma cell lines (Lala and Orucevic, 1998; Xie and Fidler, 1998; Li et al., 1991). This results in increased NO which inhibits DNA synthesis and this is inversely correlated with metastasis. Bani and colleagues (Bani et al., 1995) reported that MCF-7 cells incubated with porcine relaxin showed an increased expression of two isoforms of NOS. They reported a dose dependent, bi-phasic increase in Ca^{2+}/calmodulin dependent NOS (cNOS) and a graduate increase in iNOS activity. This implies that relaxin may indirectly attenuate tumour growth by activating the NO pathway to inhibit DNA synthesis that results in cytostasis and/or relaxin may facilitate tumorigenesis by assisting cells to avoid apoptosis.

Relaxin has been shown to activate protein kinase A (PKA) in a number of cells including the human tumour cell lines MCF-7 and THP-1 (Parsell et al., 1996; Fei et al., 1990; Hsu et al., 2000; Failli et al., 2002) and evidence in most cells confirms that the PKA pathway not PKC mediates the actions of the LGR7 and 8 receptors (Hsu et al., 2000; Hsu et al., 2002; Willcox et al., 2009) but there is one exception. It appears as if the action of relaxin in cardiac myocytes is mediated through PKC (Shaw et al., 2009). Through a complex cascade (Xi et al., 1994): increased PKAc activity results in enhanced phosphorylation of the NFkappaB p65 subunit and an increase in transcriptional activity of NFkappaB. This change in transcription has been suggested to promote tumour growth (Zhong et al., 1997).

Relaxin and cell invasion

Remodeling of connective tissue is a hallmark action of relaxin (Bathgate et al., 2006a) and the hormone has been implicated in anti-fibrotic action (Casten and Boucek, 1958). Relaxin acts directly on transforming growth factor-beta-stimulated human dermal fibroblasts (Unemori and Amento, 1990), lung fibroblasts (Unemori et al., 1996) and cardiac fibroblasts (Samuel et al., 2004) to promote both a decrease in type I and type II collagen synthesis and an increase in MMP expression and activation (Samuel et al., 2004). As a result, relaxin has actually been used in a number of animal models to alleviate fibrosis where it has been used to remodel the extracellular matrix including in the skin (Kibblewhite et al., 1992; Unemori et al., 1993), lung (Unemori et al., 1996); liver (Williams et al., 2001), liver (Bennett et al., 2003; Bennett et al., 2007; Bennett et al., 2009) and kidney (Garber et al., 2001; Garber et al., 2003). However, apart from the original report of clinical trials with porcine relaxin in humans by Casten and Boucek, (1958), a more rigorous clinical trial with genetically engineered relaxin was not successful in demonstrating an effective anti-fibrotic therapeutic action for relaxin in the human disease scleroderma (Seibold et al., 2000; Khanna et al., 2009). Nevertheless, relaxin has been reported to improve wound healing (Casten et al., 1960) although the prime site of action may not be on the connective tissue but on blood supply (see later) and in serving as a cardioprotective agent to experimentally produced ischemia (Masini et al., 1997; Bani et al., 1998).

It has been strongly suggested that loosening connective tissue may assist in tumour migration as a result of the actions of relaxin, mediated through the matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMP) (Silvertown et al., 2003a). In tumour biology, MMP/TIMP has been implicated in degradation of the extracellular matrix to facilitate cell migration, alteration in the cellular environment that fosters cell migration, and the activation of tissue specific molecules that modulate TIMPs (Vu and Werb, 2000). MMPs are also involved in angiogenesis, invasion and metastasis (Duffy et al., 2000; Hiraoka et al., 1998) and they affect tumour suppressing growth factor (TGF-beta) (Yu and Stamenkovic, 2000),

heparin-binding epidermal growth factor (HBEGF) (Pierce et al., 2001; Prenzel, 1999) various binding proteins (Fowlkes et al., 1994a; Fowlkes et al., 1994b); and proteases (Polette and Birembaut, 1998; Ugwu et al., 1998). These data clearly lead to the conclusion that by activating MMPs and TIMP, relaxin could support and enhance tumour invasion. However, at the same time there is evidence that MMPs can induce programmed cell death in anchorage-dependent cells and might defy tumour progression (Li et al., 1999: Will et al., 2000).

In a similar vein, the effects of relaxin on the MMP/TIMP system appear to be cell-type dependent: relaxin is reported to stimulate MMPs in cervical fibroblasts (Palejwala et al., 2001) but reduces pro-MMP-1 in endometrial cells (Palejwala et al., 2002). There is certainly evidence that relaxin can stimulate MMP release: Binder et al., (2002) showed that relaxin upregulated the expression of mRNA of MMP-2, -9 and -14 in MCF-7 and SK-BR3 cell lines and increased cellular migration; and Silvertown et al., (Silvertown et al., 2001; Silvertown et al., 2003a) showed that human relaxin could stimulate the migration of L6 cells and the movement of canine mammary tumour cells (CF33.Mt) respectively, through a porous membrane. Again, suggesting perhaps that the response is cell-type specific, Silvertown and colleagues, (2003a) reported that human relaxin resulted in a decreased migration of the human mammary cancer cell line MDA-MB-435.

