

Compartment-specific transcriptional programs of the gravid human myometrium underlie labor and uterine atony

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Abstract

Background

The human myometrium undergoes an extraordinary physiological transformation during pregnancy to accommodate the growing fetus and facilitate delivery. Recent single cell RNA sequencing (scRNA-seq) studies mapping the uterine landscape, are technically limited due to underrepresentation of the large, hypertrophied uterine smooth muscle cells (uSMC) essential to pregnancy and parturition. Our high-resolution single nucleus and spatial transcriptomic atlas overcomes these constraints and provides mechanistic insights into uterine function and life-threatening complications such as uterine atony, the leading cause of postpartum hemorrhage (PPH) and global maternal mortality.

Methods

The present report includes comprehensive single-nucleus RNA sequencing (snRNA-seq) data of the human gravid myometrium collected during cesarean delivery from term and preterm, laboring and nonlaboring pregnant patients. Spatial validation of cell-type identities was accomplished with MERFISH and RNAscope. These data were interrogated to define: (1) pregnancy-specific smooth muscle cell signaling; (2) transcriptomic progression during labor; and (3) the molecular underpinnings of PPH due to uterine atony. Findings were validated at the RNA, protein, and functional levels in uSMC at low passage primary culture.

Results

Myometrial samples collected from 15 patients resolved the complex myometrial landscape into two distinct smooth muscle cell and four endothelial cell subtypes. Pregnancy induced follicle stimulating hormone receptor expression (*FSHR*) in uSMC with definitive, functional FSH-FSHR signaling present in uSMC from the pregnant myometrium. With labor, we identified cell-type specific hypoxia signaling and metabolic reprogramming among the fibrovascular niche. We additionally discovered that patients with uterine atony upregulate expression of *ENAH* compared to those with preserved uterine contractility. ENAH is an actin-regulatory protein which orchestrates cytoskeletal dynamics; the physiologic significance of increased ENAH expression is illustrated by a distinct, stellate F-actin architecture in atony uSMC, consistent with diversion of actin into peripheral protrusions at the expense of the organized contractile apparatus required for sustained postpartum contractility.

Conclusions

This snRNA-seq atlas provides a foundational framework for investigation into human myometrial biology. Crucially, it characterizes previously underrepresented uterine smooth muscle cells which define the smooth muscle and endothelial cell subtypes, alongside dynamic metabolic changes in the fibrovascular niche during labor. Our work shifts the understanding of PPH from a simple failure of oxytocin signaling to, in some cases, a pre-defined mechanical state. By uncovering a pre-existing structural predisposition to uterine atony

and a functional *FSHR* axis in the myometrium, this work suggests new avenues for prediction and treatment of PPH.

Introduction

The human uterus changes dramatically during pregnancy, marked by massive expansion in tissue volume and creation of a complex maternal-fetal interface. The myometrium must remain quiescent throughout gestation to accommodate the growing fetus. With labor, the myometrium transitions to contract rhythmically, accomplishing dual goals of fetal delivery with contractions and placental perfusion between contractions. After delivery, the myometrium must again change contractility pattern and generate a powerful, sustained contraction to compress bleeding from the placental bed. Alterations in myometrial contractility at any stage can result in significant complications for the fetus and mother including preterm birth and postpartum hemorrhage (PPH), respectively. Premature birth is the leading cause of death in children under 5 and PPH is the leading global cause of maternal mortality.^{1–3}

Despite the central importance of the uterus in all mammalian biology, insight into the cellular heterogeneity and spatial architecture of the gravid human myometrium remains limited. Single cell transcriptomic studies that map the architecture of the gravid human uterus employing single cell RNA sequencing (scRNA-seq)^{4,5} are technically limited owing to the massive size of its hypertrophied smooth muscle cells: droplet based scRNA-seq have difficulty capturing large uterine smooth muscle cells, which can reach up to 600 microns in length,⁶ leading to an underrepresentation of the very cells so central to uterine function.⁶ This technical blind spot limits the fundamental understanding of the biology of uterine contractility in pregnancy and with it hinders the development of new drugs for conditions such as uterine atony, the failure of the uterine smooth muscle to contract after delivery and the most common cause of PPH.^{7,8}

We hypothesized that single nuclear RNA sequencing (snRNA-seq) would map the transcriptome of the human gravid uterus with a high degree of biologic fidelity, enabling novel findings about uterine contractility. snRNA-seq overcomes the size constraints of scRNA-seq and ensures inclusion of hypertrophied smooth muscle cells. Furthermore, this approach enables uniform processing and analysis of clinically matched, snap-frozen myometrial samples from high quality biobanks thereby reducing noise. Use of snap-frozen tissue samples also mitigates the presence of stress-induced transcriptional signatures caused by the scRNA-seq dissociation process.⁶ We analyzed samples from the preterm, term nonlaboring, and laboring myometrium, as well as patients with severe PPH due to uterine atony, a population not previously explored with single cell transcriptomic analyses.

We further integrated snRNA-seq data with MERSCOPE™ spatial transcriptomics and *in situ* hybridization data in order to generate a high-resolution map of the gravid human myometrium. We characterized two distinct smooth muscle cell and four endothelial cell subtypes, consistent with cell type identification in other organs that have been better characterized up to this point. This cell segmentation enabled several novel insights into uterine physiology and pathophysiology. First, extragonadal Follicle Stimulating Hormone Receptor (*FSHR*) expression in gravid uterine smooth muscle cells is demonstrated. Follow up functional studies underscore a role for this endocrine axis in uterine contractility. Second, a pivotal role for hypoxia in

modulating cell-cell communication during labor is identified. Finally, analysis of samples from patients with PPH due to uterine atony revealed a novel molecular mechanism underlying the contractile failure that characterizes uterine atony. These findings highlight the utility of deep transcriptomic analysis of well characterized, carefully phenotyped clinical samples.

RESULTS

To deeply profile the myometrium of pregnant patients, we combined snRNA-seq, MERSCOPE spatial transcriptomics, protein expression, and functional validation of findings in both intact tissue and cultured uSMC in low passage primary culture (workflows detailed in Fig. 1).

Single nuclear transcriptomic atlas of the gravid myometrium

snRNA-seq was applied to myometrial tissue from 15 patients, including 8 term nonlaboring, 3 term laboring, and 4 preterm nonlaboring patients (clinical metadata is included in Supplemental Table 1). After quality control, the dataset contained 70,644 nuclei. Nuclei were first clustered by canonical lineage markers into a uniform manifold approximation and projection (UMAP) space into mesenchymal, endometrial, endothelial, and immune clusters (Fig. 2A, B). Each patient contributed well over 1000 nuclei to the dataset, ranging from 1,418 to 15,644 nuclei (Fig. 2C, Supplemental Fig. 1).

Nineteen distinct cell subtypes were identified in the gravid myometrium, including two smooth muscle cell populations and four endothelial cell populations (Fig. 2D-F). All 15 patients contributed nuclei to each smooth muscle, endothelial, fibroblast, and trophoblast subpopulation, but some lower abundance cell types including many immune cell types and endometrial cells were disproportionately contributed by fewer patients (Supplemental Fig. 1).

MERSCOPE spatial transcriptomic atlas of the gravid myometrium

To spatially map cell distribution and further validate differentiating genes and cell populations, a MERSCOPE™ single cell spatial transcriptomic panel was created with 500 cell type differentiating genes identified both in the literature and in our snRNA-seq cell segmentation. This panel was applied to three fixed term, nonlaboring myometrial samples (Fig. 3A-B) to validate cell segmentation and define cellular neighborhoods. Spatial transcriptomic analysis distinguished 10 cell subpopulations with uterine smooth muscle cells most well represented (Fig. 3C-E). Consistent with the snRNA-seq data, in which some uterine biopsies contain abundant endometrium and others virtually none (Supplement 1), significant inter-sample heterogeneity was present relative to endometrial and trophoblast sampling among the 3 samples. Higher magnification inspection of gene transcript puncta (Fig. 3G) and the cells types defined by the transcripts (Fig. 3H), as well as neighborhood analysis (Fig. 3I) revealed spatial proximity of vascular, but not uterine, smooth muscle cells with arterial and venous endothelial cells as well as a fibrovascular niche characterized by colocalization of immune, endothelial, vascular smooth muscle, and stromal (fibroblast) cells.

Single-nucleus transcriptomics resolves two distinct smooth muscle cell populations in the gravid myometrium

Our snRNA-seq approach distinguished two transcriptionally distinct smooth muscle cell subtypes, uterine smooth muscle (uSMC, 76% of *ACTA2* positive nuclei) and vascular smooth muscle (vSMC, 24% of *ACTA2* positive nuclei), Fig. 4. In contrast to prior scRNA-seq reports in the human uterus that posited multiple SMC sub-clusters,^{4,5} the present phenotypic cross-validation revealed only a single transcriptionally distinct uSMC population, defined by *OXTR*, *KCNMA1*, *LINGO2*, and *DPP6* expression and a vSMC population not identified in prior gravid uterus atlases, characterized by *NOTCH3* and *PDE3A* expression. The spatial segregation of uSMC and vSMC was absolute, with vSMC localized around vascular structures and uSMC making up contractile bundles (Fig. 4D-F, Supplemental Fig. 2).

Transcriptomic reprogramming of smooth muscle cells of the gravid myometrium:

To define the smooth muscle-specific molecular changes governing the transition from the nonpregnant to gravid uterus, we next performed a cross-dataset comparative analysis. snRNA-seq transcriptomes of uSMC and vSMC populations in the present dataset were contrasted with uSMC and vSMC cell populations derived from the non-pregnant uterus samples included in the *Tabula Sapiens 2.0* scRNA-seq database⁹ (Fig. 5A, Supplemental Table 2).

uSMC change from a metabolically active, housekeeping non-pregnant state to a mechanically active, contractile state in pregnancy characterized by an active gonadotropin signaling pathway (Fig. 5B-C). The non-pregnant uSMC transcriptome was characterized by high expression of dissociation-induced stress markers such as *GADD45A* and *HSPA1B*,^{10,11} and protein synthesis machinery including numerous ribosomal proteins (*RPLP1*, *RPS8*) reflecting use of the scRNA-seq, rather than snRNA-seq technique.⁶ In contrast, the transcriptome of gravid uSMC was dominated by myofilament organization (*ACTG2*, *LMOD1*),¹² excitation-contraction coupling (*CAV3*, *SCN8A*)¹³, and an endocrine signaling interface characterized by a 20-fold increase in oxytocin receptor (*OXTR*) density. The gravid uterine smooth muscle transcriptome was also characterized by the emergence of gonadotropin receptors (*FSHR*, *LHCGR*) that are virtually absent in the uSMC from the nonpregnant uterus, indicative of a population-wide shift in uSMC identity.

vSMC, in contrast, changed from a contractile phenotype in the non-pregnant state (*TAGLN* and *CNN1* high) to a relaxed state characterized by a coordinated upregulation of the nitric oxide sensing machinery (soluble guanylate cyclase subunits *GUCY1A2*, *GUCY1B1*) and downstream effector kinase *PRKG1* to promote vasodilation, supporting the profound increase in uterine blood flow of pregnancy (Fig. 5D-F). Moreover, increased expression of basement membrane and interstitial collagen (*COL1A2*, *COL3A1*, *SLIT3*, *ITGA1*) molecules likely promote vascular expansion in pregnancy.

Follicle Stimulating Hormone Receptor (FSHR) is functionally expressed in gravid uterine smooth muscle cells

Analysis revealed cell-type specific expression of *FSHR* in (Fig. 6A) gravid uSMC, but not in non-gravid uSMC (Fig. 5B-D). This finding was particularly notable given the contested role of extragonadal FSHR in peripheral tissues.^{14,15} To confirm expression of *FSHR* transcripts in gravid uSMC, as opposed to detection of ambient RNA or transient expression, RNAscope *in situ* hybridization was performed on primary uSMC isolated from the gravid myometrium of 6 patients. These analyses confirmed high density *FSHR* mRNA presence in uSMC (Fig. 6B, Supplemental Fig. 3).

To determine physiologic activity of FSHR, we performed dynamic calcium imaging in primary human uSMC. Recombinant human FSH (rhFSH) increased cytosolic calcium in gravid uSMC to a similar degree as oxytocin (Fig. 6C, D), confirming the presence of physiologically significant FSH-FSHR signaling in gravid uSMC.

Endothelial cell subpopulations of the gravid human uterus

With snRNAseq, the endothelial lineage was defined by 4 marker transcripts (*CD34*, *CDH5*, *PCAM1*, and *VWF*). We identified four distinct endothelial cell populations representing lymphatic, arterial, venous, and capillary endothelial cell types (Fig. 7A). While the arterial (*GJA5*) and lymphatic endothelial (*RELN*, *CD36*) were defined by canonical markers that consistently identify these cell types in other tissues¹⁶, the gravid uterine venous endothelial niche was uniquely characterized by the enrichment of *CPE*, *NRG3*, and *RYR3*, suggesting a distinct regulatory program governed by neuropeptide processing, juxtacrine signaling with smooth muscle, and specialized calcium-homeostasis mediating vasomotor control. To further refine the endothelial annotation, we identified a 'macrovascular' gene signature consisting of *SULF1*, *PDE3A*, and *COL8A1*. These genes were robustly expressed in both arterial and venous endothelial populations, reflecting the structural requirements of large-caliber uterine vessels, but were notably absent in the capillary bed (Fig. 7B). Using spatial transcriptomics, we validated anatomical distribution of endothelial populations, confirming discrete localization of lymphatic, arterial, and venous/capillary signatures within the myometrium (Fig. 7C). Consistent with classic vascular architecture, spatial mapping revealed a significantly higher density of vSMC associated with arterial endothelium than its venous counterpart, corroborating the high-pressure conduit identity of the arterial niche in the gravid uterus (Fig. 7D).

