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Laryngoscope. Author manuscript; available in PMC 2024 December 01.

Published in final edited form as:

Laryngoscope. 2023 December ; 133(12): 3506–3511. doi:10.1002/lary.30856.

Localizing Hormone Receptor Expression to Cellular Compartments in Idiopathic Subglottic Stenosis

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Abstract

Objectives: Idiopathic subglottic stenosis (iSGS) is an unexplained progressive fibrosis of the upper airway. iSGS almost exclusively affects women; as a result, female hormones (estrogen and progesterone) have been proposed to participate in the pathogenesis of iSGS. Our aim was to localize cell-specific gene expression of estrogen receptors (*ESR1* & *ESR2*) and progesterone receptor (*PGR*) using an established iSGS single-cell RNA sequencing (scRNAseq) cell atlas.

Study Design: *Ex vivo* molecular study of airway scar and healthy mucosa from iSGS patients.

Methods: An established scRNAseq atlas consisting of 25,974 individually sequenced cells from subglottic scar (n=7) or matched unaffected mucosa (n=3) in iSGS patients was interrogated for RNA expression of *ESR1*, *ESR2*, and *PGR*. Results were quantified and compared across cell subsets, then visualized using Uniform Manifold Approximation and Projection (UMAP). Confirmatory protein assessment of endocrine receptors in fibroblasts from iSGS patients (n=5) was preformed via flow cytometry.

Results: The proximal airway mucosa in iSGS patients demonstrates differential expression of endocrine receptors (*ESR1*, *ESR2*, *PGR*). Within airway scar, endocrine receptors are primarily expressed by fibroblasts, immune cells, and endothelial cells. Fibroblasts show strong *ESR1* and *PGR* expression, while immune cells possess RNA for both *ESR1* and *ESR2*. Endothelial cells predominantly express *ESR2*. Epithelial cells in unaffected mucosa express all 3 receptors, which are all reduced in airway scar.

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Disclosures: The authors report no conflicts of interest.

This appeared as a podium presentation at the Triological Society 2023 Combined Sections Meeting, to be held January 26–28, 2023, in Coronado, California.

Level of Evidence: N/A; Basic science

Conclusions: scRNAseq data localized endocrine receptor expression to specific cell subsets. These results provide the foundation for future work interrogating how hormone-dependent mechanisms promote, sustain, or participate in iSGS disease pathogenesis.

Keywords

idiopathic subglottic stenosis; iSGS; estrogen; progesterone; single-cell atlas

INTRODUCTION

Idiopathic subglottic stenosis (iSGS) is an unexplained progressive obstruction of the upper airway that occurs almost exclusively in perimenopausal Caucasian women.[1] The disease is characterized by mucosal inflammation and localized fibrosis, resulting in life-threatening upper airway blockage.[2] The pathogenesis of iSGS is hypothesized to involve an environmental trigger coupled with epithelial barrier dysfunction,[3] leading to a localized inflammatory response. This inflammation then triggers robust fibroblast activation, leading to the extensive extracellular matrix (ECM) deposition and scar formation.[4,5,6] The high recurrence rate of subglottic scar in iSGS requires patients to undergo frequent invasive interventions to maintain airway patency, contributing to the significant morbidity of the disease.[7] The triggers driving both disease initiation and recurrence have not yet been defined, leaving current treatments unable to address the underlying physiologic derangement.

Female sex hormones (estrogen and progesterone) are hypothesized to play a role in both the observed inflammatory process and fibrotic response in iSGS as this disease disproportionately affects women.[1] 17β-estradiol, the natural estrogen receptor (ER) agonist, can bind to either ER-α or ER-β, which are encoded by the genes *ESR1* and *ESR2*, respectively (Figure 1A).[8] Recently, it has also been demonstrated that estrogen can also activate a membrane-bound G protein-coupled estrogen receptor (GPER1),[9] which initiates rapid intracellular signaling cascades. Both the nuclear receptors (ER-α & ER-β) and GPER1 can modulate nongenomic signaling pathways. Similarly, progesterone's effects are primarily transduced through genomic activation via progesterone receptor (PR), which is encoded by the gene *PGR* (Figure 1B). Progesterone can also exert nongenomic effects via membrane PR (mPR) activation.[10]

Although the molecular mechanisms of estrogen and progesterone signaling have been explored in myriad other disease states, their involvement in iSGS pathogenesis remains undefined. Prior reports demonstrated overexpression of ER-α, ER-β, and PR within iSGS scar tissue compared to healthy controls.[11,12] Furthermore, iSGS scar overexpress ER-α and PR compared to iatrogenic subglottic stenosis scar, supporting the potential role of female sex hormones in the pathogenesis of iSGS.[13] While the presence of these hormone receptors within iSGS scar has been established, the localization of these receptors within different cell subsets and their mechanistic role in the pathogenesis of iSGS has not been explored.

