

RESEARCH

Open Access



AXL receptor tyrosine kinase modulates gonadotropin-releasing hormone receptor signaling

Pardis Mohammadzadeh¹, Mina Roueinfar¹ and Gregory C. Amberg^{1*}

Abstract

Background Gonadotropin-releasing hormone (GnRH) receptors are essential for reproduction and are expressed in numerous urogenital, reproductive, and non-reproductive cancers. In addition to canonical G protein-coupled receptor signaling, GnRH receptors functionally interact with several receptor tyrosine kinases. AXL is a receptor tyrosine kinase expressed in numerous tissues as well as multiple tumors. Here we tested the hypothesis that AXL, along with its endogenous ligand Gas6, impacts GnRH receptor signaling.

Methods We used clonal murine pituitary α T3-1 and L β T2 gonadotrope cell lines to examine the effect of AXL activation on GnRH receptor-dependent signaling outcomes. ELISA and immunofluorescence were used to observe AXL and GnRH receptor expression in α T3-1 and L β T2 cells, as well as in murine and human pituitary sections. We also used ELISA to measure changes in ERK phosphorylation, pro-MMP9 production, and release of LH β . Digital drop-let PCR was used to measure the abundance of Egr-1 transcripts. A transwell migration assay was used to measure α T3-1 and L β T2 migration responses to GnRH and AXL.

Results We observed AXL, along with the GnRH receptor, expression in α T3-1 and L β T2 gonadotrope cell lines, as well as in murine and human pituitary sections. Consistent with a potentiating role of AXL, Gas6 enhanced GnRH-dependent ERK phosphorylation in α T3-1 and L β T2 cells. Further, and consistent with enhanced post-transcriptional GnRH receptor responses, we found that Gas6 increased the abundance of Egr-1 transcripts. Suggesting functional significance, in L β T2 cells, Gas6/AXL signaling stimulated LH β production and enhanced GnRH receptor-dependent generation of pro-MMP9 protein and promoted cell migration.

Conclusions Altogether, these data describe a novel role for AXL as a modulator of GnRH receptor signaling.

Keywords TAM receptor tyrosine kinase, Gas6, Matrix metalloproteinase 9

Background

Reproductive processes are largely governed by hormones produced by the hypothalamic-pituitary-gonadal (HPG) axis. Secretory hypothalamic neurons release

gonadotropin-releasing hormone (GnRH) into the hypophyseal portal circulation which delivers the neuropeptide to the anterior pituitary. A small population of endocrine cells called gonadotropes respond to the released GnRH via binding to cognate receptors expressed on their cell surface. GnRH receptor activation initiates several signaling cascades which culminate in the synthesis and secretion of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the general circulation [1]. Circulating gonadotropins subsequently bind

*Correspondence:

Gregory C. Amberg

Gregory.Amberg@colostate.edu

¹ Department of Biomedical Sciences, Colorado State University, 1617 Campus Delivery, Fort Collins, CO 80523, USA



© The Author(s) 2023. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

and activate receptors in the gonads to promote gametogenesis and synthesis of sex steroid hormones.

During development GnRH neurons arise in the olfactory placodes and migrate to the forebrain [2]. Mislocalization of GnRH neurons due to failed or impaired migration, or loss by some other mechanism, is associated with delayed or an absence of puberty [3, 4]. The receptor tyrosine kinase AXL, a member of the TAM family of receptor tyrosine kinases (Tyro3, AXL, and Mer), is involved with GnRH neuronal migration and survival [5, 6]. AXL receptors are activated by the high-affinity ligand Gas6 (growth arrest-specific protein 6) and by ligand-independent mechanisms [7, 8]. Gas6/AXL signaling is implicated in the development of a variety of tumors by promoting cell survival, proliferation, migration, and invasion [9]. As such, AXL receptors are currently a promising target for several novel cancer chemotherapeutics [10, 11].

Following activation, intrinsic AXL tyrosine kinase activity phosphorylates C-terminal tyrosine residues which leads to recruitment and activation of multiple intracellular signaling proteins [12–16]. Examples 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 by functional coupling with other receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR) [17–19]. AXL receptors couple to the extracellular signal-regulated kinase (ERK) branch of MAPK signaling pathways [10, 20]. MAP kinases transduce extracellular signals from plasmalemmal receptors into changes in cellular function such as differentiation, survival, and migration. Indeed, Gas6/AXL receptor promotion of the survival and migration of GnRH neurons during development involves ERK signaling-dependent processes [21].

In gonadotropes, activation of GnRH receptors leads to canonical $G\alpha_q$ protein signaling where phospholipase C (PLC) cleaves phosphatidylinositol-4-5-bisphosphate (PIP_2) to generate the classic second messengers inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [22]. IP_3 induces calcium release from the endoplasmic reticulum (ER) while DAG stimulates protein kinase C (PKC) which ultimately increases calcium influx through voltage-dependent channels [23]. Calcium release from the ER then promotes c-Jun N-terminal kinase (JNK) signaling to increase FSH expression while calcium influx through voltage-dependent channels activates ERK signaling to increase LH expression [23–25]. In addition, GnRH receptor signaling to ERK in gonadotropes has been shown to incorporate MMP9 activation [26].

As noted above, AXL receptor activation also promotes ERK- and MMP9-dependent signaling processes [16]. Despite sharing several common downstream signaling mechanisms, the impact of crosstalk between AXL and GnRH receptors is unclear.

In this study we tested the hypothesis that Gas6/AXL and GnRH receptors coordinate to produce GnRH-dependent signaling outcomes. After establishing Gas6 and AXL receptor expression in human and murine pituitary slices, we used clonal murine gonadotropes to investigate the functional consequences of AXL receptor activation on GnRH receptor function. We found that Gas6-dependent AXL activation enhances downstream GnRH receptor processes including ERK activation, transcriptional responses, luteinizing hormone (LH β) generation, pro-MMP9 levels, and cell migration. Taken together, our data supports the concept of AXL receptors as novel modulators of GnRH receptor-dependent regulation of gonadotrope function.

Methods

Reagents

Cell culture reagents were from Thermo Fisher Scientific (Waltham, MA) and chemicals were from Sigma (St. Louis, MO) unless noted otherwise. Test reagents used include: R428 (APEXBio; Houston, TX), cetrorelix acetate (Tocris Bioscience; Minneapolis, MN), Gas6 (Novus Biologicals; Centennial, CO), GnRH (Sigma), and U0126-EtOH (APEXBio; Houston, TX). All drugs used were screened for potential cell toxicity at 3 and 24 h with a tetrazolium-based colorimetric XTT Assay Kit (Abcam; Boston, MA). All drugs used were found to be non-toxic at the concentrations used.

Cell culture

Clonal L β T2 and α T3-1 (kindly provided by Dr. Pamela Mellon, University of California, San Diego) gonadotrope cells were cultured in 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% CO₂ humidified air. All experiments were performed on low passage number (6 to 8) α T3-1 and L β T2 cells.

Immunofluorescence

L β T2 or α T3-1 cells (2×10^5 cells/well) were plated onto 35 mm 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 next day, cells were treated with agonists/antagonists for various times plus 2 μ L CellMask

Deep Red Actin Tracking Stain (Invitrogen; Waltham, MA) for 30 min.