The transcriptional landscape of labor

To characterize the global transcriptional landscape of parturition, snRNA-seq data from term laboring (n = 3) and term nonlaboring (n = 8) myometrial tissue biopsies were compared. The comparative analysis was focused most squarely on the nine most abundant cell types in myometrial-vascular niche that includes smooth muscle cells, fibroblasts, endothelial cells, and myeloid cells. This analysis revealed a coordinated, tissue-wide response centered on metabolic adaptation, structural remodeling, and hypoxia-driven signaling in labor (Fig. 8, Supplemental Table 3).

Uterine smooth muscle cell activation

In uSMC, labor is characterized by a shift in membrane excitability and metabolic demand, achieved through the release of molecular brakes. This transition is marked by the downregulation of the sodium leak channel

NALCN, a gene that typically dampens spontaneous contractility to maintain quiescence in gestation.¹⁷ In labor, the uSMC also activate a suite of genes critical for energy salvage and coordinated contraction including the metabolic enzyme *NAMPT*, the calcium sensor *CPNE5*, and the embryonic myosin heavy chain *MYH3*. In the laboring myometrium samples from our patient cohort, labor is also accompanied by downregulation of the oxytocin receptor *OXTR* in laboring myometrium, representing a known negative feedback loop in which oxytocin stimulation leads to relative transcriptional silencing¹⁸ (Fig. 8A).

Vascular smooth muscle cell activation

Within the vSMC compartment, the transcriptional profile in labor shifted toward an activated, hypoxia-responsive phenotype. This included the induction of stress-responsive genes (*SHC4*, *LDLR*, and the potassium channel *KCNK3*), and contractile machinery regulators (*MYH3*, *ITGA5*, and the basement membrane component *COL15A1*), suggesting the maternal vasculature undergoes functional remodeling to manage the hemodynamic pressures of labor. This transition was underpinned by profound hypoxia signaling signatures with significant increases in *HIF1A*, the nitric oxide regulator *DDAH1*, and *NAMPT*, suggesting vSMC responsiveness to the intermittent ischemia of labor (Fig. 8B).

Myeloid-driven pro-inflammatory signaling

Myeloid cells exhibited a transcriptional shift from immune quiescence to active inflammation and tissue modification in labor. Matrix remodeling programs (including *VCAN1*, *FN1*, *ADAMTS9*, and *THBS1*), and acute immune activation reflected by increased *HSPA1A* (stress chaperone) and *IL1R2* (the decoy receptor, characteristic of myeloid activation in high-turnover inflammatory environments)¹⁹ were prominent. This pro-inflammatory surge was accompanied by significant downregulation of antigen-presentation and maternal-fetal tolerance genes, specifically *HLA-DQA1*, *HLA-DMB*, *CCL21*, and complement regulator *SERPING1*. This suppression of tolerance-related programs is consistent with the inflammatory theory of labor, where the breakdown of local immune privilege is required for the transition into an active, pro-labor state^{5,20} (Fig. 8C).

Hypoxia signaling in fibroblasts

The vessel-associated fibroblast 1 population exhibited a signature consistent with hypoxia-induced stress in labor. This was evidenced by dramatic induction of *HIF1A* and its canonical downstream effector *VEGFA*, suggesting that these cells act as primary sensors and responders to the transient ischemia induced by labor contractions (Fig. 8D).

Endothelial cell remodeling

Labor induced specialized transcriptional programs across the myometrial vasculature. Venous endothelial cells (VECs, Fig. 8E) demonstrated a robust hypoxia and proinflammatory response, significantly upregulating *HIF1A*, *ICAM1*, *NAMPT*, and *IL6* alongside a vascular remodeling module defined by *ADAMTS4*, *ADAMTS9*, and *COL4A1*. VECs also showed an increased contractile identity (*MYH11*, *CNN1*). Capillary endothelial cells (CECs, Fig. 8F) also showed upregulation of *HIF1A* as well as glycolytic regulator *PFKFB3*. CECs also downregulated steady state, quiescence-associated transcripts such as *FABP4*, indicating a shift in metabolism away from fatty acid transport and toward glycolysis and *CDKN1C*, releasing a molecular

brake on the cell cycle in laboring cells. Together, these findings suggest that while the entire endothelial bed senses hypoxia, the venous system undergoes more intensive structural and inflammatory reconfiguration compared to the metabolic shifts observed in capillaries.

Cell-specific induction of HIF1A

The thematic emergence of hypoxia signaling across multiple cell subtypes led us to specifically interrogate *HIF1A* across multiple cell types (Fig. 8F). While HIF1a protein is primarily regulated via oxygen-dependent proteasomal degradation, hypoxic stress in the laboring myometrium increases *HIF1A* transcription²¹. We observed marked increases in *HIF1A* transcripts in vSMC, both fibroblast populations, and all four endothelial cell clusters, whereas uSMC levels remained stable (Fig. 8G). Given that labor contractions cause transient but repeated myometrial hypoxia, and that hypoxia enhances myometrial contractile force, these results suggest a transcriptional response to low oxygen tension states primarily in the vascular compartment.

Intercellular coordination of the hypoxia response

To determine how this hypoxia program might be coordinated across the gravid myometrial tissue, we mapped the cell-cell communication landscape for HIF-responsive ligand-receptor pairs (Fig. 8H, Supplemental Table 4). This analysis revealed a high-density network in which fibroblasts and myeloid cells are dominant signaling hubs, utilizing HIF-driven ligands like *VEGFA*, *FN1*, and *NAMPT* to communicate with integrin receptors on the vascular and contractile machinery. This points to a tissue-wide hypoxia-response program that might synchronize metabolic adaptation and structural remodeling to meet the mechanical demands of labor and delivery.

Transcriptional and Structural Antecedents of Uterine Atony

To investigate whether baseline transcriptional states predispose the myometrium to contractile failure, we performed pseudobulk differential expression analysis on snRNA-seq data from uSMC. We compared term nonlaboring patients who developed clinical uterine atony and PPH despite prophylactic oxytocin and second line uterotonic administration (n = 4) to gestational age, maternal race/ethnicity, and maternal comorbidity-matched term nonlaboring control patients who exhibited optimal postpartum contractility (n = 4).

Differential expression analysis revealed a distinct transcriptomic signature in uSMC from patients with uterine atony characterized by upregulation of genes governing membrane stability and cytoskeletal organization (Fig. 9A, Supplemental Table 5). Notably, expression of hallmark contractility and labor-associated transcripts including *OXTR* and metabolic regulators like *NAMPT* were consistent between groups, suggesting that clinical failure to respond to oxytocin was not due to a defect in primary hormonal sensing. Instead, atonic uSMC significantly upregulated a “cytoskeletal support” gene program including DMPK (Dystrophin Myotonia Protein Kinase), which regulates myosin phosphatase and cytoskeletal stabilizing proteins, and SGCA (Sarcoglycan Alpha). This molecular profile suggests that the atonic myometrium may possess an underlying structural resistance to contractility.

Central to this structural program was the actin regulator ENAH (Fig. 9B), a member of the Ena/VASP family that binds the barbed ends of actin filaments to antagonize capping and promote elongation of long, unbranched filaments.²² Mapping of ENAH expression onto our snRNA-seq global manifold confirmed its specificity to the smooth muscle compartments (Fig. 9C). Although ENAH did not reach genome-wide significance after multiple-testing correction (adjusted $p = 0.33$), its nominal significance ($p = 0.004$), high basal expression in uSMC (> 99 th percentile of expressed genes), and membership in a coherent cytoskeletal program prompted targeted validation. Because Ena/VASP-driven actin elongation localizes to the cell periphery and competes with the organized cortical remodeling required for smooth muscle force generation,²³ we identified ENAH as a leading candidate contributing to the contractile failure of uterine atony.

To validate these findings at the RNA level, we utilized primary uSMC cultures derived from an independent, larger cohort of uterine atony ($n = 8$) and control ($n = 8$) patients. Using RNAscope™ *in situ* hybridization, we confirmed a significant increase in *ENAH* transcripts within the atony-derived uSMC (Fig. 9D-E).

Elevated ENAH protein was also apparent in both frozen myometrial tissue and uSMC in primary culture. We applied acquisition liquid chromatography tandem mass spectrometry to snap frozen myometrial tissue from 20 patients (10 with uterine atony and 10 matched controls). After normalization to smooth muscle content, ENAH was 1.44-fold higher in atony than in control myometrium ($p = 0.022$; Fig. 9F). The result was concordant in a covariate-adjusted model that estimated the relationship between ENAH and smooth muscle content from the data (1.30-fold, $p = 0.035$). The increase was not uniform across the atony group: values were distributed bimodally, with 5 of 10 atony specimens exceeding the highest control value and the remaining 5 falling within the control range, whereas control specimens were tightly clustered. Western blot analysis of uSMC also revealed higher ENAH protein in cells derived from women with uterine atony than matched controls ($n = 6$) (Supplemental Fig. 5).

Finally, we sought to determine if this molecular program altered cell phenotype. Phalloidin staining of F-actin revealed a qualitative phenotypic difference. Whereas control uSMC frequently displayed a flexible, lattice-like actin network with largely smooth edges, uSMC from patients with uterine atony displayed a markedly different F-actin architecture characterized by prominent, ENAH-positive stellate protrusions at the cell periphery (Fig. 9H), in contrast to the elongated, smoother-edged morphology of controls. This peripheral, protrusive actin distribution is the expected morphological consequence of Ena/VASP overactivity and suggests that ENAH-high atony uSMC divert actin assembly toward unbranched peripheral protrusions and away from the organized contractile apparatus, providing a structural correlate for impaired postpartum contractility.

DISCUSSION

This study presents the first high-resolution single nucleus and spatial transcriptomic atlas of the human gravid myometrium. By mapping this complex landscape, we resolve the controversy regarding extragonadal follicle-stimulating hormone receptor (FSHR) signaling in the uterus and identify transcriptional signatures that define human labor. This work also represents the first-ever single cell investigation into uterine atony and postpartum hemorrhage (PPH), the leading global cause of maternal mortality. We demonstrate that

idiopathic, non-laboring cases of PPH from uterine atony are characterized by upregulation of the actin-regulator *ENAH*, which may act as a "structural brake" that resists the dynamic remodeling required for postpartum contraction.

Our work advances on prior single-cell atlases of the gravid human myometrium both technically and biologically. The two existing single-cell atlases of human parturition relied on droplet-based scRNA-seq,^{4,5} a platform whose microfluidic size threshold (~ 50–60 µm) cannot accommodate the hypertrophied uterine smooth muscle cells of the gravid uterus; indeed, Pique-Regi and colleagues explicitly documented that a substantial fraction of smooth muscle cells exceeded the encapsulation limit and were lost.⁴ This under-capture has direct consequences for cell-type annotation. The earlier atlas resolved three "smooth muscle" subsets, yet two were delineated by immune-process signatures—neutrophil biology and interferon-γ response—rather than canonical contractile programs, a pattern more consistent with ambient-RNA contamination or doublets in dissociated, inflamed tissue than with genuine muscle subtypes, and the authors acknowledged that only a fraction of smooth muscle cells were accounted for by these phenotypes.⁴ Moreover, neither prior atlas distinguished vascular from uterine smooth muscle or resolved the arterial, venous, capillary, and lymphatic endothelium defined here. By recovering intact nuclei irrespective of cell size, our snRNA-seq approach overcomes this capture bias and resolves two transcriptionally distinct smooth muscle populations and four endothelial subtypes, providing the cellular granularity required to assign labor- and atony-associated programs to specific compartments.

Defining a discrete uSMC cell type enabled resolution of long-standing controversy surrounding extragonadal *FSHR* expression in pregnancy. Our unbiased cross-dataset comparison between the gravid myometrium and the non-pregnant *Tabula Sapiens 2.0* reference⁹ identified a robust, cell-type-specific induction of the *FSHR* axis in uSMC during pregnancy. These results build upon the conflicting findings of Stilley and colleagues¹⁵ who reported elevated FSHR protein in laboring compared to nonlaboring myometrium and that FSH promoted uterine contractility via IP3-mediated mechanisms versus Palak and colleagues¹⁴ who more recently reported a complete lack of FSHR expression in pregnant and nonpregnant myometrium and no impact of treatment with recombinant FSH upon pregnant or nonpregnant myometrial contractility. Our findings are supported by validation at the RNA, protein (~ 75kDa glycosylated receptor), and functional levels. The demonstration that rhFSH triggers cytosolic calcium transients in primary uSMC resolves a controversy in the literature and establishes a physiologically active signaling axis, identifying a new endocrine regulatory pathway critical for the pregnant state.

