Chapter 15

Methods and Model Systems Used to Study Pregnant Human Uterine Smooth Muscle

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Additional information is available at the end of the chapter

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Abstract

Successful pregnancy necessitates that the human uterus is maintained in a relaxed, quiescent state for the majority pregnancy, before ultimately transforming to a contractile phenotype capable of powerful, coordinated contractions to facilitate parturition. The exact mechanisms that regulate this transition are yet to be fully understood, and as such, we still do not understand the molecular mechanisms that trigger the onset of human labor. This is in large part due to the ethical considerations associated with human pregnancy, which, outside of clinical trials, primarily limits human studies to *in vitro* investigations on cell lines and biopsied tissues. Researchers have therefore devised numerous model systems for investigating pregnant human uterine smooth muscle, which have played vital roles in elucidating the fundamental biology and key regulatory pathways that underpin the transition from quiescence to contractility. This chapter describes in detail, those methods and model systems used to study pregnant human uterine smooth muscle, and explores the challenges associated with these model systems.

Keywords: smooth muscle, parturition, cell lines, tissue pieces, tissue strips

1. Introduction

Parturition requires that myometrial smooth muscle undergo a phenotypic transition, remaining quiescent for the majority of gestation and then transforming to a tissue capable of generating forceful, coordinated contractions to expel the fetus and the placenta [1–3]. Characterizing the regulation of key myometrial genes is essential to understanding normal human birth, as well as obstetric complications, including preterm labor. Outside of clinical trials, researchers are primarily limited to observational studies of human pregnancy, and as such, rely heavily on *in vitro* models of the uterus for performing investigational studies. Human cell cultures

are a valuable *in vitro* tool, used to gain insight into numerous physiological and pathological processes; however, concerns have been raised about the lifespan of cultured primary cells, as well as their ability to remain representative of the tissue of origin. In an attempt to address the limited lifespan of cultured primary cells, immortalized cell lines have been developed by transfecting telomerase expression vectors into primary cells. Nevertheless, multiple studies have shown that immortalization could cause fundamental changes in the cells, and therefore many studies opt to use primary cells at low passage numbers instead. More recently, 3D cell culture models have been developed to more closely mimic the *in vivo* environment of the cells. Although these 3D cell cultures recreate tissue environments, they still have multiple limitations, such as cost, long production times and often the need for specialized equipment. Incubation of tissue *ex vivo* as small pieces (explants) or strips models the *in vivo* phenotype more closely and has emerged as a popular experimental model for interrogating myometrial biology. Nonetheless, recent evidence indicates that pregnant human smooth muscle tissue undergoes rapid phenotypic changes during these *ex vivo* studies. The consequences are considerable in that findings made using 'non-laboring' tissue may in fact have been attained using tissue that had spontaneously transitioned to a laboring phenotype throughout the course of the study. Model systems of myometrium that are in a state of flux have the capacity to confound results when researchers seek to elucidate the trigger(s) for labor. This chapter describes in detail, methods and model systems used to study pregnant human uterine smooth muscle, and explores the challenges associated with these model systems.

2. Preterm birth

Preterm birth is defined by the World Health Organization as birth occurring before 37 completed weeks of gestation [4]. Estimates of global rates of preterm births suggest that of the 135 million live births worldwide in 2010, 14.9 million babies were born preterm, representing a preterm birth rate of 11.1% [5, 6]. In recent decades, the rate of preterm birth has continued to rise in most countries, despite advances in medical technology as well as the introduction of medical interventions designed to reduce preterm birth [5, 7–9]. This is alarming given that preterm birth is a leading cause of neonatal death, responsible for 44% (2.761 million) of the 6.3 million deaths of children who died before age 5 years in 2013 [10]. Additionally, survivors of preterm birth often suffer both short- and long-term morbidities. Short-term morbidities include respiratory distress syndrome, necrotizing enterocolitis, intraventricular hemorrhage, and patent ductus arteriosus, while long-term morbidities include cerebral palsy, mental retardation and learning impairment, visual and hearing problems, as well as possible increased risk of cancer [11–13]. These translate into enormous economic and societal cost [14]. The economic costs include immediate expenses associated with neonatal intensive care, as well as the long-term costs of ongoing management of disabilities and diseases [14, 15]. The societal costs include families experiencing the sudden loss of a newborn, or stressful hospitalization of newborns that often extends for months, followed by lifelong support of children with varying severities of ongoing disease(s) [14]. Achieving decreased rates of preterm birth with improved neonatal outcomes would therefore have enormous implications at the community level. Nevertheless, little progress has been made addressing preterm birth due to a poor understanding of the underlying mechanisms that initiate myometrial contractions. As

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such, the development of *in vitro* methods and model systems for studying pregnant human myometrium is critical to determining the regulatory mechanisms that underpin the onset of both term and preterm labor.

3. Human myometrial smooth muscle cell culture models

Human myometrial smooth muscle cell (SMC) cultures have been used extensively to study myometrial biology and researchers have been able to elucidate many aspects of fundamental biology, such as the regulation of gene expression and signaling pathways. In SMC cultures, myometrial biopsies are typically obtained from the lower uterine segment of term singleton pregnancies during Cesarean section, dissected into 1 mm^3 pieces and washed in a saline solution, such as Hanks' Balanced Salt Solution, to remove excess blood. Myometrial pieces are enzymatically digested down to single cells (collagenase XI, collagenase IA). The digestion suspension is then repeatedly passed through a fine sterile pipette to disperse myometrial cells. Enzymatic digestion is stopped by addition of 5% fetal bovine serum (FBS) to the medium. Myometrial cells are then purified using a cell sieve (pore diameter of ~70 μm) and centrifugation, before ultimately being plated into culture flasks containing smooth muscle medium, supplemented with 5% FBS and antibiotic-antimycotic. The isolated SMCs are typically assessed by Trypan blue exclusion method to determine cell viability. The myometrial SMCs are incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for growth and expansion, as required (**Figure 1**).

Casey *et al.* [16] established first human myometrial SMCs in monolayer culture in 1984 and reported that the cells were stable for up to a year with no morphological changes. Nevertheless, concerns were raised about the lifespan of cultured primary cells, and Hayflick and Moorhead [17] reported that cell populations are only capable of dividing a fixed number of times before reaching replicative senescence. In an attempt to address the limited lifespan of cultured primary cells, numerous strategies have been used to develop myometrium-like cell lines with greater proliferative potential.

Perez-Reyes *et al*. [18] immortalized human SMCs *in vitro* by infecting the cells with a retroviral vector that contained the E6/E7 open reading frames of human papillomavirus type 16. The immortalized SMCs had significantly increased growth rates, compared to non-immortalized control cells, and there were no signs of senescence with long-term passage [18]. These first retrovirally infected SMC lines can be in continuous tissue culture for more than 1 year [18]. However, in addition to having an increased growth rate, cell size was decreased and there were alterations in α-smooth muscle actin (α-SMA) filament distribution and staining intensity [18].

Previous studies have demonstrated that human telomerase reverse transcriptase (hTERT) can stabilize telomere length and prolong cellular proliferative capacity [19–23]. Therefore, Condon *et al*. [24] developed a SMC line from hTERT-infected myometrial cells to investigate the complex molecular, hormonal and cellular processes associated with the myometrium. hTERT-infected myometrial cells were able to be in continuous culture for more than 10 months [24]. Furthermore, these cells exhibited a number of markers of SMCs, including α-SMA, smoothelin, *h*-caldesmon and calponin expression, as well as genes associated with reproductive function and parturition,