Following completion of experiments, 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 1 µg/mL dilution and AXL primary Antibody (PA5-106,118, 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 1 h at room temperature. Finally, nuclei were stained, and cells were mounted with ProLong Glass Antifade Mountant containing NucBlue Stain (Thermo Fisher Scientific) following the manufacturer's protocol.

C57 murine (male and female) and Human (unidentified) pituitary 5 µm paraffin sections were purchased from Zyagen (San Diego, CA) and immunostained for AXL, GnRH receptor (GnRHR) and Gas6 proteins [27]. Briefly, tissues were de-waxed using five-minute washes in CitriSolv (Decon Labs, 1601), then rehydrated through a gradual alcohol series for 5-min 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 min. Slides were washed for five minutes in 1×PBS and incubated for twenty minutes followed by incubation with the quenching solution in a humidity chamber for 5 min 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 (GnRHR03, Thermo Fisher Scientific) at 1:100 dilution, AXL antibody (PA5-77,875, Thermo Fisher Scientific) at 1:200 dilution, AXL antibody (PA5-106,118, Thermo Fisher Scientific) at 1:200 dilution, GnRHR monoclonal antibody (GnRHR/768, ab220196, Abcam) at 1 µg/mL dilution, Gas6 rabbit antibody (A8545, Abclonal) at 1:100 dilution and Gas6 goat antibody (AF885SP, R&D Systems) at 5 µg/mL dilution in blocking buffer in a humidified chamber at 4 °C overnight. The following day, sections were washed three

times (5 min 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 h at room temperature. The slides were then washed in 1×PBS three times (5 min each) and the tissues dehydrated using a gradual alcohol series (50%, 70%, 90% and 100%). Finally, cells were mounted, and the nuclei were stained with ProLong Glass Antifade Mountant with NucBlue Stain (Thermo Fisher Scientific) following the manufacturer's protocol. Slides were later imaged at 40X and 63X using an LSM 800 Airyscan (Zeiss) confocal microscope and processed via Zen Blue software (Zeiss).

AXL protein quantification

LβT2 and αT3-1 cells (2×10^5 cells/well) were seeded in 6-well plates and incubated for 48 h after which GnRH (10 nM) was added for 24 h. Protein was then extracted using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Total protein was measured with Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific) and AXL protein was quantified with a murine AXL ELISA kit following the manufacturer's instructions (Thermo Fisher Scientific). Each independent experiment was performed in duplicate.

AXL and GnRH receptor distribution in human pituitary

Images of human pituitary stained for AXL (red), GnRHR (green) and nucleus (blue) were used to identify the expression and distribution pattern of AXL and GnRHR throughout the pituitary. Using Zen blue software, a total of 577 cells were masked in one 2×2 tiled and two regular images. Images were then processed and fluorescent intensities for TaRFP, AF488 and DAPI channels (corresponding to AXL, GnRHR and nuclei respectively) were extracted. The fluorescent intensities were then divided by the area for each cell (µm²) and plotted as histograms.

ERK phosphorylation measurements

Semiquantitative sandwich ELISA kit (Abcam, ab176660) was used to measure ERK phosphorylation (pERK) and total ERK (pERK/ERK) in LβT2 and αT3-1 cells in response to Gas6 (100 nM) at different time points and GnRH (10 nM, 5 min) was used as a positive control. Briefly, cells were seeded at 8×10^4 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 DMEM and incubated at 37 °C in 5% CO₂ humidified air over night (12 h). Cells were then treated with Gas6

and/or GnRH for 5 min to 2 h; cell extracts were then obtained with RIPA Lysis and Extraction Buffer (Thermo Scientific) containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) was collected in separate tubes. BCA assay was performed to determine protein concentrations prior to ELISA analysis according to the manufacturer's protocol.

Reverse transcription digital droplet PCR

Reverse transcription digital droplet PCR (RT-ddPCR) was used to measure absolute transcript levels of the immediate early gene *Egr1*. L β T2 and α T3-1 cells were seeded in 6 well plates (2×10^5 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 (Bio-Rad) RT-ddPCR was performed 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 μ L EvaGreen, and 5 μ M of primers for murine *Egr1* (Fwd 5'-GAG CGAACAACCCTATGAGC-3' and Rev 5'-AGCGGC CAGTATAGGTGATG-3'), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Fwd 5'-GGGAAGCCC ATCACCATCTT-3' and Rev 5'-GCCTTCTCCATGGTG GTGAA-3'), plus nuclease-free H₂O to reach a total of 20 μ L, 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 s (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.

LH β release measurements

LH β production and release from L β T2 cells were measured with a murine LH Beta SimpleStep ELISA Kit (Abcam). Briefly, 1×10^4 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 μ L/well serum-free DMEM and incubated at 37 °C in 5% CO₂ humidified air for 2 h. Cells were then treated with GnRHR ligand (GnRH, 10 nM), AXL ligand (Gas6, 100 nM), GnRH receptor inhibitor (cetorelix acetate, 2 nM), AXL inhibitor (R428, 50 nM) and MEK inhibitor (U0126-EtOH, 10 μ M) 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. Total LH β was calculated as (LH β quantity in cell extract + LH β quantity in the media of the same well). Each individual experiment was performed in duplicate.

Pro-MMP9 release measurements

Pro-MMP9 production and release from L β T2 and α T3-1 cells were measured with a pro-MMP-9 Murine ELISA Kit (Invitrogen). Briefly, 1×10^4 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 μ L/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 (cetorelix acetate, 2 nM), AXL inhibitor (R428, 50 nM), and MEK inhibitor (U0126-EtOH, 10 μ M) 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.

Cell migration assay

We measured the migratory response of L β T2 and α T3-1 cells to stimuli with a Cell Migration/Chemotaxis Assay Kit (Abcam). L β T2 and α T3-1 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, 100 nM), AXL ligand (Gas6, 1000 nM), combination of Gas6 and GnRH, MEK activator (PAF C-16, 100 μ M) and MEK

inhibitor (U0126-EtOH, 100 μ M) 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.

Statistical analysis

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 \pm 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; ns = not significantly different ($P \geq 0.05$).

Results

To test our hypothesis that AXL receptor activation modulates GnRH receptor function we set four requisite experimental criteria: 1) AXL receptors must be expressed in cells with GnRH receptors; 2) activation of AXL receptors should impact GnRH receptor-dependent signaling processes; 3) AXL activation should alter GnRH receptor-dependent changes in gene expression; and 4) AXL receptor activation must impact GnRH receptor-associated biological functions (e.g., gonadotropin production).

AXL receptor-like expression in the anterior pituitary

To begin our investigation of AXL receptor tyrosine kinase regulation of GnRH receptor function, we used immunohistochemistry to assess 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 (Fig. 1A). Although AXL-like and GnRH receptor-like immunoreactivities were heterogeneous, there was considerable overlap between the signals for the two proteins (Fig. 1B). Indeed, in the human pituitary sections, >99% of the cells identified as GnRH receptor positive were also positive for AXL-like immunoreactivity. Conversely, \approx 19% of the cells identified as AXL positive were also positive for GnRH receptor-like reactivity.