Binder and colleagues, (2001) reported that patients with active metastatic breast cancer have elevated circulating levels of relaxin in the serum. In an interesting study on the incidence of breast cancer in the early nineties, Lambe et al., (1994) had postulated that one pregnancy increased the risk of breast cancer but multiple pregnancies decreased the risk which was confounding. Silvertown and colleagues (2003a) suggested that perhaps the short- and long-term risks of breast cancer and pregnancy might be related to the differential action of relaxin but this remains to be explored.

Relaxin and angiogenesis

Tumour growth depends on blood supply and there is critical point in the growth phase when a switch towards an angiogenic phenotype is absolutely critical (Ellis et al., 1996; Hanahan and Folkman, 1996; Tonini et al., 2003; Kerbel, 2008). The modelling and remodeling of vascular supply depends on a balance of proangiogenic and antiangiogenic factors that are produced by neoplastic tissue or induced in the surrounding cells (Tonini et al., 2003). Proangiogenic factors include vascular endothelial growth factor (VEGF), angiopoietins and ephrins, and a variety of other molecules and transcriptional factors. A number of these have been implicated as a possible product of relaxin stimulation. Reports and claims that relaxin stimulates these angiogenic substances are provided in Table II. In contrast, although there is a wide cadre of potential antiangiogenic factors known, only a limited number have been reported to be stimulated by relaxin.

Originally identified as a single compound, it is now known that VEGF is one of the most potent angiogenic cytokines and comprises a family of related molecules VEGF A-D and placental growth factor (Ogawa, 1998; Meyer et al., 1999; Neufeld et al., 1999; Ferrar, 2002; Hicklin and Ellis, 2005; Kerbel, 2008). The critical importance of VEGF to the integrity of the vascular system is supported by knockout studies: disruption of one VEGF allele in mice results in lethal abnormalities and removal of both alleles results in a virtually complete absence of vasculature in embryos (Cameliet et al., 1996; Ferrara et al., 1996; Carmeliet, 2000). All the members of the VEGF family have overlapping abilities to interact with the different receptors expressed primarily in the vascular endothelium (Eriksson and Alitalo, 1999). The vital importance of angiogenesis in tumour growth and development and the major role of VEGF has led to a great deal of basic and clinical research directed towards the VEGF family and the receptor tyrosine kinases that mediate their proangiogenic effects (Ferrara, 2002; Hicklin and Ellis, 2005). Relaxin has been shown to upregulate VEGF in stromal and glandular epithelial cells of the endometrium in wound healing (Palejwala et al., 2002; Unemori et al., 1999; Unemori et al., 2000), and in the myelomoncytic leukemia cell line THP-1 (Parsell et al., 1996). The THP-1 cells also exhibit relaxin receptors (Unemori et al., 1999; Unemori et al., 2000) which implies there may be some autocrine function of relaxin that may be related to angiogenesis in tumour development (Silvertown et al., 2003a; Kerbel, 2008). But again, the action of relaxin may be dependent on cell type and by inference on tumour cell type - Zhang and colleagues (2002) report that human endometrial stromal cells incubated with relaxin showed a reduced level of VEGF transcription.

The major mediator of tumour angiogenesis appears to be VEGF-A (Kerbel, 2008) which acts preferentially through the VEGF receptor 2. This is highly expressed by endothelial cells engaged in angiogenesis and by circulating bone marrow-derived endothelial precursor cells (Shibuya and Claesson-Welsh, 2006). There is also a VEGF receptor 1 which has a ten-fold higher affinity with VEGF-A but its signal transducing properties are extremely weak (Shibuya and Claesson-Welsh, 2006). Consequently, the role of VEGF receptor-1 remains unknown (Kerbel, 2008).

Known Angiogenic Factor	Relaxin stimulates production
Adenosine	Chen et al. 1988
Angiogenin	Unemori et al. 1999
Angiopoetin-1 (Ang-1)	Hewitson and Samuel 2009
Collagen	Unemori et al. 1993
Epidermal growth factor	Steinetz et al. 2009
Ephrins	Davison et al. 2004
Fibroblast growth factors (a and b)	Taylor and Clark 1992
Fibronectin	McDonald et al. 2003
Follistatin	Petraglia et al. 1994
Granulocyte colony-stimulating factor	Moore et al. 2007
Heparin	Masini et al. 1994
Interleukin 8 (IL-8)	Bryant-Greenwood et al. (2009a)
Leptin	Steinetz et al. (2009)
Midkine	Sacchi et al. (1994)
Nicotinamide	Berne 2002
Proliferin	Conrad et al. 2004

Table II
Known angiogenic factors which have been linked with or claimed to be linked with relaxin.