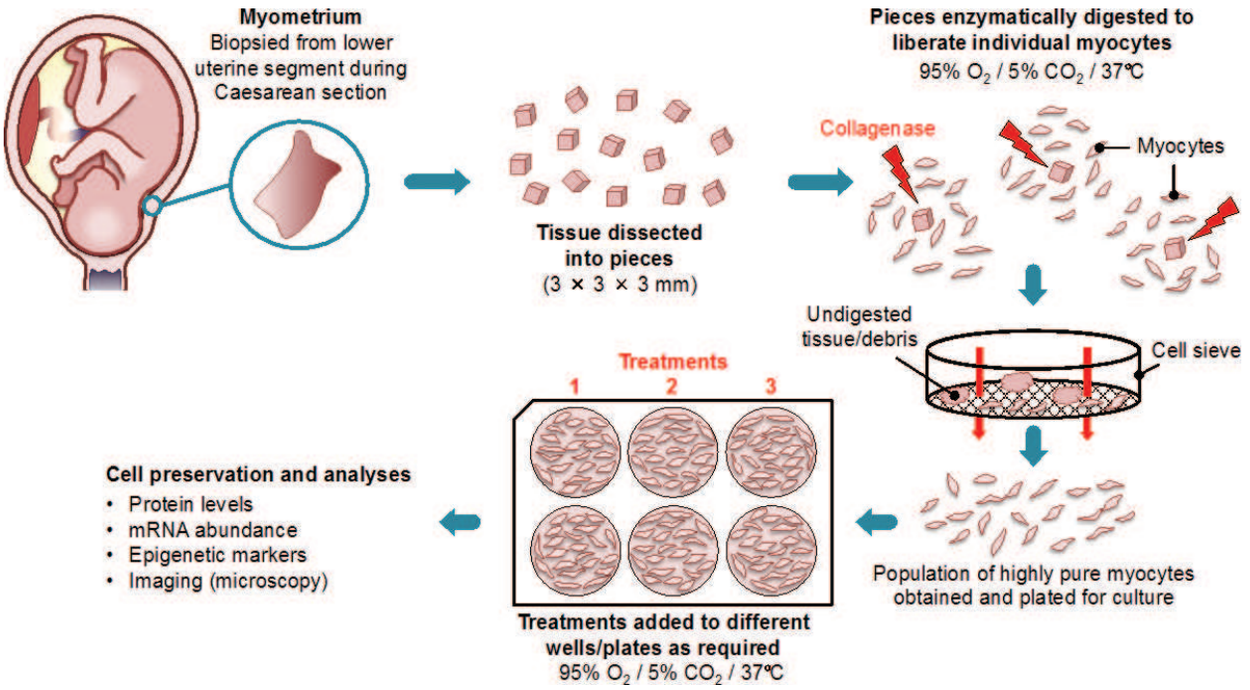

Figure 1. Human myometrial smooth muscle cell culture model. Human myometrial samples are obtained from lower uterine segment during elective Cesarean section of singleton pregnancies. The samples are cleared of serosa, fibrous or damaged tissue and visible blood vessels, and dissected into small pieces. The myometrial pieces are enzymatically digested down to single cells (collagenase XI, collagenase IA). Myometrial cells are then purified using a cell sieve and plated into culture plates containing smooth muscle medium (95% O_2/5% CO_2/37°C). The cultured myometrial cells are then subjected to desired treatments or experimental conditions, after which cells can be snap frozen or preserved for subsequent analyses.

including the oxytocin receptor (*OXTR*), estrogen receptor 1 (*ESR1*) and progesterone receptor (*PGR*) [24]. Additionally, hTERT-infected myometrial cells were responsive to both oxytocin and estrogen, thus further implying that they are a suitable *in vitro* model for investigating the molecular mechanisms that regulate uterine SMC gene expression [24].

Since hTERT cell lines have become popular among researchers, Soloff *et al*. [25] compared phenotypes of three telomerase-immortalized myometrial cell lines with the matching cells in primary culture, from which the immortalized cells were derived. Out of more than 10,000 expressed genes, only 1% of genes consistently exhibited expression changes in the telomerase-immortalized cell lines [25]. Furthermore, the comparison between primary and telomerase-immortalized cells revealed no significant differences in signaling pathways, such as epidermal growth factor (EGF)-stimulated phosphorylation of the EGF receptor, insulin-stimulated Akt phosphorylation, oxytocin and lysophosphatidic acid-stimulated extracellular signal-regulated kinase (ERK) 1 and 2 phosphorylation, myosin light chain (MYL) phosphorylation, and interleukin-1 induction of IκBα degradation [25]. Although this study found no significant difference between immortalized and primary cell lines, it has been suggested that immortalization could cause fundamental changes in the cells [26], and as such many studies use primary cells only at low passages (passage five and lower) [27].

Mosher *et al*. [26] established primary cultures of human myometrial cells isolated from paired upper and lower segment uterine biopsies for 10 passages and determined the expression of smooth muscle markers, fibroblast markers, contractile proteins or labor-associated

proteins over time. It was found that both upper and lower segment human myometrial cells stably expressed smooth muscle markers (α-SMA, calponin, caldesmon, tropomyosin) and fibroblast markers (vimentin, 1B10) to at least 10 passages [26]. Interestingly, comparison of paired upper and lower segment myometrial cells revealed that mRNA levels for Connexin 43 (*GJA1*), Prostaglandin-endoperoxide synthase 2 (*PTGS2*) and vimentin (*VIM*) were significantly higher in lower segment cells compared to upper segment cells [26]. These findings supported the concept of a functional regionalization of the upper and lower segment of the human uterus and indicated that both upper and lower segments should be examined in order to garner maximum insight into the mechanisms underlying human parturition. Furthermore, both cell populations retained their ability to respond to inflammatory stimuli from passage 1 through to passage 10, as demonstrated by increased expression of *PTGS2* and release of the pro-inflammatory chemokine, *CXCL8*, following treatment with the interleukin-1β (IL-1β) [26]. Mosher *et al*. [26] concluded that primary myometrial cells are viable and responsive for at least 10 passages, and therefore represent a useful tool for investigating human parturition. Nevertheless, this study did not to compare each passage with the fresh tissue to ascertain whether the cells were truly representative of the tissue of origin.

Recently, a study by Zaitseva *et al*. [28] compared gene expression profiles between myometrial and fibroid tissues, as well as SMCs isolated from these tissues that were cultured for up to three passages. It was found that 2055 genes were differentially expressed between all groups (fresh myometrial tissue, fresh fibroid tissue, myometrial SMCs at passage 0, fibroid SMCs at passage 0, myometrial SMCs at passage 3 and fibroid SMCs at passage 3) [28]. A total of 128 genes were found to be significantly different between fresh myometrial and fibroid tissues [28]. More than 1100 genes were significantly different between fresh tissues and cultured SMCs, with 648 genes common between both myometrial and fibroid SMCs at passage 0 and passage 3 [28]. These findings indicate that culture conditions significantly changed the gene expression profile of myometrial and fibroid SMCs, decreasing differences between the cells *in vitro* compared to the cells *in vivo* [28]. Furthermore, Zaitseva *et al*. [28] examined expression of *ESR1* and *PGR* in fresh myometrial and fibroid tissues, as well as cultured SMCs. Expression of both receptors was significantly decreased in cultured SMCs compared to fresh tissues [28]. Zaitseva *et al*. [28] concluded that myometrial and fibroid cell cultures provide a vital tool to study the uterus, but nevertheless, *in vitro* studies must be carefully planned and assessed to provide significant results.

4. Stretch-based culture systems

Throughout normal pregnancy the uterus increases several-fold in size by both hyperplasia and hypertrophy to accommodate the growing fetus and placenta [29, 30]. In 1982, Manabe *et al*. [31] applied mechanical stretching of the uterus to seven term patients who were not in labor by inserting and inflating a 150 mL rubber balloon. All seven patients showed significant cervical softening, as well as initiation and progress of labor [31]. Furthermore, analysis of amniotic fluid revealed that the uterine stretch initiated increased release of prostaglandin (PG) F (PGF) [31]. Based on these results, the *in vitro* model of pregnancy-induced uterine stretch has been used in multiple studies to investigate the effect of stretch on human SMCs [29, 30, 32, 33].