We further leveraged our dataset to define novel molecular frameworks for labor and atony. In our laboring samples, we identified a localized hypoxia-inducible program and metabolic reprogramming concentrated within the fibrovascular niche. This suggests that the physiological stress of labor triggers a HIF-mediated signaling cascade that coordinates the high-energy demands of sustained contractions with a necessary inflammatory response.

Despite methodological differences, our findings converge with prior work on the core biology of labor, lending cross-platform validity to all three datasets. Consistent with the long-recognized inflammatory signature of parturition^{5,24} and with the recent demonstration that HIF1A drives hypoxia-induced enhancer

activation to promote myometrial contractility,²¹ we identify a robust, labor-associated hypoxia-inducible program marked by upregulation of *HIF1A* together with the NAD-salvage enzyme *NAMPT*, the glycolytic regulator *PFKFB3*, *VEGFA*, and the inflammatory mediators *IL6* and *NFKB1*. Notably, *RUNX1* and *IL6*, genes experimentally validated by Ji and colleagues as HIF1A-dependent labor effectors,²¹ are among our most strongly labor-upregulated transcripts. Our cell-type resolution, however, refines the anatomy of this response: the *HIF1A*-anchored hypoxic program is concentrated within the fibrovascular niche—vascular smooth muscle, endothelium, and matrix fibroblasts—with *HIF1A* itself not significantly differentially expressed in uterine smooth muscle, even as broader metabolic remodeling (e.g., *NAMPT*) extended across compartments. This localization supports the established importance of hypoxic *HIF1A* signaling in labor while reframing it as a property of the vascular and stromal microenvironment rather than the contractile myocyte itself. This distinction was accessible only with the cellular resolution of our approach.

This work is perhaps most novel in its exploration of the transcriptomic signatures of uterine atony. Uterine atony is responsible for most cases of PPH.^{7,25} After fetal and placental delivery, sustained uterine smooth muscle contraction is essential to compress rapid hemorrhage from the placental bed which can reach 500 milliliters per minute.^{26 262726} Unfortunately, the pathophysiology underlying uterine atony remains very poorly understood, and about 50% of patients who suffer PPH due to uterine atony during cesarean delivery lack any identifiable risk factors.²⁷ The lack of understanding of the pathophysiology behind most cases of uterine atony hinders efforts to risk stratify patients for clinical care and to develop novel therapeutic agents.

For our snRNA-seq investigation into PPH, we matched low risk, nonlaboring patients who experienced PPH due to uterine atony with controls with robust contraction after fetal delivery. We found consistent upregulation of *ENAH* in uSMC in uterine atony samples, identifying a potential cytoskeletal mechanism for the failure of the postpartum uterus to transition into a sustained tonic state. Increased *ENAH* RNA was confirmed in primary uSMC cultures from an additional 16 patients, and increased protein demonstrated in both myometrial tissue (n = 20) and uSMC cultures (Fig. 9F, Supplemental Fig. 5).

The bimodal structure of this difference is informative. Control myometrium occupied a narrow range of *ENAH* abundance, consistent with a physiological state that must be reliably achieved after delivery, whereas the atony specimens separated into one group indistinguishable from controls and another well above the control range. Uterine atony is defined clinically, by the failure of the uterus to contract adequately after delivery, and a single clinical definition can encompass more than one underlying biology. Heterogeneity of this kind is now well recognized in other syndromes defined by a shared clinical endpoint, in which molecular sub-phenotypes with distinct biology and distinct treatment responses lie within an apparently uniform diagnosis. A uniform shift in *ENAH* across all atony specimens would in fact be difficult to reconcile with the clinical reality that atony likely arises through several routes. We therefore interpret elevated *ENAH* not as a signature of all cases of uterine atony, but as a candidate marker of a significant cytoskeletal sub-phenotype of uterine atony.

The *ENAH* protein functions by binding to the barbed ends of actin filaments to antagonize capping proteins, thereby facilitating the assembly of long, unbranched F-actin fibers that stabilize the cytoskeleton.²³ Rather than stiffening the cell, *ENAH* overexpression may impair contractility by mis-allocating actin: by driving

elongation of unbranched filaments into peripheral, filopodia-like protrusions, ENAH could divert actin away from the organized, force-transmitting contractile apparatus, a model supported by the stellate morphology of atony uSMC. This interpretation parallels vascular smooth muscle, where Ena/VASP proteins promote relaxation,²² and where ENAH is essential for NO-, cAMP-, and cGMP-mediated relaxation through incompletely understood effects on actin dynamics.²⁸ We therefore propose ENAH as a candidate molecular brake on uterine contraction that warrants direct functional testing. While our expression and morphologic data are concordant with this model, establishing causation will require gain- and loss-of-function studies as an important future direction.

The strength of this study lies in its multimodal approach and the rarity of its clinical cohort. Obtaining uterine biopsies from pregnant patients in both laboring and non-laboring states accompanied by robust clinical metadata, particularly those with confirmed idiopathic atony, represents a significant clinical and logistical challenge.

This work has some limitations which warrant consideration. First, our identification of the labor and uterine atony-associated differential gene expression programs derived from a discovery cohort of modest size (n = 3 labor samples compared to 8 nonlaboring samples, and n = 4 uterine atony versus n = 4 control for pseudobulk comparisons), which limits statistical power. Notably, *ENAH* did not survive genome-wide multiple-testing correction in the uterine atony discovery comparison (adjusted p = 0.33). We prioritized this target on the basis of its nominal significance, its high basal expression in uSMC, and its membership in a coherent cytoskeletal program, and we subsequently confirmed its differential expression in an independent cohort of primary uSMC cultures (n = 8 atony, n = 8 control) at the transcript, protein, and morphologic levels. The qualitative differences in F-actin architecture provide support for the notion that altered ENAH activity modulates contraction. Moreover, our cross-platform comparison of the gravid myometrium (snRNA-seq) against a non-pregnant reference (Tabula Sapiens scRNA-seq) may be subject to technical confounding, given divergent approach with single cell compared to single nucleus RNA seq. Finally, our reliance on nuclear RNA may not fully capture rapid post-transcriptional and proteomic shifts, though the concern is mitigated relative to FSHR and ENAH via validation in tissue and cells.

In conclusion, this transcriptomic atlas redefines our understanding of the human myometrium during extraordinary physiological transitions from quiescence to contractility. By uncovering a functional FSHR axis, a labor-specific hypoxia signature, and a structural predisposition to uterine atony, we have moved toward a mechanistic understanding of life-threatening obstetric complications. This work shifts the focus from simple oxytocin responsiveness to complex mechanical and metabolic predispositions, offering a new molecular foundation for the prediction and treatment of postpartum hemorrhage to improve global maternal health outcomes.

Methods

Human myometrial biopsy sample collection

Human myometrial tissue samples were collected with informed consent and study approval from the Stanford Institutional Review Board (IRB) at Lucile Packard Children's Hospital, California, United States

(Protocol 63612, February 2022). Exclusion criteria included suspicion of intraamniotic infection (maternal fever or fetal tachycardia) or known myomas (fibroids).

Myometrial biopsies (1 cm³) were obtained from the midline of the upper margin of the lower uterine segment incision during the cesarean delivery immediately following the delivery of the placenta. Lower uterine segment biopsies exhibit comparable contractile properties to segments taken from the uterine fundus and minimize risk to patients by avoiding a new uterine incision²⁹. This standard tissue procurement technique is widely employed for human uterine contractility studies, as well as cellular and molecular investigations.

Myometrial tissue was transported from the operating room to the laboratory in smooth muscle cell growth medium-2 (SmGM-2, Lonza) on ice and processed within 15 minutes as follows: tissue was sectioned into thirds, with one third immediately frozen in liquid nitrogen, one third used to isolate smooth muscle cells in primary culture, and one third fixed in paraffin.

Clinical sample selection and definitions

Samples were selected to include racially and ethnically diverse patient populations at a variety of gestational ages and contractile states to maximize generalizability of cell type classification and avoid missing cell types important in only some physiologic states. Race and ethnicity were patient-reported at the time of hospital admission.

Patients were defined as laboring if they had at least one hour of painful contractions with progressive cervical dilation and effacement over at least two examinations by obstetricians. Patients were defined as non-laboring if they underwent a scheduled cesarean delivery and had no painful contractions, no rupture of membranes, and no documentation of cervical dilation.

Term nonlaboring samples were classified as “uterine atony” because the patient met all 3 of the following criteria:

- a. The patient required a second-line uterotonic agent (methylergonovine, carboprost, or misoprostol) in addition to oxytocin after delivery.
- b. The patient experienced PPH with quantitative blood loss (QBL) greater than 1000 mL during cesarean delivery. QBL was calculated by weighing surgical pads and sponges, as well as the contents of suction canisters, upon conclusion of the cesarean delivery.
- c. Uterine tone assessment by the obstetrician 10-minutes after placental delivery was less than or equal to 6. For this assessment, the operating obstetrician directly palpates the uterine fundus and rates the tone on a validated 0-10 numeric grading scale (Cole et al., 2022; Koons et al., 2025) in which 0 represents a completely atonic uterus and 10 a firmly contracted uterus.

Uterine atony samples were compared to term nonlaboring “control patients” who met the following criteria:

- a. The patient received only a single prophylactic dose of oxytocin with no oxytocin dose escalation and no second-line uterotonic administration.
- b. The patient did not experience PPH, and QBL was less than 1000 mL at delivery.
- c. The uterine tone assessment 10 minutes after placental delivery was greater than or equal to 8.

Single nuclei RNA sequencing (snRNA-seq)

Nuclei were isolated from flash frozen lower uterine segment, gravid human myometrial tissue from our biobank. cDNA libraries were generated with the Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) according to the manufacturer's instructions (10x Genomics, User Guide CG000315 Rev E).

Sequencing reads were aligned to the Grch38 human genome using Cellranger (v7.1.0). Gene expression count tables were turned into anndata objects and processed using scanpy when not otherwise specified.³⁰ Nuclei that had less than 1000 unique molecular identifiers (UMIs) or 500 unique genes detected were removed. Genes that aligned to the mitochondrial chromosome were removed as contamination. Scrublet was used to automatically detect doublets and then were removed. Counts were then normalized to counts per ten thousand, then log transformed.³¹ To prepare for embedding and clustering, highly variable genes were selected, PCA was ran, and Harmony batch correction was then performed.³² The Leiden algorithm was used for clustering, and Uniform Manifold Approximation and Projection to embed all nuclei.^{33,34} Low quality clusters, those with high ribosomal and ambient RNA signal, were manually removed. Lineage identity was assigned to each leiden cluster based off its expression of marker genes *PECAM1* endothelial, *PAEP* endometrial, *PTPRC* immune, and *COL1A1* mesenchymal. Each lineage was then re-clustered and embedded using the above process, with cell type assigned to each cluster. Cell type markers were generated by running Wilcoxon rank sum tests between cell types. To identify differentially expressed genes (DEGs) within cell populations, pseudobulk samples were generated for each patient using decoupler and differential expression was run using pyDESeq2.^{35,36} Pathway analysis was performed using Metascape.³⁷ For more detail see https://github.com/CarstenKnutsen/Gravid_Myometrium_Atlas/single_cell

Spatial transcriptomics

Gene selection for MERFISH measurements

We used a combination of our snRNA-seq data and literature to select genes for MERFISH. 500 genes were added to a gene list uploaded to the Vizgen panel design portal and checked against all profiled tissues, removing genes that were overexpressed in any individual tissue based on Vizgen's Fragments Per Kilobase Million (FPKM) limiting design guidelines. The final custom gene panel contained a total number of 428 genes (Supplemental data file).

Sample preparation for MERFISH imaging

Myometrial tissue samples were transported in media on ice to the laboratory and immediately fixed for 24 hours in 10% neutral buffered formalin, then subsequently transferred to 70% ethanol for 24 - 36 hours. Tissue samples were processed with the Leica HistoCore Peloris 3 at Stanford Anatomic Pathology & Clinical Laboratories. Paraffin-embedded samples were subsequently processed following the Vizgen User Guide Formalin-Fixed Paraffin-Embedded Tissue Sample Preparation (91600112) at Stanford Human Pathology Histology Services Laboratory. In brief, 5µm thick sections were captured using a Leica RM2255 Automatic Rotary Microtome onto MERSCOPE slides, baked at 55°C for 15 minutes, then further dried and stored according to manufacturer instructions. Parallel 5µm thick tissue sections were deparaffinized, stained with hematoxylin and eosin, and scanned at 40X with the Leica Biosystems Aperio AT2 DX.

The MERSCOPE sample slides subsequently underwent deparaffinization and decrosslinking steps, staining with cell boundary stain (PN 10400118), and hybridization with anchoring probes overnight before being embedded in a thin 4% polyacrylamide gel. Samples were incubated for two hours with a digestion solution at 37°C and then overnight at 47°C in a detergent clearing solution and proteinase K to remove native proteins and lipids while the anchoring probes kept nucleic acids bound to the gel. After clearing for an additional 4 days at 37°C, samples were photobleached using Vizgen's MERSCOPE Photobleacher for 5 hours at room temperature in the clearing solution. Samples were hybridized with encoding probes, then washed in Formamide Wash Buffer (PN 20300002) for 30 min at 47 °C for a total of 2 times to remove excess encoding probes and polyA-anchor probes. Following the MERSCOPE Instrument User Guide (91600001), samples were then washed in Sample Prep Wash Buffer 2 times and stained with DAPI/PolyT mix for 15 min. Finally, slides were loaded into the MERSCOPE Flow Chamber and imaged with imaging kits (PN 10400006) at both 20× and 60× magnification.

