DISSERTATION

GAS6/AXL SIGNALING CONTRIBUTES TO GNRH-DEPENDENT ACTIVATION OF PITUITARY GONADOTROPES

Submitted by

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ABSTRACT

GAS6/AXL SIGNALING CONTRIBUTES TO GNRH-DEPENDENT ACTIVATION OF PITUITARY GONADOTROPES

Gonadotropin-releasing hormone (GnRH) receptor plays a fundamental role in reproduction and is prevalent in various urogenital, reproductive, and non-reproductive cancers. Beyond the conventional G protein-coupled receptor signaling, GnRH receptors interact functionally with multiple receptor tyrosine kinases. AXL, a receptor tyrosine kinase found in various tissues and numerous tumors, is the focus of this dissertation to discover its impact, along with its endogenous ligand Gas6, on GnRH receptor signaling.

In this study, clonal murine pituitary αT3-1 and LβT2 gonadotrope cell lines were utilized to evaluate the effect of AXL activation on GnRH receptor-dependent signaling pathways. A combination of ELISA and immunofluorescence techniques was employed to analyze AXL and GnRH receptor expression in αT3-1 and LβT2 cells, as well as in murine and human pituitary sections. Additionally, ELISA was used to quantify alterations in ERK phosphorylation, pro-MMP9 production, and the release of LHβ. The abundance of Egr-1 transcripts was measured using digital droplet PCR. To assess αT3-1 and LβT2 cell migration responses to GnRH and AXL, trans-well migration assay was used.

Results showed the presence of AXL, alongside GnRH receptors, in αT3-1 and LβT2 gonadotrope cell lines, as well as in murine and human pituitary sections. In line with AXL's potentiating role, Gas6 enhanced GnRH-dependent ERK phosphorylation in αT3-1 and LβT2 cells. Furthermore, Gas6 increased the abundance of Egr-1 transcripts, suggesting enhanced post-transcriptional GnRH receptor responses. Notably, in LβT2 cells, Gas6/AXL signaling not only

stimulated LHβ production but also enhanced GnRH receptor dependent pro-MMP9 protein generation and promoted cell migration, underlining its functional significance.

In summary, our findings unveil a hitherto undiscovered role for AXL as a modulator of GnRH receptor signaling.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

GNRH/GNRH RECEPTOR AND GAS6/AXL RECEPTOR SIGNALING MECHANISMS

1.1. Introduction

1.1.1. Overview of the hypothalamic-pituitary-gonadal (HPG) axis

The hypothalamic-pituitary-gonadal (HPG) axis is a crucial hormonal system responsible for regulating reproductive function in humans and other vertebrates. It involves a complex interplay between the hypothalamus, pituitary gland, and gonads (testes in males and ovaries in females). The HPG axis is regulated by a series of intricate feedback loops that ensure the proper secretion of hormones. The process begins with the hypothalamus releasing gonadotropin-releasing hormone (GnRH), which stimulates the pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (1). LH and FSH then travel through the bloodstream to the gonads, where they stimulate the production of sex hormones such as testosterone in males and estrogen and progesterone in females(2). These sex hormones play vital roles in gamete production, sexual development, and the maintenance of reproductive function. The HPG axis is essential for fertility, and disruptions within this system can lead to various reproductive disorders and hormonal imbalances. Understanding the intricacies of the HPG axis is crucial for advancing reproductive medicine and developing effective treatments for reproductive health issues(3).

1.1.2. Importance of GnRH in regulating the HPG axis

GnRH plays a pivotal role in regulating the hypothalamic-pituitary-gonadal (HPG) axis, making it a key component in the intricate orchestration of reproductive function. GnRH a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH2) produced and released by the hypothalamus in a pulsatile manner, with each pulse initiating a cascade of hormonal events(4,5). Its primary function is to stimulate the pituitary gland's gonadotrope cells to secrete LH and FSH (***Figure 1-1***). These gonadotropins, in turn, act on the gonads (testes in males and ovaries in females), prompting the production and release of sex hormones such as testosterone, estrogen, and progesterone. GnRH acts as the master regulator, controlling the timing and amplitude of LH and FSH secretion, which is crucial for the proper functioning of the reproductive system. Disruptions in GnRH secretion can lead to imbalances in the HPG axis, resulting in reproductive disorders, menstrual irregularities, infertility, or precocious or delayed puberty. Therefore, understanding the importance of GnRH in regulating the HPG axis is crucial for diagnosing and treating various reproductive conditions, as well as developing targeted therapies to restore hormonal balance and improve reproductive health(6).

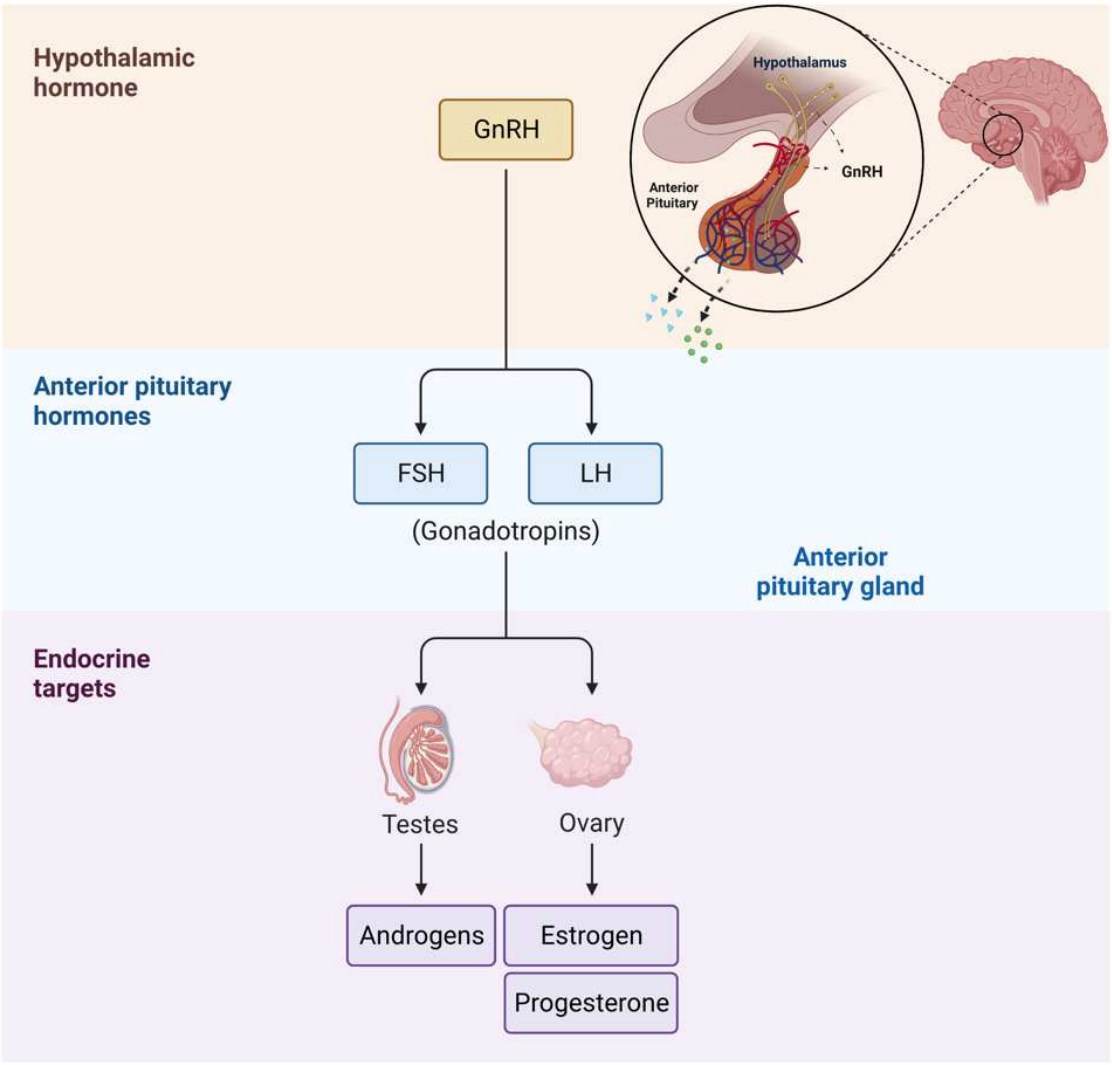

Figure 1-1. Unlocking the Intricacies of the Hypothalamic-Pituitary-Gonadal (HPG) Axis: A Visual Guide to Hormonal Regulation in the Reproductive System. HPG axis is a finely tuned hormonal system in the human body responsible for regulating sexual development and reproduction. It operates through a series of intricate feedback loops which all begins in the hypothalamus, where specialized neurons release Gonadotropin-Releasing Hormone (GnRH) into the bloodstream. GnRH travels to the anterior pituitary gland, where it stimulates the release of two key hormones: Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH). These hormones, in turn, travel to the gonads (testes in males, ovaries in females), where they prompt the production of sex hormones—testosterone in males and estrogen and progesterone in females. These sex hormones are essential for the development of secondary sexual characteristics, regulation of the menstrual cycle in females, and the maintenance of reproductive health. The HPG axis's delicate balance ensures proper sexual development and function, making it a fundamental system in human physiology. Created with BioRender.com

1.1.3. Overview of the AXL receptor and its potential role in GnRH signaling

AXL part of the TAM family of kinases including TYRO3 and MERTK and located at chromosome 19q13.2. AXL was first isolated in 1988 by Liu *et al*.(7) in an attempt to find transforming genes in patients suffering from chronic myeloid leukemia (CML) that led to the blast crisis which is the final and fetal stage of CML if not treated. Later in 1991, Janssen *et al.*(8) and O'Bryan *et al*.(9) separately characterized AXL in two different cells. Using a sensitive transfection-tumorigenicity assay, Janssen *et al.* found the transforming complementary DNA (cDNA) in NIH3T3 mouse fibroblasts transfected with a chronic myeloproliferative disorder patient's DNA. Concurrently, O'Bryan *et al*. independently identified the same transforming cDNA that was overexpressed in human myeloid leukemia cells that they named AXL (from the Greek anexelekto, meaning uncontrolled). They also recognized AXL protein as a novel receptor tyrosine kinase (RTK) subclass.

The Axl gene has two different transcript variants and remained conserved between vertebrate species throughout evolution(10). Almost all cell lines that express AXL have a 70-85kD soluble form (s-AXL) as well as a 100-140kD activated membrane-bound form of the receptor. The full-length AXL receptor is predicted to encode a 98kD protein and contains 894 amino acids(11–15), while the s-AXL, which only includes the extracellular domain of the full-length AXL receptor, exists in both human and murine plasma.

While AXL is similar to other RTKs in terms of kinase function, it has a unique structure of the extracellular region that juxtaposes two immunoglobulin-like repeats (IgL domains) and two fibronectin type 3-like repeats (FNIII domains). AXL has been identified to have six phosphorylation sites: Tyr866, Tyr82, Tyr779, Tyr703, Tyr702, and Tyr698. The three residues closest to the C-terminal (Tyr866, Tyr82, Tyr779) function as docking sites for downstream

effector proteins. While the three tyrosines that are proximal to N-terminal (Tyr703, Tyr702, and Tyr698) are putative autophosphorylation locations that reflect AXL activation(16).

Growth arrest-specific protein 6 (GAS6) belongs to the vitamin K-dependent family of proteins and serves as a high affinity ligand to AXL receptor. Once Laminin G-like (LG1) domain of GAS6 binds to IgL1 and IgL2 domains of AXL receptor(17,18), it undergoes homodimerization followed by auto-phosphorylation and trans-phosphorylation of the intracellular tyrosine residues(19). These changes help transduce signals from the extracellular matrix into the cytoplasm. Gas6 was first identified in serum-starved NIH 3T3 fibroblast cells in response to growth arrest. Although GAS6 activates each of the TAM receptors, its relative affinity to AXL is significantly higher than its affinity other two members of TAM family (with the ranking of affinity: Axl>Tyro3>Mer)(20). AXL has about 10-fold higher affinity to GAS6 compared to Mer affinity against the same target (21).

Later in 2015, Fujimori et al.(22) reported that the interaction between AXL and GAS6 might not be sufficient for subsequent activation of downstream pathways. Other studies suggest that AXL activation occur via a GAS6-independent mechanism. Evidence suggests that the extracellular domains of AXL protein are abundantly aggregated on adjacent cells(23). Additionally, AXL was reported to undergo ligand-independent homodimerization followed by downstream activation of effectors in both cases (24). AXL can also be heterodimerized with other RTKs (non-TAM) with or without their dimerization partner's ligand, eventually leading to AXL-dependent downstream cascades(25,26).

Studies have shown that AXL is expressed in various reproductive tissues, including the hypothalamus, pituitary gland, and gonads. In the hypothalamus, AXL is predominantly expressed in GnRH neurons, suggesting a potential involvement in GnRH signaling. Additionally, AXL

expression has been detected in gonadotropes. These findings raise the possibility that AXL may have a regulatory role in GnRH signaling within the HPG axis(27,28).

1.1.4. Research questions and objectives

The potential role of AXL in GnRH signaling is still an emerging field of research(29). Recent studies have provided intriguing evidence supporting its involvement. It has been suggested that AXL signaling may modulate the migration and activity of GnRH neurons, impacting GnRH release and downstream reproductive processes(30,31). Activation of AXL in GnRH neurons may contribute to the regulation of GnRH pulsatility, a critical feature of normal reproductive function. AXL signaling may also influence the sensitivity of gonadotropes to GnRH, affecting the production and secretion of LH and FSH. Moreover, fundamental mechanisms underlying integrative AXL and GnRH receptor signaling in gonadotropes, and GnRH-responsive cancers are unknown.

Here, we evaluated the hypothesis whether the receptor tyrosine kinase AXL contributes to GnRH receptor-dependent regulation of pituitary gonadotrope function using mouse clonal gonadotrope cells (αT3-1 and LβT2). These cells are an ideal model for the study of local signaling processes due to the robust localization-dependent divergence of signaling outputs following activation by the key hypothalamic peptide GnRH(32). Earlier work, including our own, uncovered that GnRH, acting via Gq-coupled receptors, stimulates a local Ca2+ signal produced by L-type Ca2+ channels specifically coupled to ERK signaling(33–35).

Interestingly, new data show that AXL receptor bound to its ligand (Gas6) also contributes to ERK activation(36). In this project we proposed a model where GnRH receptor and AXL receptor coordinate to transduce and activate signaling molecule complexes critical to ERK activation.

Experiments outlined below have been performed using clonal mouse gonadotrope α-T31 and LβT2 cells, human as well as C57 balb/c female and male mice pituitary sections.

The overall goal of this project is to 1) identify and characterize molecular components involved in the generation and activation of AXL and ERK; 2) characterize the regulatory mechanisms involved with dynamic localized AXL and ERK signaling including that of the actin cytoskeleton; and 3) achieve these goals in a physiologically and biomedically meaningful context (i.e., gonadotrope cell biology).

Results of this study has a broad impact including but not limited to: 1) conception of integrated molecular mechanisms involved in AXL and GnRH receptor signaling applicable to multiple biological systems; 2) better understanding of the effects of AXL and GnRH receptors on ERK signaling, LHβ production and gonadotrope cell's movements; 3) identification of joint AXL and GnRH receptors signaling components as potential therapeutic targets for manipulation of GnRH receptor function in fertility, obesity-related endocrine dysfunction, polycystic ovarian syndrome, and GnRH-responsive carcinomas. Therefore, better understanding of GnRH receptor signaling is practically valuable to improve human health.

This study used three main objectives to investigate the role of Gas6/AXL signaling in regulation of GnRH-dependent regulation of gonadotrope cells (***Figure 1-2***):

Objective 1: Investigate the functional expression of AXL in gonadotropes and its regulation by GnRH, employing confocal and fluorescent microscopy of fixed cells and protein analysis/immunoassays as primary methods to elucidate the interplay between GnRH and AXL receptor regulation.

Objective 2: Explore the involvement of AXL in the GnRH-dependent activation of MAP kinase signaling within gonadotropes. Utilize confocal microscopy of fixed cells/tissue, protein analysis/immunoassays, and nucleic acid quantification methods (ddPCR) to identify critical components of AXL receptor signaling complexes, shedding light on their role in MAP kinase activation and downstream signaling pathways.

Objective 3: Investigate the impact of AXL on the GnRH-directed migration of gonadotropes. Examine the influence of AXL signaling on GnRH-dependent regulation of pERK, actin dynamics, and MMP9 activities, particularly in cell movement and cell-matrix adhesions of gonadotrope cells, employing trans-well chamber migration tests and molecular biology approaches such as ELISA.

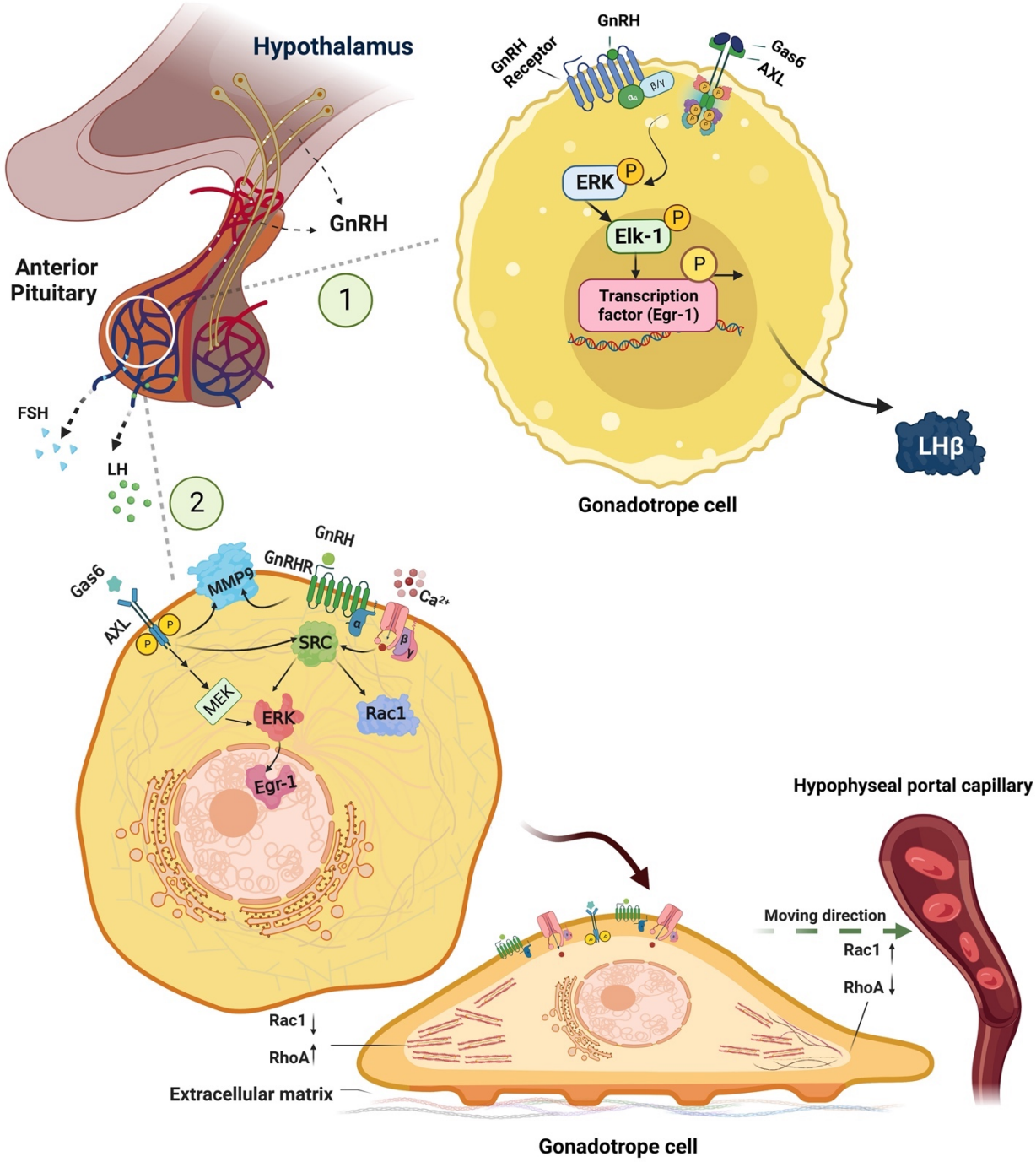

Figure 1-2. Proposed pathways associated with activation of AXL and GnRH receptors in pituitary gonadotrope cells. 1) activation of AXL and GnRH receptors lead to ERK activation and later LHβ production and release from gonadotrope cells. 2) Activation of AXL and GnRH receptors lead to morphological changes, actin cytoskeleton reorganization and movement of gonadotrope cells. Created with BioRender.com

1.2. Literature Review

1.2.1. Overview of GnRH signaling and gonadotrope function

Gonadotropin-releasing hormone (GnRH) signaling and gonadotrope function are integral components of the hypothalamic-pituitary-gonadal (HPG) axis, which governs reproductive processes in vertebrates. GnRH, produced by the hypothalamus, regulates the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland. Different studies have provided overviews of GnRH signaling and its impact on gonadotrope function, shedding light on the intricate processes that underlie reproductive regulation(37).

GnRH signaling begins with the release of GnRH from the hypothalamus in a pulsatile manner. The pulsatile nature of GnRH release is crucial for the regulation of gonadotropin secretion. GnRH binds to its receptor on the surface of gonadotrope cells in the anterior pituitary gland, activating a cascade of intracellular events(37). This leads to the activation of phospholipase C, which generates inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 triggers the release of calcium ions from intracellular stores, while DAG activates protein kinase C. These events culminate in the phosphorylation and activation of various downstream signaling molecules, including mitogen-activated protein kinases (MAPKs) and protein kinase A (PKA)(37,38).

Gonadotropes are specialized cells within the anterior pituitary gland responsible for the synthesis and secretion of LH and FSH(39). GnRH signaling plays a significant role in regulating gonadotrope function. Upon binding to the GnRH receptor, GnRH stimulates the transcription and translation of LH and FSH, as well as their storage in secretory granules(40). GnRH also promotes the secretion of LH and FSH from gonadotropes into the systemic circulation(41).

The activity of gonadotropes is tightly regulated by both positive and negative feedback mechanisms(6). Positive feedback occurs when rising levels of sex steroids, such as estrogen, promote an increase in GnRH release from the hypothalamus. This surge of GnRH then stimulates gonadotrope cells to produce and secrete a large amount of LH and FSH, triggering ovulation in females and testosterone production in males. Negative feedback occurs when elevated levels of sex steroids suppress GnRH release and later inhibit gonadotrope activity. This feedback loop helps keep hormonal balance and prevent overstimulation or underproduction of LH and FSH(42,43).

GnRH signaling does not act in isolation but interacts with various other signaling pathways to modulate gonadotrope function. For instance, GnRH signaling converges with the cyclic adenosine monophosphate (cAMP) pathway, where cAMP acts as a secondary messenger downstream of GnRH receptor activation(44). Additionally, GnRH signaling can activate MAPK pathways, leading to the phosphorylation of transcription factors involved in the regulation of α-subunit, GnRH receptor, LHβ and FSHβ synthesis(45).

1.2.2. Signaling pathways associated with Gas6/AXL activation

AXL, widely distributed across various tissues, engages in diverse intracellular signaling pathways. The outcomes of AXL receptor activation are largely context-dependent, with distinct effects in healthy cells, where AXL signaling supports functions like cell survival, proliferation, migration, and adhesion, and in transformed cells, where these signals promote abnormal cell growth, metastasis, invasion, angiogenesis, immune evasion, and drug resistance.