The *in vitro* model of pregnancy-induced uterine stretch involves growing SMCs in 6-well culture plates with a flexible collagen growth surface. The plates are placed in a computer-driven Flexercell strain unit, which generates a vacuum in order to deform the flexible growth surface. Through deforming the growth surface, cultured cells can be subjected to static stretch of 6, 11 or 16% for 1 or 6 h (**Figure 2**). Using this model, Sooranna *et al*. [30] investigated the effect of stretch on PG synthesis in non-pregnant, pregnant non-laboring and pregnant laboring primary human SMCs. Non-pregnant and pregnant laboring SMCs had significantly higher *PTGS2* mRNA levels than pregnant non-laboring SMCs [30]. When stretch was applied, *PTGS2* mRNA expression significantly increased in all three groups [30]. In additional studies using pregnant non-laboring SMCs, 6 h of stretch increased PTGS2 protein levels, increased PGI_2 metabolite and PGE_2 concentrations in the media, as well as decreased $PGF_{2\alpha}$ metabolites in the media [30]. Furthermore, following stretch there was increased activator protein-1 (AP-1) nuclear protein DNA binding activity in pregnant non-laboring SMCs [30]. These results provided evidence that increased PTGS2 activity, following stretch of human myocytes, occurs through activation of the AP-1 system [30].

Terzidou *et al*. [32] investigated the effect of stretch on the expression of *OXTR* mRNA and DNA binding of AP-1, CCAAT/enhancer binding protein (C/EBP)β, and nuclear factor-κB (NF-κB) transcription factors in non-pregnant, pregnant non-laboring and pregnant laboring primary

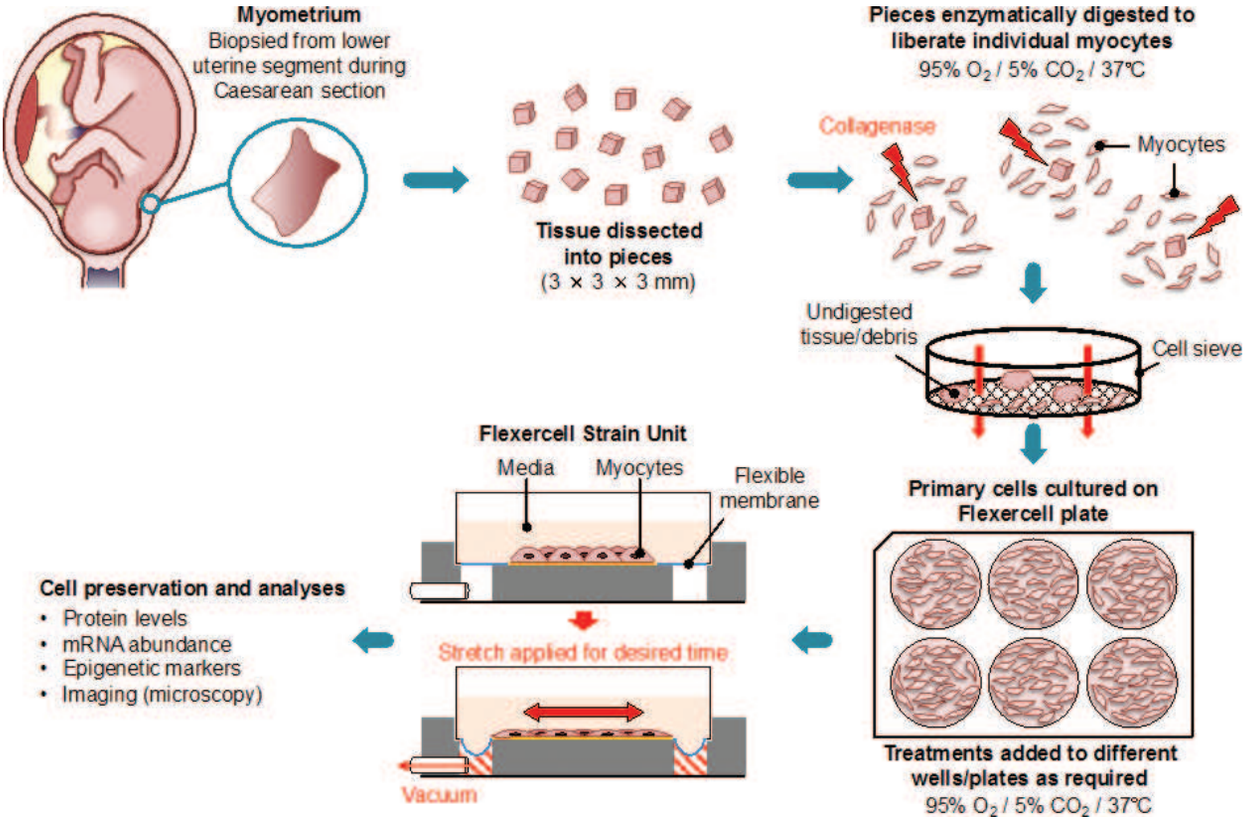

Figure 2. Flexercell stretch-based culture system. Primary myometrial cells, prepared from myometrial biopsies, are plated into 6-well culture plates with a flexible collagen growth surface (95% O_2/5% CO_2/37°C). The plates are placed in a computer-driven Flexercell strain unit capable of generating a vacuum that deforms the flexible growth surface. Through deforming the growth surface, cultured cells can be subjected to varying degrees of stretch, as well as treated with exogenous agents. The cells can then be snap frozen or preserved for subsequent analyses.

human SMCs. Pregnant laboring SMCs had significantly higher *OXTR* mRNA expression compared to non-pregnant and pregnant non-laboring SMCs [32]. Application of stretch to pregnant non-laboring human myocytes significantly increased *OXTR* mRNA levels and increased *OXTR* promoter activity [32]. Conversely, stretching of non-pregnant and pregnant laboring human myocytes did not affect *OXTR* mRNA levels [32]. Increased promoter activity within the pregnant non-laboring cells was associated with increased DNA binding of C/EBPβ and AP-1 [32]. Overexpression of C/EBPβ led to increased *OXTR* promoter activity [32]. Based on these results, the authors concluded that stretch of pregnant non-laboring SMCs increases *OXTR* expression through increased C/EBPβ DNA binding [32]. More importantly, these results imply that stretch contributes to the increase in *OXTR* expression at the time of labor during human parturition [32].

Loudon *et al*. [29] investigated the effect of stretch on the expression of interleukin-8 (IL-8) in non-pregnant, pregnant non-laboring and pregnant laboring primary human SMCs. Previous studies have shown that labor is associated with increased synthesis of pro-inflammatory cytokines, including IL-8 [34, 35]. Pregnant laboring SMCs have significantly higher *IL-8* mRNA expression than non-pregnant and pregnant non-laboring SMCs [29]. The application of stretch, via the Flexercell strain unit, significantly increased *IL-8* mRNA expression in myocytes from all three groups [29]. Furthermore, stretch-induced increase in *IL-8* mRNA expression was concurrent with increased IL-8 levels in the culture supernatant, as well as increased promoter activity [29].

Previous pregnancy-induced uterine stretch studies have shown that *PTGS2* and *IL-8* mRNA expression increases *in vitro* following mechanical stretch of uterine SMCs [29, 30]. Therefore, Sooranna *et al*. [33] further investigated whether IL-1β and mechanical stretch increase the myometrial expression of *PTGS2* and *IL-8* through mitogen-activated protein kinase (MAPK) activation, and whether these effects were synergistic. Pregnancy-induced uterine stretch increased *PGHS-2* and *IL-8* mRNA expression through ERK1/2 and p38 MAPK pathways [33]. Likewise, IL-1β increased *PGHS-2* mRNA expression through ERK1/2 and p38 MAPK pathways [33]. However, IL-1β increased *IL-8* mRNA expression through the ERK1/2 pathway only [33]. Furthermore, there was no evidence of a synergistic effect of IL-1β and stretch on *PTGS2* and *IL-8* mRNA expression [33].

These studies using the *in vitro* model of pregnancy-induced uterine stretch demonstrated that mechanical stretch is able to increase expression of various genes. Namely, stretch increased *PTGS2* and *IL-8* expression through MAPK-dependent signaling, while *OXTR* expression was increased through a MAPK-independent mechanism [29, 30, 32, 33]. As such, Lei *et al*. [36] investigated whether progesterone was able to inhibit stretch-induced MAPK activation, as well as *PTGS2* mRNA expression and protein synthesis [36]. Pre-incubation of primary SMCs with progesterone did not inhibit stretch-induced ERK1/2 activation or *PTGS2* mRNA expression [36]. Additionally, it was determined that mechanical stretch did not modify the ability of progesterone to modulate progesterone-responsive gene expression, activate a progesterone response element or inhibit IL-1β-driven *PTGS2* mRNA expression [36]. Mechanical stretch was found to decrease *PR-T* and *PR-B* mRNA expression via NF-κB activation; however, stretch does not appear to inhibit progesterone action [36]. These findings, derived from the *in vitro* model of pregnancy-induced stretch, suggest that stretch is not responsible for the functional progesterone withdrawal observed with the onset of human labor [36].