The objective of this study was to localize cell-specific gene expression of *ESR1*, *ESR2*, and *PGR* using an established iSGS scRNAseq cell atlas. We then applied flow cytometric techniques to confirm protein expression of ER-α, ER-β, and PR within specific cell subsets

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in iSGS scar and matched healthy airway mucosa. Our results highlight the potential role of female sex hormones in iSGS pathogenesis.

MATERIALS & METHODS

This study was performed in accordance with the Declaration of Helsinki and good clinical practice and was approved by the Institutional Review Board at Vanderbilt University Medical Center, Nashville, Tennessee (140429).

Sample tissue collection and fibroblast isolation:

Diagnosis of iSGS was made according to standard clinical exclusionary criteria.[2] *scRNAseq*: We used an established scRNAseq atlas, and methods for sample tissue collection were previously described.[14] *Fibroblast isolation*: Subglottic scar biopsies were collected from 5 iSGS patients undergoing routine endoscopic intervention, along with a matched healthy airway specimen at the level of the fourth-fifth tracheal ring. Primary fibroblast cell lines isolated via previously described methods[15] were then used for downstream analysis.

Flow cytometry:

Fibroblasts derived from iSGS patient subglottis biopsies were used for flow cytometry to determine presence of ER-α, ER-β, and PR. Single-cell suspensions were prepared [concentration $1*10^6$/ml]. Cells were preincubated with 5ul Fc receptor blocking solution (Human TruStain FcX™, BioLegend inc, San Diego Ca. Cat. #422301) in 50ul PBS/2%FCS (Phosphate Buffered Solution, Fetal Calf Serum) cells for 10min at 4°C to block nonspecific staining. Cells were then incubated 30 minutes at 4°C in a staining containing a pre-titrated, optimized concentration (5μg) of fluorescent monoclonal antibodies or isotype-matched control (Supplemental Table S1). After incubation, cells were washed twice, pelleted by centrifugation, and resuspended in PBS/2%FCS for flow cytometric analysis. All flow cytometry experiments were acquired with an LSR-II flow cytometer (BD Biosciences) and analyzed using FlowJo (FlowJo, LLC, Ashland, OR). *Gating*: Cells were gated by FSC/SSC, doublets were gated out, dead cells were excluded, and live cells were analyzed for ER-α, ER-β, and PR expression.

Single-cell RNA sequencing:

North American Airway Collaborative (NoAAC) iSGS Cell Atlas: We previously published[14] on the distribution and phenotype of cellular populations present in iSGS airway scar in 7 iSGS patients and 3 matched unaffected controls using scRNAseq (Supplemental Table S2). Sequencing employed the 10x Genomics Chromium platform on 34,000 cells with data integration via Harmony[16] and analysis with Seurat.[17,18] Unsupervised clustering analysis classified the tissue type of each cluster (immune, epithelial, endothelial, mesenchyme) based on PanglaoDB.[19] Identical to prior work, we analyzed our established scRNAseq dataset[14] for *ESR1*, *ESR2*, and *PGR* expression. *Mapping gene localization*: We adapted published methods to define the tissue-specificity of *ESR1*, *ESR2*, and *PGR* in the subglottic scar of iSGS patients.[20,21,22] The expression levels of candidate genes were determined from the average cell expression in the NoAAC scRNAseq cell atlas. The

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percentage of cells expressing *ESR1*, *ESR2*, and *PRG* within a cell type was also tabulated (number positive cells / total cell count for the cluster).

Statistical analysis:

Analyses were conducted with Prism v9.5.0 (GraphPad Software). Nonparametric data were analyzed by Mann-Whitney U test for two variables and by Kruskal-Wallis test for greater than two variables. P values less than 0.05 were considered statistically significant.

