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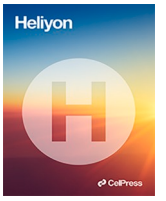

Review article

The diverse influences of relaxin-like peptide family on tumor progression: Potential opportunities and emerging challenges

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ABSTRACT

Relaxin-like peptide family exhibit differential expression patterns in various types of cancers and play a role in cancer development. This family participates in tumorigenic processes encompassing proliferation, migration, invasion, tumor microenvironment, immune microenvironment, and anti-cancer resistance, ultimately influencing patient prognosis. In this review, we explore the mechanisms underlying the interaction between the RLN-like peptide family and tumors and provide an overview of therapeutic approaches utilizing this interaction.

1. Introduction

The relaxin-like peptide family (RLPF) is classified as RLN1–3 and INSL3–6 in humans and mediates various physiological actions by binding to G-protein-coupled receptors (GPCRs) [1]. Human relaxin (H RLN) is located in the p-arm region of chromosome 9 in humans [2]. Human and other primates express both RLN1 and RLN2, but in mammals that are not primates, only RLN1 is found to be expressed. The peptide expressed by H2 RLN is identical to that encoded by RLN1 in non-primates [1,3]. H1 and H2 RLN mRNAs are co-expressed in the decidua, placental trophoblasts, and prostate. However, only H2 RLN is expressed in the ovaries [4,5]. H2 RLN is expressed at the protein level, whereas H1 RLN has not yet been detected as a native peptide, and its function remains unknown [6]. RLN is expressed in human semen and corpus luteum by the H2 RLN gene [7]. In this review, both non-primate RLN1 and human RLN2 are referred to as RLN. RLN is the cognate ligand of RXFP1, a receptor that is widely and diversely distributed including the brain, kidney, uterus, and heart. Therefore, RLN is a pleiotropic hormone that elicits various physiological effects in multiple organs [8,9].

RLN is synthesized in the corpora lutea, released into the bloodstream during pregnancy and parturition, and acts as an angiogenic factor in the endometrium/decidua [9]. Additionally, RLN promotes decidualization, regulates the uterine immune system, facilitates pelvic joint relaxation. Additionally, hyperrelaxinemia is associated with prematurity [10–12]. Furthermore, RLN mediates anti-fibrotic effects in various organs, including the liver, kidneys, heart, and lungs, thereby delaying the progression of fibrotic diseases. It also plays a role in vascular dilation and the maintenance of sperm motility. These effects occur through the activation of relaxin-mediated signaling pathways, including the pERK-nNOS-NO-cGMP, PI3K-AKT-eNOS, cyclic adenosine monophosphate (cAMP)-protein kinase A, nuclear factor kappa B (NF-κB), peroxisome proliferator-activated receptor gamma, and vascular endothelial growth factor (VEGF) signaling pathways, as well as the inhibition of the tumor growth factor (TGF)-β1/pSmad pathway [13–28]. RLN3 is a neuropeptide primarily synthesized in the brain and maps to chromosome 19p13.3 [29,30]. RXFP (GPCR135) recognizes RLN3 and is predominantly expressed in the brain, including hippocampus, hypothalamus, cortex, and septal nucleus [31].

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Abbreviations

RLPF	relaxin-like peptide family
GPCR	G-protein-coupled receptor
RLN	Relaxin
H1 RLN	Human Relaxin1
H2 RLN	Human relaxin2
cAMP	cyclic adenosine monophosphate
NF-κB	nuclear factor kappa B
VEGF	vascular endothelial growth factor
TGF	tumor growth factor
INSL	insulin-like peptide
TNF	tumor necrosis factor
MMP	matrix metalloproteinase
CNNP	chitosan nanoparticle
TRAMP	transgenic adenocarcinoma of mouse prostate
AR	androgen receptor
AI	androgen independent
PSA	prostate specific antigen
NHT	neoadjuvant hormone therapy
KD	knockdown
ARI	AR signaling inhibitor
NSE	neuron-specific enolase
NED	neuroendocrine differentiation
NEP	neural endopeptidase
t-SCNC	treatment-emergent small-cell neuroendocrine prostate cancer
MUC	melanoma cell adhesion molecule
CTRP8	C1q-tumor necrosis factor-related protein 8
ER	estrogen receptor
NO	nitric oxide
OC	ovarian cancer
IL	interleukin
TME	tumor microenvironment
EOC	epithelial ovarian cancer
OS	osteosarcoma
EC	endometrial cancer
ESCC	esophageal squamous cell carcinoma
ECM	extracellular matrix
CAF	cancer-associated fibroblast
TIL	tumor-infiltrating lymphocytes
ICI	immune checkpoint inhibitor
FGF	fibroblast growth factor
MCP-1	monocyte chemoattractant protein-1
PBMC	peripheral blood mononuclear cell
IFN	interferon
NK cell	natural killer cell
PSC	pancreatic stellate cell
HSC	hepatic stellate cell
OV	oncolytic virus
TCGA	analysis of the cancer genome atlas
NSCLC	non-small cell lung cancer
NPC	nasopharyngeal carcinoma
EBV	Epstein-Barr virus
CRC	colorectal cancer
microRNA	miRNA

RLN3-RXFP3 inhibits intracellular cAMP accumulation and activates PKC-dependent ERK1/2 [32]. RLN3-RXFP3 signaling is involved in the metabolic abnormalities observed in patients receiving antipsychotic treatment and in the neurotransmission changes in patients with depression and Alzheimer's disease [33,34]. Additionally, it is involved in regulating hippocampal theta rhythms related to

learning and memory, stress response, increased appetite, and arousal [29,35–37].

Insulin-like peptide 3 (INSL3) is a testicular Leydig cell hormone that is almost exclusively expressed in the Leydig cells of the testes. However, INSL3 is also expressed in the ovaries and corpus luteum [38–40]. INSL3 acts as a cognate ligand for RXFP2, which is present in the testes, brain, kidneys, ovaries, and bones [8]. INSL3 is essential for testicular descent, and its expression is increased in polycystic ovary syndrome [41–43]. INSL4 was first identified in early human placentas and was mapped to chromosome 9p24 [44]. INSL5 is expressed in the rectum, colon, uterus, thymus, and prostate. It is located on chromosomes 1p31.1–1p22.3. However, the receptor for INSL4 has not yet been identified, and little is known about its role. RXFP4 (GPCR142) is primarily expressed in the colon and peripheral tissues as a receptor for INSL5 [45,46]. INSL5 acts as a weak antagonist of RLN-RXPF3. Additionally, the fetal brain and pituitary gland express high levels of INSL5 mRNA but low levels of RXFP4 mRNA, suggesting the presence of additional INSL5 receptors [46]. INSL5 is an orexigenic hormone involved in glucose homeostasis in mice, promoting hepatic glucose production under energy-deprived conditions, and its expression is regulated by energy availability and gut microbiota. INSL5 is also involved in immune system regulation [47–50]. INSL6 is 43% identical to H2 RLN and is located on chromosome 9p24. It is adjacent to INSL4 and the autosomal testis-determining factor gene locus [51]. INSL6 may be involved in male reproductive functions. Furthermore, INSL6 protects the heart from cardiac fibrosis in mice [52]. Tumor necrosis factor (TNF)-α-polarized macrophages produce INSL6, which stimulates the differentiation of osteoblasts and contributes to bone metabolism [53,54].

The landscape of cancer treatment is rapidly evolving towards precision and personalized medicine, propelled by advancements in molecular genetics and the analysis of in molecular genetics and the analysis of individual patient genomes. Moreover, the paradigm of therapeutic targets is evolving from directly attacking cancer cells to leveraging the immune system, with treatment like immune checkpoint inhibitors and chimeric antigen receptor-T cell therapy yielding remarkable clinical results [55,56]. Consequently, the study of the tumor microenvironment (TME) and the characteristics of the immune system of the host has become an increasingly critical area of research. Understanding the role of the RLPF has markedly advanced with the discovery of its receptors, unraveling its physiological functions in a range of organs. RLPF modulates multifaceted roles, including angiogenesis, immune modulation, fibrosis, and energy metabolism. These functions closely correlate with disease-related mechanisms and their significance is becoming clearer within pathological contexts. Notably, mechanisms involving RLPF in cancer may act as potential regulators, either promoting or inhibiting tumor growth. Elucidating this complex interplay between RLPF and tumor progression could lead to the development of novel therapeutic strategies and provide insights into previously unknown aspects of tumor biology.