In a similar approach, Dalrymple *et al*. [37] used the Flexercell strain unit to subject SMCs to tonic stretch of 25% for 1, 4 and 14 h. Mechanical stretch for 14 h increased basal calcium entry, as well as cyclopiazonic acid-induced calcium/manganese entry [37]. Furthermore, prolonged tonic stretch increased transient receptor potential canonical (TRPC) channel gene expression and protein levels [37]. TRPC proteins are known components of store-operated calcium entry and are expressed in human myometrium during pregnancy [38–40]. Since an increase of calcium entry leads to increased contractility of smooth muscle, this study reveals a possible pathway by which uterine function can be modified in response to the growing fetus throughout the pregnancy [37].

The studies discussed thus far have utilized human primary SMCs to investigate the effects of stretch. Recently, Lee *et al*. [41] used hTERT cells to investigate whether stretch facilitates peripheral leukocyte extravasation into the term myometrium through the release of various cytokines by uterine myocytes. In the study, hTERT cells were grown on flexible-bottom culture plates and static stretch of 25% was applied for 24 h [41]. It was found that mechanical stretch resulted in secretion of multiple cytokines and chemokines, including IL-6, IL-12p70, migration inhibitory factor (MIF), C-X-C motif ligand (CXCL) 8, CXCL1, granulocyte colony-stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor subunit B (PDGF-bb) [41]. Furthermore, stretch-induced cytokines increased leukocyte adhesion to the endothelium of the surrounding uterine microvasculature by stimulating the expression of endothelial cell adhesion molecules, as well as by directing the transendothelial migration of peripheral leukocytes [41]. These results provide evidence for the mechanical regulation of leukocyte migration from the uterine blood vessels toward the myometrium [41].

Up until now, majority of *in vitro* cell-based assays have utilized traditional two-dimensional (2D) culture, where cells are grown as a monolayer on a flat surface. Although 2D culture has proven to be an effective method for cell-based studies, and researchers have been able to elucidate many aspects of fundamental biology, the limitations of 2D culture have gradually been recognized. In the *in vivo* environment, the majority of cells are surrounded by other cells and extracellular matrix in a three-dimensional (3D) arrangement. In recognition of this, studies are increasingly exploring the application of 3D culture techniques in an effort to more closely mimic the *in vivo* environment of the cells.

5. 3D culture models

3D culture is the growing of cells in an artificial environment that permits cells to grow and interact with their surroundings in all three dimensions. Often this involves growing SMCs on or in a scaffold comprised of extracellular matrix proteins, which is designed to resemble the *in vivo* tissue environment. Through mimicking the *in vivo* environment, the scaffold permits cells to attach, attain the right configuration and migrate or differentiate. Additionally, the 3D platform can provide cells with an environment comprising the growth factors, cytokines and some extracellular matrix proteins produced by the cells during their growth. Over the past several years, immense effort has been put into the creation of various 3D cell culture platforms, for instance protein gels such as Matrigel and collagen, which reconstruct extracellular

matrix composition [42]; polymer scaffolds, which mimic tissue structure and material properties [43]; hanging drop spheroids, which use water tension in liquid droplets to aggregate cells into spheroids [44–46]; round bottom plates, which use plate geometry to aggregate cells in spherical bottom wells [46]; as well as nano-patterned plates. Although these 3D cell culture platforms recreate tissue environments to varying degrees, they still have technical and cost limitations; for instance, long production times, the need for specialized equipment, as well as the produced 3D structures primarily being limited to spheroids, as opposed to more complex shapes that would better enable investigations into uterine smooth muscle contractility, such as rings or strips [47].

Research using primary SMCs to develop 3D culture systems was first performed by Young *et al*. [48] in 2003. Young *et al*. [48] performed single- and two-mesh experiments to study human myometrial physiology. In the single-mesh experiments, myometrial SMCs were seeded onto a polyglactin-910 (Vicryl) mesh after several passages [48]. In order to avoid contact with the plastic dish, each mesh was transferred to another culture dish after several days and suspended [48]. SMCs grew into and filled the pores of the mesh by repetitive proliferation, retraction, and proliferation [48]. A confluent, 3D tissue was attained 10–14 days after the initial seeding of the mesh [48]. In the two-mesh experiments, myometrial SMCs were seeded onto two layers of mesh and then cultured as described above [48]. SMCs not only grew into and filled the pores of each mesh, but also bridged between the two layers of mesh [48]. A confluent, 3D tissue was attained 2–3 weeks after the initial seeding of the mesh [48]. In the single-mesh experiments, average membrane potential of the cells was −35 ± 6 mV, and the thickness of the tissue was 9–40 μm (1–8 cells thick) within the pores of the mesh. In the two-mesh experiments, the bridging SMCs were able to sustain a tension of 5 g/cm^2 before separation of the two meshes occurred, as well as coordinated contractions of 40–200 cells [48]. SMCs grown in 3D using Vicryl mesh would therefore provide a model system for investigating the physiology of cell-to-cell interactions in human myometrium.

Malik *et al*. [49] developed human myometrium 3D cultures using a 3D collagen gel and examined the response of these cultures to external stimuli. Myometrial cells in 3D culture maintained the *in vivo* characteristics of the tissues they were derived from, as well as the characteristic fusiform of the SMCs [49]. That is, the cells exhibited long, tapering ends, resulting in a highly spindle-shaped phenotype, which stained positive for smooth muscle-specific α-actin and F-actin fibrils [49]. Previous studies have shown that TGF-β3 treatment of myometrial cells in 2D culture resulted in up-regulation of various extracellular matrix genes [50, 51]. During 3D culture of myometrial SMCs, TGF-β3 treatment produced a similar up-regulation of extracellular matrix genes, thus demonstrating that the cells in 3D culture were similarly bioactive [49]. These results indicate that cells in 3D culture can be used to investigate aspects of human parturition and to assess the effectiveness of various treatments.

More recently, Souza *et al*. [52] used magnetic 3D bio-printing to create more complex shapes, such as rings, to imitate uterine smooth muscle. The process involved magnetizing primary SMCs with a biocompatible nanoparticle assembly consisting of gold, iron oxide, and poly-L-lysine, then aggregating the cells into hollow rings using magnetic forces [52]. Once aggregated, the cells interacted and built extracellular matrix to recapitulate native tissue environments [52]. The bio-printed uterine rings contracted immediately after they were

removed off the magnet, which is consistent with the nature of the SMCs [52]. Furthermore, the tocolytic compounds, indomethacin and nifedipine, dose-dependently inhibited contractions in the myometrial smooth muscle rings [52]. Magnetic 3D bio-printing therefore constitutes a novel model system that can serve as a valuable toolfor investigating human parturition [52].