Spatial transcriptomic analysis

Cell segmentation was done using Cellpose on the DAPI channel.³⁸ Gene expression count tables were turned into anndata objects and processed using scanpy when not otherwise specified. Cells that had less than 10 puncta or 2 unique genes detected were removed. Punctate counts were normalized to the median of puncta detected per cell, then log normalized. Due to a high dropout MAGIC was used to impute counts across all cells.³⁹ To prepare for embedding and clustering, PCA was run, and Harmony batch correction was then performed. Cell types were manually assigned to Leiden clusters based upon their gene expression profile. Neighborhood graphs were generated using Squidpy.⁴⁰ For more details, see https://github.com/CarstenKnutsen/Gravid_Myometrium_Atlas/spatial

Smooth muscle cell population comparisons between gravid and nonpregnant uterus

To define the molecular transition of the human myometrium, we performed a cross-platform comparison between our single-nucleus (snRNA-seq) dataset of the gravid uterus and the Tabula Sapiens 2.0 single-cell (scRNA-seq) atlas of the non-pregnant human uterus. Functional modules were created by filtering the top 300 DEGs through traditional Metascape, Gene ontology enrichment analysis, and large language model-based filtering (Gemini 3.5 Flash, Google, 2026).

To ensure biological comparability, we compared isolated uterine and vascular smooth muscle cell (uSMC and vSMC) clusters from both datasets. Differential expression analysis was performed by Wilcoxon test. Genes were ranked by their differential scores and adjusted p-value <0.05. Biological modules were identified through a semi-supervised approach. First, the top 200 differentially expressed genes (by score) were subjected to Over-Representation Analysis (ORA) and Gene Set Enrichment Analysis (GSEA) against the GO:Biological Process and Reactome databases. Based on these results, we defined four "Labor-Ready" modules: Muscle Contraction: Genes encoding the core myofilament and regulatory proteins. Excitation-Contraction (E-C) Coupling: Components of the sarcolemmal niches (caveolae) and ion channels governing electrical excitability. Hormonal Sensing: Extragonadal receptors and downstream effectors for systemic endocrine signals. Cytoskeletal Remodeling: Structural proteins required for hypertrophied cell integrity. Conversely, a "Basal/Housekeeping" program was defined by genes upregulated in the non-pregnant state, largely comprising ribosomal biogenesis and protein translation machinery. To visualize the magnitude of change across these biological programs, we calculated Module Scores. These scores represent the mean differential score (derived from the differential expression test) of all constituent genes within a curated module. Individual representative genes were selected to display in the figure based on their ranking within the top 50 DEGs and their established roles in myometrial physiology.

Analysis of differential gene expression functional modules within the snRNAseq dataset

For comparisons between laboring and nonlaboring patients (Figure 8), pseudobulk differential gene expression (DGE) datasets derived from snRNAseq analysis were systematically categorized into functional gene programs across discrete cell lineages. Statistical filtering was first performed for each cell subtype and restricted to genes achieving statistical significance, defined as an adjusted p-value (p-adj) < 0.05. Biological directionality was established according to the dataset's log2-fold change (LFC) encoding architecture, wherein a negative LFC denoted upregulation and a positive LFC denoted downregulation.

Using these parameters, a maximum constraint of 14 key genes was enforced to distill the transcriptomic landscape into curated gene programs across both directions. Computational screening, filtering criteria validation, and the structural aggregation of these bipartite functional gene modules were assisted by the large language model Gemini (Gemini 3.5 Flash, Google LLC) using the following algorithm:

1. Directional Filtering and Thresholding: Before any functional grouping occurred, the dataset was filtered using strict statistical (p-adj <0.5) and directional cutoffs in the LFC.
2. Functional Coherence and Gene-Ontology Mapping: A semantic and functional clustering approach was used, cross-referencing Gene Ontology (GO) terms and Reactome pathways. Genes were grouped based on shared biological processes, cellular components, and molecular functions
3. Optimization filter: The LLM was trained to only output 3-4 programs with a maximum of 14 total genes for each represented cell subtype. As such, the top 3-4 statistically significant genes with the strongest functional narrative within each category were selected.

Analysis of cell-cell communication in labor

To systematically characterize ligand-receptor interactions across the diverse cell populations within the human uterine tissue atlas, we utilized the Python implementation of the LIgand-receptor ANalysis frAmework (LIANA+, version X.X.X). The fully pre-processed and annotated single-cell RNA sequencing dataset, structured as a Scanpy AnnData object, served as the primary input.

Putative signaling axes were evaluated between the identified cellular subsets, including the contractile uterine smooth muscle cells, vascular smooth muscle cells, and neighboring endothelial populations. Interactions were first filtered to include only ligand-receptor pairs expressed in a requisite minimum fraction of cells (default 10%) within the source and target clusters. Statistical significance for each putative interaction was determined via a permutation-based approach ($n = 1,000$ permutations), followed by a Benjamini-Hochberg correction for multiple comparisons. Interactions with an aggregate false discovery rate (FDR) < 0.05 were deemed statistically significant. Downstream visualization of the prioritized interaction networks and key signaling axes was generated using LIANA's native plotting utilities.

Differential Expression: Interactions were filtered based on the interaction $\log_2$ fold change (LFC) and adjusted p-value. To focus specifically on signals upregulated during the transition into labor, we restricted the dataset to interactions with a negative LFC (denoting higher expression/activity in the Labor group) and a statistical significance threshold of $\text{Padj} < 0.05$.

Selection of Ligand-Receptor Pairs: The specific ligand-receptor pairs were selected through a multi-step bioinformatic pruning process:

1. Niche-Specific Filtering: We focused on a defined niche consisting of nine key cell types: Uterine Smooth Muscle, Vascular Smooth Muscle, Fibroblast 1 (Vessel-associated), Fibroblast 2 (Matrix-associated), Lymphatic EC, Arterial EC, Venous EC, Capillary EC, and Myeloid cells.
2. Hierarchical Ranking: Out of thousands of potential interactions, the top-ranking interactions (by LFC magnitude) were selected to represent the most robust signaling shifts occurring during labor.
3. Cell Subtype-based Ordering: To visualize the flow of information, the "Source" (ligand-secreting) and "Target" (receptor-expressing) cells were ordered to be concordant with the rest of the manuscript and according to their physiological roles, moving from the primary contractile engine (smooth muscle) to the structural support (fibroblasts) and the responding vascular/immune systems.

Analysis of differential gene expression in uterine atony

To investigate baseline transcriptional states associated with contractile failure, we performed pseudobulk differential expression analysis on snRNA-seq data from uterine smooth muscle cells (uSMC), comparing term nonlaboring patients who developed clinical uterine atony and PPH despite prophylactic oxytocin and second-line uterotonic administration ($n = 4$) with term nonlaboring control patients who exhibited optimal postpartum contractility ($n = 4$), matched for gestational age, self-reported maternal race/ethnicity, and maternal comorbidity. Pseudobulk profiles were generated as described above. Consistent with the dataset's

log2-fold-change encoding architecture, a negative LFC denoted upregulation and a positive LFC denoted downregulation in uterine atony relative to control.

In contrast to the labor analysis, in which functional gene programs were restricted to transcripts meeting an adjusted p-value < 0.05, the uterine atony comparison was treated as a focused, hypothesis-generating screen given its limited sample size and correspondingly reduced statistical power. Candidate genes defining the atony-associated structural program were therefore prioritized by integrating multiple criteria including adjusted p-value, nominal p-value, effect size, baseline expression magnitude (DESeq2 baseMean), and biological coherence rather than by an adjusted-p threshold alone. On this basis, ENAH was selected as the primary candidate for orthogonal validation despite not reaching genome-wide significance after multiple-testing correction (adjusted p = 0.33), supported by its nominal significance (p = 0.004), its high baseline expression in uSMC (baseMean in the >99th percentile of expressed genes), and its membership in a coherent program of upregulated cytoskeletal and membrane-stabilizing transcripts. Prioritized candidates were subsequently validated in an independent cohort of primary uSMC cultures at the transcript, protein, and morphologic levels, as described below.

Cell isolation and primary culture of human myometrial smooth muscle cells

Human myometrial tissue was sliced into 2- to 3-mm-long pieces and subjected to enzymatic digestion in 1 mg/mL collagenase type II (Sigma-Aldrich), 0.15 mg/mL deoxyribonuclease I (Roche Applied Sciences), 1 mg/mL BSA (Sigma-Aldrich), 0.1 mg/mL soybean trypsin inhibitor (Sigma-Aldrich), and 10% fetal bovine serum (FBS; Gibco) at 37°C with agitation. After a 30-minute incubation, an equivalent volume of smooth muscle cell growth medium-2 bullet kit (SmGM-2, Lonza) was introduced to the digestion mixture. The digestion and incubation procedure was systematically repeated three times on the remaining tissues. The resulting cell suspension from each digestion was maintained on ice and combined post the digestion process. This combined mixture underwent a centrifugation step at 250 g for 5 minutes, and the resultant cell pellet was collected, resuspended in SmGM-2, and plated in a 100 mm plate.

In order to enhance the purity of isolated uSMC, an additional attachment step was facilitated at 37°C with 5% CO₂, following established protocols. Primary uSMC were characterized through immunofluorescence for markers such as Alpha Smooth Muscel Actin (ACTA2), Myosin Heavy Chain 11 (MYH11), and calponin. Human uSMC at early passage (1-4) were used for all experiments.

RNAscope in situ hybridization

RNAscope *in situ* hybridization was performed using the RNAscope Multiplex Fluorescent Detection Reagent v2 kit (Advanced Cell Diagnostics, ACD; 323110) according to the manufacturer's instructions.

For myometrial tissue, samples were fixed in 10% neutral buffered formalin, paraffin embedded, and sectioned at 5 µm onto SuperFrost Plus slides (Fisherbrand). Slides were baked at 60°C for 1 h, deparaffinized in xylene, and rehydrated through graded ethanol. Endogenous peroxidase activity was quenched using RNAscope Hydrogen Peroxide (ACD; 322335). Target retrieval was performed using RNAscope 10X Target Retrieval Reagent (ACD; 322000) at ≥ 99°C, followed by treatment with RNAscope Protease Plus (ACD; 322331). Sections were hybridized with probes targeting OXTR (ACD; 422651-C2) and

NOTCH3 (ACD, 558991) for 2 h at 40°C in a HybEZ oven. Signal amplification was carried out using RNAscope Multiplex FL v2 AMP1–AMP3 and HRP-C1/C2/C3 reagents (included in 323110), followed by fluorophore development using TSA Vivid Fluorophore (ACD): TSA Vivid Fluorophore 520 (323271), Vivid Fluorophore 570 (323272), and Vivid Fluorophore 650 (323273), diluted in RNAscope Multiplex TSA Buffer (ACD; 322809). Slides were counterstained with RNAscope Multiplex FL v2 DAPI (included in 323110) and mounted using ProLong Gold Antifade Mountant (Invitrogen, P36934).

For isolated smooth muscle cells (SMC), low-passage primary uSMC (passage 1-3) cells were plated on collagen coverslips and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were dehydrated through graded ethanol and processed according to the manufacturer's protocol for adherent cells. Hydrogen peroxide treatment, Protease III (ACD; 322337) digestion, probe hybridization (ENAH (ACD, 831711-C2) and FSHR (ACD, 400501)), amplification, and fluorescent detection were performed as described above. Coverslips were mounted using ProLong Gold Antifade Mountant (Invitrogen, P36934).

Each experiment included a 3-plex Positive Control Probe (ACD, 320861) and a 3-plex Negative Control Probe (ACD, 320871) processed in parallel to assess RNA integrity and background signal.

Fluorescent images were acquired using a Leica SP8 confocal microscope or a Leica Thunder fluorescence microscope. Acquisition parameters were held constant across samples within each experiment.

ENAH RNAscope signal quantification was performed using Fiji (ImageJ). Nuclei were identified using DAPI staining and counted for each corresponding image. The RNAscope signal was normalized to the number of nuclei per image and expressed as puncta per nucleus. Because two rounds of the experiment, each with n=4 uterine atony and n=4 control uSMC samples, were run months apart using a different lot number of *ENAH* probe, each count was divided by the mean of the four control values for additional normalization. Statistical analysis was then performed using Welch's t-test.

Immunofluorescent staining

Primary human uterine smooth muscle cells (uSMC) were cultured on glass coverslips and fixed with 4% methanol-free formaldehyde for 30 min at room temperature. Cells were washed in PBS, permeabilized/blocked for 1 h at room temperature using VSVG blocking buffer (Triton X-100, glycine, fetal bovine serum in 1× PBS). Cells were incubated overnight at 4°C with primary rabbit monoclonal anti-ENAH (1:75; Cell Signaling Technology, 57248) antibody diluted in blocking buffer. Coverslips were washed three times in PBS (10 min each) and incubated for 1 h at room temperature with appropriate fluorochrome-labeled secondary antibodies at a 1:200 dilution for 1 hour: Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568.