Upon receptor activation, intrinsic kinase activity-dependent phosphorylation of specific tyrosine residues in the AXL C-terminus (Y779, Y821, and Y866) helps the recruitment of signaling proteins, creating docking sites for protein-protein interactions (**Figure 1-*3***)(46–49).

Phosphorylated Y821 binds to proteins with SH-2 domains, including the regulatory subunit p85 of phosphatidylinositol 3-kinase (PI3K), c-SRC cellular tyrosine kinase (c-SRC), and phospholipase C-γ. Phosphorylated Y821 also interacts with the SH-2 containing adaptor protein GRB2, which links receptor tyrosine kinases to multiple signaling cascades like RAS/RAF/MAPK and Rho GTPase Rac1. To further diversify signaling outcomes, AXL forms clusters and interacts functionally with other receptor tyrosine kinases such as EGFR (epidermal growth factor receptor) and HER2 (human epidermal growth factor receptor 2)(50,51).

PI3K/Akt Signaling

PI3K/Akt signaling orchestrates various cellular processes, including protein synthesis, metabolism, and motility. Activation of receptors, including AXL, amplifies PI3K/Akt signaling, promoting growth, proliferation, and survival(51,52). In numerous healthy cells (such as vascular smooth muscle and endothelial cells, hepatic stellate cells, and neurons) and cancer cells (e.g., chronic lymphocytic leukemia, prostate(53), and breast carcinomas(54), and non-small cell lung cancers(55)), AXL-dependent PI3K/Akt signaling contributes to cell survival, proliferation, and drug resistance(56–60).

AXL activation, either through Gas6 binding or ligand-independent mechanisms, leads to the recruitment of p85, the regulatory subunit of PI3K. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2), generating phosphatidylinositol trisphosphate (PIP3)(61). Increased PIP3 levels boost the activity of the serine/threonine kinase Akt/protein kinase B (PKB), which phosphorylates pro-survival proteins like MDM2, IKK, and mTOR(62,63). This phosphorylation inactivates pro-apoptotic signaling proteins (e.g., BAD) and apoptosis-executing proteins (e.g., caspases 3 and 9).

Akt activity also triggers the nuclear translocation of the transcription factor NF-kB(64). NF-kB upregulates the expression of several cell survival genes, including those encoding anti-apoptotic proteins, cell cycle regulators, proliferation-associated proteins, and other pro-survival transcription factors. NF-kB also increases the expression of matrix metalloproteins that impact cell migration, proliferation, and survival(65,66).

Small GTPase Signaling

Small GTPases belonging to the Ras superfamily, such as RhoA, Rac1, and Cdc42, are frequent targets of tyrosine kinase receptors like AXL. These GTPases contribute to actin cytoskeletal remodeling and cellular protrusion formation(67). AXL receptor activation influences small GTPases like Rac1 and RhoA through mechanisms involving Ras-dependent and independent PI3K/Akt signaling(52,68). Additionally, AXL function and downstream signaling can be regulated by NADPH oxidase, a source of reactive oxygen species(69–72). The downstream targets of Rac1 and RhoA encompass PAK/JNK as well as the MAP kinase signaling pathways. These signaling processes typically result in the restructuring of the actin cytoskeleton and facilitate cell movement and migration(73,74).

MAP Kinase Signaling

Mitogen-activated protein kinases (MAPKs) send extracellular signals to regulate cellular processes like proliferation, survival, migration, and differentiation. AXL receptors are known to activate the extracellular signal-regulated kinase (ERK) branch of MAPK signaling pathways(75,76). In healthy cells, AXL-dependent ERK signaling supports cell survival, proliferation, and differentiation(77), while in cancer cells, it contributes to poor clinical outcomes by reducing apoptotic potential and promoting therapy resistance.

In summary, AXL receptor activation is intricate, involving both ligand-dependent and ligand-independent mechanisms. AXL signaling complexity is further increased by the extensive crosstalk among redundant and non-redundant signaling cascades (***Figure 1-3***). Due to the multitude of signaling protein interactions and variable protein expression profiles, the effects of AXL activation are likely to differ between cell types and can change as proteomic landscapes evolve. Additionally, the functional relationship between AXL and other distantly related receptor tyrosine kinases, such as EGFR, stays poorly understood.

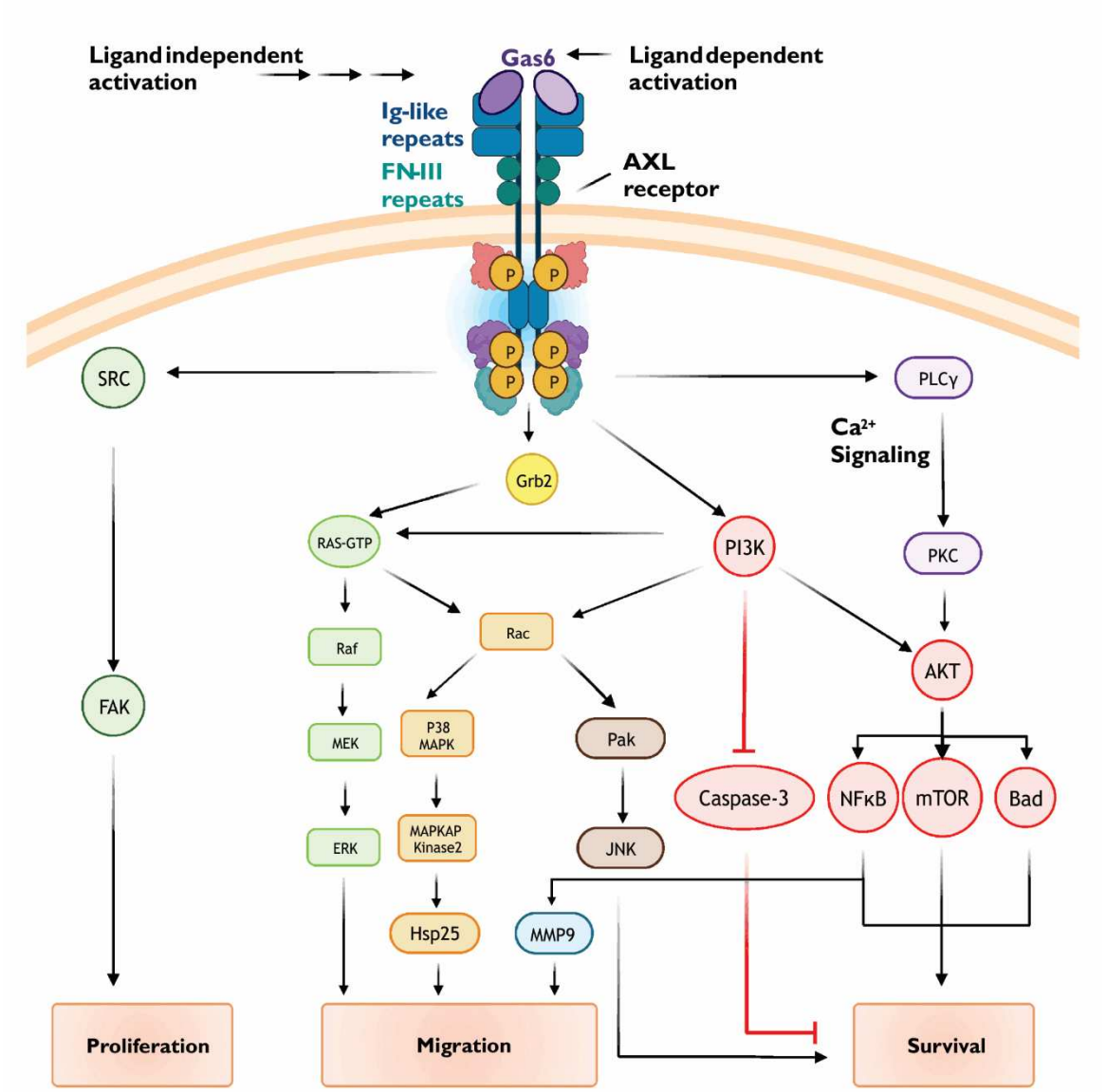

Figure 1-3. Typical Signaling Pathways of the AXL Receptor. AXL receptor function is linked to various signaling pathways, such as the PI3K/Akt pathway, small GTPase activation, and MAP kinase cascades. Created with BioRender.com(27)

1.2.3. Role of AXL receptor in other physiological systems

The involvement of the AXL receptor in various physiological systems extends beyond the reproductive system. Here, we explore its role in other bodily functions:

Immune System Regulation:

AXL plays a crucial role in immune regulation. It is expressed in immune cells, such as dendritic cells and macrophages, where it can modulate inflammatory responses.

By interacting with its ligand Gas6, AXL can dampen excessive immune activation, preventing an overzealous immune response that could lead to autoimmune disorders(78–80).

Central Nervous System:

AXL is expressed in various cells within the central nervous system (CNS), including microglia, astrocytes, and neurons. In the CNS, AXL engages in processes like synaptic pruning and neural plasticity, which are essential for normal brain development and function.

Dysregulation of AXL in the CNS has been linked to neuroinflammatory conditions and neurodegenerative diseases(81,82).

Cardiovascular System:

AXL has implications in cardiovascular health. It is expressed in endothelial cells, smooth muscle cells, and cardiomyocytes. AXL activation can protect blood vessels by promoting endothelial cell survival and inhibiting inflammation, contributing to vascular homeostasis. It may also play a role in cardiac remodeling after injury(83).

Hematopoiesis:

Hematopoietic stem cells and progenitor cells express AXL, suggesting a role in hematopoiesis. AXL has been implicated in regulating the differentiation and survival of certain blood cell lineages(84–86).

Cancer:

In addition to its role in reproductive system cancers (e.g., breast, ovary, etc.), AXL is also associated with various other cancer types. AXL activation in cancer cells can promote metastasis, chemoresistance, and immune evasion(76).

Vision:

AXL is expressed in the retina(87) and has been linked to Proliferative diabetic retinopathy (PDR). Axl inhibition is a promising potential for PDR therapy(88).

Bone Metabolism:

AXL is expressed in osteoblasts(89) and osteoclasts(90), suggesting a role in bone remodeling and metabolism.

Metabolic Regulation:

AXL has been implicated in metabolic processes, including glucose homeostasis(91) and adipocyte function(92).

In summary, the AXL receptor's influence extends across multiple physiological systems, playing diverse roles in immune regulation, CNS function, cardiovascular health, hematopoiesis, cancer, vision, bone metabolism, and metabolic regulation. Its involvement in these systems highlights its significance in maintaining overall bodily homeostasis and health.

1.2.4. Previous research on AXL receptor in the reproductive system

The hypothalamic-pituitary-gonadal (HPG) axis plays a pivotal role in regulating hormones related to sexual differentiation and reproduction. The signaling cascade of the HPG axis starts with the secretion of gonadotropin-releasing hormone (GnRH) by specialized hypothalamic neurons into the hypothalamic-hypophysial-portal circulation. GnRH then activates receptors on gonadotrope cells in the anterior pituitary (adenohypophysis). The precise location

and alignment of GnRH neurons and pituitary gonadotropes are necessary for efficient interaction in the HPG axis.

In this context, AXL/Gas6 signaling is necessary for the proper development of hypothalamic GnRH neurons(27). These neurons, crucial for HPG axis function, are located in the olfactory placode and migrate to the forebrain(93). AXL contributes to the Rac1-dependent chemotactic migration of GnRH neurons(94). Rac1 activity is essential for actin cytoskeletal remodeling and migration, a process caused by Gas6-dependent AXL activation. Additionally, Rac1 enhances p38 MAPK/MAPKAP kinase 2 activity, resulting in the phosphorylation of HSP25, a small heat shock protein that regulates actin polymerization and cell movement. AXL-dependent ERK and PI3K/Akt signaling enhance the survival of migrating cells through anti-apoptotic processes(95). Remarkably, AXL signaling serves a dual purpose by suppressing GnRH expression while supporting GnRH migration from the olfactory placode to the forebrain(96). Additionally, Rac1 is involved in AXL-dependent inhibition of GnRH production.

Beyond their shared ligand, Gas6, the three TAM receptors (Tyro3, AXL, and Mer) display structural similarities and functional redundancy(97). Consequently, the study of AXL receptor biology often encounters complexities due to potential influences from Tyro3 and Mer receptors. To address this issue, mice with various combinations of TAM receptor null mutations were created(98). Notably, only TAM receptor triple knockout mice showed clear abnormalities in reproductive phenotypes.

Subsequently, investigations into AXL/Tyro3-null (double knockout) mice showed distinct, though nuanced, roles for these two TAM receptors in female reproductive function(99). These mice showed impaired GnRH neuronal survival and migration along the migratory route, leading to delayed time to first estrus and abnormally prolonged estrous cycles(95,96). Despite

normal vaginal opening and pituitary responses, ovariectomized AXL/Tyro3-null mice did not respond to exogenous estradiol, showing compromised GnRH neuronal function(100). Collectively, these findings imply that AXL/Tyro3 signaling contributes to normal female reproductive cyclicity and function.

All three TAM receptors (Tyro3, AXL, and Mer) are believed to play roles in normal male reproductive function(96). However, elucidating specific functions for each receptor is challenging due to their intricate interplay. Male TAM receptor triple knockout mice were found to be sterile, producing only immature sperm due to the progressive death of differentiating germ cells(101). These effects were seen postnatally, with no clear impact on embryonic or neonatal testes. Although variations in expression patterns were reported, Tyro3, AXL, Mer, and Gas6 are differentially expressed in testicular somatic support cells (e.g., Leydig and Sertoli cells) and spermatogonia(101–104). Expression disparities may arise from measurement methods (e.g., measured protein vs mRNA), cell types studied, and developmental considerations (e.g., postnatal vs perinatal). Interestingly, Gas6, potentially produced by Leydig cells, was upregulated in TAM triple knockout mice, suggesting a self-regulating feedback mechanism(102–104).

Observations from TAM triple knockout mice underscore the cooperative role of these three receptors in supporting spermatogenesis. Among somatic support cells, Sertoli cells displayed the most significant impairment in TAM triple knockout mice, while Leydig cells were less affected. Sertoli cells in TAM triple knockout and Mer-null mice showed dysfunctional phagocytic activity, although cell survival and differentiation appeared unaffected(105,106). Spermatogenesis was more severely impaired in TAM triple knockout mice compared to those lacking only Mer receptors. Intriguingly, AXL receptor expression was upregulated in Mer-null mice(105). The compromised phagocytic function of Sertoli cells negatively impacted

spermatogenesis, likely by impeding the removal of apoptotic cells and reducing nutrient availability(107–109).

While AXL is commonly associated with the development of various cancers, *Axl* mutations are seldom identified as direct causative factors. Instead, AXL seems to play a secondary role in cancer development, often linked to increased expression levels(77,110,111). In alignment with this proposed role in cancer, Axl mutations and malfunctioning AXL proteins are not widely implicated in the onset of reproductive disorders. However, there is a connection between AXL dysfunction and specific forms of Kallmann Syndrome, a subtype of idiopathic congenital hypogonadotropic hypogonadism.

Emerging evidence shows that certain rare variants of idiopathic hypogonadotropic hypogonadism can be attributed to *Axl* mutations that hinder AXL's normal function(112). Idiopathic hypogonadotropic hypogonadism involves abnormalities in either the release of neuronal GnRH or the action of GnRH(113,114). This aligns with AXL's documented role in helping the migration of GnRH neurons from the medial olfactory placode to the hypothalamus. Consequently, these mutations result in impaired survival and migration of neurons, including GnRH neurons, from the medial olfactory placode to the hypothalamus. This diminished population of hypothalamic GnRH neurons is associated with reduced levels or the absence of circulating testosterone, luteinizing hormone, and follicular stimulating hormone (FSH).

In summary, these observations collectively suggest that alterations in AXL function, as well as other components of the TAM receptor family (such as Tyro3, Mer, and Gas6), can contribute to disruptions in endocrine function and the development of related diseases.

1.2.5. Knowledge gaps and rationale for the study

Understanding the potential role of the AXL receptor in GnRH signaling has significant implications for some cancers and reproductive health and fertility. Dysregulation of GnRH signaling can lead to reproductive disorders, such as hypogonadism, infertility, or disorders of sexual development. Elucidating the involvement of AXL in GnRH signaling pathways may provide new insights into the underlying mechanisms of these conditions and open avenues for targeted therapeutic interventions. Further research is needed to unravel the specific molecular mechanisms by which AXL influences GnRH signaling and its interactions with other key molecules within the HPG axis.

CHAPTER 2

GAS6/AXL SIGNALING MEDIATES ERK ACTIVITY AND PLASTICITY OF GONADOTROPES

2.1. Summary

2.1.1. Background

Gonadotropin-releasing hormone (GnRH) receptors play a crucial role in reproductive processes and are expressed in various urogenital, reproductive, and even non-reproductive cancer types. Apart from their typical signaling through G protein-coupled receptors, GnRH receptors also engage in functional interactions with multiple receptor tyrosine kinases. AXL, a receptor tyrosine kinase found in numerous tissues and various tumors, is of particular interest. In this study, we aimed to investigate whether AXL, in conjunction with its natural ligand Gas6, exerts an influence on GnRH receptor signaling.

2.1.2. Methods

We conducted our experiments using clonal murine pituitary cell lines, specifically αT3-1 and LβT2 gonadotrope cells, to assess how AXL activation affects the outcomes of GnRH receptor-dependent signaling. Our methods included the use of ELISA and immunofluorescence techniques to observe the expression of both AXL and GnRH receptors in αT3-1 and LβT2 cells, as well as in pituitary sections from mice and humans. We also employed ELISA to quantify changes in ERK phosphorylation, pro-MMP9 production, and the release of LHβ. The abundance

of Egr-1 transcripts was measured using digital droplet PCR. To evaluate cell migration responses to GnRH and AXL, we employed a trans-well migration assay.

2.1.3. Results

Our observations revealed the presence of AXL, alongside GnRH receptors, in αT3-1 and LβT2 gonadotrope cell lines, as well as in pituitary sections from both murine and human sources. In line with a potential potentiating role of AXL, Gas6 was found to enhance GnRH-dependent ERK phosphorylation in αT3-1 and LβT2 cells. Moreover, indicative of enhanced post-transcriptional GnRH receptor responses, Gas6 was associated with an increase in the abundance of Egr-1 transcripts. Demonstrating functional significance, in LβT2 cells, Gas6/AXL signaling not only stimulated LHβ production but also heightened the GnRH receptor-dependent generation of pro-MMP9 protein and promoted cell migration.

2.1.4. Conclusions

In summary, our findings unveil a novel role for AXL as a regulator of GnRH receptor signaling. This discovery sheds light on the intricate mechanisms at play in the regulation of reproductive processes and opens new avenues for understanding the role of these receptors in both normal and pathological contexts.

2.2. Introduction

Reproductive processes are predominantly regulated by hormones generated within the hypothalamic-pituitary-gonadal (HPG) axis. Specialized hypothalamic neurons release gonadotropin-releasing hormone (GnRH) into the hypophyseal portal circulation, transporting this neuropeptide to the anterior pituitary. A specific subset of endocrine cells, known as gonadotropes,

respond to GnRH by binding to receptors expressed on their cell surfaces. Activation of GnRH receptors starts a series of signaling cascades culminating in the synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), into the bloodstream. Subsequently, these circulating gonadotropins engage with receptors in the gonads, promoting processes like gametogenesis and the synthesis of sex steroid hormones.

During development, GnRH neurons originate in the olfactory placodes and migrate to the forebrain. Misplacement of GnRH neurons, either due to migration issues or other factors, is linked to delayed or absent puberty. A pivotal player in GnRH neuronal migration and survival is the receptor tyrosine kinase AXL, belonging to the TAM family of receptor tyrosine kinases, which includes Tyro3, AXL, and Mer. AXL receptors can be activated by the high-affinity ligand Gas6 (growth arrest-specific protein 6) as well as through ligand-independent mechanisms. Gas6/AXL signaling plays a role in the development of various tumors by promoting cell survival, proliferation, migration, and invasion. Consequently, AXL receptors are currently considered promising targets for novel cancer chemotherapeutics.

Upon activation, the intrinsic tyrosine kinase activity of AXL phosphorylates C-terminal tyrosine residues, leading to the recruitment and activation of numerous intracellular signaling proteins. These proteins include phosphatidylinositol 3-kinase (PI3K), Src tyrosine kinase, phospholipase C-γ, protein kinase C (PKC), Rho GTPases, matrix metalloprotease 9 (MMP9), and mitogen-activated protein kinases (MAPK). AXL signaling is further diversified through its functional interaction with other receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). AXL receptors are linked to the extracellular signal-regulated kinase (ERK) branch of MAPK signaling pathways, which send external signals from cell membrane receptors to elicit changes in cellular functions such as

differentiation, survival, and migration. Notably, during development, Gas6/AXL receptor-mediated promotion of the survival and migration of GnRH neurons involves processes dependent on ERK signaling.

In gonadotropes, activation of GnRH receptors starts canonical Gαq protein signaling. This pathway involves phospholipase C (PLC) cleaving phosphatidylinositol-4,5-bisphosphate (PIP2) to generate the classic second messengers, inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces calcium release from the endoplasmic reticulum (ER), while DAG activates protein kinase C (PKC), ultimately leading to increased calcium influx through voltage-dependent channels. Calcium release from the ER then triggers c-Jun N-terminal kinase (JNK) signaling, enhancing FSH expression, while calcium influx through voltage-dependent channels activates ERK signaling, augmenting LH expression. Additionally, GnRH receptor signaling in gonadotropes has been shown to incorporate matrix metalloprotease 9 (MMP9) activation. As mentioned earlier, AXL receptor activation also promotes ERK- and MMP9-dependent signaling processes. Nevertheless, the precise impact of crosstalk between AXL and GnRH receptors is still unclear.

In our study, we put forward the hypothesis that Gas6/AXL and GnRH receptors collaborate to produce GnRH-dependent signaling outcomes(115). After confirming the expression of Gas6 and AXL receptors in human and murine pituitary slices, we conducted clonal murine gonadotropes to investigate the functional implications of AXL receptor activation on GnRH receptor function. Our findings revealed that Gas6-dependent AXL activation enhances various GnRH receptor processes, including ERK activation, transcriptional responses, luteinizing hormone (LH β) generation, pro-MMP9 levels, and cell migration. In summary, our data lends

support to the concept of AXL receptors acting as innovative regulators of GnRH receptor-dependent modulation of gonadotrope function.

2.3. Materials and Methods

2.3.1. Animal model and experimental design

Animal Model:

In this study two distinct gonadotrope cell lines were used, murine clonal LβT2 and αT3-1, which were generously provided by Dr. Pamela Mellon from the University of California, San Diego. These cell lines were chosen as the primary model system for investigating specific biological processes.

C57 murine (male and female) and Human (unidentified) pituitary 5 µm paraffin sections were purchased from Zyagen (San Diego, CA).

Reagents:

The following reagents and chemicals were employed in the experiments:

Cell Culture:

All reagents were sourced from Thermo Fisher Scientific, (Waltham, MA.) Passage Number: All experiments were conducted on low passage number cells, specifically ranging from passage 6 to 8, for both αT3-1 and LβT2 cell lines.

The use of these well-characterized gonadotrope cell lines in a controlled cell culture environment allowed for the precise investigation of the effects of the tested reagents and chemicals on gonadotrope cell biology. This experimental design ensures the reproducibility and reliability of the study's results and conclusions.