In conducting the literature review, a comprehensive search was performed using the PubMed database. The search terms employed were ‘Relaxin’ and ‘INSL’. The time frame for the literature search spanned from 1980 to 2023, focusing specifically on paper related to cancer. To ensure a focused and relevant collection of literature, the search was restricted to articles reported in this period. In the current scientific landscape, while the physiological roles of RLN and INSL are relatively well-documented, their specific functions in cancer biology are not fully understood. This review addresses this gap by focusing on the roles of RLN in various cancer types and its impact within the tumor microenvironment. Additionally, it explores the role of RLPF in cancer, focusing on its association with bone complications in cancer patients. The literature to date presents limited and scattered information on these topics, indicating a need for an integrated analysis of existing studies. This review aims to collate and critically evaluate the current evidence, aiming to provide a clearer understanding of the potential roles of RLN and INSL in cancer. By doing so, it seeks to highlight areas where further research is necessary and to suggest possible directions for future investigations. This approach is expected to contribute to the development of new therapeutic strategies and to improve our understanding of cancer pathophysiology.

2. Influence of relaxin across different cancer types

This section focuses on exploring the impact of RLN on certain cancers, specifically prostate and ovarian cancers, which are directly related to the reproductive system, and breast cancer, which, while not a reproductive system cancer, is also influenced by reproductive hormones. This is grounded in the biological role of RLN and its significance in these specific types of cancer. RLN is actively expressed in reproductive tissues, and its role in these tissues is believed to influence the development and progression of cancer. Additionally, abnormal expression of RLN has been observed in other types of cancers, indicating that RLN could play an important role in various cancer types. These findings provide new insights into the pathological functions of RLN and its implications in cancer research.

2.1. Prostate cancer

mRNA expression of RXFP1 is comparable between normal and prostate cancer clinical samples, whereas mRNA expression of RLN is significantly higher in recurrent cancer than in normal tissues [57]. In addition, RXFP1 expression is comparable between normal prostate and prostate cancer cell lines (PC3, LnCaP, LAPC4, and DU145). In contrast RLN expression markedly differ between normal and cancer cells and even among prostate cancer cell lines themselves. Particularly, RLN is highest in LnCaP cells but lowest in PC3 cells. Moreover, the expression levels of RLN in the aggressive tumor cell lines C4-2 and CWR22Rv1 are significantly higher than those in the normal prostate cell line RWPE1 [57,58].

in vitro studies, upon exposure to RLN, LnCaP and PC3 cells exhibited slight cell proliferation and dose-dependent effects on cell adhesion and increased the invasiveness. However, this invasiveness was completely counteracted by FN439, a matrix metalloproteinase (MMP) inhibitor, which effectively neutralized RLN-induced invasiveness [57]. Conversely, other studies reported that exogenous RLN did not affect the proliferation of PC3 cells and exhibited concentration-dependent biphasic MMP activity. In addition, the overexpression of RLN in PC3 cells downregulated proMMP-9 activity approximately twofold [59]. Inhibition of RXFP1 or RLN

expression in LnCaP cells led to a slight suppression of cell proliferation, increased apoptosis, and substantial inhibition of cell invasion. Similarly, the inhibition of RXFP1 increased apoptosis and suppressed cell invasion in PC3 cells. This suggests that anti-apoptotic effects and tumor invasion are regulated through RLN-RXFP1 signaling in prostate cancer [57,60].

In vivo studies, the administration of siRXFP-chitosan nanoparticle (CNNP) via CNNP delivery suppressed the growth of LnCaP by inhibiting the expression of RXFP1 in LnCaP xenograft models. Similarly, RXFP1 inhibition in PC3 xenograft models, which were highly metastatic prostate cancer cell lines, suppressed tumor growth, and this suppression was transient and reversible. Moreover, RXFP1 inhibition reduced the metastasis rate. This indicated that the RLN-RXFP1 signaling pathway was involved in tumor metastasis. In transgenic adenocarcinoma of mouse prostate (TRAMP) mice, RLN overexpression resulted in decreased survival time, increased serum RLN levels, and reduced apoptosis [57,60]. RLN overexpression in PC3 xenograft models promoted tumor growth, increased VEGF mRNA expression, and enhanced intracellular microvessel density. Additionally, RLN facilitated the formation of a more extensive vascular network and induced an angiogenic phenotype [59]. Conversely, the RXFP1 antagonist AT-001 inhibited tumor growth by up to 60% in PC3 xenograft models and had no impact on microvessel density; however, it significantly reduced the average microvessel area, indicating the suppression of the angiogenic pathway mediated by RLN-RXFP1 signaling through AT-001 [61]. *In vitro*, RLN has minimal or no effect on cell proliferation in PC3 cells; however, *in vivo*, tumor growth is stimulated, suggesting that RLN-induced angiogenesis signaling may indirectly influence tumor growth.

mRNA expression of RLN in LnCaP cells is time- and dose-dependently regulated by androgens *in vitro*. Treatment of LnCaP cells with R1881 (an androgen receptor (AR) agonist) markedly decreases RLN expression [62]. Under androgen-deprived conditions, RLN

