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Histone deacetylase inhibitors: Providing new insights and therapeutic avenues for unlocking human birth

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Abstract:	The pregnant uterus remains relaxed throughout fetal gestation before transforming to a contractile phenotype at term to facilitate birth. Despite ongoing progress, the precise mechanisms that regulate this phenotypic transformation are not yet understood. This knowledge gap limits our understanding of how dysregulation of uterine smooth muscle biology contributes to life threatening obstetric complications, including preterm birth, and hampers our ability to develop effective therapeutic intervention strategies. Protein acetylation plays a vital role in regulating protein structure, function, and subcellular localization, as well as gene transcription availability through regulating chromatin condensation. Histone deacetylase inhibitors (HDACis) are a class of compounds that block the removal of acetyl functional groups from proteins, and as such, have profound effects on important cellular events, including phenotypic transformation. A large body of data now demonstrates that HDACis have profound effects on pregnant human myometrium. Studies to date show that HDACis operate through both genomic and non-genomic mechanisms to affect myometrial function and phenotype. Interestingly, the effects of HDACis on pregnant myometrium are largely 'pro-relaxation', including the direct inhibition of contractile machinery as well as repression of pro-labor genes. The 'dual action' effects of HDACis make them a powerful tool for unlocking the regulatory processes that underpin myometrial phenotypic transformation and raises prospects of their therapeutic applications. Here we review the new insights into human myometrial biology that have garnered through the application of HDACis and explore their potential therapeutic application toward the development of novel preterm birth prevention strategies.
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Abstract (244 words)

The pregnant uterus remains relaxed throughout fetal gestation before transforming to a contractile phenotype at term to facilitate birth. Despite ongoing progress, the precise mechanisms that regulate this phenotypic transformation are not yet understood. This knowledge gap limits our understanding of how dysregulation of uterine smooth muscle biology contributes to life-threatening obstetric complications, including preterm birth, and hampers our ability to develop effective therapeutic intervention strategies.

Protein acetylation plays a vital role in regulating protein structure, function, and subcellular localization, as well as gene transcription availability through regulating chromatin condensation. Histone deacetylase inhibitors (HDACis) are a class of compounds that block the removal of acetyl functional groups from proteins, and as such, have profound effects on important cellular events, including phenotypic transformation. A large body of data now demonstrates that HDACis have profound effects on pregnant human myometrium. Studies to date show that HDACis operate through both genomic and non-genomic mechanisms to affect myometrial function and phenotype. Interestingly, the effects of HDACis on pregnant myometrium are largely 'pro-relaxation', including the direct inhibition of contractile machinery as well as repression of pro-labor genes.

The 'dual action' effects of HDACis make them a powerful tool for unlocking the regulatory processes that underpin myometrial phenotypic transformation and raises prospects of their therapeutic applications. Here we review the new insights into human myometrial biology that have garnered through the application of HDACis and explore their potential therapeutic application toward the development of novel preterm birth prevention strategies.

Keywords (6 keywords)

Parturition, progesterone, epigenetics, histone deacetylase, histone deacetylase inhibitors, Trichostatin A.

Background

Despite continued research during recent decades, we still do not understand fully the process of human birth. Particularly, we have no clear understanding of the regulatory processes, biochemical signaling pathways, and associated physiological changes that ultimately culminate in the initiation of contractions within the smooth muscle layer of the pregnant uterus. This knowledge gap limits our understanding of how dysregulation of myometrial cell function leads to life-threatening obstetric complications, such as preterm birth, which is the most common cause of death among newborns. There remains a pressing need to elucidate key elements of the regulatory processes that underpin the transition of the pregnant uterus from quiescence to generating forceful, coordinated contractions necessary for normal birth.

Research to understand human birth is subject to ethical considerations that tightly constrain experimental intervention. Researchers rely heavily on the use of *in vitro* and *ex vivo* models such as pregnant human myometrial cell lines, uterine tissue biopsies, and animal models of pregnancy complications [1]. Within these models, researchers routinely treat cells with different classes of chemical compounds to observe their effects on gene expression, phenotype, and in the case of uterine myocytes, contractile properties. One class of compounds that have led to significant insights into myometrial biology are histone deacetylase (HDAC) inhibitors (HDACis).

Acetylation is the addition of an acetyl functional group (CH_3CO) to a compound, which is catalyzed by histone acetyltransferases (HATs), while deacetylation is the process of removing an acetyl functional group, which is catalyzed by HDACs. The classification of HDACs is outlined in Table 1.