Chemicals:

Unless stated otherwise, chemicals were obtained from Sigma (St. Louis, MO).

Test Reagents:

The specific test reagents used in the experiments were:

Cetrorelix Acetate (Tocris Bioscience; Minneapolis, MN), R428 (APExBio; Houston, TX) Gas6 (Novus Biologicals; Centennial, CO), U0126-EtOH (APExBio; Houston, TX), and GnRH (Sigma).

Cell toxicity assay:

All these drugs underwent rigorous screening based on a previously described method(116) for potential cell toxicity at both 3 hours and 24 hours using a tetrazolium based colorimetric XTT Assay Kit from Abcam, situated in Boston, MA. Importantly, it was found that all drugs used in the study were non-toxic at the concentrations employed.

2.3.2. Techniques for studying GnRH and AXL receptor signaling

Understanding the signaling pathways associated with GnRH and AXL receptors is crucial for unraveling their roles in various physiological and pathological processes. Several techniques are employed to investigate these receptor signaling pathways. Here are some key methods commonly used:

1. Immunofluorescence and Immunohistochemistry:

a) Description: These techniques involve using specific antibodies to visualize the presence and localization of GnRH and AXL receptors in cells and tissues.

b) Application: Determine the cellular and subcellular distribution of these receptors in various tissues and cell lines.

2. ELISA (Enzyme-Linked Immunosorbent Assay):

a) Description: ELISA quantifies the concentration of specific proteins or phosphorylated proteins in a sample using antibodies and enzyme-catalyzed reactions.

b) Application: Measure the levels of GnRH, AXL, or downstream signaling molecules, such as phosphorylated ERK, in response to ligand activation.

3. Digital Droplet PCR (ddPCR):

a) Description: ddPCR is a highly precise method for quantifying the absolute abundance of specific RNA transcripts in a sample.

b) Application: Assess changes in gene expression, such as Egr-1, in response to GnRH or AXL receptor activation.

4. Western Blotting:

a) Description: Western blotting detects and quantifies specific proteins in a sample by separating them based on size through gel electrophoresis and then transferring them to a membrane for antibody detection.

b) Application: Analyze protein expression levels, post-translational modifications, and activation of signaling molecules like ERK in response to receptor activation.

5. Trans-well Migration Assay:

    a) Description: This assay measures the migratory ability of cells in response to various stimuli.

    b) Application: Assess the impact of AXL activation and GnRH receptor signaling on cell migration, providing insights into their functional roles.

6. Co-immunoprecipitation (Co-IP):

    a) Description: Co-IP is used to study protein-protein interactions by isolating a target protein and its binding partners from a complex protein mixture.

    b) Application: Investigate potential interactions between AXL, GnRH receptors, and downstream signaling molecules to identify signaling complexes.

7. Phosphorylation Profiling Arrays:

    a) Description: These arrays simultaneously detect the phosphorylation status of multiple signaling molecules using specific antibodies.

    b) Application: Identify changes in phosphorylation patterns of key signaling proteins in response to GnRH or AXL activation.

8. Fluorescence Resonance Energy Transfer (FRET):

    a) Description: FRET measures interactions between molecules in close proximity by detecting energy transfer between donor and acceptor fluorophores.

    b) Application: Investigate protein-protein interactions and conformational changes within signaling complexes in live cells.

9. Small Interfering RNA (siRNA) Knockdown:

    a) Description: siRNA is used to selectively knock down the expression of specific genes, allowing the study of their functional roles.

    b) Application: Determine the effects of depleting GnRH or AXL receptor expression on downstream signaling pathways and cellular responses.

10. Pharmacological Inhibition:

    a) Description: Use of specific inhibitors to block the activity of key signaling molecules or pathways.

    b) Application: Assess the impact of inhibiting specific signaling components on GnRH and AXL receptor-mediated responses.

By employing these techniques, researchers can gain a comprehensive understanding of the signaling events associated with GnRH and AXL receptors, shedding light on their roles in normal physiology and disease states.

For this research we used a combination of pharmacological inhibition/activation followed by ELISA, ddPCR, Immunofluorescence and Trans-well Migration Assay(117).

2.3.3. Cell culture and treatment of αT3-1 and LβT2 cells with phospho-ERK, GnRH and AXL receptor agonists and antagonists

Clonal LβT2 and αT3-1 gonadotrope cells were cultured in High Glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum and 1% (v/v) Antibiotic-Antimycotic and maintained at 37°C in 5% CO_2 humidified air.

2.3.4. Immunofluorescence for visualizing GnRH and AXL receptors expression and localization in αT3-1, LβT2 cells, mouse, and human pituitaries

αT3-1 or LβT2 cells ($2x10^5$ cells/well) were plated on 35mm glass bottom dishes (Matsunami Glass; Bellingham, WA) coated with Geltrex (Thermo Fisher Scientific). Cells were cultured overnight in 2 mL media (as above) and, on the day prior to fixation, media were replaced with 2 mL of media containing 10% charcoal stripped FBS. The following day, cells were incubated with agonists/antagonists for various times plus 2 µl CellMask Deep Red Actin Tracking Stain (Invitrogen; Waltham, MA) for 30 mins.

Following completion of the experiment, cells were washed with 1X D-PBS with calcium and magnesium three times and fixed in freshly prepared 4% paraformaldehyde in PBS. After fixation, cells were washed with 1X PBS three times, and blocked in eBioscience IHC/ICC Blocking Buffer - High Protein (Thermo Fisher Scientific) for 30 min. Samples were then incubated overnight at 4°C with GnRHR Monoclonal primary antibody (GNRHR/768, ab220196, Abcam) at 1µg/mL dilution and Axl primary Antibody (PA5-106118, Thermo Fisher Scientific) at 1:200 dilution in blocking buffer. The next day, cells were washed three times with 1X eBioscience TBS Wash Buffer for IHC/ICC (Thermo Fisher Scientific) and Alexa Fluor 546 (A-11035, Thermo Fisher Scientific) along with Alexa Fluor 488 (A-11029, Thermo Fisher Scientific) diluted 1:500 in blocking Buffer, were added to the cells for 1h at room temperature. Finally, nuclei were stained, and cells were mounted by ProLong Glass Antifade Mountant with NucBlue Stain (Thermo Fisher Scientific) following the manufactures protocol.

C57 mouse (male and female) and Human (unidentified) pituitary 5µm paraffin sections were purchased from Zyagen (San Diego, CA) and immunostained for AXL, GnRH receptor (GnRHR) and Gas6 proteins based on the protocol described before(118). Briefly, tissues were de-waxed using five-minute washes in CitriSolv (Decon Labs, 1601), then rehydrated through a gradual alcohol series for 5-mins each (100%, 90%, 70%, 50% and 30% ethanol) and finally were washed in deionized water for two minutes. Afterwards, the tissue sections were microwaved in citric acid-based Antigen Unmasking Solution (pH 6.0; Vector Laboratories, H-3300-250) for 20 minutes. Slides were washed for five minutes in 1x PBS and incubated for twenty minutes followed by incubation with the quenching solution in a humidity chamber for 5 mins using Vector TrueView Autofluorescence Quenching Kit (Vector Laboratories). To prevent nonspecific binding, tissue sections were incubated for thirty minutes with eBioscience IHC/ICC Blocking Buffer - High Protein (Thermo Fisher Scientific). After blocking, tissue sections were incubated with GnRHR Monoclonal Antibody (GNRH03, Thermo Fisher Scientific) at 1:100 dilution, Axl Antibody (PA5-77875, Thermo Fisher Scientific) at 1:200 dilution, Axl Antibody (PA5-106118, Thermo Fisher Scientific) at 1:200 dilution, GnRHR Monoclonal antibody (GNRHR/768, ab220196, Abcam) at 1µg/mL dilution, Gas6 Rabbit Antibody (A8545, ABclonal) at 1:100 dilution and Gas6 Goat Antibody (AF885SP, R&D Systems) at 5µg/mL dilution in Blocking buffer in a humidified chamber at 4°C overnight. The following day, sections were washed three times (5mins each) in 1X eBioscience TBS Wash Buffer for IHC/ICC (Thermo Fisher Scientific) and incubated for with appropriate secondary antibodies (Alexa Fluor 546 (A-11035, Thermo Fisher Scientific), Alexa Fluor 488 (A-11029, Thermo Fisher Scientific and NC0679377, Abcam)) all at 1:500 dilution in blocking buffer for 1 hour at room temperature. The slides were then washed in 1x PBS three times (5mins each) and the tissues dehydrated using a gradual alcohol series (50%,

70%, 90% and 100%). Cells were mounted using ProLong Glass Antifade Mountant with NucBlue Stain (Thermo Fisher Scientific) following the manufactures protocol to stain the nuclei simultaneously. Slides were later imaged at 40X and 63X using an LSM 800 Airyscan (Zeiss) confocal microscope and processed via Zen Blue software (Zeiss).

2.3.5. AXL and GnRH receptor distribution in human pituitary

We also used human pituitary images that had been stained to highlight AXL (shown in red), GnRHR (displayed in green), and the cell nuclei (depicted in blue). These images were employed to determine how AXL and GnRHR are expressed and distributed within the pituitary. To achieve this, we employed Zen blue software to analyze a total of 577 cells present in one 2x2 tiled image and two standard images. Subsequently, we processed these images and extracted the fluorescent intensities from the TaRFP, AF488, and DAPI channels (which correspond to AXL, GnRHR, and nuclei, respectively). The obtained fluorescent intensities were then divided by the individual cell areas measured in square micrometers (μm2), and these results were represented as histograms.

2.3.6. ELISA for quantification of phosphorylated ERK/total ERK

To measure total ERK (pERK/ERK) and phosphorylated ERK (pERK) in αT3-1 and LβT2 cells in response to Gas6 (100nM) and GnRH (10nM) at different time points a semiquantitative sandwich ELISA kit (Abcam, ab176660) was used. GnRH (10nM, 5min) was used as a positive control. Briefly, cells were seeded at $8x10^4$ cells/well in 6-well plates and incubated for 2 days until 60-70 % confluent. Cells then were washed with 1X PBS two times and media was changed to 2 mL/well charcoal stripped DEMEM and incubated at 37°C in 5% CO2 humidified air over

night (~12h). Cells were then treated with Gas6 and/or GnRH for 5min-2h and cell extracts using RIPA Lysis and Extraction Buffer (Thermo Scientific™) containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo ScientificTM) were taken into separate tubes. Bicinchoninic acid (BCA) assay was performed to determine protein concentrations prior to ELISA analysis according to the manufactures protocol (n=2, mean ± SEM).

2.3.7. ddPCR for quantification of early responsive Egr-1 transcription factor mRNA levels

Reverse transcription digital droplet PCR (RT-ddPCR) was used to measure absolute transcript levels of the immediate early gene Egr1. LβT2 and αT3-1 cells were seeded in 6 well plates ($2x10^5$ cells/well) in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic. Media were changed after 24 h to DMEM supplemented with 10% charcoal stripped FBS and serum starved overnight. Cells were treated with Gas6, GnRH or Gas6 + GnRH for 0, 5, 10, 20, 40 and 60 min, then collected and preserved in 1X DNA/RNA Shield (Zymo Research; Irvine, CA). Collected RNA was purified with the Quick-RNA Miniprep Plus Kit (Zymo Research) and cDNA was synthesized from 40 ng RNA of each sample using iScript cDNA Synthesis Kit (Bio-Rad; Hercules, CA).

EvaGreen ddPCR (Bio-Rad) was used on a QX200 system (DG8 cartridge, QX200 droplet generator, PX1 PCR plate sealer, C1000 Touch thermal cycler and QX200 droplet reader (Bio-Rad). Briefly, EvaGreen ddPCR supermix was prepared using 10 ng cDNA, 10 µL EvaGreen, and 5 µM of primers for murine Egr1 (Fwd 5'-GAGCGAACAACCCTATGAGC-3' and Rev 5'-AGCGGCCAGTATAGGTGATG-3'), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Fwd 5'-GGGAAGCCCATCACCATCTT-3' and Rev 5'-

GCCTTCTCCATGGTGGTGAA-3'), plus nuclease-free H_2O to reach a total of 20 µL, which was then used to generate droplets.

Droplets were transferred into a ddPCR Semi-Skirted 96-Well Plate (Bio-Rad), sealed with Pierceable Foil Heat Seal, and amplified with the following thermal protocol: polymerase activation (initial denaturation) at 95°C for 5 min, 40 cycles of amplification at 95°C for 30 sec (denaturation) and 60°C for 1 min (annealing/elongation) with a ramp of 2 °C/s for each step. Following amplification, droplets were stabilized at 4°C for 5 min followed by 95 °C for 5 min and then an infinite hold at 4°C. For analysis, the PCR plate containing the droplets was placed in the QX200 droplet reader for data acquisition and absolute quantification analysis with QuantaSoft (Bio-Rad) software. Transcript copy numbers were adjusted post hoc to GAPDH transcript levels for each sample.

2.3.8. ELISA for quantification of gonadotropin hormone LHβ levels

The LH Beta SimpleStep ELISA Kit (Abcam) was used to measure LHβ production and release from LβT2 cells. Briefly, $1x10^4$ cells/well were seeded in 96-well plates and incubated for 2 days until 60-70 % confluent. Cells then were washed with 1X PBS two times and media were changed to 200 µL/well serum-free DMEM and incubated at 37°C in 5% CO_2 humidified air for 2 h. Cells were then treated with GnRHR ligand (GnRH, 10 nM), AXL ligand (Gas6, 100 nM), GnRH receptor inhibitor (cetrorelix acetate, 2 nM), AXL inhibitor (R428, 50 nM) and MEK inhibitor (U0126-EtOH, 10 µM) for 2 h and both cell extracts using RIPA Lysis and Extraction Buffer and media were collected in separate tubes containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). ELISA was done according to the manufacturer's

instructions. The total LHβ quantity was calculated as (LHβ quantity in cell extract + LHβ quantity in the media of the same well). Each individual experiment was performed in duplicate.

2.3.9. ELISA for pro-MMP9 release from LβT2 and αT3-1 cells measurements

Pro-MMP9 production and release from LβT2 and αT3-1 cells were measured with a pro-MMP-9 Murine ELISA Kit (Invitrogen). Briefly, $1x10^4$ cells/well were seeded in 96-well plates and incubated for 2 days until 60-70 % confluent. Cells then were washed with 1X PBS two times and media were changed to 200 µL/well DMEM containing 10% stripped FBS and incubated at 37°C in 5% CO2 humidified air for 24 h. Cells were then treated with GnRH (10 nM), AXL ligand (Gas6, 100 nM), GnRH receptor inhibitor (cetrorelix acetate, 2 nM), AXL inhibitor (R428, 50 nM), and MEK inhibitor (U0126-EtOH, 10 µM) for 24 h and both cell extracts using RIPA Lysis and Extraction Buffer and media were collected in separate tubes containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). ELISA was performed following the manufacturer's instructions.

2.3.10. Trans-well Migration Assay for measuring the migration of LβT2 and αT3-1 cells

The migratory response of αT3-1 and LβT2cells to agonists/antagonists was measured with a Cell Migration/Chemotaxis Assay Kit (Abcam). LβT2 and αT3-1 cells were seeded in 100 mm plates at 60-70% confluency in DMEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic. Media were changed after 24 h to DMEM supplemented with 10% charcoal stripped FBS and serum starved for 24 h and then were seeded in the upper chamber of the kit and assembled based on the manufacturer's instructions. GnRH receptor ligand (GnRH, 100nM), AXL ligand (Gas6, 1000nM), combination of Gas6 and GnRH, MEK activator (PAF C-16, 100 µM)

and MEK inhibitor (U0126-EtOH, 100μM) were then added to the upper and bottom chambers of the kit. Cells were then incubated for 48 h with the stimulators/inhibitors and migration percentage was measured following the manufacturer's instructions.

2.4. Data analysis and statistical methods

Figures were created and data were analyzed with GraphPad Prism and MathWorks MATLAB. Two-sample comparisons were performed using either a paired or unpaired (as appropriate) two-tailed Student's t-test; comparisons between more than two groups were performed using one- or two-way (as appropriate) ANOVA with Dunnett's multiple comparison post-test. All data are presented as mean ± SEM, with $n \geq 3$ for all comparisons; n indicates the number of independent biological replicates unless stated otherwise. $P < 0.05$ was considered significant and asterisks (*) used in the figures are included to indicate significance.

2.5. Results

To examine our hypothesis that the activation of the AXL receptor influences the functionality of the GnRH receptor, we established four essential experimental conditions:

3. AXL receptors should be present in cells expressing GnRH receptors.
4. The activation of AXL receptors should influence signaling processes dependent on the GnRH receptor.
5. Activation of AXL should lead to changes in gene expression dependent on the GnRH receptor.
6. Activation of the AXL receptor must have an effect on biological functions associated with the GnRH receptor, such as the production of gonadotropins.

2.5.1. Characterization of AXL receptor expression in the pituitary gland of C-57 mouse and human

AXL receptor-like expression in the anterior pituitary

To start our study of AXL receptor tyrosine kinase regulation of GnRH receptor function, immunohistochemistry was used to investigate AXL expression in commercially available murine and human pituitary paraffin sections. Gonadotropes were identified by co-immunostaining for the GnRH receptor. Consistent with AXL expression in gonadotropes, we observed AXL-like and GnRH receptor-like immunoreactivities in murine (C57 male and female) human (sex unknown) pituitary sections (**Figure 2-*1***A). While AXL-like and GnRH receptor-like immunoreactivities were heterogeneous, there was substantial overlap between the signals for the two proteins (**Figure 2-*1***B). Interestingly, more than 99% of the cells which were identified as GnRH receptor positive were also positive for AXL-like immunoreactivity in the human pituitary sections. On the other hand, ≈ 19% of the cells detected as AXL positive were also positive for GnRH receptor-like reactivity.

To evaluate expression of the endogenous AXL ligand Gas6, immunohistochemistry was performed on murine and human pituitary paraffin sections. Together with GnRH receptor-like staining, we observed robust Gas6-like immunoreactivity in murine pituitary sections and weak but noticeable Gas6-like immunoreactivity in human pituitary sections (**Figure 2-*1***C&D). In contrast to AXL-like immunoreactivity, there was little to no overlap between the Gas6 and GnRH receptor signals in any of the pituitary sections imaged (**Figure 2-*1***C, *right*). Hence, and unlike AXL, Gas6 seems to be expressed in non-gonadotrope cells more. Altogether, our immunohistochemical data suggest that in the anterior pituitary, AXL expression is associated with

GnRH receptor-positive cells (i.e., gonadotropes) and that the endogenous ligand Gas6 is expressed in surrounding GnRH receptor-negative cells.

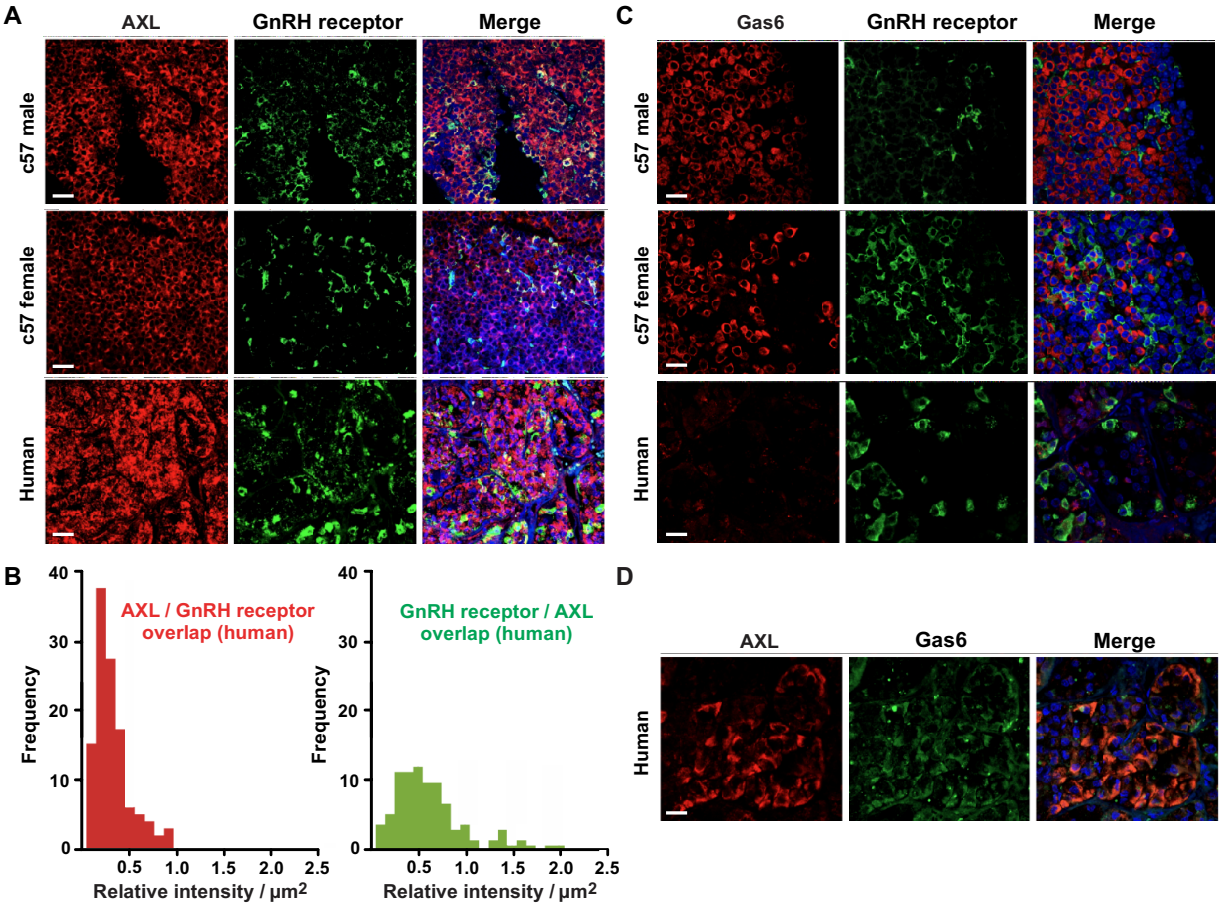

Figure 2-1. AXL and GnRH receptor-like immunoreactivity in murine and human pituitaries. (A) Representative images showing AXL-like (red) and GnRH receptor-like (green) immunostaining in human, male C57 murine, and C57 female murine pituitary sections. Cell nuclei (blue) blue are shown in the merged images. (**B)** Frequency of overlapping AXL- and GnRH receptor-like immunoreactivity in a human pituitary; > 100 cells were analyzed from 7 different images for both AXL- and GnRH receptor-like immunoreactivity. **(C)** Representative images showing Gas6-like (red) and GnRH receptor-like (green) immunostaining in human, male C57 murine, and C57 female murine pituitary sections. Cell nuclei (blue) blue are shown in the merged images. **(D)** Representative images showing AXL-like (red) and Gas6-like (green) immunostaining in a human pituitary section. Cell nuclei (blue) blue are shown in the merged images. Images are representative of ≥ 3 paraffin sections; scale bars = 20µm.