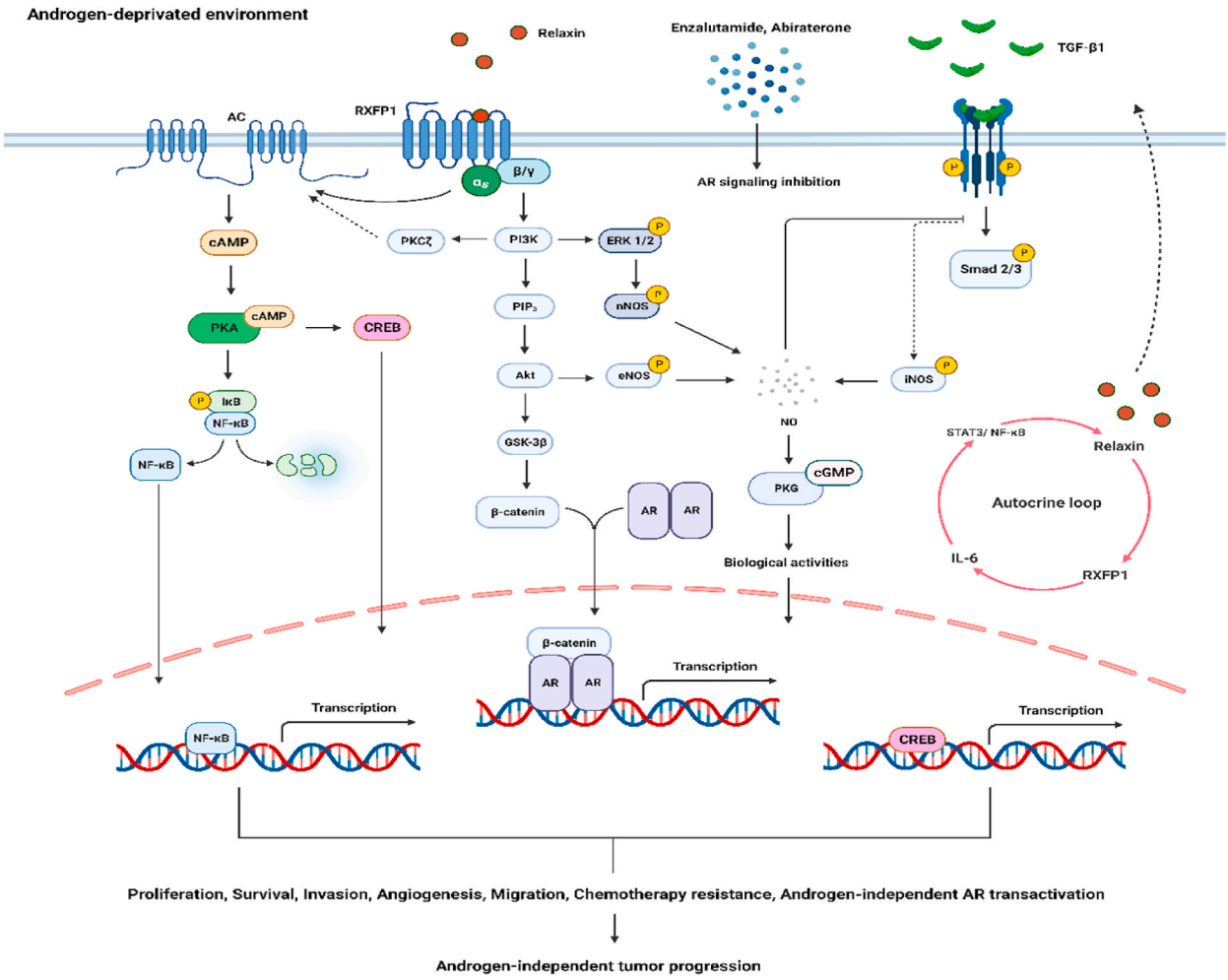

Fig. 1. Androgen-dependent prostate cancer is treated with an AR signaling inhibitor (ARI) such as Enzalutamide or Abiraterone. Continuous treatment of ARI stimulates the expression of RLN. RLN can activate β-catenin through RXFP1. In the absence of androgens, β-catenin accumulated through RLN signaling co-localizes with AR into the nucleus, leading in inappropriate expression of AR target genes. Furthermore, RLN-RXFP1 signaling stimulates the expression of genes associated with cell proliferation, survival, metastasis, and resistance to anticancer drugs through cAMP, cGMP, NF-κB, and ERK pathway. Consequently, this promotes AI growth in androgen-depleted environment. IL-6 exhibits constitutive expression in prostate cancer. This can stimulate the expression of RLN through similar autocrine loop as observed in ovarian cancer. Created with BioRender.com.

promotes androgen-independent (AI) growth in LnCaP cells, and RLN overexpression in LnCaP cells stimulates the upregulation of various survival genes, including cyclin D [63,64]. Androgen deprivation caused by castration reduces prostate-specific antigen (PSA) levels in the LnCaP xenograft model. However, as they reached a stable state, they regained the ability to express PSA and exhibited AI growth, resuming growth in an androgen-deprived environment. In contrast, mRNA levels of RLN are higher in the androgen-deprived environment. Similarly, RLN expression increases during the transition from precastration to postcastration. Additionally, RLN expression is high in tissue samples from patients with prostate cancer undergoing long-term neoadjuvant hormone therapy (NHT). However, patients with AI prostate cancer have levels similar to those of patients who do not undergo the NHT. This suggests that RLN expression in androgen-dependent prostate cancer is stimulated by androgen deprivation, suggesting that RLN may be clinically important in prostate cancer [62,65].

mRNA expression of RLN is increased in p53 gain of function (GOF) LnCaP transfectants (G245S, R248W, R273H, and R273C) under androgen deprivation conditions; specifically, there are increased protein expression in LnCaP-p53$^{R273H.}$ Furthermore, the p53 GOF mutant induces the expression of RXFP1 under androgen deprivation conditions, whereas the knockdown (KD) of RLN or RXFP1 by RNAi reduces the AI growth of LnCaP-p53^{R273H}. This suggests that RLN-RXFP1 signaling may be a component of the autocrine signaling mechanism involved in anchorage-independent growth and survival [63]. p53 mutations occur in approximately 70% of hormone-independent prostate cancer cases, and this, promotes AI growth. R273 is a hotspot for missense mutations in p53 and represents DNA contact and conformational mutations. This mutation increases genomic instability and promotes chemoresistance [66,67]. In an androgen-deprived environment, cytokines and growth factors produced by LnCaP-p53^{R273H} cells stimulate AI growth and activate the AR-mediated PSA promoter construct. This suggests the involvement of RLN in the process [63].

The mechanism underlying RLN-mediated AI growth in prostate cancer is not well defined, but the activation of β-catenin by RLN has been implicated in AI growth. RLN activates the β-catenin pathway through the phosphorylation of AKT and GSKβ in LnCaP. Increased levels of β-catenin interact with AR and stimulate its colocalization into the nucleus. In addition, β-catenin/AR complex activates the expression of AR target genes [68]. RLN activates the Wnt11/β-catenin pathway by upregulating protocadherin Y. In addition, RLN overexpression in LnCaP cells lead to decreased IκB-κ and increased nuclear NF-κB, resulting in increased expression of its downstream target, BCL-xL. This is suppressed by RNAi-mediated knockdown of RXFP1. RLN overexpression in LnCaP confers resistance to therapeutic agents such as perifosine, rapamycin, and docetaxel [64,69]. This suggests that the PI3K/AKT and Wnt pathways and the NF-κB pathway are activated through RLN-mediated signaling in an androgen-depleted environment, thereby promoting cell survival and contributing to anti-cancer resistance (Fig. 1).

According to next-generation sequencing followed by gene ontology analysis, RLN overexpression in LnCaP cells is associated with key processes including proliferation (12.6 %), transcription (18.6 %), metabolism (16.4 %), signal transduction (11.7 %), and proteolysis (6.2 %). RLN inappropriately regulates AR activity without directly affecting AR expression. Furthermore, neuron-specific enolase (NSE), a key marker of neuroendocrine differentiation (NED), is upregulated, while neural endopeptidase (NEP) is downregulated. These findings suggest a correlation between increased RLN expression and NED progression, indicating its association with a more aggressive prostate cancer phenotype [64,70]. Treatment-emergent small-cell neuroendocrine prostate cancer (t-SCNC), which morphologically resembles de novo small-cell prostate cancer, occurs in less than 1% of patients with therapeutic resistance resulting from exposure to AR signaling inhibitors, such as abiraterone and enzalutamide. t-SCNCs exhibit pure small- or mixed-cell histology and are characterized by an abundance of neural development genes. Despite low canonical AR transcriptional activity, continuous expression of nuclear AR is observed, indicating the potential existence of an epigenetically regulated alternative AR transcription program. Although the molecular pathogenesis of t-SCNC remains unclear, these characteristics are similar to those of RLN overexpression in LnCaP [71,72]. Therefore, RLN requires further investigation to determine its potential involvement in these processes. RXFP1 suppression in PC3 is associated with the downregulation of molecules involved in prostate cancer growth, metastasis, and invasion, including melanoma cell adhesion molecule (MUC) 1, MUC18, tetraspanin 8, glucose phosphate isomerase, and angiopoietin-like 4 [60]. This suggests that RLN signaling promotes prostate cancer progression.

RLN and its receptors are expressed in prostate cancer cells, suggesting that autocrine and/or paracrine functions may be more important than endocrine functions. An increase in serum RLN level is potentially associated with prostate cancer progression [59,69]. However, RLN is not detected in PC3 cells. This suggests that cancers with characteristics similar to those of PC3 may exhibit limited *in vivo* biological responses to RLN. Although, RXFP1 affects the growth of PC3 cells, it remains inconclusive whether this modulation *in vivo* is mediated through its typical ligand, RLN. A novel partner of RXFP1, that is, C1q-tumor necrosis factor-related protein 8 (CTRP8)-RXFP1 signaling, has been recently identified in brain cancer [73,74]. This indicates the potential involvement of RXFP1 in tumor growth in PC3 through interactions with factors other than RLN.

2.2. Breast cancer

Short-term exposure of MCF-7 cells (an estrogen receptor (ER) + human breast cancer cell line) to RLN regulates cell proliferation in a biphasic manner *in vitro*. At low concentrations, RLN exhibits mitogenic effects, whereas at high concentrations, it has no significant effect on cell proliferation. Similar patterns are observed for cAMP concentrations [75,76]. In contrast, prolonged exposure to low concentrations of RLN suppresses cell proliferation, which is distinct from the growth-promoting effects observed after short-term exposure. This inhibitory effect is not associated with apoptosis but is rather anti-proliferative. Additionally, RLN enhances the expression of E-cadherin and promotes cell differentiation and cell-cell adhesion [76,77]. RLN stimulates iNOS activity and activates the L-arginine-nitric oxide (NO) pathway in MCF-7 cells, leading to elevated intracellular cGMP levels, which may be associated with the anti-mitogenic effect of RLN and upregulation of E-cadherin [77].