To assess expression of the endogenous AXL ligand Gas6, we again performed immunohistochemistry on

murine and human pituitary paraffin sections. Along with GnRH receptor-like staining, we observed robust Gas6-like immunoreactivity in murine pituitary sections and weak but distinct Gas6-like immunoreactivity in human pituitary sections (Fig. 1C and 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 (Fig. 1C, right). Therefore, and unlike AXL, Gas6 appears to be expressed in non-gonadotrope cells. 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.

AXL receptor expression in murine gonadotrope cell lines

To investigate the functional interactions between AXL and GnRH receptor function we took advantage of two different murine gonadotrope cell lines. α T3-1 and L β T2 cells, both of which express functional GnRH receptors, are widely used experimental surrogates for native gonadotropes [28]. α T3-1 cells are considered to have a relatively immature gonadotrope phenotype while L β T2 cells represent a more mature form [29, 30]. First, using immunohistochemistry, we confirmed AXL- and GnRH receptor-like immunoreactivity in each cell line. Similar to our results in pituitary sections, we observed overlapping AXL and GnRH receptor signal in α T3-1 and L β T2 cells (Fig. 2A). While the biological significance requires further investigation, using quantitative AXL ELISA we found that incubating α T3-1 and L β T2 cells with GnRH (10 nM) slightly increased AXL protein levels after 24 h (Fig. 2B).

AXL receptor activation enhances canonical GnRH receptor-dependent signaling processes

Gonadotrope GnRH receptor activation promotes MAP kinase-dependent changes in immediate-early gene expression which ultimately increases gonadotropin synthesis [31]. We examined the impact of Gas6-dependent AXL receptor activation on ERK phosphorylation, expression of the transcription factor Egr-1 (early growth response factor 1) in α T3-1 and L β T2 cells, and production of the gonadotropin subunit LH β in L β T2 cells. Using a semiquantitative ELISA approach, we found that Gas6 (100 nM for 3 h) increased the proportion of phosphorylated ERK relative to total ERK (pERK/ERK) in clonal gonadotrope cell cultures (Fig. 3A). To further characterize Gas6/AXL-dependent ERK phosphorylation, we incubated L β T2 cells with Gas6 (100 nM) for increasing amounts of time; we incubated L β T2 cells with GnRH (10 nM for 5 min) as a comparative positive control. Unlike GnRH, which produced an effect at

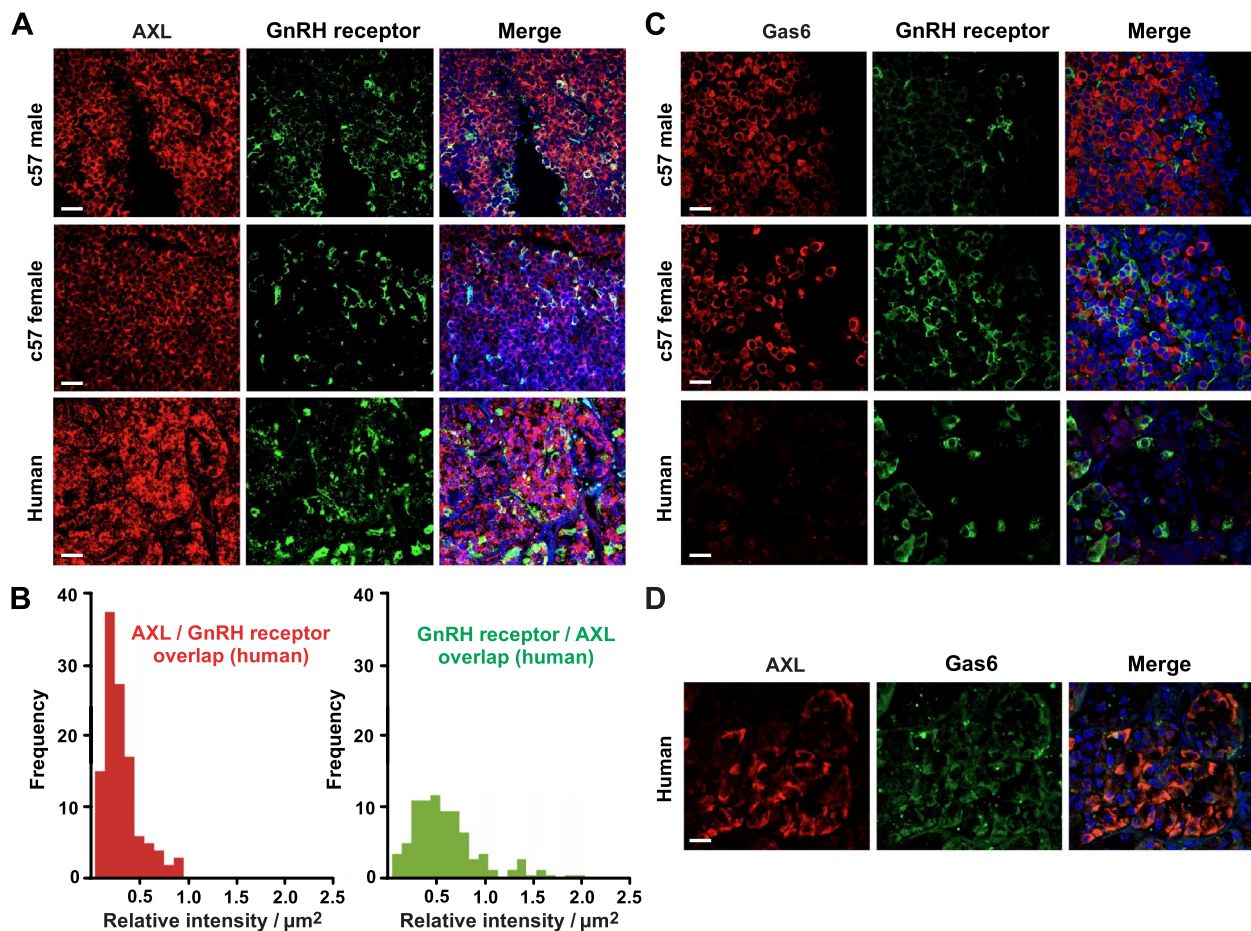


Fig. 1 AXL and GnRH receptor-like immunoreactivity in murine and human pituitaries. **A** Representative images showing AXL-like (red) and GnRH receptor-like (green) immunostaining in human, male C57 murine, and C57 female murine pituitary sections. Cell nuclei (blue) are shown in the merged images. **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) 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) are shown in the merged images. Images are representative of ≥ 3 paraffin sections; scale bars = 20 μm

5 min, Gas6 did not increase pERK/ERK for incubation periods < 2 h (Fig. 3B). The increase in pERK/ERK elicited by Gas6 at 2 h was not different from that produced by GnRH at 5 min. Of note, although Gas6 at 5 min had no observable effect, simultaneous incubation of L β T2 cells with Gas6 and GnRH for 5 min increased pERK/ERK to a greater extent than GnRH alone (Fig. 3B).