AXL receptor expression in murine gonadotrope cell lines

To explore the functional connections between GnRH and AXL receptor function we took advantage of two different murine gonadotrope cell lines. LβT2 and αT3-1 cells, both of which produce functional GnRH receptors, are widely utilized experimental surrogates for native gonadotropes. αT3-1 cells are considered to have a relatively immature gonadotrope phenotype while LβT2 cells represent a more mature form. Primarily, AXL and GnRH receptor-like immunoreactivity in each cell line was confirmed using immunohistochemistry. Overlapping AXL and GnRH receptor signal in αT3-1 and LβT2 cells were observed similar to the results obtained in pituitary sections (**Figure 2-*2***A). While the biological significance requires further investigation, using quantitative AXL ELISA we found that incubating αT3-1 and LβT2 cells with GnRH (10 nM) slightly increased AXL protein levels after 24 h (**Figure 2-*2***B).

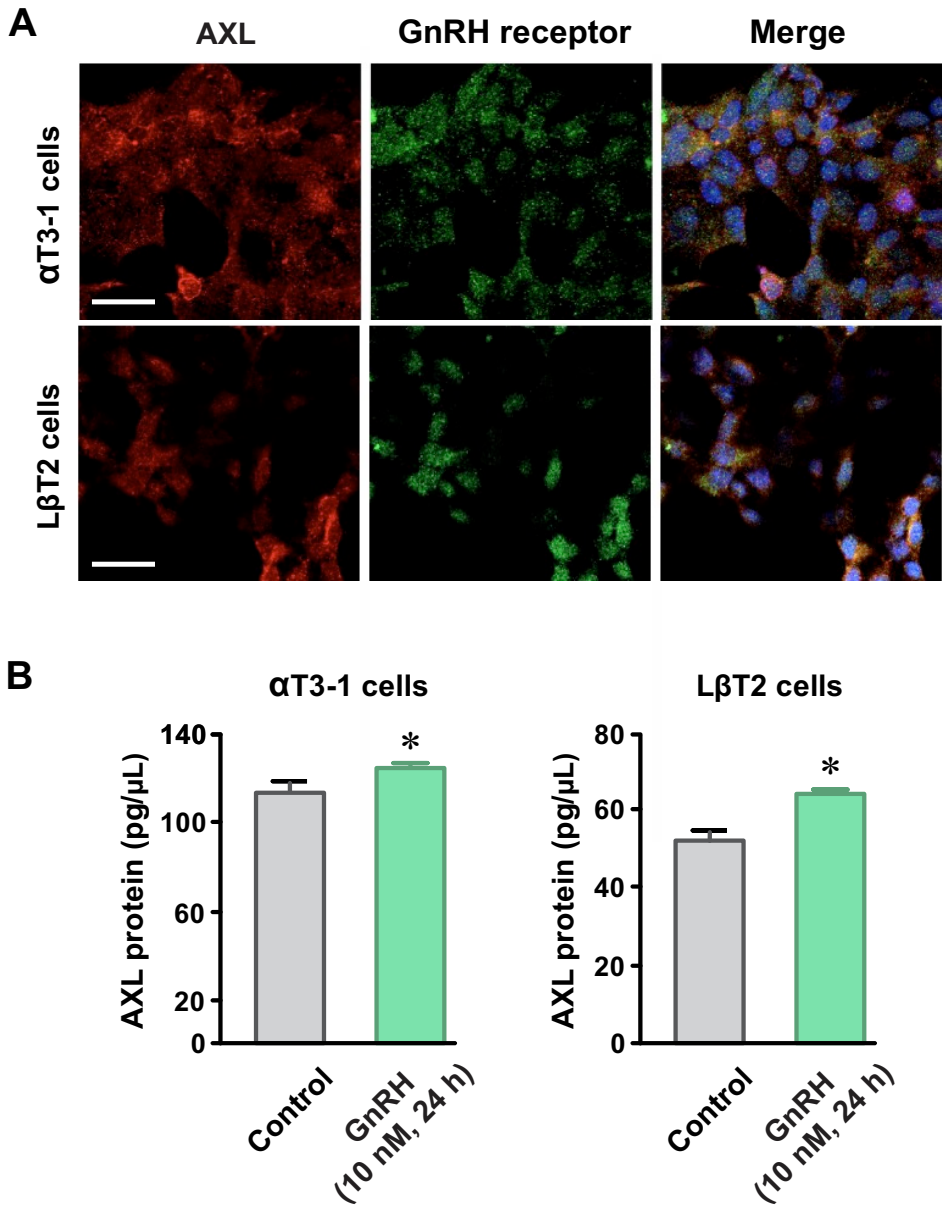

Figure 2-2. AXL receptor expression in murine gonadotrope cell lines. AXL-like immunoreactivity (**A**) and protein abundance (**B**) in αT3-1 and LβT2 murine gonadotrope cell lines and AXL protein abundance in control cells and cells incubated with GnRH (10 nM for 24h; n = 3 independent cultures). Images representative of ≥ 3 αT3-1 and LβT2 cell cultures; n = number of independent experiments; numerical data presented as mean ± SEM; * $P < 0.05$.

2.5.2. Effects of AXL receptor activation on gonadotrope function in αT3-1and LβT2 cells

AXL receptor activation enhances canonical GnRH receptor-dependent signaling processes

Gonadotrope GnRH receptor activation promotes MAPK-dependent changes in immediate-early gene expression, which ultimately leads to the increase of gonadotropin production. The effect of Gas6-dependent AXL receptor activation on ERK phosphorylation, expression of the transcription factor Egr-1 (early growth response factor 1) in LβT2 and αT3-1 cells, and the production and release of the gonadotropin subunit LHβ in LβT2 cells were examined. Using a semiquantitative ELISA technique, it was found that Gas6 (100 nM for 3 h) boosted the proportion of phosphorylated ERK relative to total ERK (pERK/ERK) in clonal gonadotrope cell lines (**Figure 2-*3***A). To further characterize Gas6/AXL-dependent ERK phosphorylation, we incubated LβT2 cells with Gas6 (100 nM) for increasing amounts of time; we incubated LβT2 cells with GnRH (10 nM for 5 min) as a comparative positive control. Unlike GnRH, which produced an effect at 5 min, Gas6 did not increase pERK/ERK for incubation periods < 2 h (**Figure 2-*3***B). The increase in pERK/ERK caused by Gas6 at 2 h was similar to that produced by GnRH at 5 min. Notable, though Gas6 at 5 min had no detectible effect, concurrent incubation of LβT2 cells with Gas6 and GnRH for 5 min elevated pERK/ERK to a greater extent than GnRH alone (**Figure 2-*3***B).

To find the impact of AXL receptor activation on the expression of Egr-1, we incubated αT3-1 and LβT2 cells with Gas6 (100 nM) for durations ranging from 5 min to 1 h and measured Egr-1 transcript levels with droplet digital PCR (ddPCR). For αT3-1 cells, Gas6 increased Egr-1 transcript abundance becoming clear at 20 min and peaking at 40 min (**Figure 2-*3***C). In LβT2 cells, the time course of Gas6-dependent changes in Egr-1 transcripts was faster than αT3-1 cells

with an observable increase in Egr-1 transcripts at 5 min and followed quickly with a peak at 10 min.

LβT2 (but not αT3-1) cells produce the gonadotropin subunit LHβ. The impact of AXL receptor signaling on LHβ expression by measuring protein levels in LβT2 culture media and cell lysates were assessed with ELISA. Similar to GnRH (10 nM for 2 h), Gas6 (100 nM for 2 h) increased the abundance of LHβ protein in the culture media and LβT2 cell lysates (**Figure 2-*3***D). Gas6 induced a more robust increase in LHβ protein amounts than GnRH in the culture media but not in LβT2 cell lysates. To test for possible constitutive GnRH and AXL receptors and MAP kinase activities on LHβ expression, the effects of the GnRH receptor antagonist (cetrorelix; 2 nM), the AXL receptor antagonist (R428; 50 μM), and the MEK (MAPK kinase) inhibitor (U0126; 10 μM) on LHβ protein abundance were examined (**Figure 2-*3***E). Inclusion of all three inhibitors reduced basal levels of LHβ protein in the media of LβT2 culture media to ≈ 50 % of control.

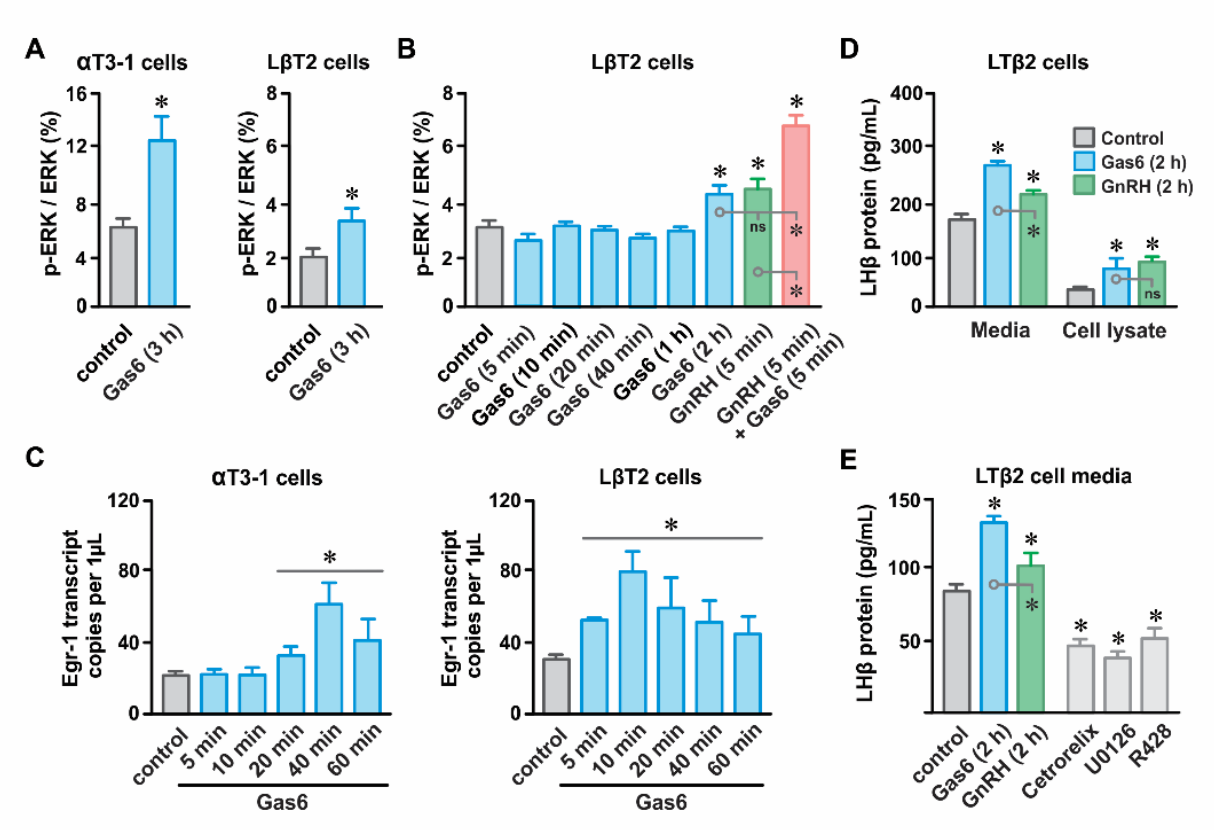

Figure 2-3. Gas6-dependent AXL receptor activation enhances GnRH receptor signaling processes. (**A)** Proportion of phosphorylated ERK relative to total ERK (pERK/ERK) in αT3-1 and LβT2 cells under control conditions and after incubation with Gas6 (100 nM for 3 h); n = 3 for each condition. (**B)** Time course of Gas6-dependent changes (100 nM) in pERK/ERK in LβT2 cells (from left); effect of incubating LβT2 cells with GnRH (10 nM) and GnRH plus Gas6 (100 nM) for 5 min on pERK/ERK (at right); n = 3 for each condition. (**C)** Time course of Gas6-dependent (100 nM) changes on Egr-1 transcript abundance in αT3-1 and LβT2 cells; n = 3. (**D)** LHβ protein abundance in the culture media and lysates of LβT2 cells under control conditions and after 2 h incubations with Gas6 (100 nM) and GnRH (10 nM); n = 3. € LHβ protein abundance in LβT2 culture media under control conditions and after 2 h incubations with Gas6 (100 nM), GnRH (10 nM), the GnRH receptor antagonist cetrorelix (2 nM), the AXL receptor antagonist R428 (50 μM), and the MEK inhibitor U0126 (10 μM); n = 3 for each condition. n = number of independent experiments; numerical data presented as mean ± SEM; * $P < 0.05$.

AXL enhances gonadotrope matrix metalloproteinase 9 activity and cell migration

GnRH stimulation of ERK involves the convergence of multiple signaling processes, including MMP9 (metalloproteinase 9)-dependent transactivation of EGF receptors. As MMP9 and EGF receptors are known downstream mediators of AXL receptor signalling, and our data

showing AXL-mediated potentiation of ERK above (**Figure 2-*4***A&B), we examined the effects of Gas6 on MMP9 activity in αT3-1 and LβT2 cells. To do so, we measured the abundance of the inactive pro-form of MMP9 (pro-MMP9) in lysates and culture media of αT3-1 and LβT2 cells (**Figure 2-*4***). Incubating αT3-1 cells for 24 h with Gas6, GnRH, and the MEK/ERK activator PAF C-16 produced no observable changes in pro-MMP9 levels in the culture media and a slight increase in cell lysates (**Figure 2-*4***A). For LβT2 cells, GnRH increased pro-MMP9 abundance in the culture media and in cell lysates; MEK inhibition with U0126 blocked these effects (**Figure 2-*4***B). Although Gas6 did not affect, treating LβT2 cells with GnRH and Gas6 elevated pro-MMP9 levels in the culture media than GnRH alone. Coincubation with R428 (AXL receptor antagonist) attenuated GnRH-dependent increases in pro-MMP9 in LβT2 cell lysates and culture media.

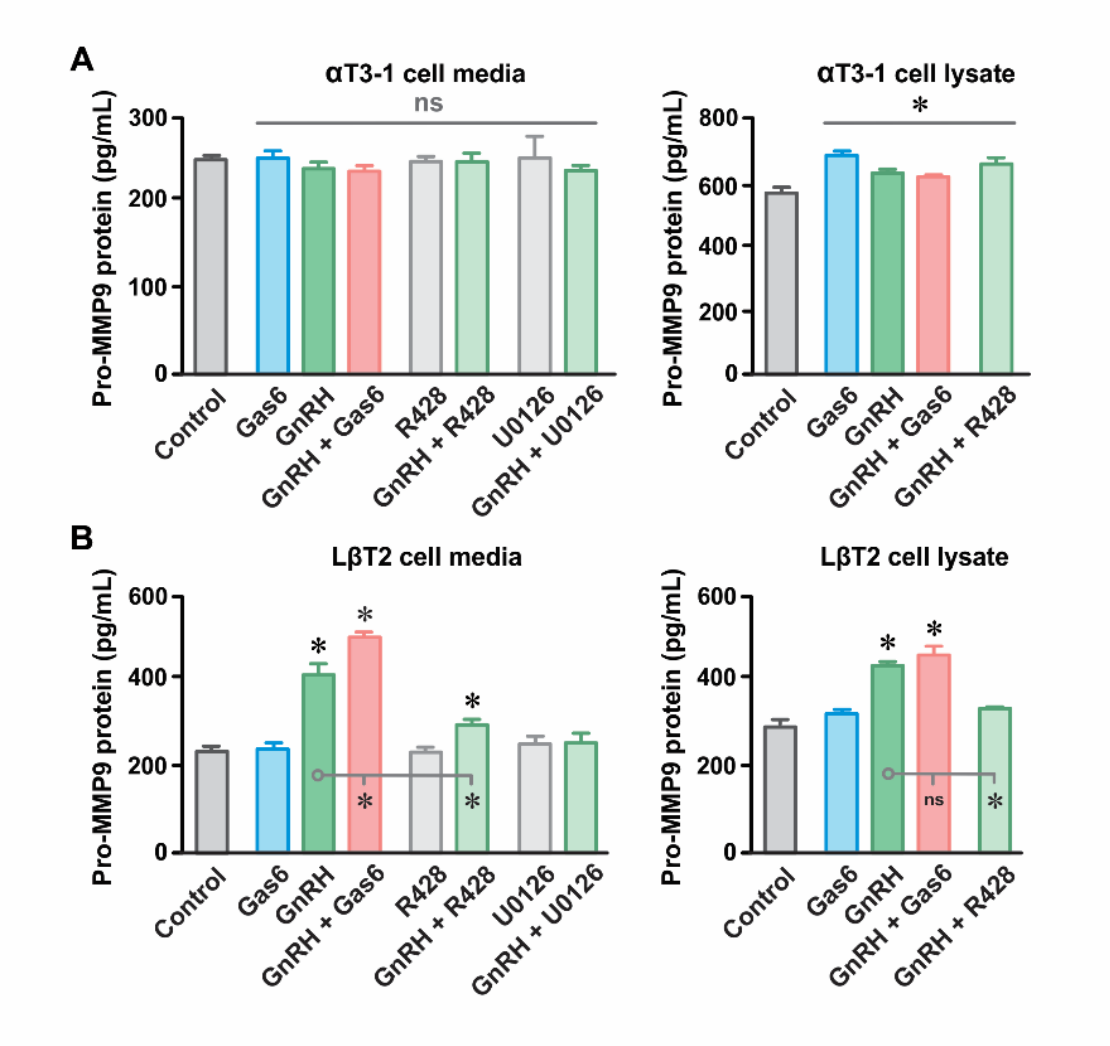

Figure 2-4. AXL and GnRH receptor stimulation promotes pro-MMP9 release. Pro-MMP9 abundance in the culture media and lysates of αT3-1 (**A**) and LβT2 (**B**) cells following incubation with Gas6 (100 nM), GnRH (10 nM), Gas6 plus GnRH, and the AXL receptor antagonist R428 (50 µM), the MEK inhibitor U0126 (10 µM). n = 4 for each condition. n = number of independent experiments; numerical data presented as mean ± SEM; ns = $P > 0.05$; * $P < 0.05$.

Modified by secretory endopeptidases like MMP9, the extracellular matrix influenced the migratory behavior of cells. Accumulated evidence suggested that cellular movement constituted a part of the gonadotrope response to GnRH. Consequently, a trans-well cell migration assay was employed to assess how AXL-dependent modulation affected the chemotactic responses of αT3-1 and LβT2 cells to GnRH.

Our observations indicated that when subjected to GnRH (at a concentration of 10 nM) for a duration of 48 hours, αT3-1 and LβT2 cells showed no discernible effects on cell migration, as illustrated in ***Figure 2-5***A and B. In the absence of GnRH, incubation of αT3-1 cells with Gas6 provoked an increase in cell migration. In contrast, Gas6 did not stimulate the migration of LβT2 cells unless GnRH was present.

Furthermore, the activation of MEK/ERK with PAF C-16 (utilized as a positive control at a concentration of 100 μM) resulted in a significant increase in the migration of both αT3-1 and LβT2 cells, while the inhibition of MEK with U0126 (used at a concentration of 10 μM as a negative control) had no observable effects.

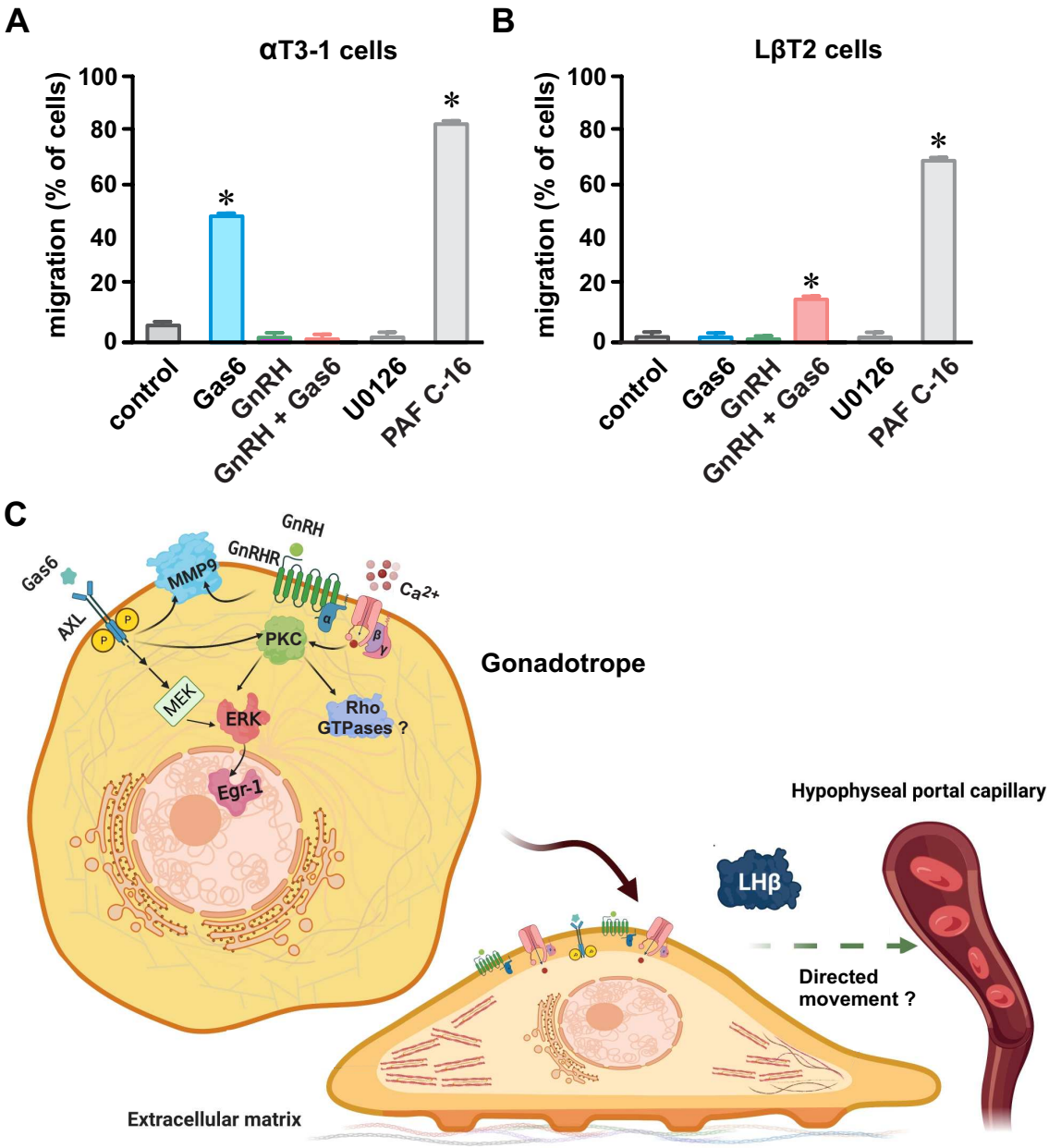

Figure 2-5. AXL and GnRH receptor activation promotes migration of clonal gonadotropes. Trans-well migratory responses (% cells) of αT3-1 (**A**) and LβT2 (**B**) cells following 48 h incubations with Gas6 (100 nM), GnRH (10 nM), Gas6 plus GnRH, the MEK inhibitor U0126 (10 μM), and the MEK activator PAF C-16 (100 μM). n = 4 for each condition. n = number of independent experiments; numerical data presented as mean ± SEM; ns = $P > 0.05$; * $P < 0.05$. **C** Working model of AXL and GnRH receptor signaling integration in gonadotropes. Created with BioRender.com

CHAPTER 3

UNRAVELING THE COMPLEXITIES OF AXL RECEPTOR-MEDIATED EFFECTS ON GONADOTROPE FUNCTION: INSIGHTS, MECHANISMS, AND FUTURE DIRECTIONS

3.1. Discussion

In this research, our primary aim was to investigate the hypothesis that AXL, a member of the TAM receptor tyrosine kinase family, plays a modulatory role in Gonadotropin-Releasing Hormone (GnRH) receptor signaling. Our findings have provided substantial support for this hypothesis, and we have highlighted several key observations that support the role of AXL receptors in influencing gonadotrope function.