RESULTS

Differential expression of *ESR1*, *ESR2*, and *PGR* within distinct cell types from iSGS patients

Cell types were manually grouped into 4 broad tissue classes: (Immune/Epithelial/Endothelial/Mesenchymal) based on their identity and canonical lineage markers (Figure 2A).[14] RNA was quantified in each cell within a cluster and used to determine the expression of *ESR1, ESR2,* and *PGR* within iSGS scar tissue (Figure 2B). Female sex hormone receptors are primarily expressed by fibroblasts, immune cells, and endothelial cells (Figure 2C). Epithelial cells in unaffected mucosa express RNA for all three hormone receptors, but expression for these receptors was reduced in airway scar (Supplemental Figure S1). Fibroblast subsets demonstrated strong *ESR1* and *PGR* expression. Endothelial cells predominantly express *ESR2,* while also expressing modest *ESR1* and *PGR*. While immune cells broadly possess RNA for *ESR1* and *ESR2*, closer inspection of different types of immune cells reveals varying levels of gene expression for hormone receptors. Notably, both CD4+ T_{Eff} and CD8+ T_{Eff} express *ESR1* and *ESR2*. Macrophages primarily express *ESR1*. Plasma cells express both *ESR1* and *ESR2*, while B cells primarily express *ESR2*.

iSGS fibroblast expression of ER-α, ER-β, and PR

Fibroblast activation is a key driver in extracellular matrix deposition and scar formation. Among the four broad tissue classes, fibroblast subsets demonstrated significant *ESR1* and *PGR* expression compared to other cell types (Figure 3A). We then used flow cytometry to confirm protein expression of ER-α, ER-β, and PR in iSGS fibroblasts (Figure 3B). We found that fibroblasts from iSGS scar and unaffected mucosa demonstrated strong expression of ER-α and PR, while ER-β protein expression was not detected. Fibroblasts from 5 distinct iSGS lines confirm the presence of ER-α and PR and limited expression of ER-β (Figure 3C).

DISCUSSION

Idiopathic subglottic stenosis (iSGS) is a progressive, life-threatening upper airway obstruction characterized by mucosal inflammation and localized fibrosis, almost exclusively affecting perimenopausal Caucasian women.[1] This molecular study localized the gene expression of female hormone receptors (*ESR1*, *ESR2*, *PGR*) to the different cell types found in iSGS airway scar and normal mucosa. Epithelial cells in normal mucosa express RNA for all three hormone receptors, particularly *ESR2*, but expression of these receptors was reduced in airway scar. Scar endothelial cells demonstrated increased *ESR2* expression

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relative to unaffected mucosa. iSGS fibroblasts demonstrated significant *ESR1* and *PGR* expression compared to other cell types, and protein assessment confirmed strong expression of ER-α and PR. Immune cells broadly possess RNA for *ESR1* and *ESR2*, especially CD4+ and CD8+ T_{Eff} cells. Notably, macrophages primarily express *ESR1*, while B cells showed strong *ESR2* expression.

In vitro data and animal models of iSGS suggest that epithelial barrier dysfunction is a key contributor to scar formation in the proximal airway by allowing microbial invasion deep into the airway mucosa, triggering dysregulated immune activation and fibrotic remodeling.[3,4,6] Interestingly, the airway scar epithelium showed reduced expression of all three receptors compared to unaffected mucosa, suggesting that estrogen may have a protective role in epithelial cell function in the proximal airway. These findings are consistent with cutaneous epidermal injury, wherein activation of ER-α and ER-β provide an anti-inflammatory effect during skin repair, and ER-β has an additional role of maintaining epithelial barrier integrity.[23] ER-β signaling also limited epithelial permeability in animal models of colitis as well as patients with inflammatory bowel disease.[24] Furthermore, estrogen reinforces the epithelial barrier and has a protective effect against inflammation triggered by tumor necrosis factor alpha in oral epithelial cells.[25] Taken together, these studies suggest that estrogen may play an important role in maintaining epithelial barrier integrity in the proximal airway and will be an important focus of future research.