While lipids and carbohydrates can be acetylated, it is the acetylation of proteins that has received the most attention, as acetylation is an important post-translational modification impacting protein structure and function (Figure 1) [2]. Indeed, approximately 85% of human proteins are acetylated on their N-terminus, which plays an important role in regulating the synthesis, stability, and localization of the protein [3]. N-terminal amino acids that may be acetylated by N-terminal acetyltransferases (NATs) include glycine, alanine, serine, methionine, and aspartic acid [4].

Within eukaryotic cell nuclei, ε-acetylation of lysines on histone proteins by HATs plays a vital role in regulating gene expression [5]. That is, through reducing the positive charge of histones, acetylation decreases the interaction between histones and the negatively charged phosphate backbone of DNA, which promotes the decondensation of chromatin, which in turn enables greater levels of gene transcription [5]. Lysine ε-acetylation is therefore primarily associated with gene activation while deacetylation is primarily associated with gene silencing (Figure 2)[6].

Given that the transition from not-in-labor to in-labor is associated with protein post-translational events and differential gene expression, HDACis have been used to explore the role of protein acetylation in myometrial biology. The HDACis examined throughout these investigations include Trichostatin A (TSA), Suberoylanilide hydroxamic acid (SAHA), Valproic acid (VPA), Suberic bis-hydroxamate (SBHA), Compound 2, and Resveratrol (Table 2). The structure of these agents are shown in Figure 3. Studies utilizing these agents have led to significant findings, which we review here to obtain an up-to-date picture of the non-genomic and genomic effects of HDACis in the context of parturition.

New Insights into Myometrial Biology

Genomic effects of HDACis

Progesterone signaling

The steroid hormone progesterone plays a central role in maintaining pregnancy by promoting myometrial quiescence and relaxation [7-9]. The withdrawal of progesterone action signals the end of pregnancy, and in most non-primate mammals labor is initiated by a fall in maternal circulating levels of progesterone [10-14]. In humans and higher primates, however, maternal, fetal, and amniotic concentrations of progesterone remain elevated up to and during labor [15-17]. Nonetheless, blocking the actions of progesterone at any stage of pregnancy (e.g., by the progesterone antagonist RU486) induces labor and stimulates cervical ripening [18, 19]. This indicates that progesterone action is essential in maintaining human pregnancy and that the onset

of human labor involves a decrease of progesterone responsiveness ("functional progesterone withdrawal"), rather than a decline in circulating progesterone levels.

In 2016, Nadeem *et al.* [20] published important evidence of the molecular mechanism contributing to functional progesterone withdrawal in human myometrium. They found that found that during pregnancy, progesterone-liganded PR-B associates with Jun/Jun homodimers and the repressor complex P54nrb/Sin3a/HDAC to repress the transcription of *GJA1*, which encodes the key contraction-associated protein, connexin-43 [20]. In contrast, during labor PR-A, a truncated PR isoform, dissociates from progesterone, and in this unliganded state interacts with Fos/Jun heterodimers to activate the transcription of *GJA1*, thereby promoting term labor [20]. In 2018, Nadeem *et al.* [21] used mouse models of preterm labor to show that a similar myometrial AP-1 protein composition, from Jun/Jun homodimer during most of gestation to Fos/Jun heterodimer during labor, occurs during preterm labor, thus suggesting that similar molecular machinery is involved in the induction of preterm and term labor. This finding builds upon a vast body of previous work demonstrating that PR-B is the principal mediator of progesterone's relaxatory actions through repressing the expression of genes that promote uterine contractility [22]. PR-A represses the transcriptional activity of PR-B, and, in doing so, decreases progesterone responsiveness [7, 8, 19, 23]. The amount of PR-A relative to PR-B in myometrial cells is therefore a key determinant of progesterone responsiveness. A low PR-A/PR-B ratio (a PR-B dominant state) is consistent with responsiveness to progesterone's relaxatory effects, while a high PR-A/PR-B ratio (a PR-A dominant state) is consistent with a decrease of progesterone responsiveness [7, 8, 19, 23]. In accordance with this, the PR-A/PR-B protein ratio in pregnant human myometrium was 0.5 (a PR-B dominant state) at 30-weeks gestation, which increased to 1.0 at term prior to the onset of labor and increased further to 3.0 (a PR-A dominant state) at the time of labor [24]. Furthermore, the *PR-A/PR-B* mRNA ratio has been directly correlated with estrogen receptor α (*ERα*) mRNA levels, which is indicative of a functional link between the *PR-A/PR-B* ratio and the action of estrogens, which drive the expression of contraction-associated proteins [25].