First, our research showed the presence of AXL receptor expression in GnRH-expressing cells, specifically gonadotropes. This initial observation underscores the relevance of AXL receptors within the context of the hypothalamus-pituitary-gonadal (HPG) axis, where GnRH plays a vital role in regulating reproductive function.

One of the pivotal findings in our study was the enhanced GnRH receptor-dependent phosphorylation of Extracellular Signal-Regulated Kinase (ERK) in response to the AXL receptor agonist Gas6. ERK activation is a key step in the GnRH signaling cascade, and the potentiation of this pathway by AXL receptors suggests a direct influence on gonadotropin release. This observation points to the intricate interplay between AXL and GnRH signaling pathways.

Furthermore, our research revealed that Gas6, when introduced, increased the transcriptional expression of Egr-1, an immediate early gene known to be responsive to GnRH.

This finding implies that AXL receptor activation can modulate gene expression patterns in gonadotropes, potentially impacting hormone synthesis and release.

In addition to gene expression, we observed an increase in the production of the gonadotropin subunit LHβ following Gas6 exposure. This observation is highly significant, as it suggests that AXL receptor activation can directly influence the synthesis of key reproductive hormones. This finding not only emphasizes the potential role of AXL receptors in gonadotrope function but also has implications for the regulation of gonadal activity.

Another intriguing aspect of our research was the observation that Gas6 exposure facilitated the migration of clonal gonadotropes. Cellular migration is a critical aspect of gonadotrope function, enabling these cells to reach their target locations within the anterior pituitary and regulate hormone secretion effectively. The connection between AXL receptor activation and cell migration adds another layer of complexity to our understanding of AXL-mediated effects on gonadotropes.

While our research has provided valuable insights, several aspects call for further investigation in future research endeavors. One key area of interest is elucidating the precise mechanisms through which AXL receptors modulate GnRH receptor signaling. The crosstalk between AXL and GnRH signaling pathways is intricate and multifaceted, necessitating in-depth studies to uncover the molecular events and signaling molecules involved.

Additionally, understanding the potential role of AXL receptors in the pathogenesis of reproductive disorders, such as polycystic ovary syndrome (PCOS) and hypothalamic amenorrhea, is a promising avenue for future research. Investigating the links between AXL receptor dysregulation and these conditions may yield insights into disease mechanisms and potential therapeutic interventions.

Moreover, the impact of AXL receptor-mediated effects on gonadotrope function in the context of gender-affirming care is an area that deserves attention. Optimizing hormone therapies for transgender individuals undergoing gender transition by considering the influence of AXL receptors on hormone levels and physiological responses can be crucial.

Lastly, the development of novel therapeutic targets within the AXL receptor signaling pathway is an exciting prospect. Identifying compounds that selectively modulate AXL receptors or exploring combination therapies that target both AXL and GnRH pathways can potentially revolutionize the treatment of reproductive disorders.

In conclusion, our research has unveiled intriguing insights into the role of AXL receptors in gonadotrope function and their potential impact on the HPG axis. While we have made significant strides in understanding the interplay between AXL and GnRH signaling, the field is ripe for further exploration. Future research endeavors should focus on elucidating the molecular mechanisms, investigating AXL's involvement in reproductive disorders, optimizing gender-affirming care, and exploring novel therapeutic avenues. Collaborative efforts across disciplines will be essential to advance our understanding of AXL receptor-mediated effects on gonadotrope function and their broader implications for reproductive health.

3.2. Significance of the findings in the context of GnRH signaling and gonadotrope function

The intricate dance of hormones within the human body orchestrates a myriad of physiological processes, and the hypothalamus-pituitary-gonadal (HPG) axis is a prime example of this complex interplay. At the core of the HPG axis lies Gonadotropin-Releasing Hormone (GnRH), a critical regulator of reproductive function. Gonadotropin-Releasing Hormone (GnRH),

a decapeptide produced in the hypothalamus, plays a pivotal role in the regulation of the hypothalamus-pituitary-gonadal (HPG) axis.

The anterior pituitary comprises five distinct cell types, with gonadotrope cells representing a minority population, typically ranging from 5% to 15%. Despite their relatively small numbers, these cells play a crucial role in reproductive health by synthesizing and releasing hormones into the bloodstream, ultimately regulating gonadal functions.

GnRH, secreted by the hypothalamus, stimulates the anterior pituitary gland to release gonadotropins, namely luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn, govern gonadal activity. Understanding the precise mechanisms underlying GnRH signaling and its impact on gonadotrope function is essential for understanding human reproduction and addressing reproductive disorders. Recent research has shed light on the role of AXL receptors and extracellular matrix modification in this context, revealing significant insights that hold promise for advancing our knowledge of this crucial hormonal pathway.

3.2.1. Significance of the findings in this study

Integration of AXL Receptors into GnRH Signaling: One of the key findings in the recent research is the integration of AXL receptors into the GnRH signaling cascade. AXL receptors have previously been associated with various cellular processes, including cell migration and proliferation, but their involvement in GnRH signaling was not well understood. The study demonstrates that AXL receptors play a role in modulating the ERK signaling pathway, a crucial component of GnRH signaling. This revelation opens new avenues for exploring the crosstalk between different signaling pathways and the potential for therapeutic interventions in reproductive disorders.

1. **Extracellular Matrix Modification:** The research also highlights the importance of extracellular matrix modification, specifically the role of secretory endopeptidases like MMP9, in influencing cell migration. This is particularly significant in the context of gonadotrope function because cell migration is a fundamental aspect of the response to GnRH. Understanding how the extracellular matrix is changed and how it affects cell behavior sheds light on the intricate processes involved in GnRH signaling.

2. **AXL-Mediated Potentiation of ERK:** Another critical finding is the AXL-mediated potentiation of ERK (Extracellular Signal-Regulated Kinase), a key player in the GnRH signaling pathway. ERK activation is known to be essential for gonadotrope function, and the research shows that AXL receptors can enhance ERK activation. This discovery suggests that targeting AXL receptors may have therapeutic potential in manipulating GnRH signaling to treat reproductive disorders.

3. **Gas6 and MMP9 Interactions:** The study delves into the interactions between Gas6, an AXL receptor ligand, and MMP9. Understanding these interactions is crucial because they have the potential to influence the activity of AXL receptors and, by extension, GnRH signaling. The research shows that Gas6 can impact MMP9 activity, further emphasizing the complex regulatory network governing reproductive function.

3.2.2. Implications for gonadotrope function and reproductive health

The significance of these findings in the context of GnRH signaling and gonadotrope function is profound. First and foremost, they provide a deeper understanding of the intricate mechanisms underlying the HPG axis, particularly how extracellular matrix modification and AXL receptors contribute to GnRH signaling. This knowledge can pave the way for the development of targeted therapies for reproductive disorders such as infertility, polycystic ovary syndrome (PCOS), and hypothalamic amenorrhea.

Additionally, the findings have implications for fertility preservation and assisted reproductive technologies. By gaining insights into the regulation of gonadotropin release and the factors that influence it, clinicians and researchers may be better equipped to optimize fertility treatments and minimize side effects.

Furthermore, these discoveries underscore the importance of considering the broader context of hormonal signaling in the body. GnRH signaling is not isolated but interconnected with various other pathways. AXL receptors, through their influence on ERK and cell migration, bridge the gap between different signaling cascades, highlighting the need for a holistic approach in studying hormonal regulation.

The recent research findings regarding the integration of AXL receptors, extracellular matrix modification, and their impact on GnRH signaling and gonadotrope function have significant implications for our understanding of reproductive health. These insights illuminate previously uncharted territories in the intricate web of hormonal regulation, offering potential therapeutic avenues and promising prospects for addressing reproductive disorders and improving fertility treatments. As our understanding of these mechanisms deepens, we move closer to unraveling the mysteries of human reproduction and enhancing the quality of life for individuals facing reproductive challenges.

3.3. Implications for understanding reproductive disorders

Research into Gonadotropin-Releasing Hormone (GnRH) signaling, and the clinical applications of GnRH analogues has far-reaching implications for our comprehension of reproductive disorders. Reproductive health is a cornerstone of human well-being, and disorders in this domain can have profound physical, psychological, and social impacts. In this essay, we will explore the implications of recent findings in GnRH research for our understanding of reproductive disorders.

3.3.1. Understanding the Basis of Reproductive Disorders

1. **Precocious Puberty:** GnRH signaling research provides insights into the underlying mechanisms of precocious puberty. This condition, characterized by early onset of puberty, can be attributed to dysregulation within the hypothalamus-pituitary-gonadal (HPG) axis. Understanding how GnRH analogues modulate this axis sheds light on potential treatment avenues for precocious puberty, ultimately improving the quality of life for affected children.
2. **Infertility:** Reproductive disorders often manifest as infertility, which can result from a range of hormonal imbalances and dysfunctions in the HPG axis. GnRH analogues play a pivotal role in addressing these underlying issues. By offering precise hormonal control, they facilitate fertility treatments, such as in vitro fertilization (IVF), intrauterine insemination (IUI), and ovulation induction. Continued research in this area can lead to refinements in infertility management, benefiting couples struggling to conceive.

3. **Uterine Fibroids and Endometriosis:** Conditions like uterine fibroids and endometriosis can disrupt normal reproductive function, causing pain and fertility challenges. GnRH analogues are employed to mitigate the hormonal influences contributing to these disorders. Research into their mechanisms of action can provide valuable insights into developing targeted therapies for uterine fibroids and endometriosis, improving the lives of affected individuals.
4. **Gender Dysphoria:** Gender dysphoria, while not a reproductive disorder in the traditional sense, is profoundly linked to hormonal regulation. GnRH analogues are instrumental in gender-affirming care by suppressing endogenous hormone production, allowing individuals time to explore their gender identity. Research in this area informs the safe and effective use of GnRH analogues in transgender healthcare.

GnRH Analogues: Balancing Benefits and Risks

1. **Side Effects:** GnRH analogues are not without side effects, some of which mimic symptoms of hypogonadism. Understanding these side effects is crucial for patients and healthcare providers alike. Research helps identify strategies to manage side effects effectively, enhancing patient compliance and treatment outcomes.
2. **Long-Term Implications:** GnRH analogues can have long-term consequences, including metabolic abnormalities, weight gain, and osteoporosis. Investigating the long-term effects of these analogues is essential to minimize risks, especially in patients requiring extended treatment durations.
3. **Rare Adverse Effects:** The rare but severe adverse effects of GnRH analogues, such as the temporary worsening of prostate cancer and pituitary apoplexy, underscore the importance of continued research to identify at-risk populations and develop strategies for risk mitigation.

In conclusion, recent advancements in GnRH signaling research and the clinical applications of GnRH analogues hold significant promise for our understanding and management of reproductive disorders. These findings provide a foundation for elucidating the underlying mechanisms of conditions like precocious puberty, infertility, uterine fibroids, endometriosis, and gender dysphoria.

Moreover, the research underscores the importance of striking a careful balance between the benefits and potential risks of GnRH analogues. By comprehensively studying the side effects, long-term implications, and rare adverse effects associated with these therapies, we can refine treatment protocols, enhance patient care, and ultimately improve the lives of individuals grappling with reproductive disorders.

As we continue to explore the intricate world of GnRH signaling and its clinical applications, we move closer to realizing the full potential of this knowledge in addressing reproductive disorders and advancing the field of reproductive medicine.

3.4. Effects of AXL receptor activation on gonadotrope function

The intricate regulation of the hypothalamus-pituitary-gonadal (HPG) axis is crucial for the proper functioning of the reproductive system. At the heart of this axis lies Gonadotropin-Releasing Hormone (GnRH), a pivotal neuropeptide that orchestrates the synthesis and secretion of gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), from the anterior pituitary. These gonadotropins, in turn, play a leading role in regulating the gonadal functions of both males and females. Recent research has shed light on the role of AXL receptors in modulating gonadotrope function, uncovering a novel layer of complexity in the regulation of the HPG axis.

3.4.1. AXL Receptors and Their Significance in Reproductive Physiology

AXL receptors, part of the TAM (Tyro3, AXL, Mer) family of receptor tyrosine kinases, have primarily been associated with processes like cell proliferation, survival, and migration. However, their involvement in reproductive physiology, particularly within the context of gonadotropin regulation, has gained recognition in recent years. Understanding the effects of AXL receptor activation on gonadotrope function holds significant implications for comprehending reproductive health and disorders.

3.4.2. Mechanisms of AXL Receptor Activation on Gonadotrope Function

Our studies have elucidated several mechanisms through which AXL receptor activation influences gonadotrope function:

1. **AXL-Mediated Potentiation of ERK:** AXL receptor activation has been shown to potentiate the ERK (Extracellular Signal-Regulated Kinase) signaling pathway within gonadotrope cells. ERK activation is a critical step in the GnRH signaling cascade, and AXL receptors enhance this process. Consequently, AXL receptor activation can augment the production and secretion of LH and FSH, thus influencing gonadal function.
2. **Impact on Cell Migration:** AXL receptors, known for their role in cell migration, can also affect the migratory behavior of gonadotrope cells. Cell migration is a fundamental aspect of gonadotrope response to GnRH, enabling these cells to reach their target location within the pituitary gland and regulate hormone secretion.

3.4.3. Implications for Reproductive Health and Disorders

The effects of AXL receptor activation on gonadotrope function have profound implications for reproductive health and the understanding of reproductive disorders:

1. **Potential Therapeutic Targets:** AXL receptors, given their newfound importance in regulating gonadotropin release, represent potential therapeutic targets for managing reproductive disorders. Modulating AXL receptor activity may offer a novel approach to treating conditions such as infertility, precocious puberty, and reproductive hormone imbalances.

2. **Gender-Affirming Care:** Research into AXL receptor activation can have implications for gender-affirming care. Understanding how AXL receptors influence gonadotrope function can aid in the development of hormone therapies for transgender individuals undergoing gender transition.

3. **Reproductive Disorders:** Investigating the role of AXL receptors in the pathogenesis of reproductive disorders like polycystic ovary syndrome (PCOS) and hypothalamic amenorrhea may yield valuable insights into disease mechanisms and potential therapeutic interventions.

In a nutshell, the effects of AXL receptor activation on gonadotrope function represent a fascinating frontier in reproductive physiology research. By elucidating the mechanisms through which AXL receptors modulate gonadotropin release and gonadotrope cell behavior, we gain valuable insights into the intricate regulation of the HPG axis. These findings hold promise for the development of novel therapeutic strategies for reproductive disorders and may contribute to more effective gender-affirming care. As research in this area continues to evolve, our understanding of reproductive health and disorders will undoubtedly deepen, offering new avenues for improving reproductive outcomes and overall well-being.

3.5. Comparison of AXL receptor signaling to other known signaling pathways in the gonadotropes

Understanding the intricacies of signaling pathways in gonadotrope cells is essential for unraveling the complex regulatory mechanisms governing reproductive function. Among the various signaling pathways that impact gonadotrope activity, the AXL receptor signaling pathway has emerged as a novel player. This discussion aims to compare AXL receptor signaling with other well-known signaling pathways in gonadotropes, shedding light on the similarities, differences, and potential crosstalk that govern gonadotrope function.

3.5.1. GnRH Signaling Pathway

The GnRH signaling pathway is the cornerstone of gonadotrope function. Gonadotropin-Releasing Hormone (GnRH) binds to its receptor (GnRHR) on gonadotrope cell surfaces, leading to intracellular signaling cascades. This process triggers the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Key components of the GnRH pathway include phospholipase C (PLC), inositol trisphosphate (IP3), and protein kinase C (PKC).

3.5.2. AXL Receptor Signaling Pathway

The AXL receptor signaling pathway, relatively less studied in gonadotropes, has gained our attention for its role in modulating gonadotrope function. AXL receptors belong to the TAM (Tyro3, AXL, Mer) family of receptor tyrosine kinases and have been primarily associated with processes like cell proliferation, survival, and migration. Recent research has revealed their impact on gonadotropin regulation and cell migration within the HPG axis (i.e., hypothalamus GnRH-neurons.)

3.5.3. Comparative Analysis

1. **Signal Transduction Mechanisms:**

- GnRH Pathway: The GnRH pathway predominantly relies on G protein-coupled receptor (GPCR) signaling. Upon GnRH binding, GnRHR activates G proteins, leading to the stimulation of PLC, IP3 production, and PKC activation, which, in turn, trigger LH and FSH secretion.
- AXL Receptor Pathway: AXL receptors function as receptor tyrosine kinases (RTKs), suggesting a different mode of signaling compared to GPCRs. Activation of AXL receptors results in the phosphorylation of downstream signaling proteins, including ERK. This variation in signal transduction mechanisms distinguishes AXL receptor signaling from the classic GPCR-based GnRH pathway.

2. **Role in Cell Migration:**

- GnRH Pathway: While the GnRH pathway primarily focuses on hormone secretion, it also plays a role in the migration of gonadotrope cells within the pituitary gland. This migratory behavior is essential for proper hormone release into the blood stream.
- AXL Receptor Pathway: AXL receptors have a well-established role in cell migration. Recent research suggests that AXL receptor activation influences the migratory behavior of gonadotrope cells, emphasizing a potential connection between AXL signaling and cell migration within the pituitary.

3. **Gonadotropin Regulation:**

- GnRH Pathway: The GnRH pathway is the principal regulator of gonadotropin production. It controls the synthesis and release of LH and FSH, which are critical for gonadal function and fertility.
- AXL Receptor Pathway: Emerging body of evidence (including our research) indicates that AXL receptor activation can potentiate ERK signaling, a principal component of the GnRH pathway. This suggests that AXL receptors may exert a direct influence on gonadotropin regulation.

4. Crosstalk and Synergy:

GnRH and AXL Receptor Pathways: While the two pathways are distinct, there is potential for crosstalk and synergy. The influence of AXL receptor activation on ERK signaling, a key element in the GnRH pathway, suggests possible interactions between these pathways. Further research is needed to elucidate the extent of this crosstalk and its physiological implications.

In conclusion, the comparison of AXL receptor signaling with the well-established GnRH signaling pathway in gonadotropes reveals both similarities and differences. While the GnRH pathway predominantly relies on GPCR-based signaling mechanisms for gonadotropin regulation, the AXL receptor pathway operates through receptor tyrosine kinase activation and influences cell migration. These distinctions underscore the complexity of signaling in gonadotropes and open avenues for further investigation into the potential crosstalk and synergistic interactions between these pathways. Understanding the interplay between AXL receptor signaling and the classical GnRH pathway has the potential to enhance our comprehension of gonadotrope function and may offer novel insights for the management of reproductive disorders and therapies.

3.6. Potential mechanisms of AXL receptor-mediated effects on GnRH signaling

1. **AXL Receptors and ERK Activation:**

A key mechanism through which AXL receptors influence GnRH signaling is the potentiation of the Extracellular Signal-Regulated Kinase (ERK) pathway. ERK activation is a central component of the GnRH signaling cascade, facilitating the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

- **Activation of ERK**: AXL receptor activation has been shown to phosphorylate and activate ERK, thereby augmenting ERK signaling within gonadotrope cells. This activation potentially enhances the responsiveness of gonadotrope cells to GnRH stimulation, leading to increased gonadotropin release.
- **Integration with GnRH Pathway**: The convergence of AXL receptor mediated ERK activation with the GnRH pathway suggests a potential synergistic relationship between these two signaling cascades. Further investigation is needed to clarify the exact nature of this interaction.

2. **Influence on Intracellular Calcium Levels:**

Calcium signaling is a crucial component of GnRH-induced gonadotropin release. AXL receptor activation might affect calcium dynamics within gonadotrope cells, thereby influencing GnRH signaling.

- **Calcium Mobilization**: AXL receptor activation could potentially modulate intracellular calcium mobilization in response to GnRH stimulation. This modulation might affect downstream events in the GnRH signaling pathway, including gonadotropin secretion.

3. **Regulation of Downstream Signaling Molecules:**

AXL receptor signaling may also influence other key signaling molecules downstream of GnRH receptors.

- **Phospholipase C (PLC) Activation**: AXL receptor activation could potentially modulate the activity of phospholipase C (PLC), an essential component of the GnRH signaling pathway. PLC is responsible for generating inositol trisphosphate (IP3) and diacylglycerol (DAG), which mediate intracellular calcium release and protein kinase C (PKC) activation.
- **Protein Kinase C (PKC) Activation**: The AXL receptor pathway may influence PKC activation, which is crucial for gonadotropin secretion in response to GnRH. Modulating PKC activity could alter the dynamics of the GnRH signaling cascade.

4. **Cell Migration and Localization:**

AXL receptors are known for their involvement in cell migration. Understanding how AXL receptor-mediated effects on cell migration impact the localization of gonadotrope cells within the anterior pituitary is essential, as proper cell positioning is crucial for efficient GnRH signaling and hormone release.

- **Migration Dynamics**: AXL receptor activation may influence the migratory behavior of gonadotrope cells, thereby affecting their positioning and interactions within the pituitary gland. Proper cell migration is integral to the coordination of gonadotropin release.

The potential mechanisms underlying AXL receptor-mediated effects on GnRH signaling represent a dynamic area of research in reproductive physiology. These mechanisms may involve

ERK activation, modulation of intracellular calcium dynamics, regulation of downstream signaling molecules, and effects on cell migration and localization. Understanding the intricate interactions between AXL receptors and the GnRH signaling pathway will advance our knowledge of reproductive regulation and may have implications for the development of novel therapeutic interventions for reproductive disorders. Further research is necessary to elucidate the exact mechanisms at play and their physiological significance in the context of gonadotrope function and the HPG axis.

3.7. Exploring Future Avenues in AXL Receptor-Mediated Gonadotrope Function Research

The study of AXL receptor-mediated effects on gonadotrope function is a burgeoning field in reproductive physiology research. As we continue to uncover the intricate mechanisms by which AXL receptors influence Gonadotropin-Releasing Hormone (GnRH) signaling, several promising avenues for future research emerge. This discussion explores potential directions for advancing our understanding of this critical aspect of reproductive regulation.

3.7.1. Elucidating the Intracellular Signaling Crosstalk

One promising direction for future research involves unraveling the intricate crosstalk between AXL receptor signaling and the classic GnRH pathway. While recent studies have hinted at the potentiation of the Extracellular Signal-Regulated Kinase (ERK) pathway by AXL receptors, a deeper exploration of the exact molecular interactions is warranted.

1. **Mechanistic Insights:** Investigating the precise molecular events that link AXL receptor activation to ERK activation within gonadotrope cells can provide mechanistic insights. Identifying the specific signaling molecules involved and the points of convergence between

these pathways will enhance our understanding of the interplay between AXL and GnRH signaling.

2. **Feedback Loops:** Exploring potential feedback loops and regulatory mechanisms that modulate AXL receptor-mediated signaling can shed light on the dynamic nature of gonadotrope function. Understanding how these feedback mechanisms maintain homeostasis in response to changing hormonal cues is crucial.