To determine the impact of AXL receptor activation on the expression of Egr-1, we incubated α T3-1 and L β T2 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 α T3-1 cells, Gas6 increased Egr-1 transcript abundance becoming evident at 20 min and peaking at 40 min (Fig. 3C). In L β T2 cells, the time course of Gas6-dependent changes in Egr-1

transcripts was faster than α T3-1 cells with an observable increase in Egr-1 transcripts at 5 min and followed quickly with a peak at 10 min.

L β T2 (but not α T3-1) cells express the gonadotropin subunit LH β [30]. We assessed the effect AXL receptor signaling on LH β expression by measuring protein levels in L β T2 culture media and cell lysates with ELISA. Similar to GnRH (10 nM for 2 h), Gas6 (100 nM for 2 h) increased the abundance of LH β protein in the culture media and L β T2 cell lysates (Fig. 3D). Gas6 produced a greater increase in LH β protein than GnRH in the culture media but not in L β T2 cell lysates. To test for potential constitutive GnRH and AXL receptors and MAP kinase activities on LH β expression, we examined the effects of the GnRH receptor antagonist (cetrorelix; 2 nM), the

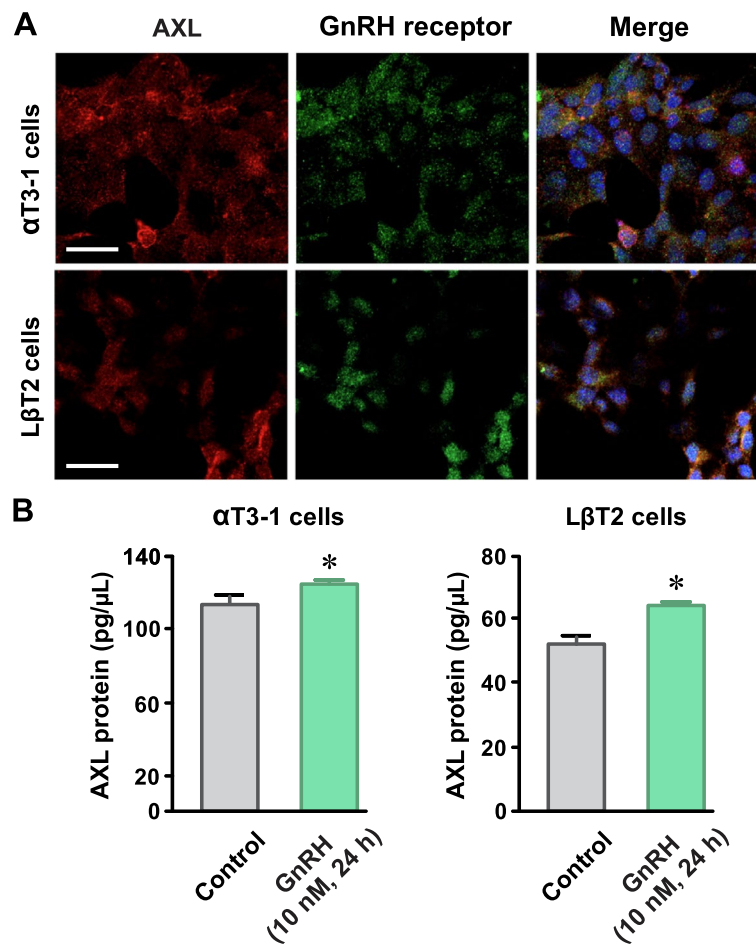


Fig. 2 AXL receptor expression in murine gonadotrope cell lines. AXL-like immunoreactivity (A) and protein abundance (B) in α T3-1 and L β T2 murine gonadotrope cell lines and AXL protein abundance in control cells and cells incubated with GnRH (10 nM for 24 h; $n=3$ independent cultures). Images representative of ≥ 3 α T3-1 and L β T2 cell cultures; n =number of independent experiments; numerical data presented as mean \pm SEM; * $P < 0.05$

AXL receptor antagonist (R428; 50 μ M), and the MEK (MAPK kinase) inhibitor (U0126; 10 μ M) on LH β protein abundance (Fig. 3E). Inclusion of all three inhibitors reduced basal levels of LH β protein in the media of L β T2 culture media to $\approx 50\%$ of control.

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 [26]. As MMP9 and EGF receptors are known downstream mediators of AXL receptor signalling [32], and our data showing AXL-mediated potentiation of ERK above (Fig. 3A and B), we examined the effects of Gas6 on MMP9 activity in α T3-1 and L β T2 cells. To do so, we measured the abundance of the inactive pro-form of MMP9 (pro-MMP9) in lysates and culture media of

α T3-1 and L β T2 cells (Fig. 4). Incubating α T3-1 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 (Fig. 4A). For L β T2 cells, GnRH increased pro-MMP9 abundance in the culture media and in cell lysates; MEK inhibition with U0126 blocked these effects (Fig. 4B). While Gas6 produced no effect, incubating L β T2 cells with GnRH and Gas6 increased pro-MMP9 levels in the culture media than GnRH alone. Coincubation with the AXL receptor antagonist R428 attenuated GnRH-dependent increases in pro-MMP9 in L β T2 culture media and cell lysates.

Extracellular matrix modification by secretory endopeptidases such as MMP9 influence the migratory behavior of cells [33]. Evidence suggests that cell movement is a component of gonadotrope response to GnRH [34, 35]. Accordingly, we used a transwell cell migration assay to