Epithelial barrier dysfunction mediates microbial infiltration within the proximal airway, triggering a localized inflammatory response mediated by host immunity.[26] One key component in immune-mediated inflammation is the adaptive immune response, involving both T and B cells.[6,27] T lymphocyte infiltration is significantly increased in iSGS scar tissue and thought to play a critical role in stimulating fibroblast deposition of scar.6 Also, the subglottis in iSGS patients is enriched for adaptive immunologic memory for viral and intracellular pathogens.6 Interestingly, our study revealed that iSGS scar T cells express *ESR1* and *ESR2*, suggesting a possible mechanism in which the T cell response may be modulated by estrogen signaling. Although the role of B cells in iSGS has not been extensively studied, iSGS B cells demonstrate increased *ESR2* expression, providing another potential link between hormonal signaling and adaptive immunity in iSGS. Given the role of B cells (as evidenced by the therapeutic effect of B cell depleting Rituximab therapy) in an alternate etiology of subglottic scar, granulomatosis with polyangiitis (GPA),[28] estrogen signaling in B cell function is also an important future question in iSGS.

Antigen presenting cells (APCs), including dendritic cells (DC) and macrophages, demonstrated differential expression of female hormone receptors in this study, and they have a clear role in airway inflammation and fibrosis. Prior studies described an increased presence of profibrotic M2 macrophages in proximal airway scar, and our observation of increased *ESR1* expression in macrophages provides a potential mechanistic link between hormonal dysregulation and alterations in APC function.[29,30] While proinflammatory M1 macrophages are activated in response to bacterial and viral pathogens, anti-inflammatory M2 macrophages play a key role in normal wound healing and tissue remodeling, linking host immunity and fibrotic response.[29] Active study is currently investigating the role of

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endocrine signaling in macrophages, but the influence of estrogen on macrophage phenotype and function remains undefined.

Fibroblasts are key effector cells responsible for ECM deposition and scar formation.[4,6] The significant *ESR1* and *PGR* expression (with confirmatory protein quantification) provides a potential link between female hormones and pathogenic fibroblast function in iSGS. Abnormal fibroblasts may be influenced by direct hormonal stimulation. Alternatively, local immune cell populations activated by endocrine hormones may drive pathogenic fibroblast function through local contact. Additional functional studies are mandatory to elucidate the hormonal contribution to iSGS fibroblast activity.

While this study provides valuable insight into the expression patterns of female hormone receptors in iSGS, there are several limitations that may be addressed with future research. Specifically, our sample size was limited as we only used 7 patients for scRNAseq and 5 iSGS fibroblast cell lines for protein expression confirmation. Furthermore, control specimens were obtained from matched unaffected tracheal mucosa, a potentially histologically distinct anatomic region.

Also, this study evaluated RNA expression of *ESR1*, *ESR2*, and *PGR* within different cell types but only confirmed protein expression in fibroblasts. Further studies are needed to confirm the protein production of the receptors within each cell type. Fiz et al. utilized immunohistochemistry to demonstrate an imbalance between ER-α, ER-β, and PR in iSGS patients.[12] Fibrotic scar showed ER-α and PR overexpression versus peristenotic tissue and unaffected controls. There was no expression of ER-β in stenosis, whereas it was normally expressed in peristenotic tissue and controls. Using an alternate methodology (immunofluorescence), Damrose et al. showed stenotic tissue expressed both ER-α and ER-β in iSGS airway scar. However, this study suggested significantly greater staining intensity of ER-α in the epithelium, while ER-β was localized to the glands and ducts compared to controls.[11] These discrepancies may relate in the tissues sampled, patient variability, or technical aspects of protein assessments. Both works support the need for continued work into the influence of endocrine hormones and their receptors on the tissue remodeling seen in iSGS.

Functional studies elucidating the effects of estrogen and progesterone on fibroblasts, epithelial cells, and endothelial cells from iSGS patients would provide insight to changes in ECM matrix production, barrier dysfunction, and vascular permeability. Also, studying the hormonal effects on immune cells may identify important contributions to aberrant local inflammation. These experiments could be achieved via direct hormone stimulation of immune cell subtypes *ex vivo* or via interrogation of endocrine influences with *in vitro* model systems. Future research may include downstream mechanisms of hormone signaling, such as genetic variants of ER and PR, stimulation of GPER1 and mPR, and ER-/PR-independent signaling.

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CONCLUSION

scRNAseq data localized female hormone receptor expression to specific cell subsets in iSGS patients, and flow cytometry confirmed ER-α and PR expression in iSGS fibroblasts. These results suggest an unappreciated complexity to estrogen and progesterone signaling given the differential expression of hormone receptors in the distinct cell types that constitute iSGS airway scar. They provide the foundation for future work interrogating how hormone-dependent signaling contributes to epithelial barrier dysfunction, vascular integrity, fibroblast function, and host immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This was a North American Airway Collaborative (NoAAC) Study. Research in the North American Airway Collaborative is supported by Patient-Centered Outcomes Research Institute under award number 1409-22214. This work was supported in part by the NIH/NHLBI grant no. R01HL146401 (Alexander Gelbard, MD) and by a 2018 Burroughs Wellcome Fund Physician-Scientist Institutional Award to Vanderbilt University (ID: 1018894) (Edward Talatala). The content is solely the responsibility of the authors.