3.7.2. Role of AXL Receptors in Reproductive Disorders

Understanding the involvement of AXL receptors in the pathogenesis of reproductive disorders is another promising avenue for future research. Investigating the links between AXL receptor dysregulation and conditions such as polycystic ovary syndrome (PCOS) and hypothalamic amenorrhea can have significant clinical implications.

1. **Molecular Mechanisms:** Delving into the molecular mechanisms through which AXL receptor dysregulation contributes to reproductive disorders can provide a foundation for potential therapeutic interventions. Identifying specific AXL receptor variants or downstream effectors involved in these conditions can inform targeted therapies.

2. **Biomarker Development:** Exploring the utility of AXL receptors as potential biomarkers for reproductive disorders can aid in early diagnosis and risk assessment. Investigating AXL receptor expression patterns and their correlation with disease progression can offer valuable clinical insights.

3.7.3. Impact on Gender-Affirming Care

In the context of gender-affirming care, further research into AXL receptor-mediated effects on gonadotrope function can enhance hormone therapy options for transgender individuals.

1. **Optimizing Hormone Therapies:** Investigating how AXL receptor signaling influences the response to hormone therapies can help optimize treatment regimens for transgender individuals undergoing gender transition. Understanding AXL receptor-mediated effects on hormone levels and their physiological consequences is essential.
2. **Safety and Efficacy:** Assessing the long-term safety and efficacy of hormone therapies that target AXL receptor signaling pathways is crucial. This research can help mitigate potential side effects and ensure the well-being of transgender patients.

3.7.4. Exploration of Novel Therapeutic Targets

Identifying novel therapeutic targets within the AXL receptor signaling pathway is a promising direction for research. Developing pharmacological interventions that selectively modulate AXL receptors may offer innovative approaches to managing reproductive disorders.

1. **AXL Receptor Modulators:** Investigating compounds that selectively activate or inhibit AXL receptors can open up new avenues for therapeutic development. These modulators may provide more precise control over gonadotrope function.
2. **Combination Therapies:** Exploring combination therapies that target both AXL receptor signaling, and traditional GnRH pathways may offer synergistic effects and enhanced treatment outcomes. Research in this area can optimize therapeutic strategies for reproductive disorders.

3.7.5. Conclusion

The study of AXL receptor-mediated effects on gonadotrope function is a dynamic field with exciting opportunities for future research. Investigating the crosstalk between AXL and GnRH signaling, elucidating the role of AXL receptors in reproductive disorders, optimizing gender-affirming care, and exploring novel therapeutic targets are all promising directions. As our understanding of these mechanisms deepens, we have the potential to improve reproductive health outcomes and enhance the quality of care for individuals affected by reproductive disorders. Collaborative efforts across disciplines will be key to advancing research in these directions and translating findings into meaningful clinical applications.

CHAPTER 4

UNVEILING AXL RECEPTOR-MEDIATED MODULATION OF GONADOTROPE FUNCTION AND ITS IMPLICATIONS FOR REPRODUCTIVE ENDOCRINOLOGY

4.1. Conclusion

4.1.1. Summary of Key Findings

Throughout our research, we have uncovered a series of key findings that collectively shed light on the role of AXL receptors in modulating Gonadotropin-Releasing Hormone (GnRH) receptor signaling. These findings represent a significant step forward in understanding the complexities of gonadotrope function and the broader context of the hypothalamus-pituitary-gonadal (HPG) axis.

One of the central observations of our study is the consistent expression of AXL receptors in GnRH-expressing cells, particularly gonadotropes. This initial discovery underscores the relevance of AXL receptors within the reproductive regulation framework, positioning them as potential players in the orchestration of hormonal responses.

Furthermore, our research has provided compelling evidence that AXL receptor activation, facilitated by the AXL agonist Gas6, enhances GnRH receptor-dependent signaling processes. This enhancement is exemplified by the increased phosphorylation of Extracellular Signal-Regulated Kinase (ERK), which is a critical component of the GnRH signaling cascade. Additionally, Gas6 exposure led to heightened transcriptional expression of the GnRH-responsive

immediate early gene Egr-1, further suggesting that AXL receptors influence gene expression patterns in gonadotropes.

Moreover, we observed a significant increase in the production of the gonadotropin subunit LHβ following Gas6 exposure. This finding is of paramount importance, as it signifies the potential for AXL receptor activation to directly impact the synthesis of essential reproductive hormones, thereby influencing gonadal activity.

The research also revealed that Gas6 exposure facilitated the migration of clonal gonadotropes, emphasizing the potential role of AXL receptors in cellular positioning within the anterior pituitary, which is crucial for efficient GnRH signaling and hormone release.

However, it is essential to acknowledge that our findings are not without complexities, as evidenced by temporal discordances between changes in ERK signaling and Egr-1 transcription and disparities between αT3-1 and LβT2 cells. These intricacies emphasize the need for further investigations to elucidate the precise mechanisms through which AXL receptors modulate GnRH receptor signaling.

4.1.2. Unlocking the Nexus of Kisspeptin, AXL Receptors, and GnRH Signaling: Implications for Reproductive Function and Cancer Therapeutics

This discussion delves into the intricate interplay between hypothalamic kisspeptin neurons, AXL receptors, and gonadotropin-releasing hormone (GnRH) receptors, shedding light on their roles in the regulation of gonadotropin generation and signaling, both in physiological and pathological contexts.

Here we would like to emphasize the crucial role of hypothalamic kisspeptin neurons as upstream regulators of GnRH neurons. These neurons play a pivotal role in mediating estradiol-dependent feedback control over gonadotropin production. The activation of both presynaptic and postsynaptic kisspeptin receptors (Kiss1R) on kisspeptin and GnRH neurons, respectively, is imperative for orchestrating the intricate patterns of gonadotropin secretion, including the crucial estradiol-dependent luteinizing hormone (LH) surge that precedes ovulation(119).

A noteworthy link between kisspeptin and AXL receptors is unveiled, particularly in the context of cancer. It has been observed that kisspeptin has the capacity to upregulate the transcriptional expression of AXL in certain cancer scenarios(120,121). However, we should acknowledge that the role of AXL in the post-developmental hypothalamus remains largely unknown territory. Nonetheless, it posits that kisspeptin-induced modulation of AXL function could potentially contribute to the maintenance and fine-tuning of synaptic communication between kisspeptin and GnRH neurons.

Expanding beyond the developmental aspect, we would like to turn this discussion's attention to the potential effects of AXL receptor function on the neuroendocrine system, extending beyond the migration of GnRH neurons during development. Compelling evidence suggests that gonadotropes residing in the anterior pituitary exhibit a tendency to move toward the hypophyseal portal capillaries(122,123). This migration hypothesis proposes that this movement may serve to facilitate the efficient transfer of gonadotropins into the bloodstream, yet the intricate molecular mechanisms underpinning GnRH receptor-dependent changes in gonadotrope chemotaxis and plasticity remain elusive. Nevertheless, it is pertinent to note that AXL transcripts have been detected in mouse gonadotropes, hinting at a potential role for AXL in these processes(124).

We would also like to underscore the expanding role of AXL receptors in the context of cancer development, positioning AXL as a promising candidate for novel targeted chemotherapeutic interventions(125,126). Currently, an array of AXL receptor inhibitory compounds is undergoing clinical investigation for the treatment of various cancers, including those with neuroendocrine origins, such as breast, ovarian, and pancreatic tumors(127). This diverse set of investigational agents encompasses small molecule inhibitors, monoclonal antibodies, antibody-cytotoxic drug conjugates, CAR-T cell therapeutics, and soluble AXL receptor fusion proteins. It is noteworthy that some of these agents inhibit AXL receptor signaling, while others harness AXL to selectively target cytotoxic agents to specific cellular populations.

However, the multifaceted nature of these distinct mechanisms renders the prediction and management of off-target effects associated with AXL-directed chemotherapeutics a complex challenge. This challenge is exacerbated by the lack of comprehensive information regarding AXL receptor expression and function post-developmentally (**Figure 4-*1***). There is prevailing lack of knowledge concerning the importance of AXL signaling within the hypothalamic-pituitary-gonadal (HPG) axis during adulthood. It should be emphasized that the consequences of therapeutic AXL receptor inhibition hinge not only on the extent of AXL function but also on the co-expression and activity of Tyro3 and Mer, two other TAM receptors. Conversely, AXL-targeted cytotoxic therapeutics may introduce unexpected outcomes that deviate from the effects observed with conventional AXL receptor inhibition. Therefore, the discussion underscores the urgent need for an enhanced understanding of AXL expression and its multifaceted function within the reproductive system.

Our data demonstrated the AXL expression within GnRH-expressing cells, specifically gonadotropes. It was revealed that the AXL agonist Gas6 enhances GnRH receptor-dependent extracellular signal-regulated kinase (ERK) phosphorylation, boosts the transcriptional expression of the GnRH-responsive immediate early gene Egr-1, and augments the production of LHβ, a vital gonadotropin subunit. Additional observations hint at the potential involvement of AXL in GnRH receptor function, encompassing increased synthesis and secretion of pro-matrix metalloproteinase 9 (pro-MMP9) and facilitated migration of clonal gonadotropes when exposed to Gas6.

Nonetheless, the exact mechanisms by which AXL modulates GnRH receptor signaling remain enigmatic. Prior reports have elucidated instances of crosstalk between GnRH receptors and receptor tyrosine kinases, particularly exemplified by GnRH-dependent epidermal growth factor receptor (EGFR) transactivation in immortalized mouse gonadotropes and GnRH neurons(128–130). It is noteworthy that AXL receptors have also been associated with the transactivation of receptor tyrosine kinases, including EGFR(131). Intriguingly, EGFR transactivation by GnRH necessitates the release of matrix metalloproteinases 2 and 9 (MMP2 and MMP9)(128), and AXL receptor activation has been linked to the release of these matrix metalloproteinases(132,133). Consequently, it is proposed that the generation of pro-MMP9 may contribute to the integration of AXL and GnRH receptor signaling. Gas6, when administered in isolation, fails to induce alterations in pro-MMP9 production by LβT2 cells. However, Gas6 significantly enhances GnRH-dependent pro-MMP9 release by these cells. Furthermore, the inhibition of AXL receptors with R428 mitigates the stimulatory impact of GnRH on pro-MMP9 generation. Collectively, these results postulate a potential mechanism underpinning the functional interactions between AXL and GnRH receptor signaling processes.

The temporal aspects of Gas6's effects on signaling are also scrutinized. Gas6 is shown to amplify ERK signaling independently of GnRH, but this effect manifests only after a 2-hour incubation period. Notably, acute short-term (5-minute) exposure to Gas6 does not yield significant alterations in phosphorylated ERK (pERK) levels. However, when combined with GnRH, the same 5-minute Gas6 exposure enhances pERK levels beyond those observed with GnRH stimulation alone. The precise molecular basis governing this facilitation of GnRH ERK signaling by Gas6 remains elusive and necessitates further experimental exploration. Along similar lines, the time course experiments involving Gas6 reveal observable changes in pERK/ERK ratios within LβT2 cells only after a 2-hour incubation period. In contrast, the measurement of Egr-1 transcripts using digital droplet polymerase chain reaction (ddPCR) indicates that a mere 5-minute exposure to Gas6 is sufficient to significantly elevate transcript levels. Existing evidence underscores that Egr-1 transcripts in gonadotropes, including LβT2 cells, are modulated by ERK activation(134–138). Hence, it is suggested that the disparities between these observations arise from the inherent differences in sensitivity between pERK/ERK measurements via enzyme-linked immunosorbent assay (ELISA) and Egr-1 measurements using ddPCR.

The intricate landscape of GnRH receptor signaling should also be considered, particularly regarding variations in cell types. In gonadotropes, the classic origin of GnRH receptor signaling involves Gαq protein-dependent activation of phospholipase Cβ (PLCβ)(139). However, it is acknowledged that clonal mouse gonadotropes utilized in this study emulate numerous but not all aspects of GnRH receptor signaling attributed to native gonadotropes(140,141). Conversely, in the context of human cancers, some of which exhibit AXL overexpression(142), GnRH receptor signaling predominantly relies on $G\alpha_i$ proteins(143). This leads to antiproliferative effects via increased protein tyrosine phosphatase activity and subsequent dephosphorylation and inactivation

of epidermal growth factor receptors (EGFRs). As such, it is posited that the effects of augmenting or inhibiting AXL-dependent modulation of GnRH receptor signaling mechanisms are likely to diverge in various cell types, warranting dedicated investigations.

Suggesting the potential functional relevance of AXL in GnRH receptor signaling, data showed that incubating LβT2 gonadotropes with Gas6 for 2 hours potentiates GnRH-dependent release of LHβ into the culture medium. This effect is not observed with Gas6 alone, thereby emphasizing the necessity of GnRH co-stimulation. Remarkably, AXL inhibition with R428, even without Gas6, induces a substantial reduction in basal LHβ release, comparable to the reductions engendered by the GnRH receptor antagonist cetrorelix and the MEK inhibitor U0126. This observation leads to the hypothesis that basal AXL signaling, perpetuated through AXL receptor interactions between adjacent LβT2 cells, may underlie the attenuation of basal LHβ release following AXL inhibition. Building upon this premise, it is noted that Gas6 prompts a chemotactic migration response in LβT2 cells when combined with GnRH. This suggests that Gas6 may foster AXL activation both directly and indirectly, by enhancing cell-cell interactions. In this scenario, the concurrent exposure of LβT2 cells to Gas6 and GnRH is expected to potentiate downstream signaling cascades more substantially than either compound alone. This aligns with the observed data indicating enhanced GnRH-dependent ERK signaling and pro-MMP9 generation by Gas6, particularly in scenarios where Gas6 alone exhibits minimal impact.

While the well-established role of AXL in facilitating the migration of GnRH neurons from the olfactory placode to the forebrain during development is acknowledged, its role in the pituitary remains unknown. Nevertheless, our data together with previous studies furnish compelling evidence suggesting a functional connection between AXL and GnRH receptors. Notably, incubating clonal gonadotropes with GnRH leads to an increase in AXL expression. Furthermore,

imaging experiments unveil clear AXL-like immunoreactivity within GnRH receptor-positive cells in both human and mouse pituitary sections. Interestingly, nearly all cells exhibiting GnRH receptor-like immunoreactivity are also identified as AXL-positive. Additionally, Gas6-like immunoreactivity is observed in adjacent cells of unknown identity within these pituitary sections, hinting at potential Gas6-dependent paracrine modulation of AXL-expressing gonadotropes.

In summation, this dissertation provides a comprehensive explanation of the intricate relationships among kisspeptin, AXL receptors, and GnRH receptors in the regulation of gonadotropin production and signaling. It underscores the potential significance of AXL in diverse physiological and pathological contexts, including its pivotal role in cancer development and its promise as a target for novel therapeutic agents. However, they also illuminate the complexities and challenges inherent in comprehending the multifaceted functions and interactions of AXL, particularly within the context of the reproductive system and GnRH receptor signaling. Further research endeavors are undeniably necessary to unravel the precise mechanisms and functional implications of AXL modulation in these intricate processes.

4.1.3. Implications for Advancing Our Understanding of the HPG Axis and Reproductive Disorders

The implications of our research extend far beyond the immediate findings and hold significant promise for advancing our comprehension of the hypothalamus-pituitary-gonadal (HPG) axis and related reproductive disorders. By revealing the involvement of AXL receptors in gonadotrope function and GnRH signaling, we contribute valuable insights to the field.

Our observations open up new avenues of inquiry into the intricate crosstalk between AXL and GnRH signaling pathways. Understanding the molecular events that link AXL receptor

activation to GnRH receptor signaling can provide mechanistic insights into the regulation of gonadotropin release and reproductive function. Furthermore, investigating the feedback loops and regulatory mechanisms that modulate AXL receptor-mediated signaling may reveal how homeostasis is maintained in response to hormonal cues.

The potential role of AXL receptors in the pathogenesis of reproductive disorders, such as polycystic ovary syndrome (PCOS) and hypothalamic amenorrhea, presents an exciting opportunity for further research. Exploring the connections between AXL receptor dysregulation and these conditions can provide a foundation for potential therapeutic interventions, addressing unmet clinical needs.

Moreover, our research has implications for gender-affirming care, where optimizing hormone therapies for transgender individuals undergoing gender transition is of paramount importance. Understanding how AXL receptor signaling influences the response to hormone therapies can contribute to more precise and effective treatment regimens.

4.1.4. Significance of the Study for the Field of Reproductive Endocrinology

In the context of reproductive endocrinology, our study holds substantial significance. It not only contributes to our understanding of the intricate regulatory mechanisms governing gonadotrope function but also introduces AXL receptors as novel players in the field.

By demonstrating the influence of AXL receptors on GnRH receptor signaling, we expand the repertoire of factors that can potentially be targeted for therapeutic interventions. Current medications manipulating GnRH receptor-expressing cells have demonstrated suboptimal efficacy and tolerability, highlighting the need for novel pharmacotherapeutic targets. The development of

innovative approaches guided by our findings can enhance treatment outcomes for individuals with conditions related to gonadotrope dysregulation, providing much-needed improvements in clinical care.

Furthermore, our research addresses the clinical imperative of understanding AXL receptor biology in non-neoplastic cells, given the ongoing clinical trials involving novel anticancer agents targeting AXL receptors. A comprehensive understanding of AXL receptor function outside the realm of cancer is vital for ensuring the safety and efficacy of these therapies, underlining the clinical relevance and implications of our study.

In conclusion, our research not only uncovers important findings regarding AXL receptor-mediated effects on gonadotrope function but also sets the stage for future investigations that promise to deepen our understanding of the HPG axis, reproductive disorders, and the field of reproductive endocrinology as a whole.

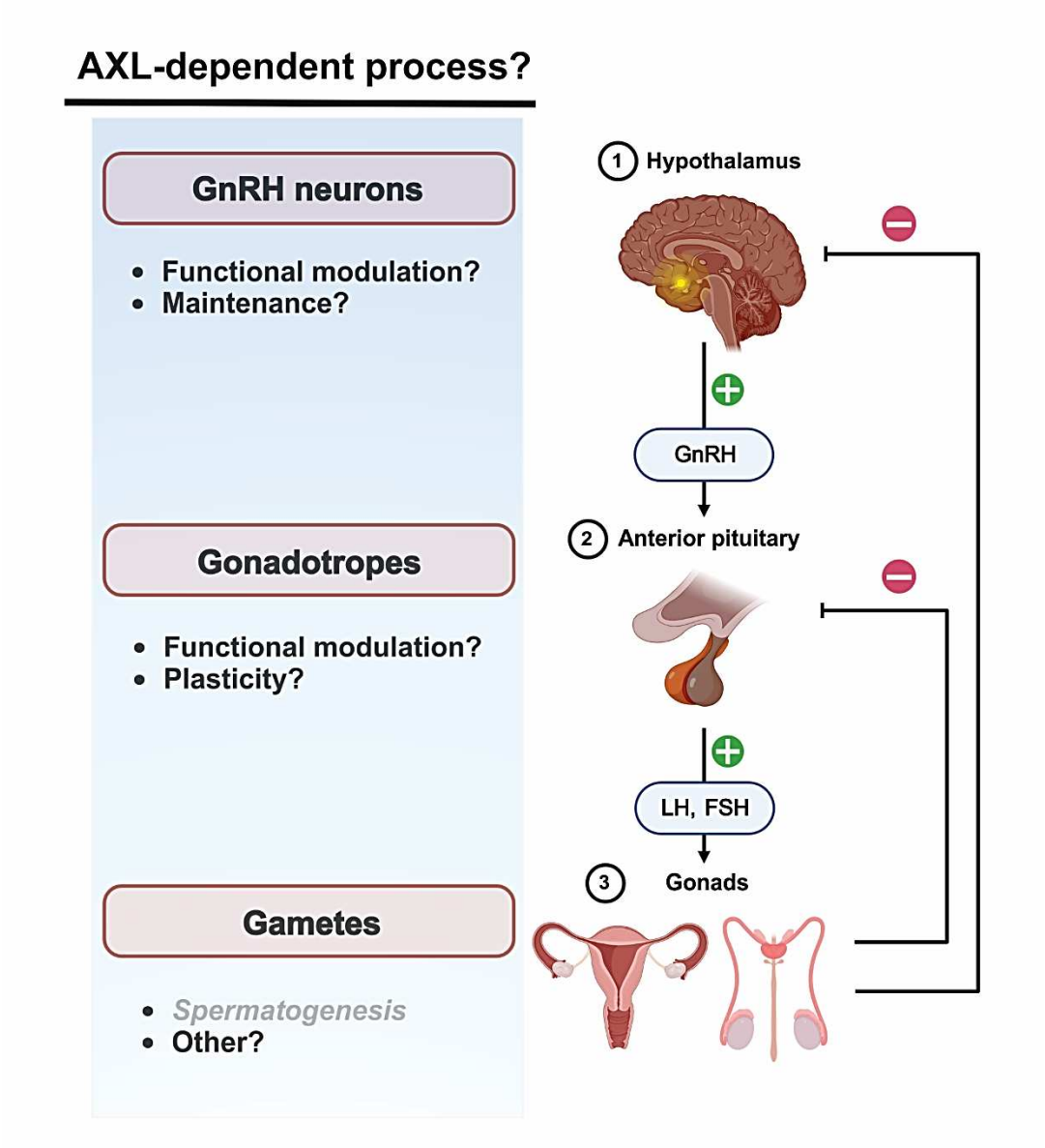

Figure 4-1 The functions of AXL/Gas6 in the neuroendocrine system after development remain inadequately understood. There is a possibility of AXL/Gas6 signaling exerting an impact on the HPG axis after development. It is well-established that AXL/Gas6 supports spermatogenesis. Made with BioRender.com

REFERENCES

1. Kanda S. Evolution of the regulatory mechanisms for the hypothalamic-pituitary-gonadal axis in vertebrates–hypothesis from a comparative view. Gen Comp Endocrinol. 2019 Dec 1;284:113075.

2. Meethal SV, Atwood CS. The role of hypothalamic-pituitary-gonadal hormones in the normal structure and functioning of the brain. Cell Mol Life Sci [Internet]. 2005 Feb [cited 2023 Sep 18];62(3):257–70. Available from: https://pubmed.ncbi.nlm.nih.gov/15723162/

3. Mills EG, Yang L, Nielsen MF, Kassem M, Dhillo WS, Comninos AN. The Relationship between Bone and Reproductive Hormones beyond Estrogens and Androgens. Endocr Rev. 2021 Dec 1;42(6):691–719.

4. Baba Y, Matsuo H, Schally A V. Structure of the porcine LH- and FSH-releasing hormone. II. Confirmation of the proposed structure by conventional sequential analyses. Biochem Biophys Res Commun [Internet]. 1971 Jul 16 [cited 2023 Sep 18];44(2):459–63. Available from: https://pubmed.ncbi.nlm.nih.gov/4946067/

5. Schally A V., Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, et al. Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. Science [Internet]. 1971 [cited 2023 Sep 18];173(4001):1036–8. Available from: https://pubmed.ncbi.nlm.nih.gov/4938639/

6. Marques P, Skorupskaite K, Rozario KS, Anderson RA, George JT. Physiology of GnRH and Gonadotropin Secretion. Endotext [Internet]. 2022 Jan 5 [cited 2023 Sep 18]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK279070/

7. Liu E, Hjelle B, Bishop JM. Transforming genes in chronic myelogenous leukemia. Proc Natl Acad Sci U S A [Internet]. 1988 Mar;85(6):1952–6. Available from: https://pubmed.ncbi.nlm.nih.gov/3279421

8. Janssen JWG, Schulz AS, Steenvoorden ACM, Schmidberger M, Strehl Ambros SPF, Bartram CR. A novel putative tyrosine kinase receptor with oncogenic potential. Oncogene. 1991;6(11).