F-actin was visualized using Alexa Fluor 568 Phalloidin (Invitrogen, A12380) diluted 1:400 in PBS, and incubated for 30 min at room temperature protected from light, following the manufacturer's recommendations.

Nuclei were counterstained with NucBlue Fixed Cell ReadyProbes reagent (Invitrogen, R37606) and mounted using ProLong Gold Antifade Mountant (Invitrogen, P36934). Fluorescence images were acquired using a

Leica SP8 confocal microscope at the Stanford Cell Sciences Imaging Facility (RRID: SCR_017787).

Western Blot analysis

Total protein was extracted from uSMC in low-passage primary culture using RIPA Lysis and Extraction Buffer (Thermo Scientific) supplemented with protease inhibitors. Lysates were clarified by centrifugation and protein concentration was determined. Samples were mixed with 4x LDS sample buffer (Invitrogen) and heat-denatured at 95°C for 10 min. Equal amounts of protein were separated by SDS-PAGE on 4–12% Bis-Tris gradient gels (Novex, Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore). Membranes were blocked for 1 h at room temperature in PBS containing 5% non-fat dry milk. Blots were incubated overnight at 4°C with primary antibodies diluted in blocking buffer: rabbit monoclonal anti-ENAH (1:200; Cell Signaling Technology, 57248. Mouse anti- β -actin (1:10,000; Sigma-Aldrich, A5441) was incubated for one hour as a loading control. Following primary incubation, membranes were washed in TBS containing 0.1% Tween-20 (TBS-T) and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit or anti-mouse secondary antibody (1:5000) diluted in TBS-T. After additional washes, immunoreactive bands were detected using enhanced chemiluminescence substrate (Pierce, Thermo Scientific) and visualized using the ChemiDoc Imaging Systems (Bio-Rad) chemiluminescent imaging system.

Dynamic calcium imaging in primary human uSMC

Intracellular calcium dynamics were assessed in low-passage primary uSMC and human pulmonary smooth muscle cells (HpSMCs), used as a negative control, using the ratiometric calcium indicator Fura-2 AM (0.5 μ M; Thermo Fisher Scientific). Cells were cultured onto 25-mm collagen-coated glass coverslips (Neuvitro) and incubated with Fura-2 AM prepared in Tyrode's solution supplemented with 0.02% Pluronic F-127 (Invitrogen) for 45 min at room temperature in the dark. Following dye loading, cells were washed and allowed to de-esterify in dye-free Tyrode's solution for an additional 45 min. Coverslips were transferred to an imaging chamber mounted on an inverted Nikon Eclipse Ti microscope equipped with a Lambda DG4 high-speed wavelength switcher (Sutter Instruments) for alternating excitation at 340 and 380 nm. Emitted fluorescence was captured using a Prime BSI Express CMOS camera (Teledyne Photometrics), and image acquisition was controlled with MetaFluor software (version 7.10).

After a 50-s baseline recording, cells were stimulated with recombinant human follicle stimulating hormone (FSH) α/β (300 nM; R&D Systems, 5925-FS/CF) or oxytocin (OXT; 1 μ M). FSH responses were additionally evaluated at 50, 100, and 300 nM to establish a dose-response curve. Intracellular calcium levels were calculated as the 340/380 fluorescence ratio, and responses were quantified either as $\Delta(340/380)$ relative to baseline or as peak amplitude over baseline.

For HuSMCs, n represents the number of independent patient samples as experimental unit: n = 5 for FSH (300 nM) and OXT (1 μ M), and n = 3 for establishment of the dose-response relationship at each FSH concentration (50, 100, and 300 nM, supplemental figure). For the negative-control HpSMCs, n represents independent experiments: n = 3 for each treatment and FSH concentration. For each sample, 30–40 individual cells were analyzed, and the mean response per sample was used for statistical comparisons.

Myometrial tissue proteomics and smooth muscle adjusted analysis

Protein extraction, digestion and LC-MS/MS

Myometrial tissue specimens from women with uterine atony (n = 10) and matched controls with normal postpartum bleeding and uterine tone scores (n=10) were analyzed by data-independent acquisition (DIA) mass spectrometry at the Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry (SUMS).

All 20 samples were prepared in a single batch using the standard workflow of the core facility, without modification. Tissue was lysed in 10% sodium dodecyl sulfate (SDS)/100 mM triethylammonium bicarbonate (TEAB) for protein extraction, and 1- 5 μ L of Pierce Universal Nuclease was added for DNA disruption. Ceramic beads were added as needed to aid with the lysis, and the sample was shaken on a bead beater in 5-10 second cycles until homogenized, and centrifuged at 10,000 RPM for ten minutes at 4 °C. A 50 μ L aliquot was transferred to a 1.7 mL Eppendorf tube. Samples were reduced with 20 mM dithiothreitol (DTT) for 30 minutes at 55 °C, cooled to room temperature, and alkylated with 40 mM iodoacetamide for 90 minutes at room temperature in the dark. Samples were acidified to a pH of 1 by adding 2.6 μ L of 27% phosphoric acid, followed by 165 μ L of suspension trap (S-trap) loading buffer (90% methanol with 10% 1 M TEAB). Samples for protein digestion were then loaded onto S-trap microcolumns (Protifi, USA) and washed sequentially with 150 μ L aliquots of successive wash solutions (90% methanol with 10% 100 mM TEAB, 90% methanol with 10% 20 mM TEAB, and 90% methanol with 10% 5 mM TEAB). Afterwards, digestion was performed at 47 °C for two hours using 300 ng of mass spectrometry-grade Trypsin/LysC mix (Promega, USA) per sample.

The digested peptides were eluted with two 30 μ L aliquots of 0.2% formic acid in water, followed by two additional 35 μ L aliquots of 90% acetonitrile with 0.2% formic acid in water, into Eppendorf tubes. Finally, the samples were dried using a SpeedVac (ThermoFisher Scientific, USA) and reconstituted with 750 μ L of 0.015% n-Dodecyl β -D-maltoside (DDM; Sigma USA) in 0.1% formic acid.

Samples were analyzed on the timsTOF Ultra (Bruker Daltonics, Germany) coupled to a nanoElute 2 (Bruker Daltonics, Germany) with 25 ng sample input. Samples were eluted off a PepSep Ultra C18 column (25 cm length x 75 μ m ID x 1.5 μ m particle size, P/N 1893484; Bruker Daltonics, Germany) at 50 °C connected to a fused silica 10 μ m emitter (Bruker Daltonics, Germany) inside a nanoelectrospray Captive Spray source (Bruker Daltonics, Germany) with a 60 min active gradient (2-26% Solvent B in 48-mins, 26-35% Solvent B in 12 mins, 35-95% Solvent B in 0.10 mins, wash at 95% for 7.90 mins; Solvent B: 0.1% formic acid in acetonitrile, Solvent A: 0.1% formic acid). The TIMS 1/K0 mobility range was 0.75-1.30 V•s/cm², with a ramp time of 100 ms and 100% duty cycle. The source was set to 1600 V, 3.0 l/min dry gas, and 200°C dry temp. diaPASEF windows were created with py_DIA⁴¹ with a mass range of 350-1250 Da and a mobility range of 0.60-1.45 1/K0 giving an estimated cycle time of 2.25 s. The collision energy ramped from 20 to 59 eV at 0.60 to 1.60 V•s/cm².

DIA data processing: The .d files acquired in data-independent mode were analyzed using Spectronaut v 20.5 (Biognosys AG, Schlieren, Switzerland) with the directDIA+ feature against the Uniprot Homo sapiens protein database. Proteolysis with Trypsin was assumed to be specific, allowing for N-ragged cleavage with up to

two missed cleavage sites, with a PSM false discovery rate (FDR) of 1%. Data filtering was based on the Q Value, and global normalization was utilized. Cysteine modified with iodoacetamide was set as a fixed modification. Variable modifications included oxidation of methionine and tryptophan, deamidation of asparagine and glutamine, and phosphorylation of serine, threonine, and tyrosine, acetylation of N-terminal and lysine, and pyro-glutamic acid conversion from glutamine and glutamic acid.

Quantitative analysis of ENAH protein and adjustment for smooth muscle content: Analyses used protein log2 quantities exported from Spectronaut.

Myometrial specimens differ in the proportion of smooth muscle relative to decidua, vasculature and blood, and this variation is large enough to obscure or create between-group differences. A per-sample smooth muscle content score was therefore derived from ten smooth muscle proteins: DES, CNN1, MYH11, CALD1, TAGLN, ACTG2, SMTN, LMOD1, MYLK and SYNM. Each marker's log2 quantity was centered on its median across all samples, and the score was defined as the median of the ten centered values. A score of 0 corresponds to typical smooth muscle content and a difference of 1 corresponds to a two-fold difference. The ten markers were highly concordant across specimens (mean pairwise Pearson $r = 0.93$).

Differential abundance was assessed by analysis of covariance, fitting log2 protein quantity as a function of group (control or atony) and the smooth muscle content score. The exponentiated group coefficient gives the fold change between groups at equivalent smooth muscle content, and its significance was assessed by a two-sided t test on the coefficient. Two additional models were computed for comparison: an unadjusted comparison of log2 quantities between groups, and a ratio model in which the smooth muscle score was subtracted from each protein's log2 quantity before comparison, which constrains the relationship between abundance and smooth muscle content to unity. Group comparisons in the unadjusted and ratio models used Welch's two-sided t test, with the Mann-Whitney U test as a nonparametric confirmation. Analyses of ENAH was a pre-specified comparison of a single protein and thus reported with unadjusted p values.

Robustness to specimen composition was assessed in two ways. First, the analysis was repeated after excluding three specimens whose smooth muscle content score fell below 55% of the cohort median. Second, relative fibroblast, endothelial and trophoblast/decidual content indices were computed by the same median-centering approach from DCN, LUM, DPT, OGN and COL14A1; PECAM1, CDH5, VWF, ESAM and NOS3; and KRT8, KRT18, KRT19 and PAPPA, respectively, and ENAH abundance was modeled as the sum of contributions from each cell type weighted by its content index, with the smooth muscle contribution allowed to differ by group. Confidence intervals for the group ratio were obtained by stratified bootstrap resampling (2,000 iterations).

All statistical analyses were performed in Python 3.12.3 using NumPy 2.4.4, pandas 3.0.2 and SciPy 1.17.1.

Declarations

Ethics approval and consent to participate

This study was approved by the Stanford University Institutional Review Board (protocol 63612, approved February 2022) and was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent before tissue collection, including consent for research use of their tissue and de-identified clinical data.

Competing interests

The authors declare no competing interests.

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Author contributions

J.R.A and D.N.C conceived and designed the study. J.R.A recruited participants, clinically phenotyped the myometrial samples, established the biobank, designed experiments, and performed data analysis. D.D.F. performed experiments including tissue processing, primary cell culture, RNAScope, immunofluorescence, western blotting, and calcium imaging. L.Y. led spatial transcriptomic design, processing, and analysis. C.K. performed the computational analyses of the snRNA-seq and spatial transcriptomic data. A.K. performed experiments, including tissue processing and RNAScope validation studies in cells and tissue. G.M. recruited participants, performed informed consent, entered clinical metadata into the secure REDCap database, and conducted tissue processing and primary cell culture. D.H. assisted with spatial transcriptomic runs and analysis. C.M.A. and S.Q. provides resources, expertise, and supervision for single nucleus and spatial transcriptomic studies. J.R.A. and D.N.C. interpreted the data and wrote the manuscript. All authors reviewed, edited, and approved the final manuscript.

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Data availability

The processed snRNA-seq count matrices and cell-type annotations, and the processed MERFISH cell-by-gene count matrices and cell coordinates, generated in this study have been deposited in the Gene Expression Omnibus (GEO) under accession code [GSEpending]. The raw snRNA-seq sequencing data (FASTQ files) and raw MERFISH imaging data are not publicly available because public deposition is restricted by the Stanford University Institutional Review Board to protect participant privacy. These data are available to researchers from the corresponding author (davidco@stanford.edu) upon request for academic research purposes, subject to a data use agreement. Requests will be answered within 4 weeks. Clinical metadata are provided in Supplementary Table 1. All other data supporting the findings of this study are available within the article and its Supplementary Information. Source data are provided with this paper.

Code availability

Code used for the snRNA-seq and spatial transcriptomic analyses is available at https://github.com/CarstenKnutsen/Gravid_Myometrium_Atlas.