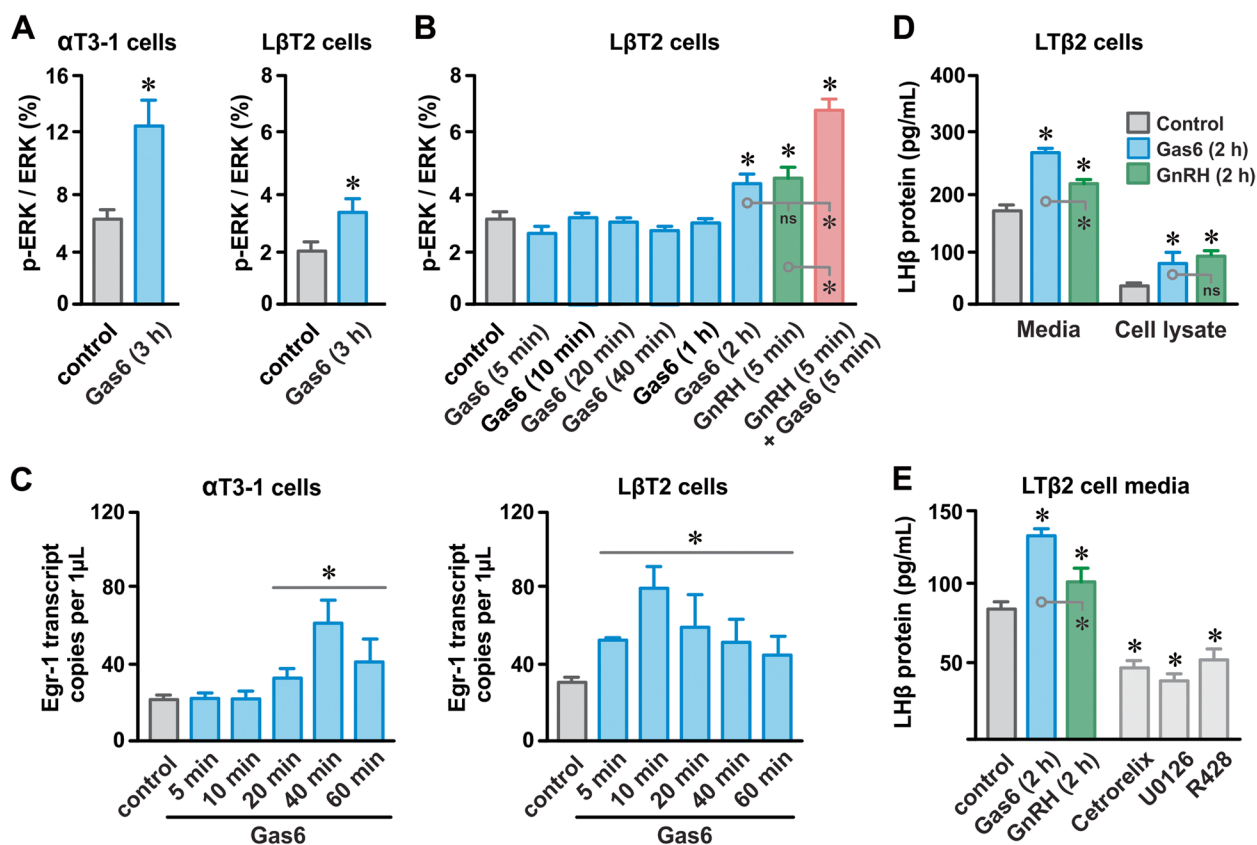


Fig. 3 Gas6-dependent AXL receptor activation enhances GnRH receptor signaling processes. **A** Proportion of phosphorylated ERK relative to total ERK (pERK/ERK) in α T3-1 and L β T2 cells under control conditions and after incubation with Gas6 (100 nM for 3 h); $n = 3$ for each condition. **B** Time course of Gas6-dependent changes (100 nM) in pERK/ERK in L β T2 cells (from left); effect of incubating L β T2 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 α T3-1 and L β T2 cells; $n = 3$. **D** LH β protein abundance in the culture media and lysates of L β T2 cells under control conditions and after 2 h incubations with Gas6 (100 nM) and GnRH (10 nM); $n = 3$. **E** LH β protein abundance in L β T2 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 μ M), and the MEK inhibitor U0126 (10 μ M); $n = 3$ for each condition. $n =$ number of independent experiments; numerical data presented as mean \pm SEM; * $P < 0.05$

assess AXL-dependent modulation of α T3-1 and L β T2 cell chemotactic responses to GnRH [36]. We found that exposing α T3-1 and L β T2 cells to GnRH (10 nM) for 48 h produced no observable effects on cell movement (Fig. 5A and B). In the absence of GnRH, incubation of α T3-1 cells with Gas6 provoked an increase in cell migration. In contrast, Gas6 did not promote L β T2 cell migration except in the presence of GnRH. MEK/ERK activation with PAF C-16 (100 μ M as a positive control) produced a robust increase in migration of α T3-1 and L β T2 cells while MEK inhibition with U0126 (10 μ M as a negative control) had no observable effect.

Discussion

In this paper we tested the hypothesis that AXL, one of the three TAM receptor tyrosine kinases, modulates GnRH receptor signaling. Supportive findings include: 1)

demonstrable AXL expression in GnRH expressing cells (i.e., gonadotropes); 2) the AXL agonist Gas6 enhanced GnRH receptor-dependent ERK phosphorylation; 3) Gas6 increased transcriptional expression of the GnRH-responsive immediate early gene Egr-1; and 4) Gas6 enhanced production of the gonadotropin subunit LH β . Additional observations suggesting potential of GnRH receptor function by AXL include increased pro-MMP9 synthesis and secretion and facilitated migration of clonal gonadotropes following Gas6 exposure. Altogether, our data are consistent with the concept of AXL as a novel modulator of GnRH receptor signaling.

The mechanisms by which AXL modulates GnRH receptor signaling in this study are unclear. Previous reports demonstrated crosstalk between GnRH receptors and receptor tyrosine kinases, notably GnRH-dependent EGFR transactivation in immortalized murine

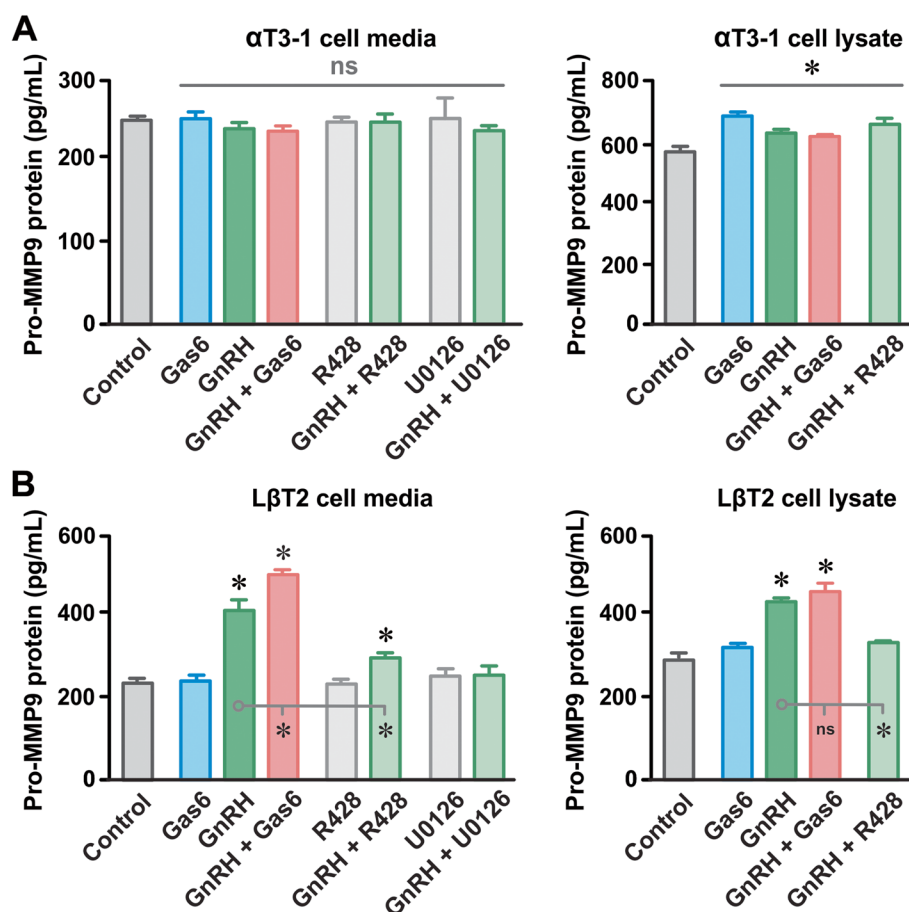


Fig. 4 AXL and GnRH receptor stimulation promotes pro-MMP9 release. Pro-MMP9 abundance in the culture media and lysates of αT3-1 (A) and LβT2 (B) cells following incubation with Gas6 (100 nM), GnRH (10 nM), Gas6 plus GnRH, and the AXL receptor antagonist R428 (50 μM), the MEK inhibitor U0126 (10 μM).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

gonadotropes and GnRH neurons [26, 37, 38]. AXL receptors have also been associated with transactivation of receptor tyrosine kinases, including EGFR [17]. EGFR transactivation by GnRH required release of MMP2 and MMP9 [26] and AXL receptor activation is associated with release of MMP2 and MMP9 [32, 39]. Our observations regarding pro-MMP9 generation by LβT2 cells suggests a potential contributory mechanism to integration of AXL and GnRH receptor signaling. Although Gas6 alone did not alter pro-MMP9 production by LβT2 cells, Gas6 enhanced GnRH-dependent release of pro-MMP9 by LβT2 cells. Further, inhibition of AXL receptors with R428 attenuated the stimulatory effect of GnRH on pro-MMP9 generation. Together, these results provide a potential mechanism contributing to functional interactions between AXL and GnRH receptor signaling processes.