9. O'bryan JP, Frye RA, Cogswell PC, Neubauer A, Kitch B, Prokop C, et al. axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. Mol Cell Biol [Internet]. 1991 Oct [cited 2022 May 14];11(10):5016–31. Available from: https://journals.asm.org/journal/mcb

10. AXL AXL receptor tyrosine kinase [Homo sapiens (human)] - Gene - NCBI [Internet]. [cited 2022 May 14]. Available from: https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=558

11. Valverde P. Effects of Gas6 and hydrogen peroxide in Axl ubiquitination and downregulation. Biochem Biophys Res Commun [Internet]. 2005 Jul 22 [cited 2022 Jun 11];333(1):180–5. Available from: https://pubmed.ncbi.nlm.nih.gov/15958209/

12. Sather S, Kenyon KD, Lefkowitz JB, Liang X, Varnum BC, Henson PM, et al. A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. Blood [Internet]. 2007 Feb 1 [cited 2022 May 14];109(3):1026–33. Available from: https://pubmed.ncbi.nlm.nih.gov/17047157/

13. Lu Q, Gore M, Zhang Q, Camenisch T, Boast S, Casagranda F, et al. Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. Nature [Internet]. 1999 Apr 22 [cited 2022 May 14];398(6729):723–8. Available from: https://pubmed.ncbi.nlm.nih.gov/10227296/

14. Mosesson Y, Shtiegman K, Katz M, Zwang Y, Vereb G, Szollosi J, et al. Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. J Biol Chem [Internet]. 2003 Jun 13 [cited 2022 May 14];278(24):21323–6. Available from: https://pubmed.ncbi.nlm.nih.gov/12719435/

15. K.A. A, R.M. R, G. V, J. H, K. S, D. D, et al. Noninsulin-dependent diabetes mellitus occurs in mice ectopically expressing the human Axl tyrosine kinase receptor [Internet]. Vol. 181, Journal of Cellular Physiology. 1999 [cited 2022 May 14]. p. 433–47. Available from: https://onlinelibrary.wiley.com/doi/10.1002/(SICI)1097-4652(199912)181:3%3C433::AID-JCP7%3E3.0.CO;2-Y

16. Graham DK, Deryckere D, Davies KD, Earp HS. The TAM family: phosphatidylserine-sensing receptor tyrosine kinases gone awry in cancer. Nature Reviews Cancer 2014 14:12 [Internet]. 2014 Nov 24 [cited 2022 Jun 20];14(12):769–85. Available from: https://www.nature.com/articles/nrc3847

17. Sasaki T, Knyazev PG, Clout NJ, Cheburkin Y, Göhring W, Ullrich A, et al. Structural basis for Gas6–Axl signalling. EMBO J [Internet]. 2006 Jan 11 [cited 2022 May 14];25(1):80. Available from: /pmc/articles/PMC1356355/

18. Fisher PW, Brigham-Burke M, Wu SJ, Luo J, Carton J, Staquet K, et al. A novel site contributing to growth-arrest-specific gene 6 binding to its receptors as revealed by a human monoclonal antibody. Biochemical Journal. 2005 May 1;387(3):727–35.

19. Zhu C, Wei Y, Wei X. AXL receptor tyrosine kinase as a promising anti-cancer approach: functions, molecular mechanisms and clinical applications. Molecular Cancer 2019 18:1 [Internet]. 2019 Nov 4 [cited 2022 May 14];18(1):1–22. Available from: https://molecular-cancer.biomedcentral.com/articles/10.1186/s12943-019-1090-3

20. Hoehn HJ, Kress Y, Sohn A, Brosnan CF, Bourdon S, Shafit-Zagardo B. Axl-/- mice have delayed recovery and prolonged axonal damage following cuprizone toxicity. Brain Res [Internet]. 2008 Nov 13 [cited 2022 May 16];1240:1–11. Available from: https://pubmed.ncbi.nlm.nih.gov/18804096/

21. Wu X, Liu X, Koul S, Lee CY, Zhang Z, Halmos B. AXL kinase as a novel target for cancer therapy. Oncotarget [Internet]. 2014 [cited 2022 Jun 19];5(20):9546. Available from: /pmc/articles/PMC4259419/

22. Fujimori T, Grabiec AM, Kaur M, Bell TJ, Fujino N, Cook PC, et al. The Axl receptor tyrosine kinase is a discriminator of macrophage function in the inflamed lung. Mucosal Immunology 2015 8:5 [Internet]. 2015 Jan 21 [cited 2022 May 14];8(5):1021–30. Available from: https://www.nature.com/articles/mi2014129

23. Bellosta P, Costa M, Lin DA, Basilico C. The receptor tyrosine kinase ARK mediates cell aggregation by homophilic binding. Mol Cell Biol [Internet]. 1995 Feb [cited 2022 May 14];15(2):614–25. Available from: https://journals.asm.org/doi/abs/10.1128/MCB.15.2.614

24. Burchert A, Attar EC, McCloskey P, Fridell YWC, Liu ET. Determinants for transformation induced by the Axl receptor tyrosine kinase. Oncogene 1998 16:24 [Internet]. 1998 Jun 17 [cited 2022 May 14];16(24):3177–87. Available from: https://www.nature.com/articles/1201865

25. Meyer AS, Miller MA, Gertler FB, Lauffenburger DA. The receptor AXL diversifies EGFR signaling and limits the response to EGFR-targeted inhibitors in triple-negative breast cancer cells. Sci Signal [Internet]. 2013 Aug 6 [cited 2022 May 14];6(287). Available from: https://www.science.org/doi/abs/10.1126/scisignal.2004155

26. Vouri M, Croucher DR, Kennedy SP, An Q, Pilkington GJ, Hafizi S. Axl-EGFR receptor tyrosine kinase hetero-interaction provides EGFR with access to pro-invasive signalling in cancer cells. Oncogenesis 2016 5:10 [Internet]. 2016 Oct 24 [cited 2022 May 14];5(10):e266–e266. Available from: https://www.nature.com/articles/oncsis201666

27. Mohammadzadeh P, Amberg GC. AXL/Gas6 signaling mechanisms in the hypothalamic-pituitary-gonadal axis. Front Endocrinol (Lausanne). 2023 Jun 15;14:1212104.

28. Burstyn-Cohen T. TAM receptor signaling in development. Int J Dev Biol [Internet]. 2017 [cited 2023 Sep 18];61(3-4–5):215–24. Available from: https://pubmed.ncbi.nlm.nih.gov/28621419/

29. Goyette MA, Côté JF. AXL Receptor Tyrosine Kinase as a Promising Therapeutic Target Directing Multiple Aspects of Cancer Progression and Metastasis. Cancers (Basel) [Internet]. 2022 Feb 1 [cited 2023 Sep 18];14(3). Available from: /pmc/articles/PMC8833413/

30. Wierman ME, Kiseljak-Vassiliades K, Tobet S. Gonadotropin Releasing Hormone (GnRH) Neuron Migration: Initiation, Maintenance and Cessation as Critical Steps to Ensure Normal Reproductive Function. Front Neuroendocrinol [Internet]. 2011 Jan [cited 2023 Sep 18];32(1):43. Available from: /pmc/articles/PMC3008544/

31. Salian-Mehta S, Xu M, Wierman ME. AXL and MET Crosstalk to Promote Gonadotropin Releasing Hormone (GnRH) Neuronal Cell Migration and Survival. Mol Cell Endocrinol

[Internet]. 2013 Jul 7 [cited 2023 Sep 18];374(0):92. Available from: /pmc/articles/PMC3690482/

32. Ruf-Zamojski F, Ge Y, Pincas H, Shan J, Song Y, Hines N, et al. Cytogenetic, Genomic, and Functional Characterization of Pituitary Gonadotrope Cell Lines. J Endocr Soc [Internet]. 2019 May 5 [cited 2023 Sep 18];3(5):902. Available from: /pmc/articles/PMC6469952/

33. Dang AK, Chaplin NL, Murtazina DA, Boehm U, Clay CM, Amberg GC. Subplasmalemmal hydrogen peroxide triggers calcium influx in gonadotropes. Journal of Biological Chemistry. 2018 Oct 12;293(41):16028–42.

34. Dang AK, Murtazina DA, Magee C, Navratil AM, Clay CM, Amberg GC. GnRH Evokes Localized Subplasmalemmal Calcium Signaling in Gonadotropes. Molecular Endocrinology [Internet]. 2014 [cited 2023 Sep 18];28(12):2049. Available from: /pmc/articles/PMC4250365/

35. Edwards BS, Dang AK, Murtazina DA, Dozier MG, Whitesell JD, Khan SA, et al. Dynamin is required for GnRH signaling to L-type calcium channels and activation of ERK. Endocrinology [Internet]. 2016 Feb 1 [cited 2023 Sep 18];157(2):831–43. Available from: /pmc/articles/PMC4733113/

36. Korshunov VA. Axl-dependent signaling: A clinical update. Clin Sci (Lond) [Internet]. 2012 Apr [cited 2023 Sep 18];122(8):361. Available from: /pmc/articles/PMC3609429/

37. Bliss SP, Navratil AM, Xie J, Roberson MS. GnRH signaling, the gonadotrope and endocrine control of fertility. Front Neuroendocrinol [Internet]. 2010 Jul [cited 2023 Sep 18];31(3):322. Available from: /pmc/articles/PMC2923852/

38. Chidiac P, Ross EM. Phospholipase C-beta1 directly accelerates GTP hydrolysis by Galphaq and acceleration is inhibited by Gbeta gamma subunits. J Biol Chem [Internet]. 1999 Jul 9 [cited 2023 Sep 18];274(28):19639–43. Available from: https://pubmed.ncbi.nlm.nih.gov/10391901/

39. Zhu X, Gleiberman AS, Rosenfeld MG. Molecular physiology of pituitary development: signaling and transcriptional networks. Physiol Rev [Internet]. 2007 Jul [cited 2023 Sep 18];87(3):933–63. Available from: https://pubmed.ncbi.nlm.nih.gov/17615393/

40. The differential secretion of FSH and LH: regulation through genes, feedback and packaging - PubMed [Internet]. [cited 2023 Sep 18]. Available from: https://pubmed.ncbi.nlm.nih.gov/14635955/

41. Scheiber MD, Liu JH. The Use of Gonadotropin-Releasing Hormone to Induce Ovulation. The Global Library of Women's Medicine [Internet]. 2011 [cited 2023 Sep 18]; Available from: http://www.glowm.com/section-view/heading/The Use of Gonadotropin-Releasing Hormone to Induce Ovulation/item/339

42. Adams C, Stroberg W, Defazio RA, Schnell S, Moenter SM. Gonadotropin-Releasing Hormone (GnRH) Neuron Excitability Is Regulated by Estradiol Feedback and Kisspeptin. Journal of Neuroscience [Internet]. 2018 Jan 31 [cited 2023 Sep 18];38(5):1249–63. Available from: https://www.jneurosci.org/content/38/5/1249

43. Shaw ND, Histed SN, Srouji SS, Yang J, Lee H, Hall JE. Estrogen Negative Feedback on Gonadotropin Secretion: Evidence for a Direct Pituitary Effect in Women. J Clin Endocrinol Metab [Internet]. 2010 [cited 2023 Sep 18];95(4):1955. Available from: /pmc/articles/PMC2853991/

44. Thompson IR, Kaiser UB. GnRH pulse frequency-dependent differential regulation of LH and FSH gene expression. Mol Cell Endocrinol [Internet]. 2014 Mar 25 [cited 2023 Sep 18];385(1–2):28–35. Available from: https://pubmed.ncbi.nlm.nih.gov/24056171/

45. Stamatiades GA, Carroll RS, Kaiser UB. GnRH—A Key Regulator of FSH. Endocrinology [Internet]. 2019 Jan 1 [cited 2023 Sep 19];160(1):57. Available from: /pmc/articles/PMC6304106/

46. Majumder A, Hosseinian S, Stroud M, Adhikari E, Saller JJ, Smith MA, et al. Integrated Proteomics-Based Physical and Functional Mapping of AXL Kinase Signaling Pathways and Inhibitors Define Its Role in Cell Migration. Mol Cancer Res [Internet]. 2022 Apr 1 [cited 2023 Sep 19];20(4):542–55. Available from: https://pubmed.ncbi.nlm.nih.gov/35022314/

47. Zhou J, Yang A, Wang Y, Chen F, Zhao Z, Davra V, et al. Tyro3, Axl, and Mertk receptors differentially participate in platelet activation and thrombus formation. Cell Communication and Signaling [Internet]. 2018 Dec 12 [cited 2023 Sep 19];16(1):1–14. Available from: https://biosignaling.biomedcentral.com/articles/10.1186/s12964-018-0308-0

48. Cavet ME, Smolock EM, Ozturk OH, World C, Pang J, Konishi A, et al. Gas6-Axl receptor signaling is regulated by glucose in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol [Internet]. 2008 May 1 [cited 2023 Sep 19];28(5):886–91. Available from: https://www.ahajournals.org/doi/abs/10.1161/ATVBAHA.108.162693

49. Braunger J, Schleithoff L, Schulz AS, Kessler H, Lammers R, Ullrich A, et al. Intracellular signaling of the Ufo/Axl receptor tyrosine kinase is mediated mainly by a multi-substrate docking-site. Oncogene [Internet]. 1997 [cited 2023 Sep 19];14(22):2619–31. Available from: https://pubmed.ncbi.nlm.nih.gov/9178760/

50. Antony J, Tan TZ, Kelly Z, Low J, Choolani M, Recchi C, et al. The GAS6-AXL signaling network is a mesenchymal (Mes) molecular subtype-specific therapeutic target for ovarian cancer. Sci Signal [Internet]. 2016 Oct 4 [cited 2023 Sep 19];9(448). Available from: https://www.science.org/doi/10.1126/scisignal.aaf8175

51. Goyette MA, Côté JF. AXL Receptor Tyrosine Kinase as a Promising Therapeutic Target Directing Multiple Aspects of Cancer Progression and Metastasis. Cancers (Basel)

[Internet]. 2022 Feb 1 [cited 2023 Sep 19];14(3). Available from: https://pubmed.ncbi.nlm.nih.gov/35158733/

52. Goruppi S, Ruaro E, Varnum B, Schneider C. Gas6-mediated survival in NIH3T3 cells activates stress signalling cascade and is independent of Ras. Oncogene [Internet]. 1999 Jul 22 [cited 2023 Sep 19];18(29):4224–36. Available from: https://pubmed.ncbi.nlm.nih.gov/10435635/

53. Sainaghi PP, Castello L, Bergamasco L, Galletti M, Bellosta P, Avanzi GC. Gas6 induces proliferation in prostate carcinoma cell lines expressing the Axl receptor. J Cell Physiol [Internet]. 2005 Jul [cited 2023 Sep 19];204(1):36–44. Available from: https://pubmed.ncbi.nlm.nih.gov/15605394/

54. Li Y, Jia L, Ren D, Liu C, Gong Y, Wang N, et al. Axl mediates tumor invasion and chemosensitivity through PI3K/Akt signaling pathway and is transcriptionally regulated by slug in breast carcinoma. IUBMB Life [Internet]. 2014 Jul 1 [cited 2023 Sep 19];66(7):507–18. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/iub.1285

55. Huang L, Fu L. Mechanisms of resistance to EGFR tyrosine kinase inhibitors. Acta Pharm Sin B [Internet]. 2015 Sep 1 [cited 2023 Sep 19];5(5):390–401. Available from: https://pubmed.ncbi.nlm.nih.gov/26579470/

56. Lee WP, Wen Y, Varnum B, Hung MC. Akt is required for Axl-Gas6 signaling to protect cells from E1A-mediated apoptosis. Oncogene [Internet]. 2002 Jan 17 [cited 2023 Sep 19];21(3):329–36. Available from: https://pubmed.ncbi.nlm.nih.gov/11821945/

57. Hasanbasic I, Cuerquis J, Varnum B, Blostein MD. Intracellular signaling pathways involved in Gas6-Axl-mediated survival of endothelial cells. Am J Physiol Heart Circ Physiol [Internet]. 2004 Sep [cited 2023 Sep 19];287(3). Available from: https://pubmed.ncbi.nlm.nih.gov/15130893/

58. Lafdil F, Chobert MN, Couchie D, Brouillet A, Zafrani ES, Mavier P, et al. Induction of Gas6 protein in CCl4-induced rat liver injury and anti-apoptotic effect on hepatic stellate cells. Hepatology [Internet]. 2006 Jul [cited 2023 Sep 19];44(1):228–39. Available from: https://pubmed.ncbi.nlm.nih.gov/16799993/

59. Allen MP, Zeng C, Schneider K, Xiong X, Meintzer MK, Bellosta P, et al. Growth arrest-specific gene 6 (Gas6)/adhesion related kinase (Ark) signaling promotes gonadotropin-releasing hormone neuronal survival via extracellular signal-regulated kinase (ERK) and Akt. Mol Endocrinol [Internet]. 1999 [cited 2023 Sep 19];13(2):191–201. Available from: https://pubmed.ncbi.nlm.nih.gov/9973250/

60. Ghosh AK, Secreto C, Boysen J, Sassoon T, Shanafelt TD, Mukhopadhyay D, et al. The novel receptor tyrosine kinase Axl is constitutively active in B-cell chronic lymphocytic leukemia and acts as a docking site of nonreceptor kinases: implications for therapy.

Blood [Internet]. 2011 Feb 10 [cited 2023 Sep 19];117(6):1928–37. Available from: https://pubmed.ncbi.nlm.nih.gov/21135257/

61. Kay JG, Grinstein S. Phosphatidylserine-mediated cellular signaling. Adv Exp Med Biol [Internet]. 2013 [cited 2023 Sep 19];991:177–93. Available from: https://pubmed.ncbi.nlm.nih.gov/23775696/

62. Zhong Z, Wang Y, Guo H, Sagare A, Fernández JA, Bell RD, et al. Protein S protects neurons from excitotoxic injury by activating the TAM receptor Tyro3-phosphatidylinositol 3-kinase-Akt pathway through its sex hormone-binding globulin-like region. J Neurosci [Internet]. 2010 Nov 17 [cited 2023 Sep 19];30(46):15521–34. Available from: https://pubmed.ncbi.nlm.nih.gov/21084607/

63. Scaltriti M, Elkabets M, Baselga J. Molecular Pathways: AXL, a Membrane Receptor Mediator of Resistance to Therapy. Clin Cancer Res [Internet]. 2016 Mar 15 [cited 2023 Sep 19];22(6):1313–7. Available from: https://pubmed.ncbi.nlm.nih.gov/26763248/

64. Demarchi F, Verardo R, Varnum B, Brancolini C, Schneider C. Gas6 anti-apoptotic signaling requires NF-kappa B activation. J Biol Chem [Internet]. 2001 Aug 24 [cited 2023 Sep 19];276(34):31738–44. Available from: https://pubmed.ncbi.nlm.nih.gov/11425860/

65. Tai KY, Shieh YS, Lee CS, Shiah SG, Wu CW. Axl promotes cell invasion by inducing MMP-9 activity through activation of NF-kappaB and Brg-1. Oncogene [Internet]. 2008 Jul 3 [cited 2023 Sep 19];27(29):4044–55. Available from: https://pubmed.ncbi.nlm.nih.gov/18345028/

66. Li X, Chen M, Lei X, Huang M, Ye W, Zhang R, et al. Luteolin inhibits angiogenesis by blocking Gas6/Axl signaling pathway. Int J Oncol [Internet]. 2017 Aug 1 [cited 2023 Sep 19];51(2):677–85. Available from: https://pubmed.ncbi.nlm.nih.gov/28627676/

67. Haga RB, Ridley AJ. Rho GTPases: Regulation and roles in cancer cell biology. Small GTPases [Internet]. 2016 Oct 1 [cited 2023 Sep 19];7(4):207–21. Available from: https://pubmed.ncbi.nlm.nih.gov/27628050/

68. Allen MP, Linseman DA, Udo H, Xu M, Schaack JB, Varnum B, et al. Novel mechanism for gonadotropin-releasing hormone neuronal migration involving Gas6/Ark signaling to p38 mitogen-activated protein kinase. Mol Cell Biol [Internet]. 2002 Jan 1 [cited 2023 Sep 19];22(2):599–613. Available from: https://pubmed.ncbi.nlm.nih.gov/11756555/

69. Huang JS, Cho CY, Hong CC, Yan M De, Hsieh MC, Lay JD, et al. Oxidative stress enhances Axl-mediated cell migration through an Akt1/Rac1-dependent mechanism. Free Radic Biol Med [Internet]. 2013 [cited 2023 Sep 19];65:1246–56. Available from: https://pubmed.ncbi.nlm.nih.gov/24064382/

70. Bekhite MM, Müller V, Tröger SH, Müller JP, Figulla HR, Sauer H, et al. Involvement of phosphoinositide 3-kinase class IA (PI3K 110α) and NADPH oxidase 1 (NOX1) in regulation of vascular differentiation induced by vascular endothelial growth factor

(VEGF) in mouse embryonic stem cells. Cell Tissue Res [Internet]. 2016 Apr 1 [cited 2023 Sep 19];364(1):159–74. Available from: https://pubmed.ncbi.nlm.nih.gov/26553657/

71. Ushio-Fukai M. Redox signaling in angiogenesis: role of NADPH oxidase. Cardiovasc Res [Internet]. 2006 Jul 15 [cited 2023 Sep 19];71(2):226–35. Available from: https://pubmed.ncbi.nlm.nih.gov/16781692/

72. Lim JKM, Leprivier G. The impact of oncogenic RAS on redox balance and implications for cancer development. Cell Death Dis [Internet]. 2019 Dec 1 [cited 2023 Sep 19];10(12). Available from: https://pubmed.ncbi.nlm.nih.gov/31852884/

73. Ridley AJ. Rho GTPase signalling in cell migration. Curr Opin Cell Biol [Internet]. 2015 Oct 1 [cited 2023 Sep 19];36:103–12. Available from: https://pubmed.ncbi.nlm.nih.gov/26363959/

74. Zdzalik-Bielecka D, Poswiata A, Kozik K, Jastrzebski K, Schink KO, Brewinska-Olchowik M, et al. The GAS6-AXL signaling pathway triggers actin remodeling that drives membrane ruffling, macropinocytosis, and cancer-cell invasion. Proc Natl Acad Sci U S A [Internet]. 2021 Jul 13 [cited 2023 Sep 19];118(28). Available from: https://pubmed.ncbi.nlm.nih.gov/34244439/

75. Fridell YWC, Jin Y, Quilliam LA, Burchert A, McCloskey P, Spizz G, et al. Differential activation of the Ras/extracellular-signal-regulated protein kinase pathway is responsible for the biological consequences induced by the Axl receptor tyrosine kinase. Mol Cell Biol [Internet]. 1996 Jan 1 [cited 2023 Sep 19];16(1):135–45. Available from: https://pubmed.ncbi.nlm.nih.gov/8524290/

76. Zhu C, Wei Y, Wei X. AXL receptor tyrosine kinase as a promising anti-cancer approach: functions, molecular mechanisms and clinical applications. Mol Cancer [Internet]. 2019 Nov 4 [cited 2023 Sep 19];18(1). Available from: https://pubmed.ncbi.nlm.nih.gov/31684958/

77. Axelrod H, Pienta KJ. Axl as a mediator of cellular growth and survival. Oncotarget [Internet]. 2014 [cited 2023 Sep 19];5(19):8818–52. Available from: https://pubmed.ncbi.nlm.nih.gov/25344858/

78. Kurowska-Stolarska M, Alivernini S, Melchor EG, Elmesmari A, Tolusso B, Tange C, et al. MicroRNA-34a dependent regulation of AXL controls the activation of dendritic cells in inflammatory arthritis. Nat Commun [Internet]. 2017 Jun 22 [cited 2023 Sep 19];8. Available from: https://pubmed.ncbi.nlm.nih.gov/28639625/

79. Lemke G, Rothlin C V. Immunobiology of the TAM receptors. Nat Rev Immunol [Internet]. 2008 May [cited 2023 Sep 19];8(5):327–36. Available from: https://pubmed.ncbi.nlm.nih.gov/18421305/

80. Rothlin C V., Lemke G. TAM receptor signaling and autoimmune disease. Curr Opin Immunol [Internet]. 2010 Dec [cited 2023 Sep 19];22(6):740–6. Available from: https://pubmed.ncbi.nlm.nih.gov/21030229/

81. Colonna M, Butovsky O. Microglia Function in the Central Nervous System During Health and Neurodegeneration. Annu Rev Immunol [Internet]. 2017 Apr 4 [cited 2023 Sep 19];35:441. Available from: /pmc/articles/PMC8167938/

82. Burstyn-Cohen T, Hochberg A. TAM Signaling in the Nervous System. Brain Plasticity [Internet]. 2021 May 18 [cited 2023 Sep 19];7(1):33. Available from: /pmc/articles/PMC8461745/

83. McShane L, Tabas I, Lemke G, Kurowska-Stolarska M, Maffia P. TAM receptors in cardiovascular disease. Cardiovasc Res [Internet]. 2019 Jul 7 [cited 2023 Sep 19];115(8):1286. Available from: /pmc/articles/PMC6587925/

84. Huey MG, Minson KA, Shelton Earp H, Deryckere D, Graham DK. Targeting the TAM Receptors in Leukemia. Cancers 2016, Vol 8, Page 101 [Internet]. 2016 Nov 8 [cited 2023 Sep 19];8(11):101. Available from: https://www.mdpi.com/2072-6694/8/11/101/htm

85. Shiozawa Y, Pedersen EA, Taichman RS. GAS6/Mer axis regulates the homing and survival of the E2A/PBX1-positive B-cell precursor acute lymphoblastic leukemia in the bone marrow niche. Exp Hematol. 2010 Feb 1;38(2):132–40.