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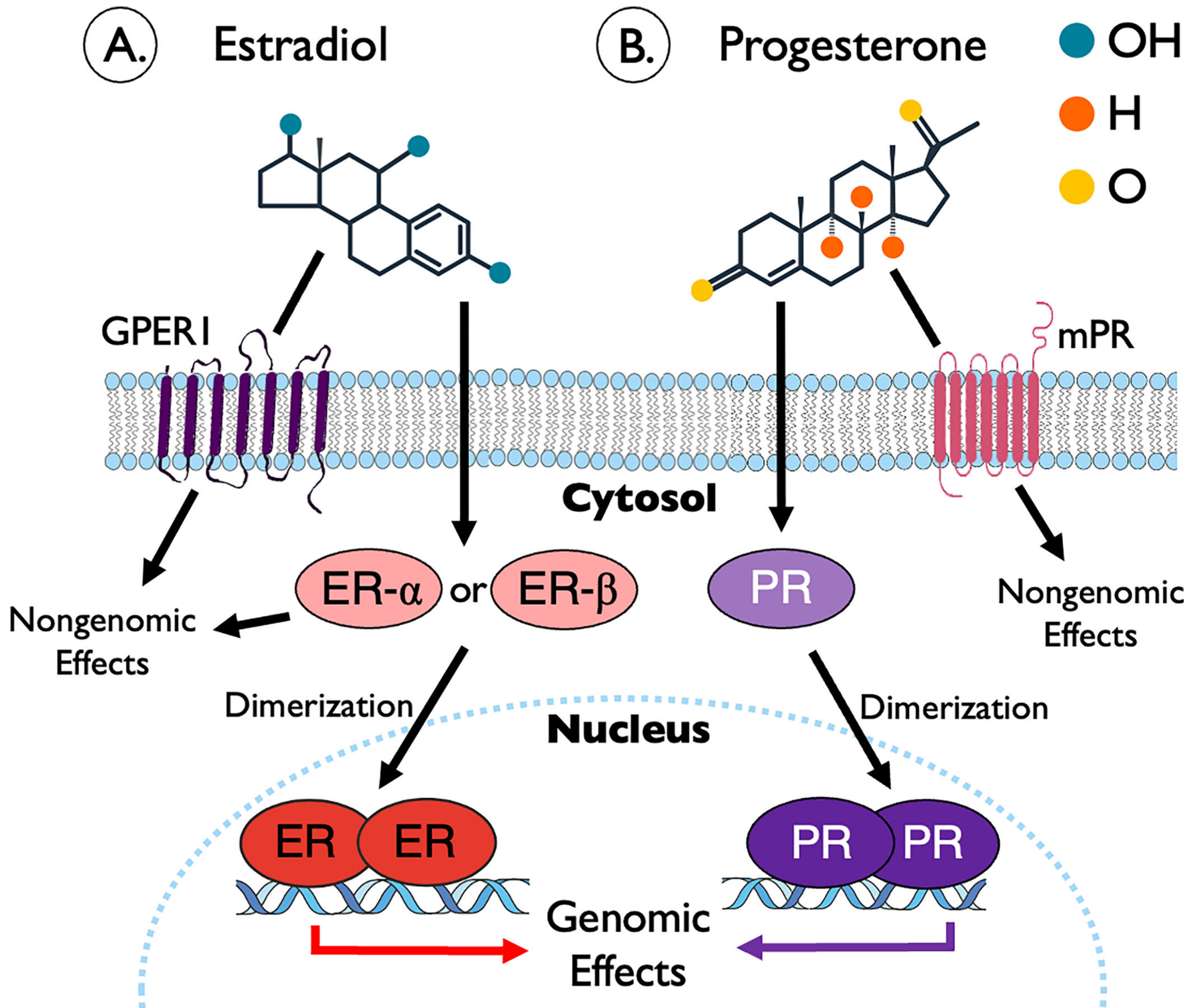

Figure 1. Estrogen and progesterone signaling.