Short-term exposure to RLN in MDA-MD-231, an ERα-independent human breast cancer cell line, stimulated cell motility *in vitro*. In

contrast, the inhibition of RXFP1 expression by RNAi abolished the increase in cell motility induced by RLN. Conversely, prolonged exposure to RLN decreased cell motility. However, this effect was not a consequence of RXFP1 downregulation. These results suggest that enhanced motility of MDA-MB-231 cells by RLN may be a transient phenomenon [78]. Furthermore, RLN downregulated the mRNA and protein expression of S100A4 (mst1). Despite short-term exposure to RLN, S100A4 KD through RNAi did not affect cell motility, indicating that the short-term effects of RLN in breast cancer may be dependent S100A4. Similarly, a xenograft model of MDA-MD-231 cells transfected with RLN exhibited slow tumor growth and decreased S100A4 mRNA expression. Additionally, RLN exposure suppressed angiogenesis. This suggests that prolonged exposure to RLN may contribute to the suppression of tumor growth by inhibiting angiogenesis through S100A4 downregulation [78,79]. MMC-RLNs transduced with a lentiviral vector to enable the expression of RLN in MMC, a spontaneous mammary tumor in Neu-transgenic mice, substantially reduced tumor growth in a xenograft model [80]. Collectively, prolonged exposure to RLN reduced cell motility, promoted cell differentiation, and induced anti-invasive effects in breast cancer cells.

In vitro and *in vivo* studies have shown that RLN inhibits the growth of breast cancer cells. However, RLN possesses a promoter sequence for ERα and is influenced by estrogen [81]. This indicates the possible involvement of RLN in the progression of ER-positive breast cancer. In addition, the analysis of serum RLN levels in patients with breast cancer showed a different pattern. Serum RLN levels were higher in patients with cancer than in healthy individuals and those with other diseases. RLN-positive patients (> 0.4 ng/ml) had shorter survival than RLN-negative patients (< 0.4 ng/ml). However, in this study, RLN-related diseases such as congestive heart failure may have affected the RLN levels in these assessments [82]. Therefore, excluding patients with diseases that can influence RLN levels and evaluating them under controlled conditions is important for understanding the relationship between RLN and breast cancer progression. This approach allows the removal of these confounding factors and should be applied when investigating the relationship between RLN and other types of cancer.

In summary, current evidence indicates a limited understanding of the impact of RLN on breast cancer. Although RLN generally exhibits antitumor effects in breast cancer, some studies have shown contrasting results. These inconsistencies may arise from the limitations of current experimental models, which do not accurately reflect human physiology; thus, different outcomes may occur in humans.

2.3. Ovarian cancer

In ovarian cancer (OC), RLN ensures tumor cell survival and induces chemoresistance. Suppression of RXFP1 via RNAi in OC cell lines (OVCAR8, SKOV3, PEO4, and PEO6) resulted in marked impairment of proliferation and induction of apoptosis. RXFP1 inhibition does not induce apoptosis in OVCAR5, an OC cell line that barely expresses RXFP1. Suppression of RLN or RXFP1 inhibits OC tumor growth and angiogenic effects *in vivo*. The pro-inflammatory cytokines interleukin (IL)-6 and TNF-α are present in ascites resulting from OC and induce the expression of RLN, whereas RLN expression is blocked by JAK/STAT3 or NF-κB inhibition. Serum RLN levels are increased after carboplatin/paclitaxel therapy. In addition, cisplatin treatment induces RLN expression in OC cells, and RXFP1-dependent OC cells show higher resistance to cisplatin. Bioinformatics analysis conducted through RNA-seq revealed that RLN upregulates 766 mRNAs and downregulates 73 mRNAs in OC. This analysis identified the activation of key signaling pathway including MAPK, AKT, and Notch pathways and VEGF signaling in OC and upregulates various secreted factors, including macrophage migration inhibitory factors and MMPs. This has the potential to stimulate cell survival and alter the tumor immune microenvironment [83].

Elevated serum IL-6 levels are associated with poor relapse-free survival and overall survival in patients with epithelial ovarian cancer (EOC). IL-6 is abundant in the serum of patients with EOC and activates the Ras/Raf/MEK/ERK and PI3K/AKT pathways, which increase the expression of Bcl-2, Bcl-xL, and XIAP. Furthermore, sensitivity to chemotherapy is diminished through the upregulation of MDR1 and GSTpi, which are associated with multidrug resistance. This suggests that IL-6 can induce chemotherapy resistance by upregulating RLN and activating the RLN/RXFP1 autocrine loop [83–85]. Serum levels of RLN are higher in patients with EOC than in healthy individuals or those with benign ovarian disease. Furthermore, higher levels of RLN have been detected in patients with lymph node metastasis, poor response to anti-cancer drugs, and recurrent cancer. Additionally, expired patients with OC show higher serum levels of RLN during the observation period [86]. Furthermore, increased serum levels of RLN have been observed not only in EOC but also in other OC types, such as clear cell carcinomas. Collectively, these findings indicate that RLN participates in tumor progression in various histological subtypes of OC [83].

2.4. Other cancers

RLN is expressed exclusively in papillary, follicular, and undifferentiated thyroid carcinoma tissues (referred to as PTC, FTC, and UTC, respectively). However, it is not expressed in benign thyroid diseases such as goiter disease and Graves' disease. RXFP1 is expressed in all human thyroid tissues, with higher expression in thyroid carcinomas than in benign thyroid diseases. RLN increases the motility and growth of FTC cells *in vitro*. In contrast, impaired RLN-RXFP1 signaling results in a reduced cAMP response and tumor invasiveness. RLN increases cath-L activity and stimulates the extracellular secretion of heavy-chain cath-L. RLN also increases the expression and secretion of pro-cath-D among cath-D forms. This indicates that cath-D activity requires additional processing in the extracellular environment [87]. In addition, RLN stimulates the expression of MT-MMP and MMP2 but downregulates that of TIMP3 [88]. Moreover, S100A4 is upregulated in thyroid cancer and is associated with RLN-induced cell motility. However, RLN downregulates S100A4 expression in breast cancer. This suggests that RLN regulates biological responses through different mechanisms, depending on the cancer type [78,89]. RLN overexpression in FTC-133 (an FTC cell line with barely detectable RLN) stimulates tumor

growth and induces angiogenesis *in vivo* [87]. Collectively, RLN-RXFP1 signaling acts in thyroid cancer via paracrine/autocrine mechanisms to promote cancer progression by inducing the expression and secretion of factors involved in metastasis, invasion, and tumor growth.

Osteosarcoma (OS) is the most common primary malignant tumor of the bone and predominantly occurs in adolescents and young adults. However, improvements in survival outcomes have been limited over the past 40 years [90]. OS tissues exhibit higher mRNA levels of RLN than non-tumor tissues, and RLN expression is very low or absent in non-tumor tissues. Furthermore, the serum levels of RLN are higher in OS patients than in healthy individuals [91]. Elevated RLN levels are more frequently detected in advanced-stage cancers and hematogenous metastases. Inhibition of RLN expression in OS not only suppresses cell proliferation, invasiveness, and VEGF-mediated angiogenic effects *in vitro* but also increases apoptosis. These effects are mediated by the AKT signaling pathway [92, 93]. Administration of an anti-RLN monoclonal antibody in the MG-63 xenograft model significantly reduced tumor growth by inducing apoptosis and inhibiting angiogenesis. Additionally, the number of tumor nodules in the lungs decreased, indicating suppression of lung metastasis, a common site of metastasis in osteosarcoma [91].

Endometrial cancer (EC) is the most common gynecological malignancy that affects the female reproductive system. High RLN expression is associated with high-grade malignancy, myometrial invasion, and shorter overall survival in EC. RLN stimulates the expression of MMP2 and MMP9 and induces the migration and invasion of EC cells *in vitro*. RLN also downregulates the expression of E-cadherin and N-cadherin. RLN-mediated invasion of EC cells is induced through the β-catenin pathway. However, RLN-induced invasion of EC cells is independent of estrogen stimulation and does not stimulate cell proliferation *in vitro* [94,95].