86. Neubauer A, Fiebeler A, Graham DK, O'Bryan JP, Schmidt CA, Barckow P, et al. Expression of axl, a Transforming Receptor Tyrosine Kinase, in Normal and Malignant Hematopoiesis. Blood. 1994 Sep 15;84(6):1931–41.

87. Tissue expression of AXL - Staining in retina - The Human Protein Atlas [Internet]. [cited 2023 Sep 19]. Available from: https://www.proteinatlas.org/ENSG00000167601-AXL/tissue/retina

88. Wu W, Xu H, Meng Z, Zhu J, Xiong S, Xia X, et al. Axl Is Essential for in-vitro Angiogenesis Induced by Vitreous From Patients With Proliferative Diabetic Retinopathy. Front Med (Lausanne) [Internet]. 2021 Dec 23 [cited 2023 Sep 19];8. Available from: /pmc/articles/PMC8734562/

89. Khoo WH, Ledergor G, Weiner A, Roden DL, Terry RL, Mcdonald MM, et al. A niche-dependent myeloid transcriptome signature defines dormant myeloma cells. 2019 [cited 2023 Sep 19]; Available from: https://ashpublications.org/blood/article-pdf/134/1/30/1553831/blood880930.pdf

90. Tanaka M, Dykes SS, Siemann DW. Inhibition of the Axl pathway impairs breast and prostate cancer metastasis to the bones and bone remodeling. Clin Exp Metastasis [Internet]. 2021 Jun 1 [cited 2023 Sep 19];38(3):321. Available from: /pmc/articles/PMC8179919/

91. Cheng LC, Chen YL, Cheng AN, Lee AYL, Cho CY, Huang JS, et al. AXL phosphorylates and up-regulates TNS2 and its implications in IRS-1-associated metabolism in cancer cells. J Biomed Sci [Internet]. 2018 Nov 12 [cited 2023 Sep 19];25(1). Available from: /pmc/articles/PMC6233515/

92. Efthymiou V, Ding L, Balaz M, Sun W, Balazova L, Straub LG, et al. Inhibition of AXL receptor tyrosine kinase enhances brown adipose tissue functionality in mice. Nature Communications 2023 14:1 [Internet]. 2023 Jul 13 [cited 2023 Sep 19];14(1):1–22. Available from: https://www.nature.com/articles/s41467-023-39715-8

93. Schwanzel-Fukuda M, Pfaff DW. Origin of luteinizing hormone-releasing hormone neurons. Nature 1989 338:6211 [Internet]. 1989 [cited 2023 Sep 19];338(6211):161–4. Available from: https://www.nature.com/articles/338161a0

94. Allen MP, Linseman DA, Udo H, Xu M, Schaack JB, Varnum B, et al. Novel Mechanism for Gonadotropin-Releasing Hormone Neuronal Migration Involving Gas6/Ark Signaling to p38 Mitogen-Activated Protein Kinase. Mol Cell Biol [Internet]. 2002 Jan 1 [cited 2023 Sep 19];22(2):599–613. Available from: https://www.tandfonline.com/doi/abs/10.1128/MCB.22.2.599-613.2002

95. Allen MP, Zeng C, Schneider K, Xiong X, Meintzer MK, Bellosta P, et al. Growth Arrest-Specific Gene 6 (Gas6)/Adhesion Related Kinase (Ark) Signaling Promotes Gonadotropin-Releasing Hormone Neuronal Survival via Extracellular Signal-Regulated Kinase (ERK) and Akt. Molecular Endocrinology [Internet]. 1999 Feb 1 [cited 2023 Sep 19];13(2):191–201. Available from: https://dx.doi.org/10.1210/mend.13.2.0230

96. Allen MP, Xu M, Linseman DA, Pawlowski JE, Bokoch GM, Heidenreich KA, et al. Adhesion-related Kinase Repression of Gonadotropin-releasing Hormone Gene Expression Requires Rac Activation of the Extracellular Signal-regulated Kinase Pathway. Journal of Biological Chemistry. 2002 Oct 11;277(41):38133–40.

97. Linger RMA, Keating AK, Earp HS, Graham DK. TAM Receptor Tyrosine Kinases: Biologic Functions, Signaling, and Potential Therapeutic Targeting in Human Cancer. Adv Cancer Res. 2008 Jan 1;100:35–83.

98. Lu Q, Gore M, Zhang Q, Camenisch T, Boast S, Casagranda F, et al. Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. Nature 1999 398:6729 [Internet]. 1999 Apr 22 [cited 2023 Sep 19];398(6729):723–8. Available from: https://www.nature.com/articles/19554

99. Pierce A, Bliesner B, Xu M, Nielsen-Preiss S, Lemke G, Tobet S, et al. Axl and Tyro3 Modulate Female Reproduction by Influencing Gonadotropin-Releasing Hormone Neuron Survival and Migration. Molecular Endocrinology [Internet]. 2008 Nov 1 [cited 2023 Sep 19];22(11):2481–95. Available from: https://dx.doi.org/10.1210/me.2008-0169

100. Pierce A, Xu M, Bliesner B, Liu Z, Richards JA, Tobet S, et al. Hypothalamic but not pituitary or ovarian defects underlie the reproductive abnormalities in Axl/Tyro3 null mice. Mol Cell Endocrinol. 2011 Jun 6;339(1–2):151–8.

101. Wang H, Chen Y, Ge Y, Ma P, Ma Q, Ma J, et al. Immunoexpression of Tyro 3 family receptors--Tyro 3, Axl, and Mer--and their ligand Gas6 in postnatal developing mouse

testis. J Histochem Cytochem [Internet]. 2005 Nov [cited 2023 Sep 22];53(11):1355–64. Available from: https://pubmed.ncbi.nlm.nih.gov/15956026/

102. Lu Q, Gore M, Zhang Q, Camenisch T, Boast S, Casagranda F, et al. Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. Nature [Internet]. 1999 Apr 22 [cited 2023 Sep 22];398(6729):723–8. Available from: https://pubmed.ncbi.nlm.nih.gov/10227296/

103. Chen Y, Wang H, Qi N, Wu H, Xiong W, Ma J, et al. Functions of TAM RTKs in regulating spermatogenesis and male fertility in mice. Reproduction [Internet]. 2009 Oct [cited 2023 Sep 22];138(4):655–66. Available from: https://pubmed.ncbi.nlm.nih.gov/19602523/

104. CHAN MCW, MATHER JP, MCCRAY G, LEE WM. Identification and Regulation of Receptor Tyrosine Kinases Rse and Mer and Their Ligand Gas6 in Testicular Somatic Cells. J Androl [Internet]. 2000 Mar 4 [cited 2023 Sep 22];21(2):291–302. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/j.1939-4640.2000.tb02107.x

105. Shi J, Gao S, Chen Z, Chen Z, Yun D, Wu X, et al. Absence of MerTK disrupts spermatogenesis in an age-dependent manner. Mol Cell Endocrinol [Internet]. 2023 Jan 15 [cited 2023 Sep 22];560. Available from: https://pubmed.ncbi.nlm.nih.gov/36379275/

106. Chen Y, Wang H, Qi N, Wu H, Xiong W, Ma J, et al. Functions of TAM RTKs in regulating spermatogenesis and male fertility in mice. Reproduction [Internet]. 2009 Oct [cited 2023 Sep 22];138(4):655–66. Available from: https://pubmed.ncbi.nlm.nih.gov/19602523/

107. Ren Y, Savill J. Apoptosis: the importance of being eaten. Cell Death Differ [Internet]. 1998 [cited 2023 Sep 22];5(7):563–8. Available from: https://pubmed.ncbi.nlm.nih.gov/10200510/

108. Xu J, Sang M, Cheng J, Luo C, Shi J, Sun F. Knockdown of disheveled-associated activator of morphogenesis 2 disrupts cytoskeletal organization and phagocytosis in rat Sertoli cells. Mol Cell Endocrinol [Internet]. 2023 Mar 1 [cited 2023 Sep 22];563. Available from: https://pubmed.ncbi.nlm.nih.gov/36681175/

109. Wang H, Wang H, Xiong W, Chen Y, Ma Q, Ma J, et al. Evaluation on the phagocytosis of apoptotic spermatogenic cells by Sertoli cells in vitro through detecting lipid droplet formation by Oil Red O staining. Reproduction [Internet]. 2006 Sep [cited 2023 Sep 22];132(3):485–792. Available from: https://pubmed.ncbi.nlm.nih.gov/16940289/

110. Xu MZ, Chan SW, Liu AM, Wong KF, Fan ST, Chen J, et al. AXL receptor kinase is a mediator of YAP-dependent oncogenic functions in hepatocellular carcinoma. Oncogene [Internet]. 2011 Mar 10 [cited 2023 Sep 22];30(10):1229–40. Available from: https://pubmed.ncbi.nlm.nih.gov/21076472/

111. Ozyurt R, Ozpolat B. Therapeutic Landscape of AXL Receptor Kinase in Triple-Negative Breast Cancer. Mol Cancer Ther [Internet]. 2023 Jul 5 [cited 2023 Sep 22];22(7):OF1–15. Available from: https://dx.doi.org/10.1158/1535-7163.MCT-22-0617

112. Salian-Mehta S, Xu M, Knox AJ, Plummer L, Slavov D, Taylor M, et al. Functional consequences of AXL sequence variants in hypogonadotropic hypogonadism. J Clin Endocrinol Metab [Internet]. 2014 [cited 2023 Sep 22];99(4):1452–60. Available from: https://pubmed.ncbi.nlm.nih.gov/24476074/

113. Raivio T, Falardeau J, Dwyer A, Quinton R, Hayes FJ, Hughes VA, et al. Reversal of idiopathic hypogonadotropic hypogonadism. N Engl J Med [Internet]. 2007 Aug 30 [cited 2023 Sep 22];357(9):863–73. Available from: https://pubmed.ncbi.nlm.nih.gov/17761590/

114. Sykiotis GP, Plummer L, Hughes VA, Au M, Durrani S, Nayak-Young S, et al. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. Proc Natl Acad Sci U S A [Internet]. 2010 Aug 24 [cited 2023 Sep 22];107(34):15140–4. Available from: https://pubmed.ncbi.nlm.nih.gov/20696889/

115. Mohammadzadeh P, Roueinfar M, Amberg GC. AXL receptor tyrosine kinase modulates gonadotropin-releasing hormone receptor signaling. 2023 Jun 29 [cited 2023 Sep 25]; Available from: https://www.researchsquare.com

116. Mohammadzadeh P, Cohan RA, Ghoreishi SM, Bitarafan-Rajabi A, Ardestani MS. AS1411 Aptamer-Anionic Linear Globular Dendrimer G2-Iohexol Selective Nano-Theranostics. Sci Rep [Internet]. 2017 Sep 19 [cited 2022 May 30];7(1):1–16. Available from: https://www.nature.com/articles/s41598-017-12150-8

117. Mohammadzadeh P, Roueinfar M, Amberg GC. AXL receptor tyrosine kinase modulates gonadotropin-releasing hormone receptor signaling. Cell Communication and Signaling 2023 21:1 [Internet]. 2023 Oct 12 [cited 2023 Oct 11];21(1):1–13. Available from: https://biosignaling.biomedcentral.com/articles/10.1186/s12964-023-01313-y

118. Watt M, Mohammadzadeh P, Pinsinski E, Hollinshead FK, Bouma GJ. Corticotropin releasing hormone is present in the feline placenta and maternal serum. Front Endocrinol (Lausanne). 2023 Apr 14;14:1132743.

119. Stevenson H, Bartram S, Charalambides MM, Murthy S, Petitt T, Pradeep A, et al. Kisspeptin-neuron control of LH pulsatility and ovulation. Front Endocrinol (Lausanne). 2022 Nov 21;13:951938.

120. Guzman S, Brackstone M, Wondisford F, Babwah A V., Bhattacharya M. KISS1/KISS1R and Breast Cancer: Metastasis Promoter. Semin Reprod Med [Internet]. 2019 [cited 2023 Sep 22];37(4):197–206. Available from: https://pubmed.ncbi.nlm.nih.gov/31972865/

121. Blake A, Dragan M, Tirona RG, Hardy DB, Brackstone M, Tuck AB, et al. G protein-coupled KISS1 receptor is overexpressed in triple negative breast cancer and promotes drug resistance. Sci Rep [Internet]. 2017 Apr 19 [cited 2023 Sep 22];7. Available from: https://pubmed.ncbi.nlm.nih.gov/28422142/

122. Alim Z, Hartshorn C, Mai O, Stitt I, Clay C, Tobet S, et al. Gonadotrope plasticity at cellular and population levels. Endocrinology [Internet]. 2012 Oct 1 [cited 2023 Sep 22];153(10):4729–39. Available from: https://pubmed.ncbi.nlm.nih.gov/22893721/

123. Clay CM, Cherrington BD, Navratil AM. Plasticity of Anterior Pituitary Gonadotrope Cells Facilitates the Pre-Ovulatory LH Surge. Front Endocrinol (Lausanne). 2021 Feb 4;11:616053.

124. Qiao S, Nordström K, Muijs L, Gasparoni G, Tierling S, Krause E, et al. Molecular Plasticity of Male and Female Murine Gonadotropes Revealed by mRNA Sequencing. Endocrinology [Internet]. 2016 Mar 1 [cited 2023 Sep 22];157(3):1082–93. Available from: https://pubmed.ncbi.nlm.nih.gov/26677881/

125. Wu S, Liao M, Li M, Sun M, Xi N, Zeng Y. Structure-based discovery of potent inhibitors of Axl: design, synthesis, and biological evaluation. RSC Med Chem [Internet]. 2022 Jul 20 [cited 2023 Sep 22];13(10):1246–64. Available from: https://pubmed.ncbi.nlm.nih.gov/36325401/

126. Zhu C, Wei Y, Wei X. AXL receptor tyrosine kinase as a promising anti-cancer approach: functions, molecular mechanisms and clinical applications. Mol Cancer [Internet]. 2019 Nov 4 [cited 2023 Sep 22];18(1). Available from: https://pubmed.ncbi.nlm.nih.gov/31684958/

127. Bhalla S, Gerber DE. AXL Inhibitors: Status of Clinical Development. Curr Oncol Rep [Internet]. 2023 May 1 [cited 2023 Sep 22];25(5):521–9. Available from: https://pubmed.ncbi.nlm.nih.gov/36920638/

128. Roelle S, Grosse R, Aigner A, Krell HW, Czubayko F, Gudermann T. Matrix Metalloproteinases 2 and 9 Mediate Epidermal Growth Factor Receptor Transactivation by Gonadotropin-releasing Hormone. Journal of Biological Chemistry. 2003 Nov 21;278(47):47307–18.

129. Grosse R, Roelle S, Herrlich A, Höhn J, Gudermann T. Epidermal growth factor receptor tyro sine kinase mediates Ras activation by gonadotropin-releasing hormone. Journal of Biological Chemistry [Internet]. 2000 Apr 21 [cited 2023 Sep 23];275(16):12251–60. Available from: http://www.jbc.org/article/S0021925819808803/fulltext

130. Shah BH, Soh JW, Catt KJ. Dependence of gonadotropin-releasing hormone-induced neuronal MAPK signaling on epidermal growth factor receptor transactivation. J Biol Chem [Internet]. 2003 Jan 31 [cited 2023 Sep 23];278(5):2866–75. Available from: https://pubmed.ncbi.nlm.nih.gov/12446705/

131. Antony J, Tan TZ, Kelly Z, Low J, Choolani M, Recchi C, et al. The GAS6-AXL signaling network is a mesenchymal (Mes) molecular subtype-specific therapeutic target for ovarian cancer. Sci Signal [Internet]. 2016 Oct 4 [cited 2023 Sep 23];9(448). Available from: https://pubmed.ncbi.nlm.nih.gov/27703030/

132. Tai KY, Shieh YS, Lee CS, Shiah SG, Wu CW. Axl promotes cell invasion by inducing MMP-9 activity through activation of NF-kappaB and Brg-1. Oncogene [Internet]. 2008 Jul 3 [cited 2023 Sep 23];27(29):4044–55. Available from: https://pubmed.ncbi.nlm.nih.gov/18345028/

133. Vouri M, Croucher DR, Kennedy SP, An Q, Pilkington GJ, Hafizi S. Axl-EGFR receptor tyrosine kinase hetero-interaction provides EGFR with access to pro-invasive signalling in cancer cells. Oncogenesis [Internet]. 2016 Oct 1 [cited 2023 Sep 23];5(10). Available from: https://pubmed.ncbi.nlm.nih.gov/27775700/

134. Bliss SP, Miller A, Navratil AM, Xie J, McDonough SP, Fisher PJ, et al. ERK signaling in the pituitary is required for female but not male fertility. Mol Endocrinol [Internet]. 2009 Jul [cited 2023 Sep 23];23(7):1092–101. Available from: https://pubmed.ncbi.nlm.nih.gov/19372235/

135. Mayer SI, Dexheimer V, Nishida E, Kitajima S, Thiel G. Expression of the transcriptional repressor ATF3 in gonadotrophs is regulated by Egr-1, CREB, and ATF2 after gonadotropin-releasing hormone receptor stimulation. Endocrinology [Internet]. 2008 Dec [cited 2023 Sep 23];149(12):6311–25. Available from: https://pubmed.ncbi.nlm.nih.gov/18719024/

136. Burger LL, Haisenleder DJ, Aylor KW, Marshall JC. Regulation of Lhb and Egr1 Gene Expression by GNRH Pulses in Rat Pituitaries Is Both c-Jun N-Terminal Kinase (JNK)- and Extracellular Signal-Regulated Kinase (ERK)-Dependent. Biol Reprod [Internet]. 2009 [cited 2023 Sep 23];81(6):1206. Available from: /pmc/articles/PMC2788048/

137. Bliss SP, Navratil AM, Xie J, Roberson MS. GnRH signaling, the gonadotrope and endocrine control of fertility. Front Neuroendocrinol [Internet]. 2010 Jul [cited 2023 Sep 23];31(3):322. Available from: /pmc/articles/PMC2923852/

138. Thompson IR, Ciccone NA, Zhou Q, Xu S, Khogeer A, Carroll RS, et al. GnRH Pulse Frequency Control of Fshb Gene Expression Is Mediated via ERK1/2 Regulation of ICER. Mol Endocrinol [Internet]. 2016 Mar 1 [cited 2023 Sep 23];30(3):348–60. Available from: https://pubmed.ncbi.nlm.nih.gov/26835742/

139. Naor Z. Signaling by G-protein-coupled receptor (GPCR): studies on the GnRH receptor. Front Neuroendocrinol [Internet]. 2009 Jan [cited 2023 Sep 23];30(1):10–29. Available from: https://pubmed.ncbi.nlm.nih.gov/18708085/

140. Windle JJ, Weiner RI, Mellon PL. Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol Endocrinol [Internet]. 1990 [cited 2023 Sep 23];4(4):597–603. Available from: https://pubmed.ncbi.nlm.nih.gov/1704103/

141. Alarid ET, Windle JJ, Whyte DB, Mellon PL. Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. Development [Internet]. 1996 [cited 2023 Sep 23];122(10):3319–29. Available from: https://pubmed.ncbi.nlm.nih.gov/8898243/

142. Tang Y, Zang H, Wen Q, Fan S. AXL in cancer: a modulator of drug resistance and therapeutic target. Journal of Experimental and Clinical Cancer Research [Internet]. 2023 Dec 1 [cited 2023 Sep 23];42(1):1–14. Available from: https://jeccr.biomedcentral.com/articles/10.1186/s13046-023-02726-w

143. Gründker C, Emons G. The Role of Gonadotropin-Releasing Hormone in Cancer Cell Proliferation and Metastasis. Front Endocrinol (Lausanne) [Internet]. 2017 Aug 1 [cited 2023 Sep 23];8(AUG). Available from: https://pubmed.ncbi.nlm.nih.gov/28824547/

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
GnRHR	Gonadotropin-releasing hormone receptor
HPG axis	hypothalamic-pituitary-gonadal axis
LH	luteinizing hormone
FSH	follicle stimulating hormone
MMP9	metalloproteinase 9
Egr-1	early growth response factor 1
Gas6	growth arrest-specific protein 6
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
MAPK	mitogen-activated protein kinases
EGFR	epidermal growth factor receptor
PDGFR	platelet derived growth factor receptor
ERK	extracellular signal-regulated kinase
PLC	phospholipase C
PIP_2	phosphatidylinositol-4-5-bisphosphate
IP_3	inositol-1,4,5-trisphosphate
DAG	diacylglycerol
ER	endoplasmic reticulum
JNK	c-Jun N-terminal kinase

DMEM	Dulbecco's Modified Eagle Medium
Ca^{2+}	Calcium
ddPCR	Droplet digital PCR
ELISA	Enzyme-linked immunosorbent assay
PBS	Phosphate buffered saline
SEM	Standard error of the mean
cAMP	Cyclic adenosine monophosphate