Our pERK/ERK measurements revealed that Gas6 increased ERK signaling independent of GnRH only

after Gas6 incubation of 2 h. Although acute short-term (5 min) Gas6 exposure produced no observable effect on pERK levels, in the presence of GnRH, the same 5 min Gas6 exposure enhanced pERK beyond that observed with GnRH alone. The molecular basis for this facilitation of GnRH ERK signaling by Gas6 is not apparent and will require additional experimentation. Somewhat analogously, our time course experiments with Gas6 showed observable pERK/ERK changes in LβT2 cells only after 2 h of incubation. However, using ddPCR to measure Egr-1 transcripts, we found that only 5 min of Gas6 exposure was required to produce significant elevations in transcript levels. Abundant prior evidence indicates that in gonadotropes, including LβT2 cells, Egr-1 transcripts are regulated by ERK activation [31, 40–43]. Accordingly, we suggest that the underlying cause of this second apparent disconnect likely arises from large differences in the sensitivities of our pERK/ERK measurements with ELISA and Egr-1 measurements with ddPCR.

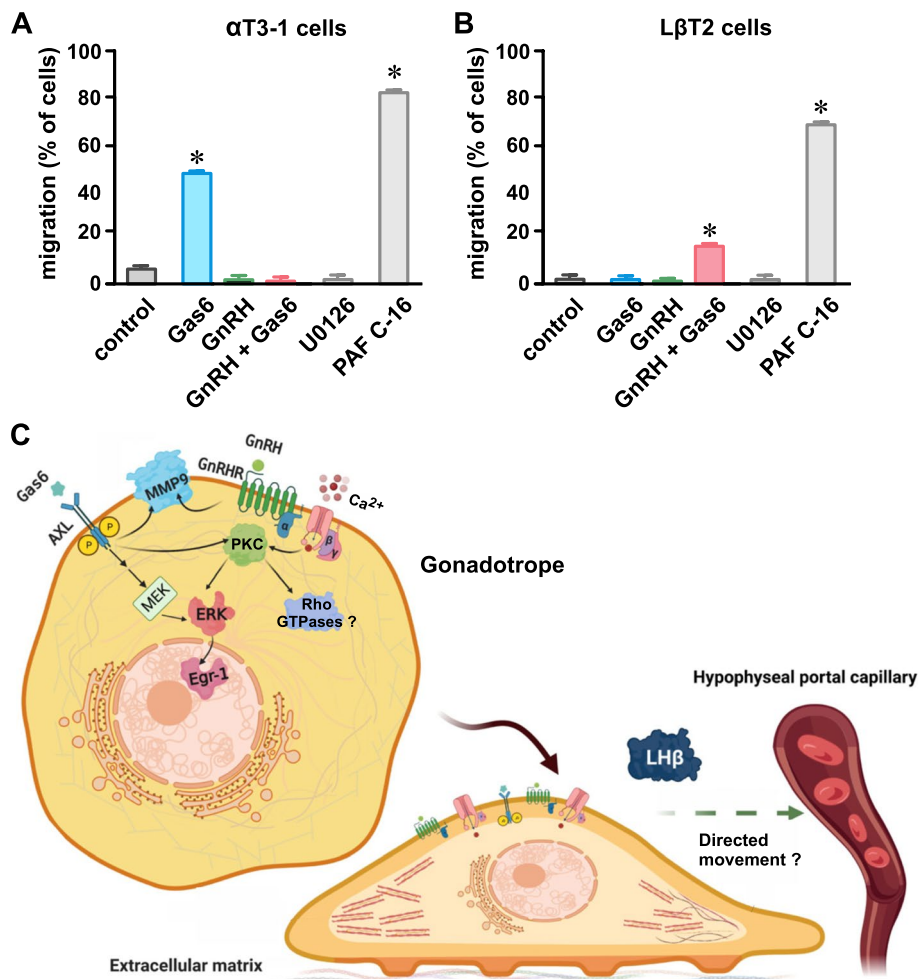


Fig. 5 AXL and GnRH receptor activation promotes migration of clonal gonadotropes. Transwell migratory responses (% cells) of α T3-1 (A) and L β T2 (B) cells following 48 h incubations with Gas6 (100 nM), GnRH (10 nM), Gas6 plus GnRH, the MEK inhibitor U0126 (10 μ M), and the MEK activator PAF C-16 (100 μ M). $n=4$ for each condition. n = number of independent experiments; numerical data presented as mean \pm SEM; ns = $P > 0.05$; * $P < 0.05$. C Working model of AXL and GnRH receptor signaling integration in gonadotropes

GnRH receptor signaling in gonadotropes classically originates with G_{α_q} protein-dependent activation of PLC β [44]. The clonal murine gonadotropes used in this study recapitulate many, but not all, of the aspects of GnRH receptor signaling ascribed to native gonadotropes [29, 30]. Thus, our observations following AXL receptor manipulation in α T3-1 and L β T2 gonadotropes need to be investigated using a more biologically relevant approach. In human cancers, some of which overexpress AXL [9], GnRH receptor signaling is predominantly mediated by G_{α_i} proteins [45]. In these cells, GnRH receptor activation produces antiproliferative effects by increasing protein tyrosine phosphatase activity leading to subsequent dephosphorylation and inactivation of EGF receptors. As such, the effects of enhancing or inhibiting AXL-dependent modulation of GnRH receptor signaling mechanisms are likely to

differ from gonadotropes and should be the focus of further study.

Suggesting potential functional importance, we found that incubating L β T2 gonadotropes with Gas6 for 2 h potentiated GnRH-dependent release of LH β into the culture medium. However, Gas6 alone produced no observable effects on the amount of pro-MMP9. Of note, we also found that AXL inhibition with R428, in the absence of Gas6, reduced basal LH β release. The decrease in LH β release was robust (\approx 50% control) and was comparable to reductions in basal release produced by the GnRH receptor antagonist cetrorelix and the MEK inhibitor U0126. AXL receptors undergo Gas6-independent activation via homotypic binding of extracellular domains of AXL receptors located on the surface of adjacent cells [46]. We postulate that basal AXL signaling, maintained by AXL receptor interactions between

adjacent L β T2 cells, could underlie the reduction in basal LH β release following AXL inhibition. Related to and expanding on this supposition, we found that Gas6 produced a chemotactic migration response in L β T2 cells in the presence of GnRH. Thus, Gas6 could promote AXL activation directly and indirectly by increasing the occurrence of cell–cell interactions. In this scenario, coincident exposure of L β T2 cells to Gas6 and GnRH would be expected to increase downstream signaling cascades to a greater extent than either compound alone. Our data showing enhanced GnRH-dependent ERK signaling and pro-MMP9 generation by Gas6 under conditions where the later showed no apparent effect on its own is consistent with this hypothesis.