Serum RLN levels are higher in patients with esophageal squamous cell carcinoma (ESCC) than in healthy patients, and higher RLN

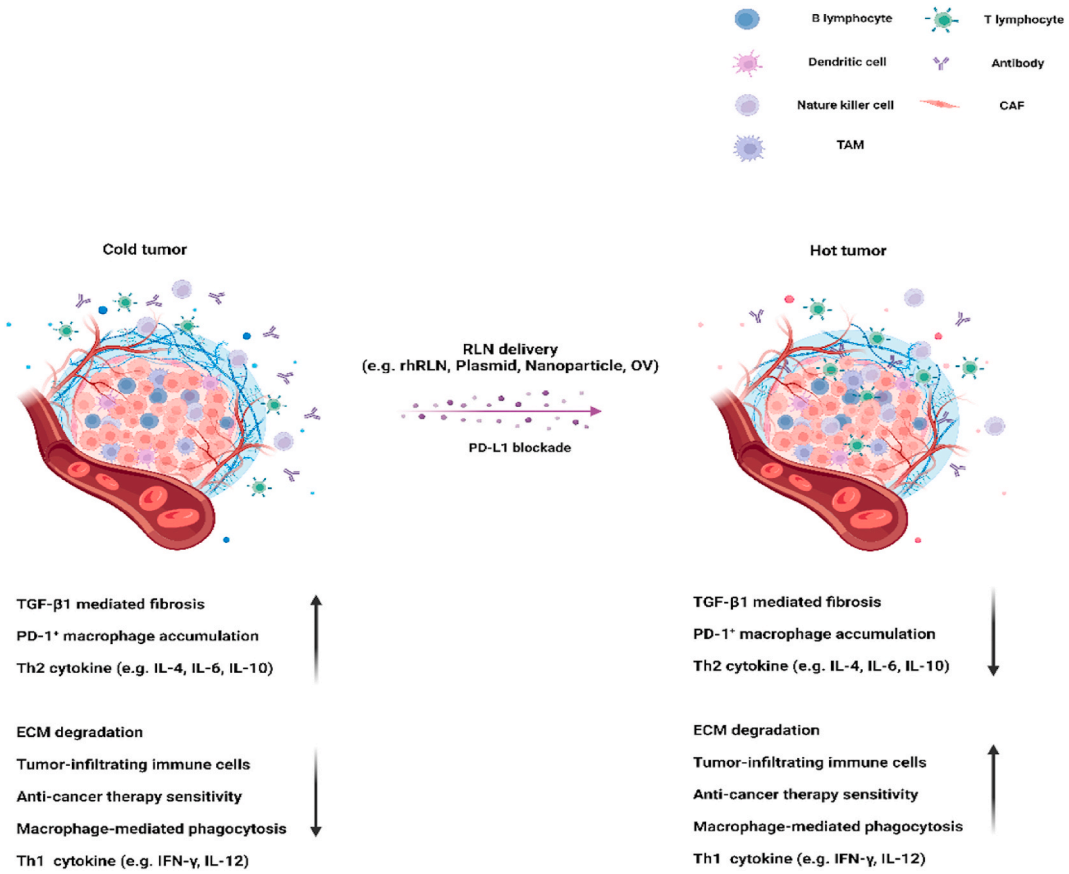

Fig. 2. Desmoplastic tumors have abundant ECM, with cancer-associated fibroblasts (CAFs) serving as a major source of ECM. The ECM acts as a physical barrier, preventing the infiltration of tumor-infiltrating lymphocytes (TILs) and inducing resistance to chemotherapy. Moreover, the accumulation of PD-1^{+} macrophages increase during tumor progression. These tumors possess characteristics of "cold tumor", characterized by immunosuppressive microenvironment facilitated by cytokines such as TGF-β1, IL-4, and IL-6. RLN delivery promotes the degradation of ECM that act as inhibits TGF-β1 mediated fibrosis and induces macrophage-mediated anti-fibrosis, therapy removing barriers and allowing improved accessibility for anti-cancer therapies. RLN downregulates immunosuppressive cytokines whereas upregulates immunostimulatory cytokines and increasing the infiltration of TILs. This leads to the transition of "cold tumor" with Immune checkpoint inhibitor (ICI) resistance into "hot tumor", thereby enable stronger anti-cancer effects. Furthermore, RLN induces the transition of "M2"-like to "M1"-like macrophages. It can also downregulate PD-1 expression and act in synergy with PD-L1 blockade, exerting anti-cancer effects through macrophage-mediated phagocytosis. Created with BioRender.com.

levels (> 0.462 ng/ml) are associated with shorter survival rates. Serum RLN levels in ESCC patients are higher for those with lymph node or distant metastases than those without such metastases. In addition, distant metastasis is frequently found in ESCC patients with persistently elevated serum RLN levels or those whose RLN levels markedly increased within a short period (1–6 months) after esophagectomy. In contrast, patients with low RLN levels remain disease-free for 12–23 months. This indicates that RLN may be involved in ESCC tumor invasion and metastasis [96].

3. Treatment strategy through ECM degradation and RLN immunomodulation in tumors

The tumor stroma contains a modified extracellular matrix (ECM) maintained by heterogeneous stromal cells. The cellular components comprising the tumor stroma include cancer-associated fibroblasts (CAFs), pericytes, endothelial cells, and immune cells, with CAFs being the main source of ECM. An abundant ECM acts as a physical barrier and triggers resistance to radiation and chemotherapy [97,98]. RXFP1 is expressed in 70 % of macrophages and CAF populations present in the TME of KPC (pancreatic cancer cell line), BPD6 (melanoma cell line), and 4T1 (triple-negative breast cancer cell line) mouse models with desmoplastic tumor characteristics and is mainly expressed by macrophages. This suggests that macrophages can play a crucial role in the TME by responding to RLN. RLN signaling has been found to induce macrophage-mediated anti-fibrotic effects in some cancers. RLN also induces anti-fibrotic effects by regulating the TGF-β pathway in fibroblasts [99]. This has led to the emergence of a novel approach to inducing RLN-mediated anti-fibrotic effects to overcome the limitations of anti-cancer therapy caused by the abundant ECM present in fibrotic tumors (Fig. 2).

3.1. RLN immunomodulation

Chemokine-like factors generated at inflammatory sites of tumors act as key mediators that promote tumor growth through tumor infiltration, leukocyte recruitment, and suppression of immune surveillance [100,101]. Macrophages stimulate angiogenesis by inducing VEGF and fibroblast growth factor (FGF) expression via RLN stimulation [102,103]. RLN stimulates migration of THP-1 (a monocyte cell line) and enhances monocyte chemoattractant protein-1 (MCP-1) chemotaxis. Meanwhile, RLN-mediated migration of THP-1 cells is induced via a cAMP-dependent pathway and is not influenced by the protein kinase C signaling pathway. This suggests that RLN and MCP-1 possess independent motility and that these two pathways may be complementary. Similar results are observed in peripheral blood mononuclear cells (PBMCs) [101]. After the delivery of plasmid-RLN (pRLN) into desmoplastic tumors, the population of $F4/80^{+}CD206^{+}$ macrophages is increased in the TME and upregulates the expressions of CCL2, CCL5, and CD206, which are associated with monocyte/macrophage recruitment. In addition, IL-1α mRNA expression is enhanced in the TME [99]. These findings suggest that RLN is involved in leukocyte recruitment.

RLN induces adenylate cyclase activity in monocytes, but not in macrophages. RAW264.7, a macrophage cell line, does not show an increase in cAMP upon RLN stimulation and inhibits migration through the RLN-induced NO pathway, independent of cAMP production. RLN suppresses IL-1β expression and upregulates VEGF in THP-1 [104]. RLN does not affect the mRNA levels of iNOS in IL-4-stimulated RAW264.7, but it induces increased expression of Arg-1 mRNA. Conversely, upon stimulation with lipopolysaccharide and interferon (IFN)-γ, RLN does not affect Arg-1 expression but promotes the upregulation of iNOS expression. This suggests a mechanism distinct from the classical mechanism of macrophage polarization [99,105]. RLN-RXFP1 signaling stimulates the secretion of IL-1β and TNF-α in PBMCs [106]. In addition, RLN has the unique action of stimulating IFN-γ production in human T lymphocyte clones with little impact on IL-4 production [107].

Collectively, tumor-derived RLN can recruit monocytes to the tumor and subsequently differentiate in the TME under the action of various cytokines. Tumor-associated macrophages accumulate within the TME as migration is suppressed through the RLN-mediated NO pathway. *In vitro,* studies suggest that macrophage stimulation with RLN can induce an "M1"-like or "M2"-like phenotype depending on the TME environment. Additionally, RLN promotes the development of "Th1"-like lymphocytes and enhances a Th1-skewed immune response. Therefore, it appears that RLN modulates the immune response by interacting with various tumor-derived factors within the TME. However, further research is required to advance our understanding of the immunoregulatory mechanisms underlying RLN. These studies will be instrumental in exploring the immunomodulatory functions of RLN and developing novel strategies for cancer treatment.