A recent development in the sphere of 3D culture is the use of biological scaffolds derived from decellularized tissues and organs. Decellularization involves devoiding a tissue or organ of its inhabiting cells, leaving behind the extracellular matrix scaffold of the original tissue. This naturally occurring 3D biologic scaffold can be then recellularized with primary SMCs that will differentiate into the original type of tissue (**Figure 3**). This approach has been successfully used in animal studies as well as in human clinical applications [53–59], and more recently, in myometrium [60]. Young *et al*. [60] used tissue-engineering techniques to reconstruct allo- and xeno-neo-myometrium from isolated rat and human myocytes and

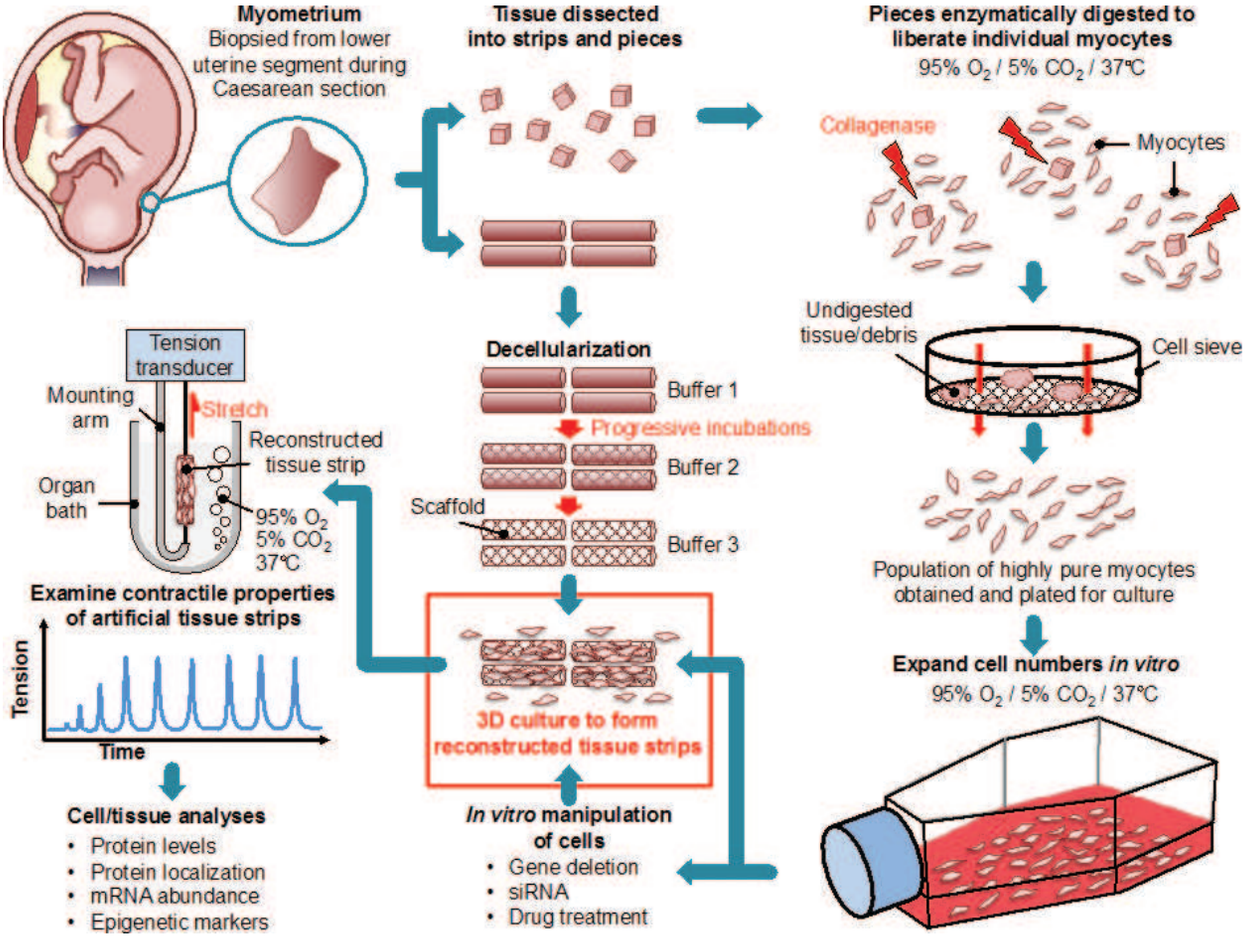

Figure 3. Application of 3D culture and decellularization to reconstruct tissue strips. Human myometrial biopsies are utilized to prepare primary myocyte cultures, as well as dissected into strips. Primary myocyte number is amplified using traditional 2D culture, while the tissue strips undergo decellularization to obtain scaffolds. Isolated primary myocytes are then seeded back into the scaffolds and the 3D culture maintained. Once the myocytes have permeated the scaffold, the contractility of the reconstructed tissue strips can be examined using contraction bioassays. To investigate signaling networks, primary myocytes can be modified while in 2D culture to observe the effect on contractility. The reconstructed strips can also be subjected to desired treatments or experimental conditions. The strips can then be snap frozen or preserved for subsequent analyses.

scaffolds. Allo-neo-myometrium was made from components of the same species (rat SMCs with rat scaffold; human SMCs with human scaffold), while xeno-neo-myometrium was made across species (human SMCs with rat scaffold) [60]. Myocytes were isolated using collagenase digestion, while scaffolds were isolated using ethanol/ trypsin protocols [60]. Isolated myocytes were amplified using monolayer culture and then cultured back into the scaffolds [60]. When human myocytes were cultured into human scaffold (allo-neo-myometrium), the myocytes overgrew each other after prolonged culture; nevertheless, they then separated, retracted, and never attained a thick layer of myocytes on the scaffold surface [60]. Rat myocytes cultured into rat scaffolds (allo-neo-myometrium) only formed multicellular layers on the surface following artificially created defects in rat scaffold [60]. Human myocytes grown on rat scaffold (xeno-neo-myometrium) formed multicellular layers on the surface [60]. These surface multicellular layers were thick, and more importantly, bundles of cells were observed to depths of 500 μm within the rat scaffold [60]. This xeno-neo-myometrium revealed structural integrity, good cellularity, as well as excellent cellular viability [58]. Interestingly, isometric contractility experiments revealed that human myocytes on rat scaffold (xeno-neo-myometrium) produced coordinated contractions, while human myocytes cultured into human scaffold (allo-neo-myometrium) did not [60]. The construction of neo-myometrium, prepared through the recellularization of decellularized scaffolds, is a model system that enables researchers to examine the role of extracellular matrices in contractility [60]. Additionally, through performing genetic manipulation of the SMCs prior to recellularization, the approach may enable researchers to investigate the role of different genes/proteins in myometrial contractility. Despite being an exciting avenue for future exploration, the production of neo-myometrium is complex, and to date, researchers have primarily utilized myometrial tissue strips or pieces to investigate the transition between uterine quiescence and contractility.

6. Tissue strip models

A model system frequently used to study pregnant human myometrium is the contraction bioassay, which uses strips of myometrium to study contractility *ex vivo*. Due to being freshly isolated intact pieces of tissue, *ex vivo* tissue strips arguably represent the *in vivo* phenotype more closely than other model systems. During the contraction bioassay, myometrial biopsies are typically obtained from the lower uterine segment of term singleton pregnancies during Cesarean section then dissected into strips (approximately 10 × 2 × 2 mm). The myometrial strips are connected to force transducers and lowered into organ baths containing a salt solution, such as Krebs–Henseleit buffer, which is continuously bubbled with 95% O_2/5% CO_2 and maintained at 37°C (pH 7.4). The contraction assay apparatus enables passive tension to be applied to individual strips. As a general protocol, myometrial strips are equilibrated for the first hour, during which the buffer is exchanged every 10 min and tension (1 g) is re-applied to strips after each wash. Following the equilibration period, strips are then left to develop spontaneous rhythmic contractions *ex vivo*. Once spontaneous rhythmic contractions have developed, the myometrial tissue strips can be used to analyze the effect of treatments on contractility in real-time [61–63], as well as capture dynamic events that occur during contraction and relaxation, such as protein phosphorylation [64, 65] (**Figure 4**).