Although the contribution of AXL to GnRH neuronal migration from the olfactory placode to the forebrain during development is established, a role for AXL in the pituitary is not evident in prior publications [5, 6, 47]. These studies, which used knockout models, conclude that AXL function in the hypothalamus, but not the pituitary, is required for reproductive function. However, our imaging experiments show clear AXL-like immunoreactivity in GnRH receptor positive cells in human and murine pituitary sections. Indeed, nearly all of the cells visualized with GnRH receptor-like immunoreactivity were also identified as AXL positive. In these pituitary sections we also visualized Gas6-like immunoreactivity, not in GnRH receptor positive cells, but in adjacent cells of unknown identity. Interestingly, published RNA sequencing data from male and female murine gonadotropes show AXL expression in these cells [48]. These data revealed apparent differential expression of AXL in female gonadotropes with greater AXL levels in cells harvested during proestrus, when GnRH pulse frequency is high, than in cells harvested during diestrus, when GnRH pulse frequency is low. Consistent with these data, we found that incubating clonal gonadotropes with GnRH produced a slight increase in AXL expression. Future investigations thoroughly examining functional relationships between GnRH receptor and AXL signaling in gonadotropes are necessary to address this issue.

Conclusions

In sum, our data consistently supports the concept of AXL receptors as a novel modulators of GnRH receptor signaling (Fig. 5C). However, our observations are not without incongruities, such as the temporal discord between changes in ERK signaling and Egr-1 transcription and disparities between in α T3-1 and L β T2 cells. We suggest that these apparent disconnects arise not only from the many differences between α T3-1 and L β T2 cells [29, 30], but also the intrinsic complexities of AXL and

GnRH receptor signaling mechanisms [16]. Additionally, the less-than-optimal efficacy and tolerability of current medications manipulating GnRH receptor expressing cells (e.g., gonadotropes) supports the need for novel pharmacotherapeutic targets. Finally, as AXL receptors are at present the target of numerous novel anticancer agents undergoing clinical trials [9, 49–51], it is clinically imperative that AXL receptor biology in non-neoplastic cells be better understood.

Abbreviations

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

Acknowledgements

The authors would like to thank Ms. Connie King for imaging assistance, Mr. John Anderson for help with ddPCR, and Mr. Eric Ron for assisting with the trans-well migration assay. We would like to thank Dr. Gerrit J Bouma and Dr. Agata Parsons Aubone for advice and assistance with immunolabeling, Dr. Michael May for assisting with image analysis and Dr. Lubna Tahtamouni for advice regarding the migration assay. We would also like to thank Drs. Stuart Tobet, Timothy Stasevich, Frederic Hoerndli, and Quinton Winger for helpful discussions regarding this manuscript.

Authors' contributions

Conceptualization: P.M.; writing—original draft preparation: P.M., M.R.; writing—review and editing: P.M., M.R., G.C.A.; supervision: G.C.A.; project administration: P.M., M.R.; funding acquisition: G.C.A.; resources: G.C.A.; All authors reviewed the manuscript.

Funding

This research was supported by National Institute of Health grant number 5R01HD087347 and the Colorado State University Department of Biomedical Sciences (G.C.A.).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 25 June 2023 Accepted: 9 September 2023