3.2. Immune infiltration through RLN-mediated ECM degradation

RLN has been demonstrated to inhibit tumor growth in breast cancer due to a marked decrease in ECM caused by RLN-induced matrix degradation. This antitumor effect was mediated by $CD8^{+}$ and natural killer (NK) cells. These findings indicate that an enhanced antitumor response is achieved by creating a favorable microenvironment for tumor-infiltrating leukocytes to access malignant cells through RLN-induced ECM degradation. Moreover, the combination of cyclophosphamide (Treg depletion) and intratumoral RLN expression exerts an additive effect on the growth delay of MMC-RLNs *in vivo* [80]. In addition, the removal of the physical barrier through tumor stroma degradation by RLN improves the therapeutic potential of trastuzumab, enhancing its efficacy [108].

Pancreatic stellate cells (PSCs) are a major source of CAFs in pancreatic ductal adenocarcinoma. Excessive secretion of ECM by activated PSCs triggers tumor-associated fibrosis, which has become a target for regulating tumor stroma [109]. RLN inhibits the expression of α-smooth muscle actin and collagen I induced by TGF-β, as well as suppression of differentiation *in vitro.* These processes are mediated by the inhibition of the pSmad2 pathway. In a mouse model of stroma-rich pancreatic cancer induced by the co-injection of Panc (a pancreatic cancer cell line) and hPSCs, intratumoral RLN delivery via RLN-superparamagnetic iron oxide nanoparticles

inhibited ECM degradation and tumor growth. Additionally, combination therapy with gemcitabine and RLN increased sensitivity to anti-cancer effects by reducing ECM [110]. RLN delivery to pancreatic cancer increased IFN-γ mRNA within the TME and reduced PD-1 expression in F4/80^{+}CD206^{+} macrophages, known as "M2"-like macrophages. However, it had little effect on F4/80^{-}PD-1^{+} cells. This suggests that the immunomodulation by RLN possesses specificity. RLN-induced PD-1 reduction cooperates with anti-PD-L1 to induce apoptosis in tumor cells. This cooperation also restored phagocytosis in F4/80^{+}PD-1^{+} macrophages, leading to T cell-independent killing. This suggested that macrophages regulated by RLN possess characteristics that diverge from those traditionally classified as M1 or M2 [99]. In the tumor environment, the majority of PD-1^{+} TAMs display "M2"-like features, whereas PD-1^{-} TAMs tend to show "M1"-like characteristics. PD-1^{+} TAMs increase over time specifically within the M2 subset as the tumor progresses. PD-L1 blockade enhances the phagocytosis of PD-1^{+} TAMs without affecting PD-1^{-} TAMs. This indicates that inhibition of the PD-1/PD-L1 axis by RLN in F4/80^{+}PD-1^{+} macrophages induce phagocytosis of "M2"-like macrophages through an unknown mechanism [111].

Activated hepatic stellate cells (aHSCs) are essential for liver metastasis. The aHSC-mediated fibrotic liver forms a favorable microenvironment for the proliferation and engraftment of metastatic tumor cells and suppresses antitumor immune responses through potent immunosuppressive activity mediated by producing TGF-β [112]. RLN inhibits liver fibrosis *in vitro* by deactivating aHSC. RLN delivery using pRLN-lipide-calcium-phosphate nanoparticle (LCP), an LCP surface modified with aminoethyl anisamide, in a CT-26 FL3 liver metastasis mouse model decreased α-SMA and collagen expression in liver metastatic lesions [113]. This effect was mediated by the RLN-mediated NO pathway, and inhibition of Smad2/3 phosphorylation reduced the expression of profibrogenic factors such as CXCL12, TGF-β, PDGF, and FGF. In addition, RLN downregulated the levels of immunosuppressive cytokines such as CCL2, CCL5, and IL-4 but upregulated those of IFN-γ and IL-1 in metastatic lesions and enhanced cytotoxic T cell killing. These changes in immune cytokines promoted the transition from the "M2"-like to the "M1"-like phenotype and decreased Treg and myeloid-derived suppressor cells. Combination therapy with PD-L1 blockade and pRLN-LCP delivery exerted effects similar to those observed in other cancer types and reduced the liver metastasis burden. These effects also mitigated the metastatic burden in 4T1 or KPC liver metastasis models [113].

The RLN-expressing oncolytic virus (RLN-OV) enhanced the antitumor effect by increasing viral spread through ECM degradation in an A375 metastatic melanoma model [114]. RLN-OV increased cell invasiveness *in vitro* but had no effect *in vivo* rather reduced metastasis levels. Similarly, the expression of RLN via the fiber chimeric-RLN-OV in PC-3-bearing mice increased the antitumor efficacy of OV [114]. RLN-induced ECM degradation and enhanced OV spread appear to mediate potent antitumor effects, surpassing RLN-mediated prostate cancer growth. E1B-deleted-RLN-OV showed potent anti-cancer activity and increased survival rates in various tumor-bearing models (C33A, U343, U87MG, HEP3B, A549, and B16BL6). Moreover, it reduced the growth of metastatic lesions in a B16BL6 spontaneous lung metastasis model [115]. This suggests that RLN-mediated ECM degradation provides a favorable environment for OV to spread into tumors and suppresses tumor growth and metastasis by enhancing antitumor effects.

However, metastasis also decreased in the non-RLN-OV group. This indicates that the reduced metastasis observed with RLN-OV cannot be attributed to RLN alone and that the possibility of it being due to the direct suppressive effect of OV cannot be ruled out. RLN-OV overcame ECM-mediated chemoresistance and restored chemotherapy sensitivity by reducing collagen I and III expression in gemcitabine-resistant pancreatic tumor-bearing mice [116]. Additionally, the antitumor efficacy was enhanced by facilitating the spread of OV and increasing apoptosis. The combined administration of RLN-OV and antibody therapy increased antibody delivery to the tumor. Furthermore, when co-treated with α-PD-1 and an RLN-OV that was engineered to carry immunostimulatory cytokines, there was an improvement the antitumor immune environment, and this synergistically created a more potent immune anti-cancer milieu (Fig. 2) [117].

In summary, the delivery of RLN via drug delivery systems effectively degrades the stroma of targeted cancers, thereby modulating the TME. This degradation exposes cancer cells and enhances the infiltration capability of immune cells. Consequently, it sensitizes the cancer cells to chemotherapy or immunotherapy agents, acting as a potent chemosensitizer. Especially, this effect of RLN is profoundly beneficial against desmoplastic tumors. However, in cancers where RLN may act as a pro-tumorigenic factor, expecting an anticancer effect solely through stromal degradation is challenging, Nonetheless, when co-treated with other anti-cancer agents (e.g., OV, pro-inflammatory cytokines), the degradation of the ECM can facilitate enhanced drug delivery, potentially leading to a more robust anti-cancer response that could outweigh the tumor-promoting effects of RLN, consequently inhibiting tumor progression. However, the anti-fibrotic mechanism mediated by RLN can provide favorable conditions for tumor invasion and metastasis. Therefore, elucidating the precise relationship between RLN and metastasis becomes an imperative step in the development of RLN-based therapeutic strategies. This consideration should be important when advancing RLN-based treatment.

4. INSL family: role in cancer

4.1. INSL3

INSL3 is not expressed in the normal thyroid glands, whereas INSL3 and LGR8 are expressed in hyperactive or neoplastic thyrocytes. INSL3 induces migration of thyroid cancer cells *in vitro* and upregulates S100A4 and the active form of cath-L. In addition, it induces ATP elevation. This suggests that INSL3 is involved in mitochondrial metabolism. In the FTC-INSL3 xenograft model, it did not affect cell proliferation *in vitro* but induced tumor growth *in vivo*, which may be due to tumor growth via INSL3-induced angiogenesis [118–120].