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Figures

Figure 1

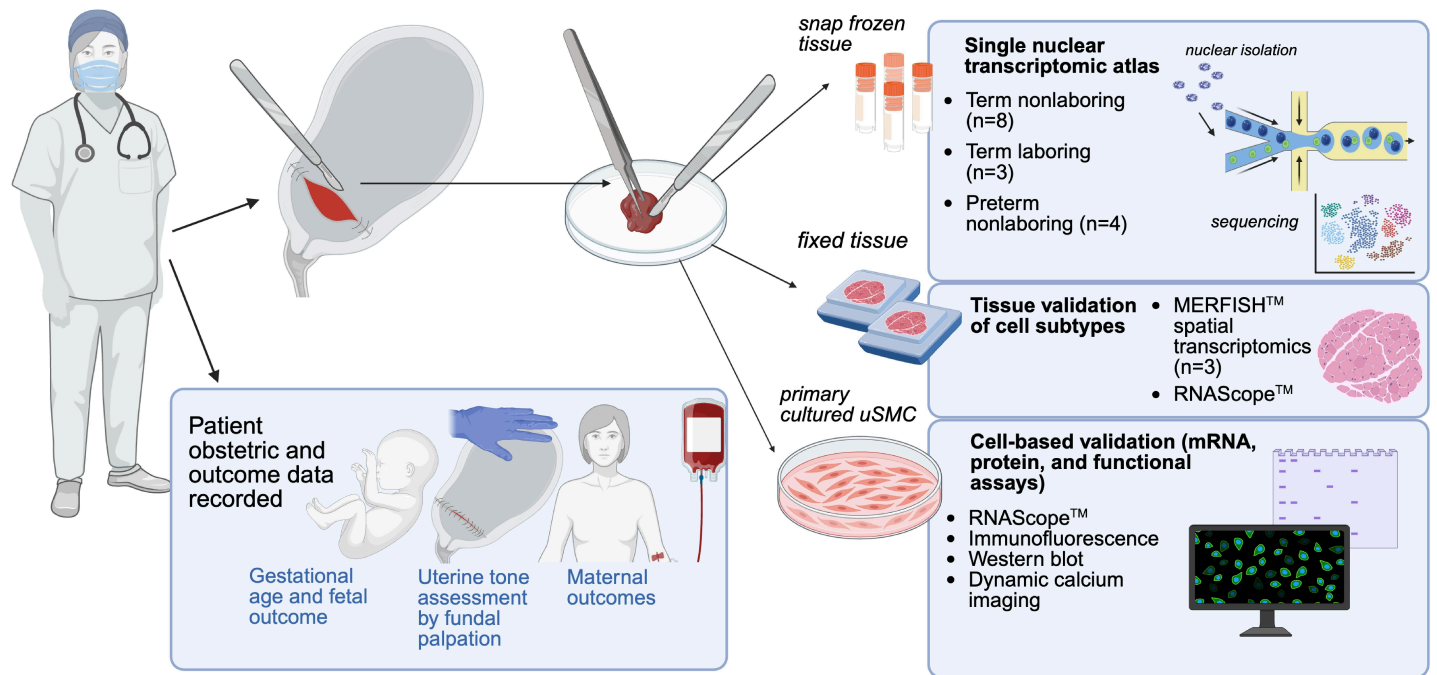


Figure 1

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Figure 2

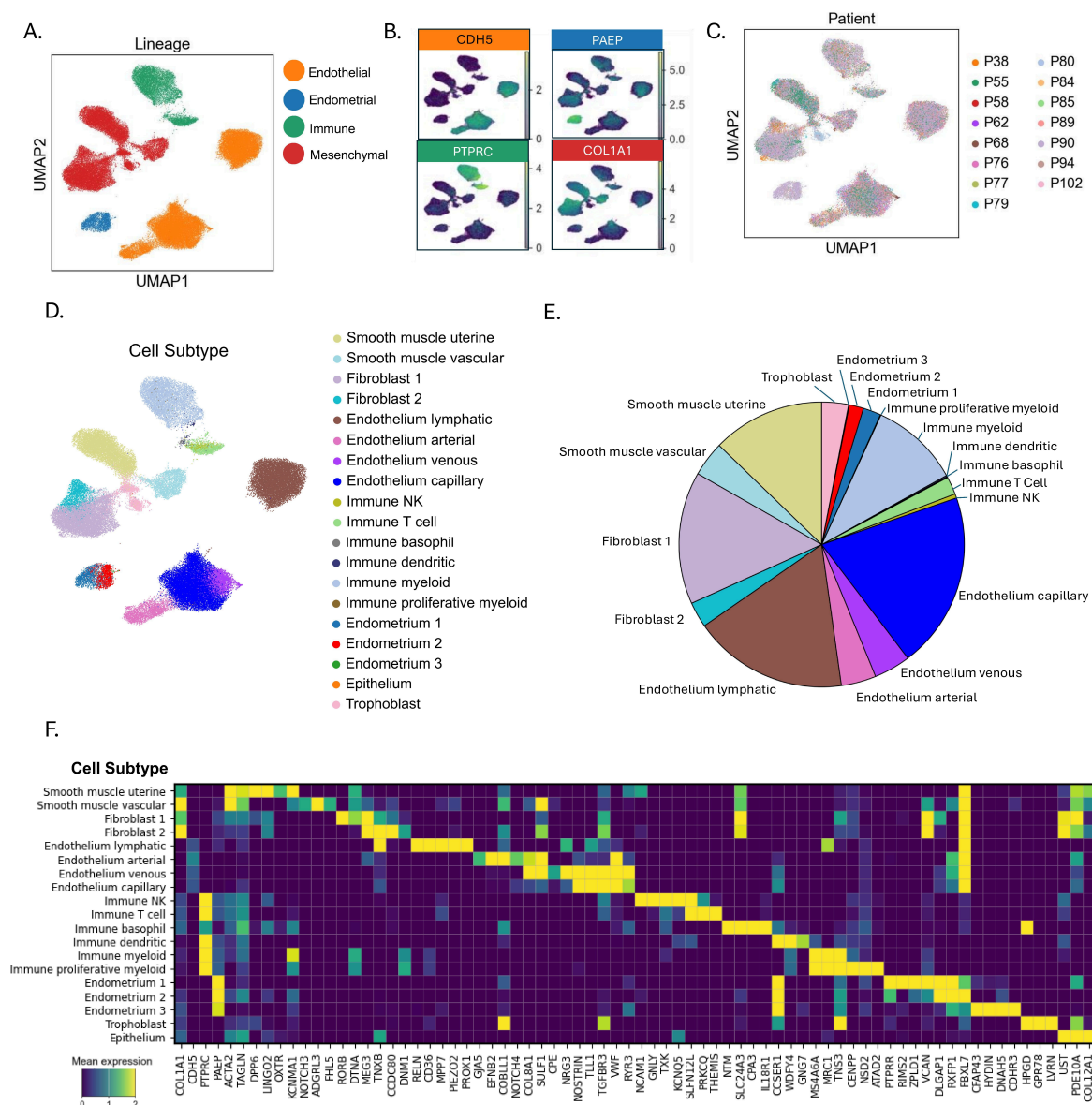


Figure 3

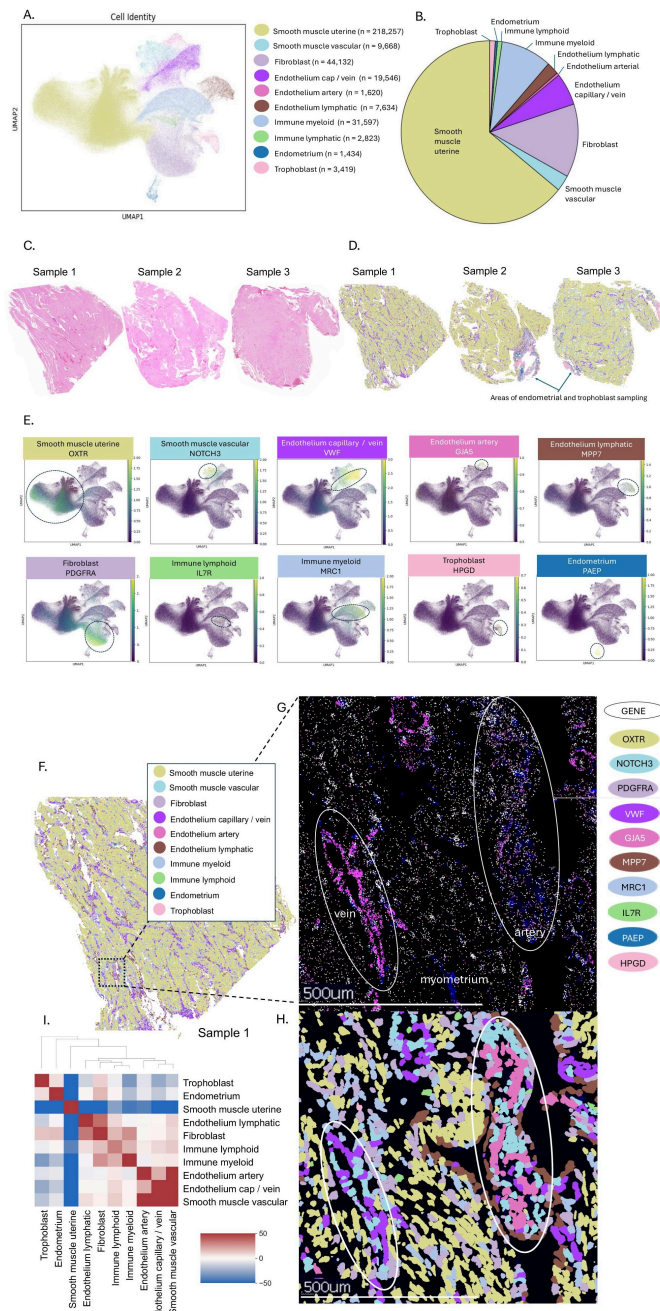


Figure 3: MERFISH single cell spatial transcriptomic analysis and cell type annotation in 3 term nonlaboring myometrial samples. A 500 gene panel was designed using cell type differentiating genes identified in our scRNAseq annotation as well as cell type differentiating canonical genes used in contemporary publications. A. UMAP displaying 10 distinct cell subtypes. n = number of cells of each cell subtype. B. Relative abundance of cell subtypes. C. Hematoxylin and eosin staining of the 3 term nonlaboring myometrial samples used in spatial transcriptomics. D. Cell types displayed on tissue samples. Colors correspond to the legend in Figure 3A. Note the areas of endometrial and trophoblast sampling on samples 2 and 3. E. Heat maps displaying expression patterns of cell type differentiating genes. F. Sample one tissue with dashed box indicating the portion magnified on the right panel. G. Select gene markers on the magnified portion of sample 1. An artery, vein, and myometrium are labeled on the image. The legend to the left displays the cell type (color matches cell types in F) and gene name is displayed in each colored oval. H. Cell types corresponding to the gene puncta are superimposed on the sample magnification. Cell types are labeled in legend for F. I. Heatmap neighborhood analysis. Red indicates colocalization within a cellular neighborhood; blue indicates lack of colocalization.