(**A**)17β-estradiol is the natural estrogen receptor (ER) agonist and can bind to either ER alpha (ERα) or beta (ERβ). Estrogen-mediated direct genomic signaling involves hormone binding to estrogen receptors in the cytosol, leading to homo/hetero dimerization (ERα:ERα / ERα:ERβ / ERβ:ERβ). The ER dimers then translocate to the nucleus and bind to the DNA on the regulatory regions of estrogen-responsive genes. Estrogen can also activate a membrane bound G protein-coupled estrogen receptor (GPER1), which initiates rapid intracellular signaling cascades such as adenylyl cyclase/cAMP and EGFR/MAPK. Both the nuclear receptors (ERα & ERβ) and GPER1 can modulate nongenomic signaling pathways. (**B**) Progesterone's effects are primarily transduced through ligand binding to progesterone receptors (PR) in the cytosol. Ligand-bound complexes dimerize and translocate to the nucleus where they bind to gene regulatory regions including progesterone response elements (PREs) to initiate transcription of progesterone-responsive

genes. Progesterone can also exert nongenomic effects via binding to a membrane progesterone receptor (mPR).

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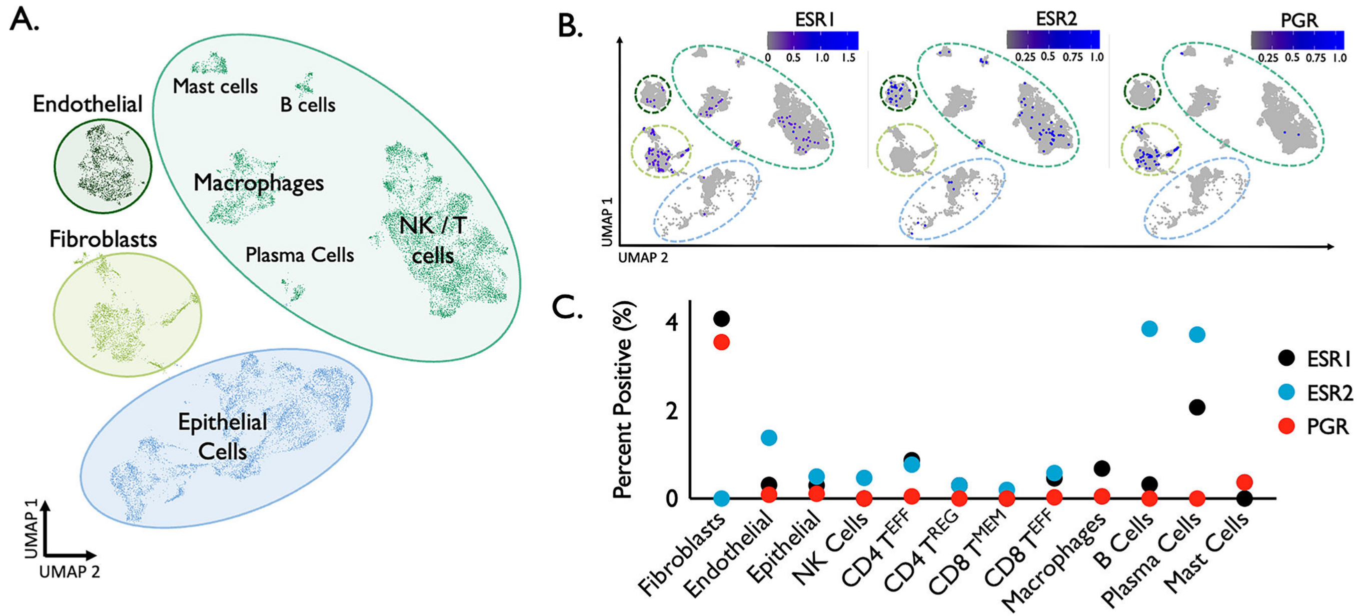

Figure 2. Cell identification and characterization in iSGS.

(**A**) UMAP of jointly analyzed single-cell transcriptomes from 25,974 cells from 7 iSGS mucosal scar and 3 healthy mucosa annotated by cell type. Cell subsets manually grouped into 4 broad tissue classes (Immune/Epithelial/Endothelial/Mesenchymal). (**B**) UMAP highlighting the differential gene expression of *ESR1*, *ESR2*, and *PGR* among the different cell type/states in iSGS airway scar. Blue dots indicate expression; color intensity denotes level of gene expression. (**C**) Graph depicting the percent positivity of expressed hormone receptors for each cell type in iSGS tissue. For cell types with multiple subsets, the average percent positivity is represented. Black dots = *ESR1*; Blue dots = *ESR2*; Red dots = *PR*.