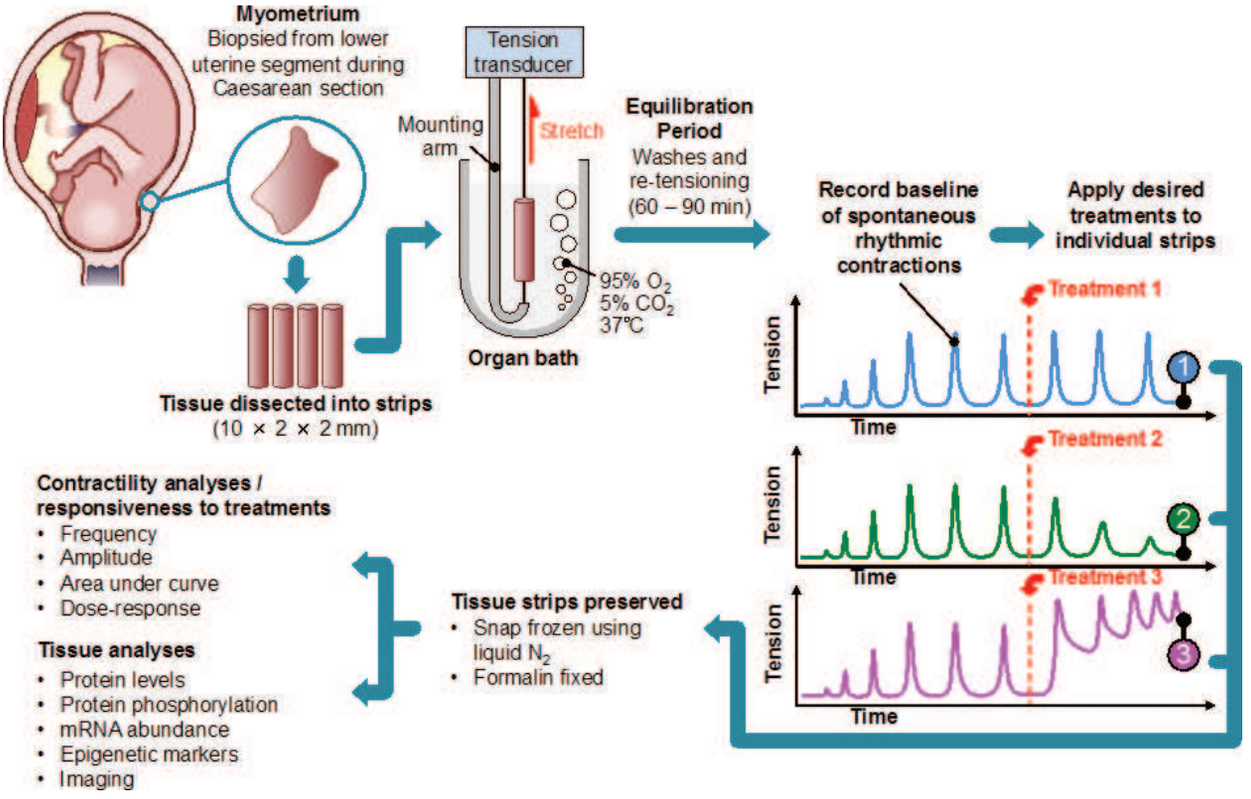

Figure 4. Myometrial tissue strip contraction bioassay. Human myometrial samples, collected during Cesarean section, are cleared of serosa, fibrous or damaged tissue and visible blood vessels then dissected into strips (~10 × 2 × 2 mm). The strips are then connected to a force transducer and lowered into organ baths containing a salt solution, which is continuously gassed with 95% O_2/5% CO_2 and maintained at 37°C (pH 7.4). The strips are equilibrated for the first hour, with washes and re-tensioning every 10 minutes. Strips are then left to develop spontaneous rhythmic contractions *ex vivo*. Upon recording a baseline of consistent contractions, desired treatments are applied to individual strips and the effects recorded. For subsequent analyses, strips can be snap frozen or preserved at the completion of the study, or at specific stages in relation treatment or contraction status.

Multiple studies have used myometrial tissue strips to analyze the effect of various treatments on contractility. Ruddock *et al*. [66] exposed contracting myometrial strips to progesterone or 17-alpha-hydroxyprogesterone caproate (17P) to determine whether these hormones directly inhibit human uterine contractility *in vitro*. Progesterone dose-dependently-inhibited uterine contractility, while 17P dose-dependently-stimulated contractility [66]. Similarly, Anderson *et al*. [61] also found that progesterone inhibited uterine contractility *in vitro*, however, in contrast to Ruddock *et al*. [66], they reported that 17P had no effect on uterine contractility [61]. Sexton *et al*. [67] also reported that 17P did not affect the contractility of pregnant or non-pregnant human myometrial strips. Although bioassay evidence suggests that 17P does not affect human uterine contractility directly, numerous clinical trials have showed that administration of 17P is successful in preventing preterm delivery in high-risk women [68–70]. This positive clinical outcome could be mediated through long-term genomic pathways that are not imitated during comparatively short-term organ bath studies. During organ bath studies, Anderson *et al*. [61] found that myometrial contractility began to decrease after 8 h incubation, presumably due to cell death and lack of energy substrates in the tissue; however, longer term studies by Young *et al*. [71] have shown that

culturing myometrial strips under tension can maintain spontaneous contractility and oxytocin responsiveness for up to 7 days.

Numerous tocolytics have also been tested using the contraction bioassay. Baumbach *et al*. [62] performed studies investigating the effects of a variety of contraction blocking drugs on term, nonlaboring myometrium *in vitro,* in both the presence and absence of progesterone. Progesterone alone had little inhibitory effect on contractility [62]. Nifedipine and indomethacin both significantly inhibited myometrial contractility alone, and to a greater extent when combined with progesterone [62]. These results indicated that combinations of progesterone with nifedipine or indomethacin might help in the prevention of preterm birth [62]. Another group also performed *in vitro* studies using myometrial strips to determine the effect of progesterone and nitric oxide alone or in a combination [72]. Previous study had shown that nitric oxide inhibits human myometrial contractions *in vitro* [73]. The combination of progesterone with nitric oxide was significantly more effective at inhibiting uterine contractility than progesterone alone or nitric oxide alone [72]. These findings suggest that administration of progesterone in combination with nitric oxide could be more successful in the treatment of preterm labor than either agent alone and demonstrates that the contraction bioassay is an effective model for elucidating synergisms between different tocolytics. This could translate into the development of more effective strategies for the prevention of preterm birth.

Recently, Paul *et al*. [74] developed the first drug delivery system targeting the pregnant uterus for preventing preterm birth. The team created liposomes loaded with various tocolytics, including nifedipine, salbutamol and rolipram, as well as the contraction-promoting agent, dofetilide, and conjugated to an antibody that specifically recognized an extracellular domain of the oxytocin receptor (OTR) [74]. Utilizing the contraction bioassay, Paul *et al*. [74] demonstrated that OTR-targeted liposomes loaded with nifedipine, salbutamol or rolipram consistently abolished human myometrial contractions *in vitro*, while OTR-targeted liposomes loaded with dofetilide increased contraction duration [74]. Non-targeted control liposomes loaded with these agents had no effect on contractility *in vitro* [74]. Paul *et al*. [74] also utilized the contraction bioassay to demonstrate that targeted liposomes were similarly effective against mouse uterine tissue *in vitro*.

The contraction bioassay model can also be used to examine tissue responsiveness and contractility after short- or long-term pre-treatment of myometrial strips. Fetalvero *et al*. [75] incubated myometrial tissue strips for 48 h in the presence or absence of the prostacyclin analog, iloprost. Initially, tissue strips displayed irregular and infrequent spontaneous contractions; as such, oxytocin had to be added to initiate rhythmic contractions [75]. The study revealed that long-term pre-treatment of myometrial strips with iloprost resulted in increased contractile responsiveness to oxytocin [75]. These results suggest that endogenous myometrial prostacyclin could play an important role in regulating myometrial activation, a crucial step in the initiation and progression of parturition [75]. Tyson *et al*. [63] revealed that corticotropin-releasing hormone (CRH) inhibited contractility in preterm and term myometrial strips *in vitro*. Pre-treatment of term myometrial tissue strips for 1 h with progesterone significantly increased CRH-induced relaxation [63]. Furthermore, pre-treatment of term myometrial tissue strips for 1 h with rolipram, a selective phosphodiesterase-4 inhibitor, significantly increased both CRH- and salbutamol-induced relaxation [63]. This implies that CRH might be a facilitator in the transition of the myometrium from relaxation to contraction.