Figure 3

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Figure 4

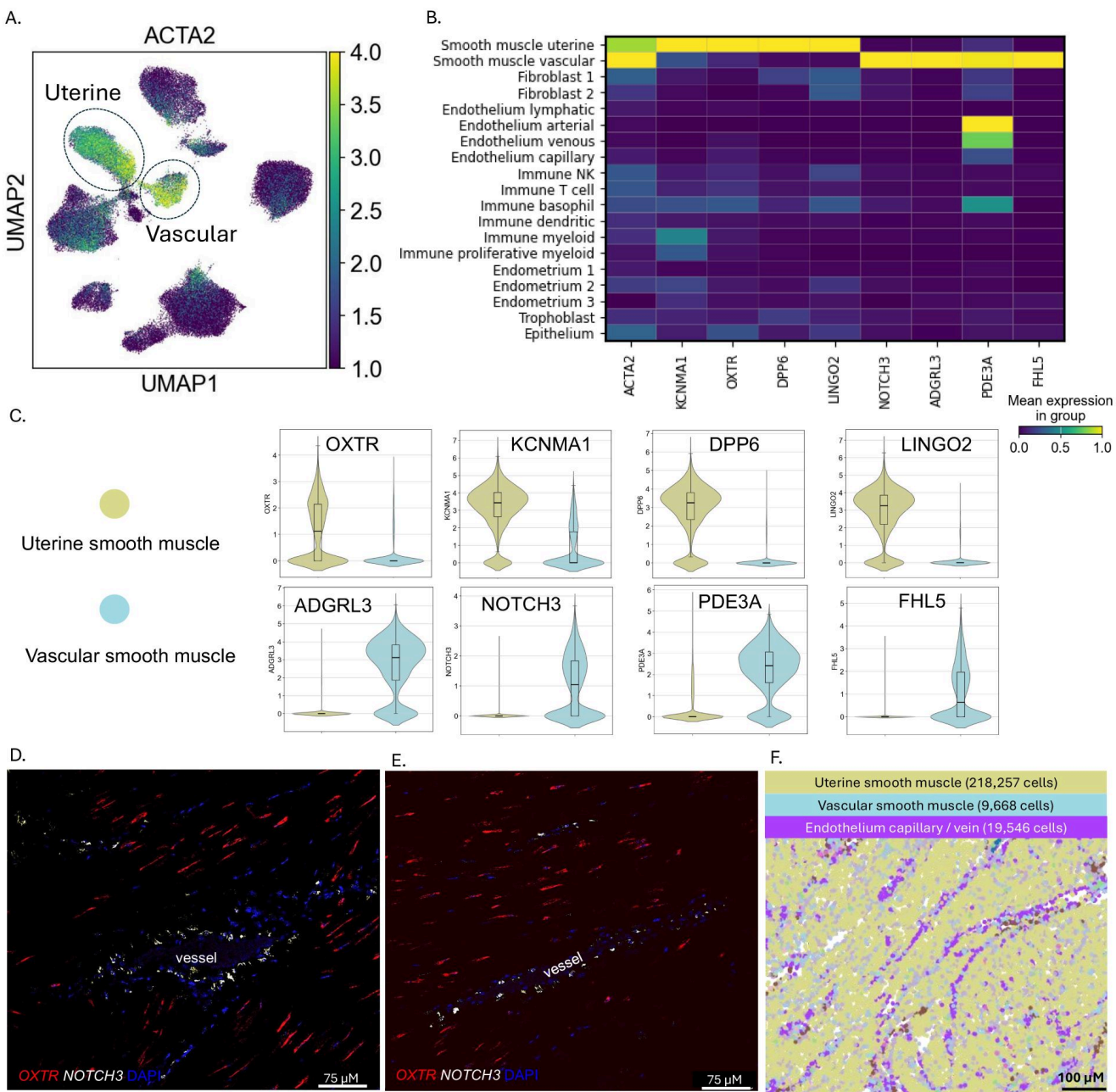


Figure 4: Two distinct smooth muscle cell types in the gravid myometrium: uterine and vascular smooth muscle. A. UMAP showing two *ACTA2*-expressing smooth muscle cell populations. B. Heatmap of smooth muscle type-differentiating genes. Uterine smooth muscle is characterized by robust *OXTR*, *KCNMA1*, *DPP6*, and *LINGO2* expression whereas vascular smooth muscle is characterized by *ADGRL3*, *NOTCH3*, *PDE3A*, and *FHL5* expression. C. Violin plots displaying differential gene expression of 8 genes in uterine versus vascular smooth muscle. D. RNAscope™ validation of uterine vs vascular smooth muscle in myometrial tissue with *OXTR* (uterine smooth muscle, red) versus *NOTCH3* (vascular smooth muscle, white), vessel in cross section in center of image. E. RNAscope™ validation of uterine vs vascular smooth muscle in myometrial tissue with *OXTR* (uterine smooth muscle, red) versus *NOTCH3* (vascular smooth muscle, white). A longitudinal vessel extends from bottom left to center right of image. F. MERFISH™ spatial transcriptomic validation: vascular smooth muscle colocalizes with endothelium around vessels whereas uterine smooth muscle makes up most of the myometrium. Counts of each labeled cell type in spatial transcriptomic analysis are included in the legend.

Figure 4

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Figure 5

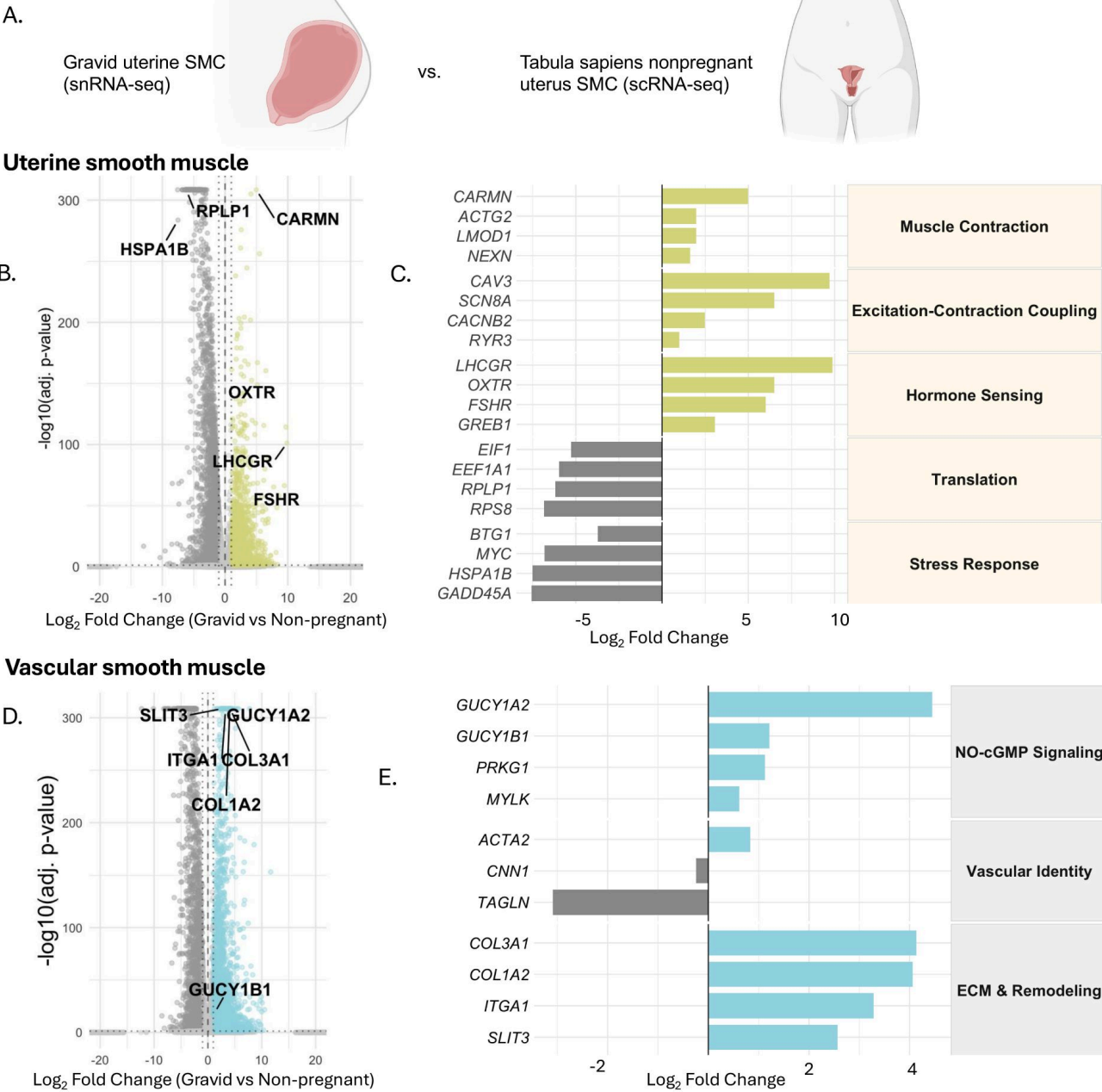


Figure 5. Transcriptomic signatures of gravid versus non-pregnant smooth muscle cells A. Our gravid human uterine snRNA-seq dataset derived from the gravid myometrium was compared with non-pregnant scRNA-seq controls included in Tabula Sapiens 2.0. B. Volcano plot identifying significant enrichment of the smooth muscle regulator *CARMN* and receptors *OXTR*, *LHCGR*, and *FSHR* define the gravid state in USMC. C. Differential module analysis reveals a functional transition from basal metabolism and translation toward specialized excitation-contraction coupling and endocrine responsiveness. D. Volcano plot identifying enrichment in nitric oxide sensing and structural reinforcement genes in gravid compared to nonpregnant vascular smooth muscle cells. E. Differential module analysis reveals upregulation genes involved in vasodilation from nitric oxide as well as extracellular matrix and vascular wall remodeling to accommodate massive increases in uterine blood flow.

Figure 5

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Figure 6

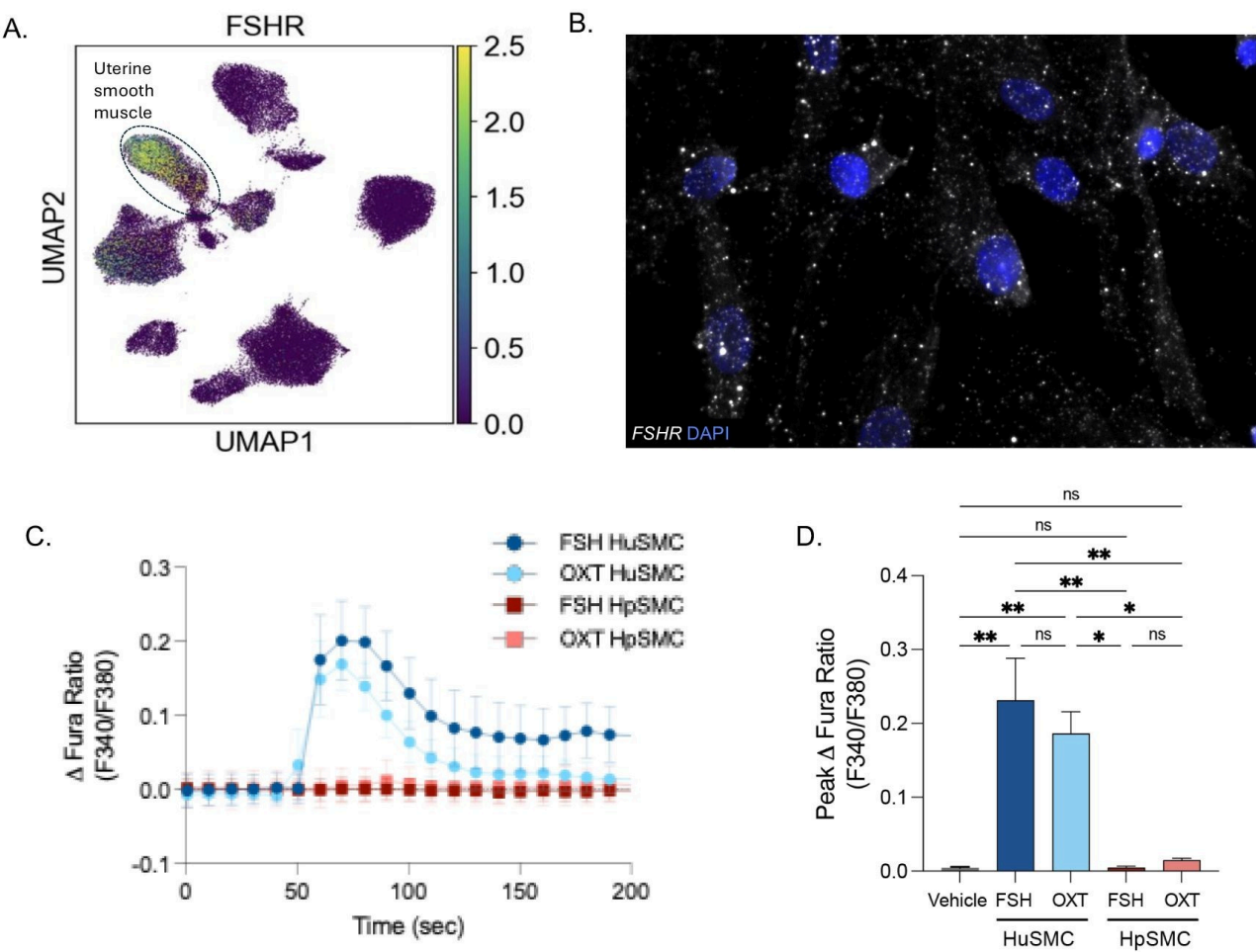


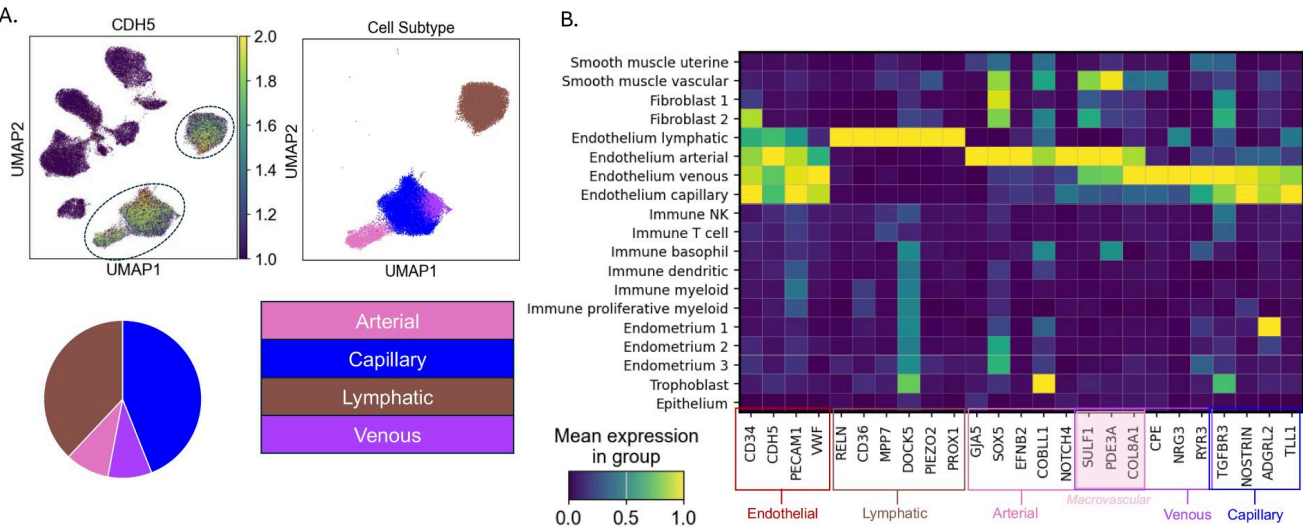
Figure 6: Gravid human uterine smooth muscle cells express functional follicle stimulating hormone receptor (FSHR) A. UMAP heatmap displays *FSHR* expression isolated to uterine smooth muscle cells. B. Confirmation of *FSHR* RNA in gravid human uterine smooth muscle via RNAscope™ in situ hybridization in n=6 low passage primary cultures of gravid human uterine smooth muscle cells. C. FSH induced a rapid increase in intracellular Ca^{2+} in gravid human uSMC (n=5 patient samples), while control HpSMCs (n=3) demonstrated no response. The peak Ca^{2+} response induced by FSH in HuSMCs was similar in magnitude to that induced by oxytocin. D. Quantification of peak change in intracellular calcium by cell type.