Tumor-derived INSL3 has been recently identified to induce anorexia, an established adverse effect of cancer. This is associated with reduced sensitivity to anti-cancer therapy and decreased survival rates [121–123]. INSL3 regulates the expression of Nucb2 (an anorexigenic gene) and Npy (an orexigenic gene) in mouse hypothalamic cells. Tumor cell lines with relatively higher levels of INSL3

expression (C26 and Capan1) showed decreased food intake and body weight *in vivo*, whereas those with lower levels of INSL3 expression (LLC and Panc1) appeared unaffected. However, intraperitoneal injection of INSL3 into LLC-bearing mice resulted in decreased food intake and increased Nucb2 mRNA expression. Furthermore, patients with pancreatic adenocarcinoma have higher serum INSL3 levels than those with benign diseases, and those with anorexia exhibit higher levels of serum INSL3 than patients without anorexia [124]. This suggests that systemic changes induced by tumors, in conjunction with INSL3-RXFP2 signaling, contribute to the development of cancer anorexia. Additionally, this implies that the RLN/INSL family may be implicated not only in tumor progression but also in the occurrence of cancer-related adverse effects.

4.2. INSL4

INSL4 is upregulated in cancer cell lines harboring LKB1 mutations. Analysis of The Cancer Genome Atlas (TCGA) reveals abnormal INSL4 expression in cervical cancer and non-small cell lung cancer (NSCLC), in which LKB1 mutations are primarily found. INSL4 expression is higher in lung tumor tissues than in normal tissues, and high INSL4 expression in patients with adenocarcinoma-NSCLC is associated with shorter overall survival. INSL4 expression is regulated by the LKB1/CRTC/CREB pathway. According to the results of Ingenuity Pathway Analysis, INSL4 promotes the growth and survival of LKB1-inactivated lung cancer cells *in vivo* through PI3K/AKT and/or MAPK signaling. However, there are differences in the transcription and translation of INSL4 among NSCLC cell lines. Some LKB1-null cell lines do not express INSL4, but this is induced upon treatment with a DNA methyltransferase inhibitor or histone deacetylase inhibitor. These differences in expression were consistent in the lung tissues of patients with NSCLC. INSL4 expression is regulated by epigenetic regulation and/or post-transcriptional modifications in tumors. Abnormal hypomethylation of the INSL4 promoter, identified through 27K Infinium methylation array analysis, has been observed in MTC, supporting previous findings [125,126].

INSL4 is exclusively expressed in breast cancer tissues but not in normal breast epithelium, and it is not expressed in stromal cells (e.g., fibroblasts) that constitute TME. INSL4 overexpression enhances breast cancer cell motility and invasiveness. Similarly, high levels of INSL4 and Her2 have been observed in the breast tissues of patients with Paget's disease of the nipple, which is associated with highly motile cells. Furthermore, INSL4 is detected in approximately 50 % of invasive breast cancer cases. Patients who were positive for both Her2 and INSL4 showed more than a 2-fold decrease in 5-year survival compared to patients who were negative for both. This co-expression indicated highly aggressive characteristics. These findings suggest a possible interaction between INSL4 and Her2. Thus, elucidating the relationship between these molecules can help us understand the mechanisms associated with breast cancer progression [127,128].

4.3. INSL5

INSL5 is highly expressed in nasopharyngeal carcinoma (NPC) and is one of the metabolism-associated genes upregulated after Epstein-Barr virus (EBV) infection. EBV infection also partly contributes to the upregulation of INSL5 in NPC. Patients with high plasma INSL5 levels have shorter overall survival and disease-free rates than those with low levels. Moreover, patients with high INSL5 expression and EBV DNA copy numbers have shorter overall survival than those with lower levels. INSL5 overexpression promotes the proliferation and invasion of NPC cells *in vitro*. Furthermore, INSL5 overexpression facilitates a shift in glucose metabolism from oxidative phosphorylation to glycolysis, promoting aerobic glycolysis and reprogramming glucose metabolism. These metabolic changes are attributed to the activation of pSTAT5 via JAK1 and ERK1/2, leading to the upregulation of HK1, GLUT3, and PFK. Additionally, INSL5 increases the expression of cyclins E, D, and c-Myc but decreases p27 expression. It also induces resistance to 5-fluorouracil or dipeptidyl peptidase-4 treatment [129].

INSL5 and RXFP4 are co-expressed in neuroendocrine/carcinoid tissues, suggesting that INSL5 may be activated in neuroendocrine tumors in an autocrine/paracrine manner, although its role has not been fully elucidated [130]. Low INSL5 expression in breast cancer is associated with a higher survival rate in the absence of distant metastasis. Treatment of MCF-7 cells with the ginsenoside Rh2 results in the induction of hypermethylation of INSL5, leading to its downregulation. This suggests that breast cancer growth may be inhibited through the epigenetic regulation of INSL5 [131]. Additionally, INSL5 is associated with the prognosis of various types of cancer. Notably, through an integrated bioinformatics analysis utilizing the Gene Expression Omnibus database, INSL5 was identified as one of the robust differentially expressed genes associated with colorectal cancer (CRC) carcinogenesis. High INSL5 expression in CRC is associated with a better prognosis, and INSL5 expression is lower in CRC tumors than in normal tissues [132,133]. Conversely, high INSL5 expression is correlated with poor overall survival in glioma, clear cell renal cell carcinoma, sarcoma, uterine carcinosarcoma, and uveal melanoma [129].

4.4. INSL6

In a pan-cancer analysis, INSL6 showed high expression in breast cancer and liver hepatocellular carcinoma in paired TCGA samples but low expression in kidney renal clear cell carcinoma, kidney renal papillary cell carcinoma, and thyroid carcinoma. The differential expression has also been observed in various cancer types. INSL6 is implicated in immune cell regulation and exhibits anti-inflammatory properties [132]. INSL6 deficiency induces infiltration of $CD4^+$ and $CD8^+$ T cells and upregulates the expression of inflammatory cytokines (i.e., TNF-α, IFN-γ) [135]. Although the exact role of INSL6 in tumor growth remains unclear, it is likely to contribute to tumor progression, directly or indirectly, by regulating the immune system.

In summary, the INSL family contributes in various manners to the development and progression of cancer. INSL3 is known to

promote the migration of thyroid cancer cells and is associated with the induction of cancer anorexia. This highlights its role in both cellular dynamics and systemic cancer-related effects. INSL4 plays a role in promoting the growth and survival in lung and breast cancer. INSL5, highly expressed in nasopharyngeal carcinoma, enhances the proliferation and invasiveness of cancer cells. Finally, INSL6, which appears to be expressed in various types of cancer, may be involved in the regulation of immune cells. Collectively, each gene within the INSL family possesses a unique role, yet they are implicated in the complex network associated with cancer. This understanding is vital for grasping the progression of cancer and contributes significantly to the development of new therapeutic strategies.

5. Bone metastases and complications

Bone metastasis is the most lethal complication of cancer. Disruption of normal bone remodeling leads to an imbalance between bone resorption and formation, resulting in bone metastasis. Bone metastases can be classified as osteolytic, osteoblastic, or mixed. They are commonly observed in breast and prostate cancers (approximately 70 %) and in thyroid and kidney cancers (30 %–40 %), but less frequently in gastrointestinal cancers. The development of bone metastasis involves a multistep process that included the escape and dissemination of tumor cells, adhesion and invasion of the bone, the formation of bone lesions, and the colonization of metastatic cells within the bone [136–138].

RLN acts as a pro-cancer or anti-cancer factor depending on the type of cancer, but it primarily contributes to the aggressive progression of cancer associated with bone metastasis. A comparative analysis of RLN expression in various metastatic sites (bone, liver, and lymph nodes) of AI prostate cancer revealed higher expression levels in bone metastases than in other metastatic sites. C4-2, a LnCaP subset with bone metastasis, also shows high RLN mRNA [62,64].

RLN is not expressed in human peripheral PBMCs or osteoclasts, although they express RXFP1. Treatment of human PBMCs with RLN alone induced osteoclast differentiation and promoted the osteoclast phenotype *in vitro*. Furthermore, RLN-induced osteoclast differentiation was capable of bone resorption and complete maturation. RLN-stimulated PBMCs showed low expression of RANK and c-FMS but high expression of NF-κB and NFATc1. This suggests that RLN-RXFP1 signaling can stimulate the expression of NF-κB and NFATc1, inducing osteoclast differentiation through mechanisms independent of the RANK and/or c-FMS pathways [139,140]. In contrast, RLN-RXFP1 signaling is also involved in osteoblast differentiation. RLN efficiently enhances BMP-2-induced osteogenic differentiation and mineralization in mouse embryonic cells and human mesenchymal stem cells. Additionally, RLN dose-dependently increases BMP-2-induced ectopic bone formation *in vivo*. RLN enhances Smad 1/3/5, p38, and TAK1 phosphorylation induced by BMP-2 and increases RunX2 expression and activity. However, these effects are not induced by RLN alone [141].