In a novel application, Paul *et al*. [64] adapted the contraction bioassay to study the molecular regulatory events associated with contraction and relaxation in term pregnant human myometrium. In order to examine rapid phosphorylation and de-phosphorylation events that occur in phase with contractions, myometrial tissue strips were snap frozen at specific stages during the development of spontaneous contractions, including: (1) prior to the onset of any contractions, (2) at peak contraction and (3) during maximum relaxation between individual contractions [64]. The results showed that Caldesmon and ERK 1/2 were both phosphorylated during contractions and de-phosphorylated during periods of relaxation [64]. This application of the contraction bioassay provided a unique insight into phasic phosphorylation events that occur during spontaneous rhythmic myometrial contractions in humans. The study emphasized the importance of considering the contractile status of the tissue (contracted versus relaxed) during experimental design and interpretation of results [64]. Hudson *et al*. [65] applied the methodology developed by Paul *et al*. [64] to measure phosphorylation of MYL kinase (MYLK) and myosin phosphatase (MYLP) during spontaneous and oxytocin receptor (OXT)-stimulated phasic myometrial contractions *in vitro*. MYLK is activated by calcium-calmodulin, while MYLP is inhibited by phosphorylation of its myosin-binding subunit (MYPT1) by calcium-independent mechanisms [65]. Hudson *et al*. [65] showed that in fresh human myometrial tissue strips, spontaneous and OXT-stimulated phasic contractions were associated with Rho-associated kinase (ROCK)-dependent increases in phosphorylation of MYL and MYPT1 [65]. These findings provide new insight into uterine physiology and increase our understanding of the control of human myometrial activity.

While tissue strip models enable the real-time assessment of contractile properties of the myometrium, assessment of contractility is not always necessary. For such studies, preparation of myometrium as small pieces, often called explants, is an attractive alternative as it represents a similar *ex vivo* model, while avoiding the complexity of subjecting the tissue to stretch.

7. Tissue pieces/explant models

Explants are pieces of tissue cut from an animal or plant and used to initiate a culture. The term 'explant' is perhaps incorrectly applied to human myometrium, at least in the context of this model, in that the small myometrial pieces are not used to establish an ongoing culture. Rather, the tissue pieces are used as an *ex vivo* model on which to directly perform relatively short-term studies (<7 days). For this reason, tissue 'pieces' is perhaps more appropriate, and will be utilized hereafter. When utilized to study human parturition, tissue piece models typically involve the collection of non-laboring or laboring pregnant human myometrium during Cesarean section delivery, which is then dissected into small pieces (~3 × 3 × 3 mm) and incubated in Petri dishes or multi-well plates containing a nutrient media, such as Dulbecco's Modified Eagle's Medium (DMEM) [76–79]. The tissue pieces are then subjected to desired treatments or experimental conditions, after which the tissue pieces are snap frozen or preserved for subsequent analyses. Utilizing the tissue piece model, researchers can examine the effect of multiple treatments on a broad range of endpoints, including gene expression, protein levels and epigenetic modifications, as well as section the tissue to perform visualization studies (**Figure 5**). The number of treatments and endpoints that can be examined is limited only by

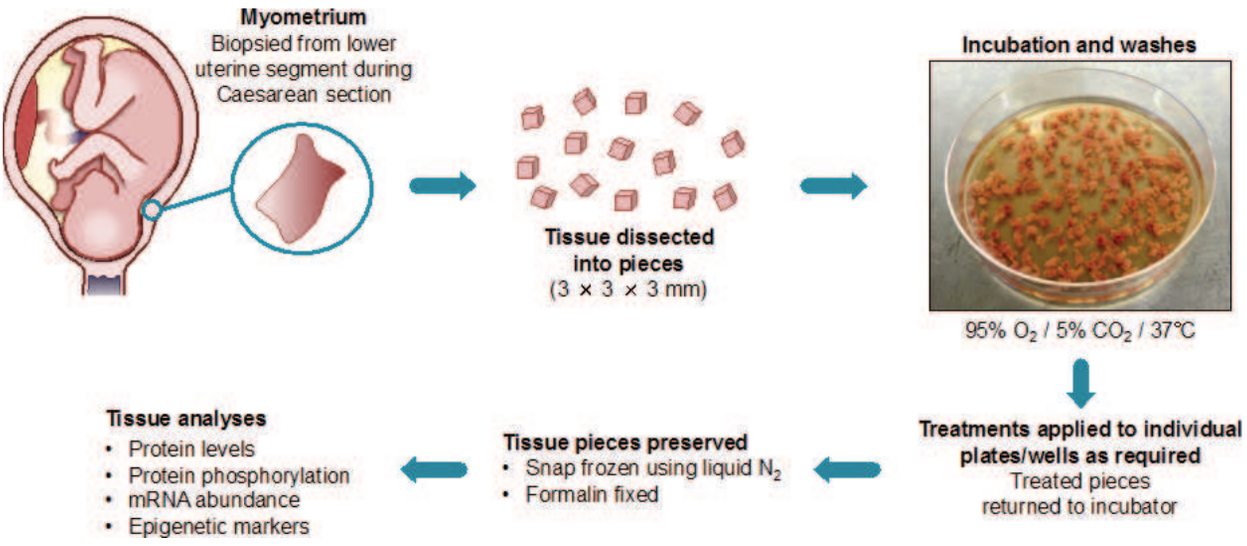

Figure 5. Myometrial tissue piece culture model. Human myometrial samples, collected during Cesarean section, are cleared of serosa, fibrous or damaged tissue and visible blood vessels, then dissected into pieces (~3 × 3 × 3 mm). The tissue pieces are incubated in Petri dishes containing a nutrient media (95% O_2/5% CO_2/37°C). Tissue pieces are then subjected to desired treatments or experimental conditions, after which the tissue pieces are snap frozen or preserved for subsequent analyses.

the amount of biopsied tissue available. The versatility and relative simplicity of the model has led to its extensive application to study human parturition. Fortunately, evidence indicates that the tissue piece model is more representative of myometrium *in vivo* than other *in vitro* models.

Georgiou *et al*. [80] compared gene expression in fresh myometrium, which was frozen at the time of Cesarean section, to *ex vivo* myometrial pieces, passage 4 primary myometrial cells, and hTERT myometrial cells. While whole-genome transcriptome analysis revealed that none of the *in vitro* models overlapped with the fresh tissue, in a principal component analysis plot [80], the gene expression profile of the tissue pieces most closely resembled that of the fresh tissue [80]. Upon direct comparison; 1444 genes varied between the *ex vivo* tissue pieces and fresh tissue; 3840 genes varied between passage 4 primary myometrial cells and fresh tissue; and 4603 genes varied between hTERT myometrial cells and fresh tissue [80]. A total of 555 genes varied commonly upon comparing all three *in vitro* groups (*ex vivo* tissue pieces, passage 4 primary myometrial cells and hTERT myometrial cells) to fresh tissue [80]. Georgiou *et al*. [80] validated the microarray results by examining genes associated with reproductive function and parturition, including *PTGS2, OXTR, PGR* and *GJA1*, as well as genes associated with the smooth muscle phenotype, including alpha smooth muscle actin (*ACTA2*) and MYL kinase (*MYLK*) [80]. *PGR*, *OXTR* and *GJA1* mRNA expression did not vary significantly between the *ex vivo* tissue pieces and fresh tissue [80]. In contrast, *PGR* mRNA expression significantly decreased in both passage four primary myometrial cells and hTERT myometrial cells, compared to fresh tissue [80]. Moreover, in passage four primary myometrial cells, *OXTR* and *GJA1* mRNA expression significantly decreased and increased, respectively, compared to fresh tissue [80], while *PTGS2* mRNA expression significantly increased in both tissue pieces and hTERT myometrial cells, compared to fresh tissue [80]. This was consistent with the gene ontology analysis, which indicated that there was an increase in genes associated with inflammation in all three *in vitro* models compared to fresh tissue [80]. *ACTA2*

and *MYLK* mRNA expression remained unchanged in *ex vivo* tissue pieces, compared to fresh tissue, whereas both were significantly decreased in hTERT myometrial cells, compared to fresh tissue [80]. Georgiou *et al*. [80] therefore concluded that myometrial *ex vivo* tissue pieces represent a superior model for studying human parturition over that of cell culture models.

It is important to note, however, that while evidence indicates that *ex vivo* tissue piece models are more representative of *in vivo* tissue than other models, recent studies demonstrate that *ex vivo* tissue pieces are not invulnerable to culture-induced changes. Ilicic *et al*. [81] examined whether myometrial tissue pieces undergo changes in key parturition-associated genes (*ESR1*, *PTGS2* and *OXTR*) upon being incubated for 48 h *ex vivo* (DMEM, 95% O_2/5% CO_2, 37°C, pH 7.4). Compared to fresh term non-laboring myometrium, the abundance of *ESR1* and *PTGS2* mRNAs significantly increased after 48 h incubation [81], while *OXTR* expression, which was high in the fresh non-laboring tissue, significantly decreased after 48 h incubation [81]. These changes are of importance as myometrial expression of *ESR1* [76, 82] and *PTGS2* [83, 84] increase with the onset of labor, while OXTR expression has been reported to decrease with advanced labor [84–87]. The gene expression changes observed during *ex vivo* incubation of term non-laboring myometrial tissue pieces are therefore consistent with the tissue transitioning to a labor-like state.