Most types of human cells have been shown to express high levels of VEGF and it appears as if hypoxia, which is a characteristic of solid tumours (Semenza, 2003) is important for inducing VEGF release. There are no data to date that indicate the hypoxia results in relaxin release but this is an intriguing possibility, especially as the appears to be conductance phenomena among branches of the microcirculation which might explain both an increased blood flow (Willcox et al., 2010) and angiogenic effect of relaxin.

It is assumed that VEGF has paracrine effects as tumour cells produce VEGF but lack cell-surface receptors for VEGF whereas endothelial cells express the receptors but produce relatively little VEGF. It has been suggested that VEGF originates from host cells in the body such as platelets and muscle cells (Kut et al., 2007) and tumour-associated tumour cells (Fukumura et al., 1998; Liang et al., 2006).

As mentioned earlier, relaxin upregulates NO through NOS in both vascular cells (Willcox et al., 2009) and neoplastic cells (Parsell et al., 1996; Fei et al., 1990; Hsu et al., 2000; Failli et al., 2002; Davel et al., 2002). Furthermore, tumour-associated angiogenic activity in vivo has been linked with increased levels of iNOS (Jadeski and Lala, 1999) and endothelial cells NOS (eNOS) (Jadeski et al., 2000) and inhibition of NOS with N-nitro-L-arginine methyl esther (L-NAME) results in a marked reduction in angiogenesis (Jadeski and Lala, 1999; Jadeski et al., 2000). Relaxin has been shown to increase microvascular arterial diameter in vitro (Bani et al., 1998) and in vivo (Willcox et al., 2009; Willcox et al., 2010). Arteriolar dilation decreases leukocyte-endothelial adhesive properties and increases vascular permeability (Fukumura and Jain, 1998). Bearing in mind that microvessel density, in both mammary and prostate tumours, is positively correlated with tumour cell survival and negatively correlated with longevity of the patient (Lissbrandt et al., 1997), Silvertown and colleagues suggested that high circulating and/or local levels of relaxin might upregulate VEGF and NO to increase blood flow to the region and stimulate an active angiogenesis to support tumour growth (Silvertown et al., 2006).

Other possible pathways for relaxin-involvement in angiogenesis

There is a body of literature supporting a pivotal new signaling pathways in angiogenesis related to tumorigenesis: notch delta-like ligand (DII) (Sainson and Harris, 2007; Noguera-Troise et al., 2006; Lobov et al., 2007; Ridgway et al., 2006; Gale et al., 2004). Notch cell-surface receptors are expressed by various cell types and generally involved in cell differentiation, proliferation and apoptosis. These receptors interact with transmembrane ligands on adjacent cells and may be involved in vital angiogenic activity which implies a possible role in vascular growth in tumorigenesis (Gale et al., 2004; Carmeliet et al., 1996; Ferrara et al., 1996). Although there are no reports to date of the possible role for relaxin in stimulating pathways that might interact with the Notch cell-surface receptors, this remains a possibility that deserves further investigation. Finally, it is known that a number of cell types can be mobilized from bone marrow that may be important in new blood vessel formation (Betolini et al., 2006).

These include various monocytic and myeloid cells that express endothelial cells markers such as VE-Cadherin, VEGF-1 and VEGF-2 (Okazaki et al., 2006; Conejo-Carcia et al., 2005; Grunewald et al., 2006). As relaxin has been reported to upregulate VEGF and bFGF in the myelomonocytic leukemia THP-1 cells (Parsell et al., 1996) this raises the interesting spectre that relaxin could also affect the responses of circulating bone-marrow derived cells in promoting angiogenesis.

IX. The next steps

The evidence that RLN2 9p24 and other members of the relaxin superfamily of peptides are involved in tumorigenesis is now unequivocal. There are data suggesting that relaxin is upregulated in tumour tissue, that receptors are present and that the hormone appears to be involved in the growth, vascularization and spread of cancer. There is a picture emerging of the signaling events induced by relaxin. Under specific conditions, relaxin appears to facilitate growth, limit apoptosis, induced angiogenesis and facilitate connective tissue remodeling that would support local and metastatic spread. This raises the spectre that inhibitors of inhibitors of relaxin could be part of the arsenal of weapons to be used in the fight against cancer. Recently, Silvertown and colleagues (Silvertown et al., 2006) showed that transfecting tumour xenografts implanted in mice with a modified relaxin cDNA not only reduced tumour size and vascularization but also reduced the incidence of metastasis raising the exciting possibility that anti-relaxin agents might suppress tumour development.

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