Young men with the $RXFP2^{T222P}$ mutation, which recognizes INSL3, are at higher risk of decreased bone density. RXFP2 is expressed in MG-63 and primary osteoblasts, and $RXFP2^{-/-}$ mice exhibit reduced bone mass and an osteoporosis-like phenotype. This imbalance between bone formation and resorption occurs because of a decrease in the rate of bone formation by osteoblasts rather than an increase in osteoclast activity. Importantly, it indicates the involvement of INSL3 in bone metabolism [142].

RLN and INSL3 regulate osteoclast and osteoblast differentiation in endocrine manner, thereby influencing bone formation. RLN is predominantly expressed in cancers associated with bone metastasis and can act as a chemokine for macrophages and PBMCs. Thus, tumor-derived RLN has the potential to regulate the expression of MMPs and cathepsins, modulate the immune microenvironment, and regulate osteoclast and osteoblast differentiation during the multistep process of bone metastasis, potentially creating a favorable microenvironment for bone colonization and adaptation. However, the current understanding of the RLN/INSL family in bone biology is limited. Elucidating its functions and mechanisms within bone tissues can play a crucial role in the development of strategies for bone metastasis and the treatment of bone complications.

6. Conclusions

The involvement of RLPF and its receptors in tumor progression has been established over the past several decades. Among them, RLN has been extensively studied. Importantly, RLN generally exhibits either pro-cancer or anti-cancer properties, depending on the type of cancer. With its pro-cancer properties, RLN promotes tumor growth and metastasis. In contrast, with its anti-cancer properties, RLN participates in anti-fibrotic and immune regulatory processes, regulating the microenvironment of desmoplastic cancer. This regulation leads to a transition to a “hot” tumor state and increases sensitivity to conventional anti-cancer therapies, suggesting its potential application as anti-cancer sensitizers. The ongoing development of various nanoparticle technologies offers the potential for consistent and stable delivery of RLN to targeted organs. Historically, the short half-life on RLN has been a significant limitation its use as a therapeutic agent. However, the advent of innovative drug delivery systems, these limitations can be overcome. As a result, there is significant progress in research focused on effective methods for RLN delivery. Therefore, the utilization of RLN delivery systems is anticipated to enhance the delivery of various anti-cancer agents and the infiltration of immune cells in fibrotic cancers by degrading the robust barrier composed of a dense ECM through RLN-mediated anti-fibrotic action, thereby increasing the responsiveness to ant-cancer drugs. Meanwhile, RLN as a pro-cancer factor contributes to tumor development and induces resistance to anti-cancer drugs, suggesting that the microenvironment in these types of cancer can be modulated in different directions. Other RLFP members are also involved in cancer progression, but further research is needed to elucidate their specific mechanisms. Recent studies have relied predominantly on *in vitro* and/or general mouse models. However, RLPF and its receptors are expressed in various organs, and their expression levels differ among species. Therefore, these results should be extrapolated to those of humans with caution. These limitations must be overcome using organoid models, humanized mouse models, and clinical studies.

RLPF is known to have the potential to be expressed in various isoforms, and the existence of multiple alternative splicing is also

possible. Traditional RNA sequencing and PCR techniques have been recognized as lacking in accuracy for analyzing these complexities. Long read sequencing technology emerges as a viable alternative. Long read sequencing produces reads much longer than the conventional 100–150 base pairs, often ranging from 1000 to 10,000 base pairs. This advantage has led to its increasing use in whole genome sequencing for various species, including humans, and in genome assembly, as well as in RNA transcript analysis. The biological significance of the numerous isoforms and alternative splicing of RLPF, especially in the tumor environment, is still largely unexplored. However, the application of this new technology show promise in broadening our understanding in this area. Furthermore, recent studies have identified microRNAs (miRNAs) involved in the regulation of RLN signaling [18,143]. Despite these advancements understanding of specific miRNAs modulated by RLN and their functional roles remains in its early stages. Utilizing advanced analytical techniques such as miRNA sequencing can potentially broaden understanding of novel physiological regulatory mechanisms mediated by RLPF. This research is crucial in elucidating complexities of intracellular signaling pathways associated with RLPF.

The RLPF recognizing receptors, known as RXFPs, belonging to the GPCR family, have recently emerged as attractive targets in various therapeutic developments. Notably, an understanding of the structural and ligand recognition mechanisms of receptors ranging from RXFP1 to RXFP4 provides critical information for the development of therapeutics targeting RXFPs. RXFP1 and RXFP2 are multi-domain GPCR, encompassing an ectodomain composed of low-density lipoprotein receptor class A (LDLa) module and leucine-rich repeats (LRR) [144–146]. In contrast, RXFP3 and RXFP4 differ structurally from RXFP1 and RXFP2, resembling more closely the class A small peptide receptors. The interaction between H2 RLN and RXFP1 primarily occurs between the B-chain of H2 RLN and LRR domain, and between the A-chain of H2 RLN and LDLa-LRR linker. These interactions are proposed to induce receptor activation, thereby promoting the activation of various signaling pathways [147]. Additionally, significant progress has been made in understanding the structural mechanisms of RXFP using cryo-electron microscopy. For instance, the binding mechanism between INSL5 and RXFP4, as reveals by cryo-electron microscopy, demonstrates a novel peptide binding mode. In this mode, the B-chain of INSL5 adopts a single α-helix that penetrates the orthosteric pocket, and the A-chain is positioned above this pocket [148]. Furthermore, the cryo-electron microscopy structure of active-state human RXFP1, bound to a single-chain version of the endogenous agonist RLN and the heterotrimeric Gs protein, has been elucidated. This study uncovers that RXFP1 signals through a mechanism of autoinhibition, adding a new layer of complexity to understanding of these receptors [149]. Understanding this interaction mechanism provides crucial information for the development of drugs targeting RXFPs. For a basic understanding of RXFPs, the review by Halls et al. is an important resource [6].

Despite advancements in understanding the ligand recognition mechanisms of RXFP1 and RXFP4, knowledge about RXFP2 and RXFP3 remains limited. This highlights the need for further research to deepen our understanding of the interactions and activation mechanisms between RLPF and RXFPs. Such understanding plays a pivotal role in the development of small molecules and provides potential clues for therapeutic development. However, the knowledge about RXFPs is relatively deficient compared to other GPCR family, and the identities of receptors recognizing INSL4 and INSL6 remain unidentified. The short half-life of the natural RLPF limits the understanding of signaling pathways in experiment using naturally occurring RLPF. To overcome these limitation, the development of small molecule agonists and antagonists for these receptors is underway, holding potential applications in the treatment of specific cancer patients.

Research into relationship between RLPF-RXFP and tumors is anticipated to significantly impact not only tumor growth but also cancer-related complications. Notably, the recent discovery that INSL3 induces anorexia highlights the potential role of INSL3 in addressing complications relate to cancer especially those associated with the nutritional status of cancer patients. This is crucial as complications arising from tumors negatively affect patient prognosis and quality of life, making the improvement of these complications a key research target. Consequently, advancements in contemporary scientific and technological fields, particularly in molecular biology and genomics, can aid in deepening our understanding about the role of RLPF. RLPF may participate in systemic changes caused by tumor development, as evidenced by its through its abnormal expression in tumors, receptor expression in various organs, and epigenetic modification in RLPF within tumor. These findings contribute to the development of new strategies for cancer treatment and the improvement of related complications, utilizing contemporary technologies such as genome editing, epigenetic regulation, and targeted drug delivery systems. In conclusion, understanding the role of RLPF and its receptors in tumor progression provides crucial evidence for developing new approaches not only for cancer treatment but also for improving cancer-related complications. This contributes to the advancement of molecular diagnostics, precision medicine, and personalized treatment strategies, which are expected to play a significant role in enhancing the prognosis and quality of life for cancer patients.

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None.

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