In light of these findings, Ilicic *et al*. [88] further examined whether myometrial tissue pieces undergo culture-induced changes in *PGR* isoform expression that are consistent with transition to a pro-contractile, labor-like phenotype. It was revealed that progesterone receptor A (*PR-A*) mRNA abundance increased after just 1 h culture, whereas progesterone receptor B (*PR-B*) mRNA abundance remained constant [88]. The net effect was a statistically significant increase in the *PR-A*/*PR-B* expression ratio after just 6 h of *in vitro* incubation [88]. This was consistent with previous reports that PR-A protein abundance increased during labor, while levels of PR-B were not altered by labor status [89]. Furthermore, it has been shown that the onset of labor is associated with increased abundance of *PR-A* mRNA, and an increase in the *PR-A*/*PR-B* expression ratio in term human myometrium [82]. The observation that *PR-A* mRNA abundance and the *PR-A*/*PR-B* expression ratio significantly increased during *in vitro* incubation is therefore once again consistent with the non-laboring tissue pieces transitioning to a labor-like state as a consequence of *in vitro* conditions [88]. Performing *in vitro* experiments using myometrium that is in a state of flux is not ideal, and raises the possibility that the relevant literature may contain findings reported for 'non-laboring' tissue that were in fact obtained with myometrium that was actively transitioning, or had already transitioned to, a laboring phenotype. To address this, the authors sought to identify culture conditions that could be implemented to maintain a non-laboring state, whereby *ex vivo* myometrial tissue pieces retained high *OXTR* mRNA expression, low *ESR1*, *PTGS2* and *PR-A* mRNA expression, as well as a low *PR-A*/*PR-B* expression ratio (<1; a *PR-B* dominant state), thereby providing a more appropriate *in vitro* model for conducting studies into myometrial biology [81, 88]. Adding progesterone to culture media prevented the culture-induced increase in *ESR1*, but failed to prevent culture-induced increases in *PTGS2* [81]. Culture-induced decrease in *OXTR* expression was prevented by supplementing media with PMA, however, this was concurrent with further upregulation of *PGTS2* expression toward a laboring phenotype [81]. Culture-induced increase of *PTGS2* expression *in vitro* was not counteracted by any of the treatments examined, including

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supplementation with NF-κB inhibitors [81]. A combination of progesterone and estrogen prevented the culture-induced increase in *PR-A*, but failed to prevent significant increase in the *PR-A*/*PR-B* expression ratio as *PR-B* expression was also reduced [88]. Stretch blocked the effects of steroids on *PR-A* expression [88]. Perhaps most importantly, supplementing media with the histone deacetylase inhibitor (HDACi), trichostatin A (TSA), prevented the culture-induced functional progesterone withdrawal phenomenon by maintaining a low *PR-A*/*PR-B* expression ratio, consistent with maintenance of a non-laboring phenotype in the *ex vivo* tissue pieces [88]. Similarly, Georgiou *et al*. [80] attempted to mimic physiological conditions as closely as possible by supplementing culture media with steroids and the adenylyl cyclase agonist, forskolin, but found that this had no effect on maintaining *PGR* levels *in vitro*. Culture-induced changes in *ESR1*, *PTGS2*, *OXTR* and *PGR* expression could not be controlled simultaneously [81, 88]. The implications are potentially significant as findings made using 'non-laboring' tissue may in fact have been generated using tissue that was no longer representative of the non-laboring phenotype, which could affect experimental outcomes.

Interestingly, rapid transition away from the non-laboring phenotype could be a requisite for contraction bioassay models. That is, upon suspending strips of freshly isolated term, non-laboring myometrium in organ baths, connected to force transducers, the strips initially show little to no contractility. Spontaneous contractions then progressively develop over time, ultimately becoming consistent and rhythmic after 1–2 h. This suggests that the tissue strips may be rapidly transitioning away from a non-laboring phenotype toward a labor-like phenotype [61, 63–67, 71, 74]. Additional studies are necessary to determine whether this indeed is the case.

Model	Advantages	Disadvantages
Primary myometrial smooth muscle cell culture model	• Cost effective • Straightforward/easy procedure • Easy downstream processing (cell extraction, imaging of cells)	• Not representative of 3D *in vivo* environment • Reduced cell-to -cell interaction • Short period of viability • Low biological relevance • Susceptible to contamination
Immortalized myometrial smooth muscle cell culture model	• Can be maintained for prolonged periods • Extended and indefinite growth *in vitro* • Cost effective • Increased growth rate • Easy downstream processing	• Not representative of 3D *in vivo* environment • Immortalization alters cell characteristics and functions • Low biological relevance • Susceptible to contamination • Decreased cell size
Stretch-based culture model (Flexercell)	• Mimics *in vivo* stretch • Defined and controlled deformations of cells *in vitro* • Straightforward/easy procedure	• Not representative of 3D *in vivo* environment • Reduced cell-to -cell interaction • Low biological relevance • Susceptible to contamination

Model	Advantages	Disadvantages
3D culture model	• Depicts 3D environment • Cell-to-cell interaction • Cells interact with extracellular matrix • More likely to represent the *in vivo* phenotype	• Expensive • Long production times • Large array of scaffold materials to trial and optimize • Requires specialized equipment • Lacks complex shapes • Long downstream processing • Extraction and imaging of cells hampered by culture depth
Tissue strip model (contraction bioassay)	• Representative of *in vivo* phenotype in the short-term • Multiple treatments can be assessed • Real-time assessment of contractility • Effects of treatments observed in real-time	• Tissue-to-tissue variation • Organ baths not sealed, therefore susceptible to contamination • Decreased myometrial contractility following prolonged incubation • Complex procedure • Requires expensive specialized equipment (organ bath contraction apparatus, analysis software) • Limited capacity to manipulate cells prior to the contraction assay
Tissue pieces model	• More representative of the *in vivo* phenotype than 2D cultured cells • Cost effective • Straightforward/easy procedure • Controlled culture conditions • Multiple treatments can be assessed simultaneously	• Tissue-to-tissue variation • Long downstream processing (mRNA/protein isolation, tissue sectioning and imaging) • Unable to assess stretch/contractility as an endpoint

Table 1. Advantages and disadvantages of model systems used to study pregnant human uterine smooth muscle.

Regardless of the whether uterine smooth muscle cells and tissues are in a state of flux during experimentation, it is apparent that each model system is associated with key advantages and disadvantages (**Table 1**). These include factors such as cost, ease of use, relevance to the *in vivo* situation, ability to be coupled with other techniques, downstream processing and more, and should be taken into consideration when selecting model systems.

8. Conclusion

Successful pregnancy necessitates that the uterus is maintained in a relaxed, quiescent state for the majority of pregnancy, before being transformed to a contractile and excitable

phenotype to facilitate parturition. Despite our rapidly advancing knowledge of myometrial biology, the exact mechanisms that regulate parturition are not yet understood. Characterizing the complex interactions that form the key regulatory pathways controlling uterine quiescence, contractility and the transition between the two states is therefore essential to understanding normal human birth, as well as obstetric complications, including preterm labor. *In vitro* models, such as myometrial SMC lines and *ex vivo* tissues, have been important tools for investigating these complex interactions. Nevertheless, recent evidence has shown that both primary cell lines and *ex vivo* tissues undergo culture-induced changes in expression of key myometrial genes. For that reason, further studies are warranted to determine appropriate culture conditions that could prevent or attenuate the changes, thus providing researchers with a stable platform on which to investigate myometrial biology. Until then, researchers should remain mindful of the limitations of myometrial cell and *ex vivo* tissue models, and be cautious when interpreting the relevance of results toward understanding human parturition.

Conflict of Interest

The Authors declare that there is no conflict of interest.

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