Figure 6

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Figure 7

Single nuclear RNA sequencing



Spatial transcriptomic validation

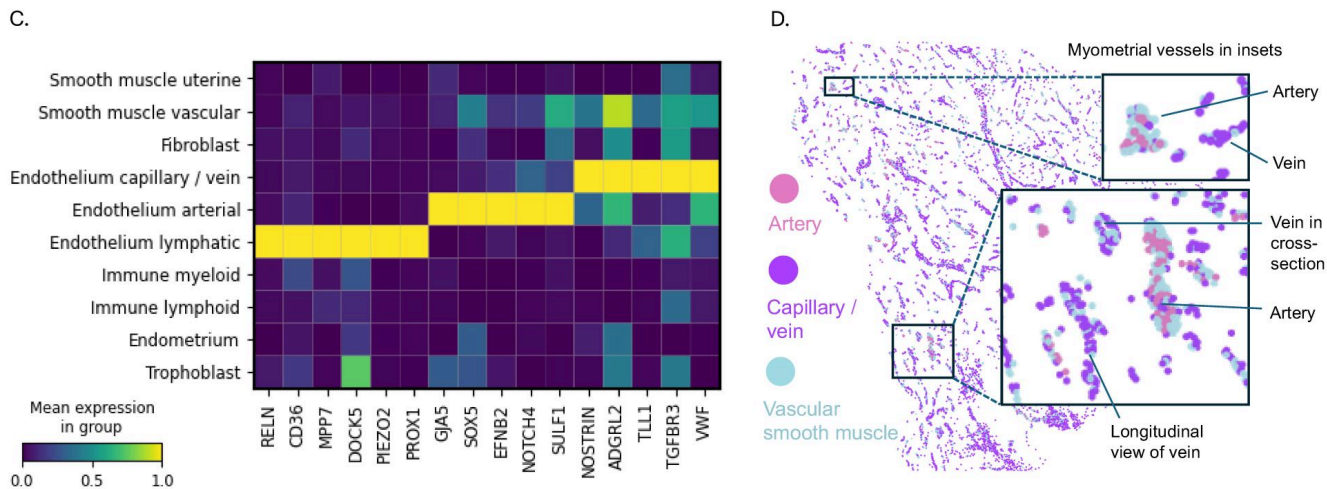


Figure 7: Four endothelial cell populations of the gravid uterus. A. UMAP of the four *CDH5*-positive endothelial cell populations in single nuclear RNA sequencing. Relative abundance of each endothelial cell subtype is represented in the pie chart. B. Heatmap displaying endothelial cell type specific gene expression. While arterial and lymphatic endothelial cells are quite distinct, venous and capillary endothelial cells have significant overlap. However, venous cells contain macrovascular markers shared with arterial endothelial cells (*SULF1*, *PDE3A*, *COL8A1*) as well as two distinct genes *CPG* and *NRG3*. C. Heatmap of endothelial cell type-specific transcripts overlaid on MERFISH™ spatial transcriptomic output. D. Spatial localization of endothelial cell types and vascular smooth muscle in sample 1. Enlarged images display arteries (pink with abundant vascular smooth muscle) compared to veins (purple with significantly less associated vascular smooth muscle).

Figure 7

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Figure 8

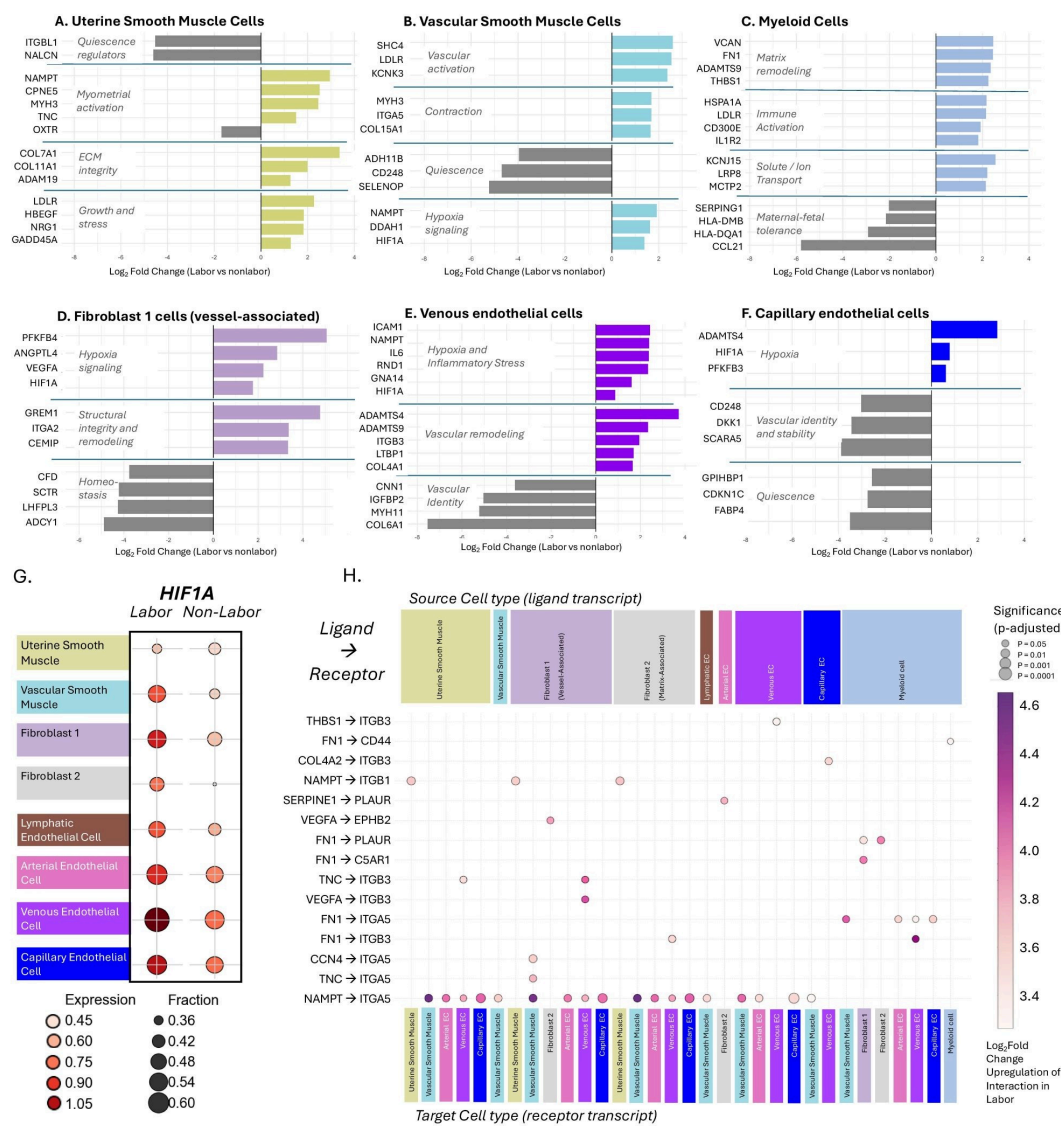


Figure 8

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Figure 9

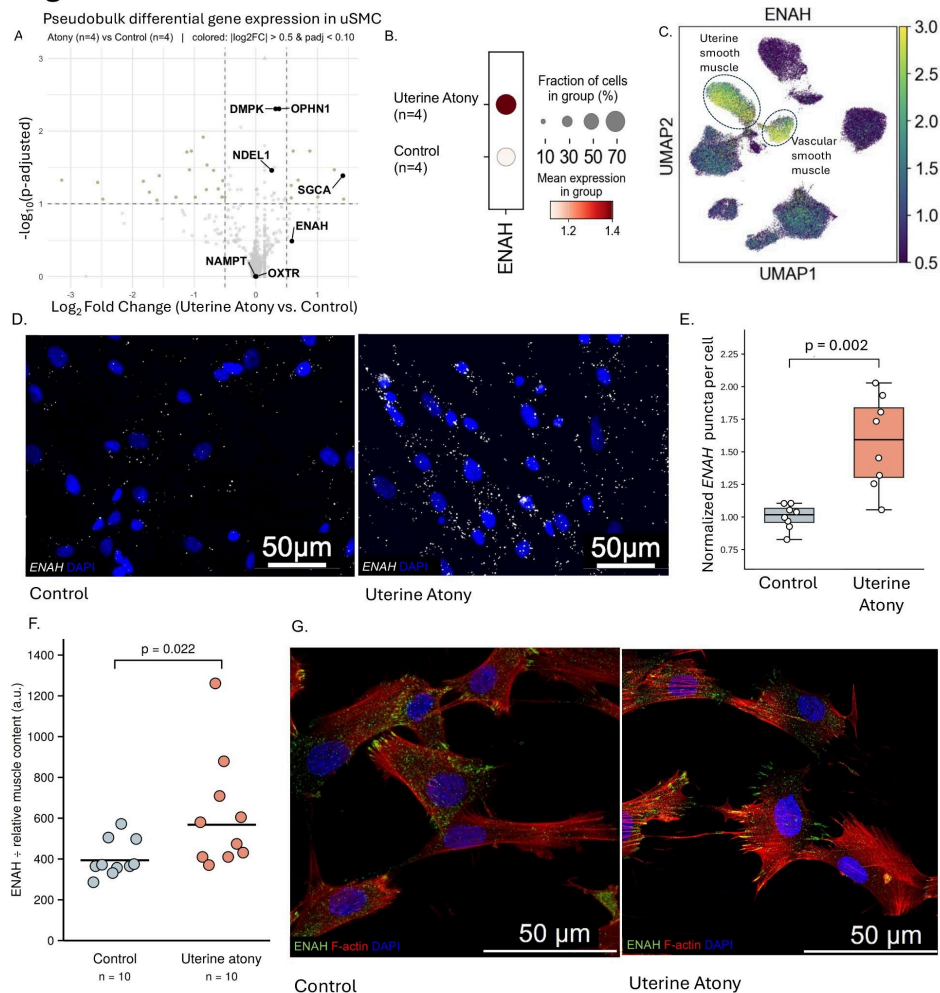


Figure 9: Differential gene expression in uterine smooth muscle cells from term nonlaboring patients with uterine atony compared to optimal contractility (controls)

A. snRNAseq pseudobulk analysis shows enriched transcription of structural stability-related genes in uSMC from patients with uterine atony.

B. Dotplot of ENAH overexpression in uSMC of the myometrium from patients with uterine atony (n=4) compared to controls (n=4)

C. Heatmap overlay on snRNAseq UMAP reveals smooth muscle cell-specific localization of ENAH in the gravid human myometrium.

D. Representative images of ENAH RNA visualized by RNAScopeTM in uterine smooth muscle cells in primary culture highlighting increased transcript puncta in patients with uterine atony compared to controls.

E. Quantification of RNAScopeTM puncta across n=16 term nonlaboring patients (8 controls with optimal contractility and 8 patients with uterine atony) in two rounds of experiments. Each uterine atony sample was matched prior to performing RNAScope experiment to a control subject matched by gestational age, self-reported race/ethnicity, maternal age, and body mass index. Statistical analysis: data were analyzed using unpaired two-tailed t tests. $p = 0.002$

F. Higher ENAH protein abundance in snap frozen myometrium from a subset of patients with uterine atony. Myometrial tissue from control (n = 10) and uterine atony (n = 10) deliveries were analyzed by data-independent acquisition liquid chromatography tandem mass spectrometry (DIA LC-MS/MS). For each specimen, a smooth muscle content score was calculated as the median of the median-centered $\log_2$ intensities of ten smooth muscle proteins (DES, CNN1, MYH11, CALD1, TAGLN, ACTG2, SMTN, LMOD1, MYLK, SYNM), and ENAH intensity was divided by relative smooth muscle content. Each circle represents one patient. Horizontal bars indicate geometric means. $*p < 0.05$, Welch's two-sided t test on $\log_2$ -transformed values.

G. Representative immunofluorescence images of ENAH protein (green) and F-actin filaments (red) visualized by immunofluorescence in n=8 samples of uterine smooth muscle cells in primary culture. ENAH localizes to cell edges. Cells from patients with uterine atony are qualitatively characterized by a more stellate and less smooth appearance at cell edges.

Figure 9

See image above for figure legend

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [S4.LaborCellCellCommunication.csv](#)
- [SupplementalTable1Patientdemographicmetadata.xlsx](#)
- [S5.Uterineatony pseudobulk comparisons by cell type.xlsx](#)
- [S3.Labor pseudobulk comparisons by cell type.xlsx](#)
- [SupplementalFiguresV2.pdf](#)
- [S2 Differential gene expression in uterine smooth muscle populations Tabula Sapiens vs gravid myometrium.xls](#)