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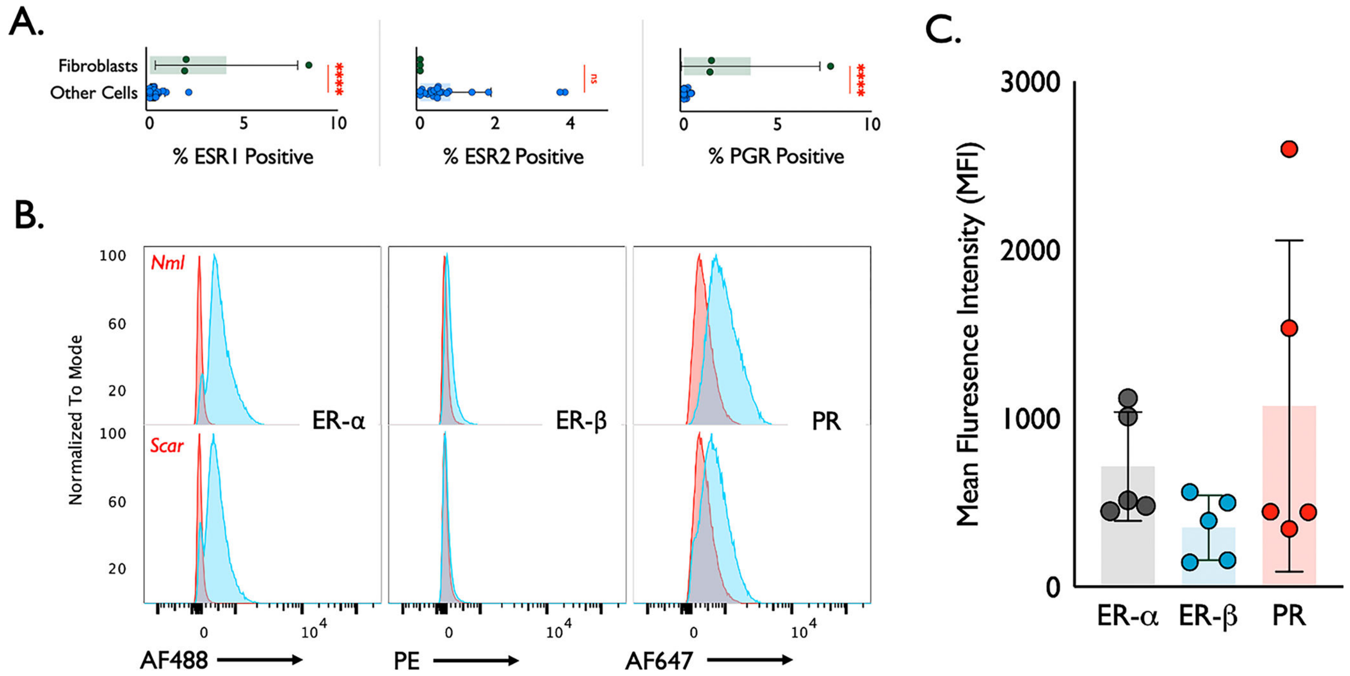

Figure 3. iSGS fibroblast expression of ER and PR.

(**A**) Differential gene expression of hormone receptors in fibroblasts compared to other cells in iSGS patients as determined by scRNAseq. iSGS fibroblasts showed significantly increased *ESR1* and *PGR* expression (both P < 0.001), while demonstrating no *ESR2* expression. Bar represents mean; error bars represent SEM; dots show individual patients. ****P < 0.001; ns = not significant (P > 0.05). (**B**) Representative histograms of fibroblast hormone receptor protein expression, confirming expression data for ER-α, ER-β, and PR in iSGS airway scar. ER-α (Abcam Alexa Fluor® 488); ER-β (Abcam PE); PR (Abcam Alexa Fluor® 647). Red = negative control; Blue = receptor expression. (**C**) Graph depicting the mean fluorescence intensity of ER-α, ER-β, and PR within fibroblasts isolated from airway scar of 5 iSGS patients via flow cytometry. Bar represents mean; error bars represent SEM; dots show individual cell lines.