Published online: 12 October 2023

References

1. Perrett RM, McArdle CA. Molecular mechanisms of gonadotropin-releasing hormone signaling: integrating cyclic nucleotides into the network. *Front Endocrinol (Lausanne)*. 2013;4:180.
2. Cho HJ, Shan Y, Whittington NC, Wray S. Nasal placode development, GnRH neuronal migration and kallmann syndrome. *Front Cell Dev Biol*. 2019;7:121.
3. Balasubramanian R, Dwyer A, Seminara SB, Pitteloud N, Kaiser UB, Crowley WF Jr. Human GnRH deficiency: a unique disease model to unravel the ontogeny of GnRH neurons. *Neuroendocrinology*. 2010;92:81–99.
4. 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*. 2011;32:43–52.
5. Pierce A, Bliesner B, Xu M, Nielsen-Preiss S, Lemke G, Tobet S, Wierman ME. Axl and Tyro3 modulate female reproduction by influencing gonadotropin-releasing hormone neuron survival and migration. *Mol Endocrinol*. 2008;22:2481–95.
6. Pierce A, Xu M, Bliesner B, Liu Z, Richards J, Tobet S, Wierman ME. Hypothalamic but not pituitary or ovarian defects underlie the reproductive abnormalities in Axl/Tyro3 null mice. *Mol Cell Endocrinol*. 2011;339:151–8.
7. Fisher PW, Brigham-Burke M, Wu SJ, Luo J, Carton J, Staquet K, Gao W, Jackson S, Bethea D, Chen C, et al. A novel site contributing to growth-arrest-specific gene 6 binding to its receptors as revealed by a human monoclonal antibody. *Biochem J*. 2005;387:727–35.
8. Sasaki T, Knyazev PG, Clout NJ, Cheburkin Y, Gohring W, Ullrich A, Timpl R, Hohenester E. Structural basis for Gas6-Axl signalling. *EMBO J*. 2006;25:80–7.
9. Tang Y, Zang H, Wen Q, Fan S. AXL in cancer: a modulator of drug resistance and therapeutic target. *J Exp Clin Cancer Res*. 2023;42:148.
10. Zhu C, Wei Y, Wei X. AXL receptor tyrosine kinase as a promising anti-cancer approach: functions, molecular mechanisms and clinical applications. *Mol Cancer*. 2019;18:153.
11. Rankin EB, Giaccia AJ. The Receptor Tyrosine Kinase AXL in Cancer Progression. *Cancers (Basel)*. 2016;8:103.
12. Braunger J, Schleithoff L, Schulz AS, Kessler H, Lammers R, Ullrich A, Bartram CR, Janssen JW. Intracellular signaling of the Ufo/Axl receptor tyrosine kinase is mediated mainly by a multi-substrate docking-site. *Oncogene*. 1997;14:2619–31.
13. Cavet ME, Smolock EM, Ozturk OH, World C, Pang J, Konishi A, Berk BC. Gas6-axl receptor signaling is regulated by glucose in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2008;28:886–91.
14. Zhou J, Yang A, Wang Y, Chen F, Zhao Z, Davra V, Suzuki-Inoue K, Ozaki Y, Birge RB, Lu Q, Wu Y. Tyro3, Axl, and Merck receptors differentially participate in platelet activation and thrombus formation. *Cell Commun Signal*. 2018;16:98.
15. Majumder A, Hosseini S, Stroud M, Adhikari E, Saller JJ, Smith MA, Zhang G, Agarwal S, Creixell M, Meyer BS, 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*. 2022;20:542–55.
16. Mohammadzadeh P, Amberg GC. AXL/Gas6 signaling mechanisms in the hypothalamic-pituitary-gonadal axis. *Front Endocrinol*. 2023;14:1212104.
17. Antony J, Tan TZ, Kelly Z, Low J, Choolani M, Recchi C, Gabra H, Thiery JP, Huang RY. The GAS6-AXL signaling network is a mesenchymal (Mes) molecular subtype-specific therapeutic target for ovarian cancer. *Sci Signal*. 2016;9:ra97.
18. Goyette MA, Cote JF. AXL receptor tyrosine kinase as a promising therapeutic target directing multiple aspects of cancer progression and metastasis. *Cancers (Basel)*. 2022;14:466.
19. Colavito SA. AXL as a target in breast cancer therapy. *J Oncol*. 2020;2020:5291952.
20. Fridell YW, Jin Y, Quilliam LA, Burchert A, McCloskey P, Spizz G, Varnum B, Der C, Liu ET. 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*. 1996;16:135–45.
21. Allen MP, Zeng C, Schneider K, Xiong X, Meintzer MK, Bellosta P, Basilico C, Varnum B, Heidenreich KA, Wierman ME. 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*. 1999;13:191–201.
22. Grosse R, Schmid A, Schoneberg T, Herrlich A, Muhn P, Schultz G, Gudermann T. Gonadotropin-releasing hormone receptor initiates multiple signaling pathways by exclusively coupling to G(q/11) proteins. *J Biol Chem*. 2000;275:9193–200.
23. Mulvaney JM, Zhang T, Fewtrell C, Roberson MS. Calcium influx through L-type channels is required for selective activation of extracellular signal-regulated kinase by gonadotropin-releasing hormone. *J Biol Chem*. 1999;274:29796–804.
24. Mulvaney JM, Roberson MS. Divergent signaling pathways requiring discrete calcium signals mediate concurrent activation of two mitogen-activated protein kinases by gonadotropin-releasing hormone. *J Biol Chem*. 2000;275:14182–9.
25. Dang AK, Murtazina DA, Magee C, Navratil AM, Clay CM, Amberg GC. GnRH evokes localized subplasmalemmal calcium signaling in gonadotrophs. *Mol Endocrinol*. 2014;28:2049–59.
26. 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. *J Biol Chem*. 2003;278:47307–18.
27. Watt M, Mohammadzadeh P, Pinski E, Hollinshead FK, Bouma GJ. Corticotropin releasing hormone is present in the feline placenta and maternal serum. *Front Endocrinol*. 2023;14:1132743.
28. Ruf-Zamojski F, Ge Y, Pincas H, Shan J, Song Y, Hines N, Kelley K, Montagna C, Nair P, Toufaily C, et al. Cytogenetic, genomic, and functional characterization of pituitary gonadotrope cell lines. *J Endocr Soc*. 2019;3:902–20.
29. Windle JJ, Weiner RI, Mellon PL. Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. *Mol Endocrinol*. 1990;4:597–603.
30. Alarid ET, Windle JJ, Whyte DB, Mellon PL. Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. *Development*. 1996;122:3319–29.
31. Bliss SP, Navratil AM, Xie J, Roberson MS. GnRH signaling, the gonadotrope and endocrine control of fertility. *Front Neuroendocrinol*. 2010;31:322–40.
32. 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*. 2008;27:4044–55.
33. Zhu Y. Metalloproteases in gonad formation and ovulation. *Gen Comp Endocrinol*. 2021;314: 113924.
34. Navratil AM, Knoll JG, Whitesell JD, Tobet SA, Clay CM. Neuroendocrine plasticity in the anterior pituitary: gonadotropin-releasing hormone-mediated movement in vitro and in vivo. *Endocrinology*. 2007;148:1736–44.
35. Clay CM, Cherrington BD, Navratil AM. Plasticity of anterior pituitary gonadotrope cells facilitates the pre-ovulatory LH surge. *Front Endocrinol (Lausanne)*. 2020;11: 616053.
36. Justus CR, Marie MA, Sanderlin EJ, Yang LV. Transwell in vitro cell migration and invasion assays. *Methods Mol Biol*. 2023;2644:349–59.
37. Grosse R, Roelle S, Herrlich A, Hohn J, Gudermann T. Epidermal growth factor receptor tyrosine kinase mediates Ras activation by gonadotropin-releasing hormone. *J Biol Chem*. 2000;275:12251–60.
38. Shah BH, Soh JW, Catt KJ. Dependence of gonadotropin-releasing hormone-induced neuronal MAPK signaling on epidermal growth factor receptor transactivation. *J Biol Chem*. 2003;278:2866–75.
39. 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: e266.
40. 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*. 2008;149:6311–25.

41. Bliss SP, Miller A, Navratil AM, Xie J, McDonough SP, Fisher PJ, Landreth GE, Roberson MS. ERK signaling in the pituitary is required for female but not male fertility. *Mol Endocrinol*. 2009;23:1092–101.
42. 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*. 2009;81:1206–15.
43. Thompson IR, Ciccone NA, Zhou Q, Xu S, Khogeer A, Carroll RS, Kaiser UB. GnRH Pulse Frequency Control of Fshb Gene Expression Is Mediated via ERK1/2 Regulation of ICER. *Mol Endocrinol*. 2016;30:348–60.
44. Naor Z. Signaling by G-protein-coupled receptor (GPCR): studies on the GnRH receptor. *Front Neuroendocrinol*. 2009;30:10–29.
45. Grundker C, Emons G. The Role of Gonadotropin-Releasing Hormone in Cancer Cell Proliferation and Metastasis. *Front Endocrinol (Lausanne)*. 2017;8:187.
46. Bellosa P, Costa M, Lin DA, Basilico C. The receptor tyrosine kinase ARK mediates cell aggregation by homophilic binding. *Mol Cell Biol*. 1995;15:614–25.
47. Allen MP, Linseman DA, Udo H, Xu M, Schaack JB, Varnum B, Kandel ER, Heidenreich KA, Wierman ME. Novel mechanism for gonadotropin-releasing hormone neuronal migration involving Gas6/Ark signaling to p38 mitogen-activated protein kinase. *Mol Cell Biol*. 2002;22:599–613.
48. Qiao S, Nordstrom K, Muijs L, Gasparoni G, Tierling S, Krause E, Walter J, Boehm U. Molecular plasticity of male and female murine gonadotropes revealed by mRNA sequencing. *Endocrinology*. 2016;157:1082–93.
49. Bhalla S, Gerber DE. AXL inhibitors: status of clinical development. *Curr Oncol Rep*. 2023;25:521–9.
50. Ozyurt R, Ozpolat B. Therapeutic landscape of AXL receptor kinase in triple negative breast cancer. *Mol Cancer Ther*. 2023;22(7):818–32.
51. Zhai X, Pu D, Wang R, Zhang J, Lin Y, Wang Y, Zhai N, Peng X, Zhou Q, Li L. Gas6/AXL pathway: immunological landscape and therapeutic potential. *Front Oncol*. 2023;13:1121130.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