The PRs interact with coactivators and corepressors to increase and decrease their transcriptional activities, respectively. The importance of these coregulators in the control of

transcriptional activity of PRs was investigated by Condon *et al*. [26]. They found that the expression of PR coactivators cAMP-response element-binding protein (CREB)-binding protein (CBP) and steroid receptor coactivators (SRC) 2 and 3 was decreased in human fundal myometrium as well as in mouse uterus during labor [26]. CBP and SRCs possess HAT activity that may contribute to PR actions at progesterone responsive genes [27, 28]. Consistent with this, Condon *et al*. [26] detected decreased histone H3 acetylation (aH3) in myometrium obtained from women in labor, and also in pregnant mouse uteri at term [26]. This indicates that a decrease in coactivator levels within the uterus leads to a decline in histone acetylation, thus closing the chromatin and inhibiting general transcription factor interaction with the target genes that maintain uterine quiescence. To determine the functional significance of the decline in coactivator levels and histone acetylation, Condon *et al*. [26] investigated the effect of TSA, a potent, reversible Class I and II HDACi (and potentially Sirt6 (Class III) [29, 30], in pregnant mice. Daily administration of TSA to pregnant mice caused hyperacetylation of uterine histone H3 on gestation day 19. Parturition was delayed by 24 – 48 h in TSA-treated mice that were allowed to deliver spontaneously [26]. TSA administration did not cause any evident toxicity to the mother and did not change maternal serum progesterone levels when compared to untreated control mice [26]. Moreover, TSA-treated mice delivered viable pups of normal litter size when compared to control mice [26]. Previous studies have shown that the administration of TSA during the crucial stages of embryonic organogenesis caused teratogenic effects [31] and neural tube defects in mouse embryos *in vitro* [32]. Nervi *et al.* [33] demonstrated that TSA administrated to pregnant mice at post-implantation was not toxic to the pregnant mice, and did not cause any obvious malformation during somitogenesis or at later stages of development. Comparable to Condon *et al*. [26], treated pups were born at a similar frequency and were of normal litter size when compared to control pups [33]. Moreover, pups developed to normal adult size and were fertile [33]. The inconsistency between these studies may be attributable to different TSA concentrations analyzed as well as different experimental methods used. These studies suggest that the overall decrease in histone acetylation within the pregnant uterus at term, associated with significantly reduced levels of steroid receptor coactivators with intrinsic HAT activity, might contribute to loss of PR function leading to birth. An important implication of these results is that HDACis, which maintain histone acetylation, may represent an avenue for preventing preterm birth.

In 2017, Ilicic *et al.* [34] found that placing non-laboring pregnant human myometrium in culture (as explants) causes the tissue to undergo a phenotypic transition toward a labor-like phenotype. During this transition, the expression of genes encoding ERα (*ESR1)*, prostaglandin-endoperoxide synthase 2 *(PTGS2),* and *PR-A* were significantly up-regulated [34, 35]. These outcomes suggest that freshly biopsied non-laboring human myometrium undergoes a transition from a pro-gestation phenotype, with a low *PR-A/PR-B* ratio, to a phenotype with a high *PR-A/PR-B* ratio, consistent with reduced progesterone sensitivity [35]. Significant elevation of the *PR-A/PR-B* ratio was observed after 6 hours of culture [35]. These changes are similar to gene regulatory events that occur during the onset of labor and are consistent with observations that strips of non-laboring human myometrium spontaneously develop rhythmic contractions *in vitro* following a period of equilibration that may take several hours [36-39]. Using this model of myometrial transformation, Ilicic *et al.* [35] investigated factors that regulate PR isoform expression in human myometrium. They showed that up-regulated *PR-A* expression and *PR-A/PR-B* ratio during myometrial transformation can be completely blocked by incubating myometrial tissue with TSA, consistent with maintaining progesterone responsiveness [35] (Figure 4). These results indicate that stabilizing histone and/or non-histone protein acetylation is critical for maintaining the progesterone-dependent quiescent phenotype of human myometrium in culture.

Consistent with these data, Li *et al*. [40] reported that *PR-A* expression was regulated by epigenetic mechanisms in term human myometrium. They found that increased *PR-A* expression was associated with decreased DNA methylation in its promoter and decreased expression of the DNA methyltransferase mRNAs encoding DNMT1 and DNMT3a (but not of *DNMT3b* mRNA) in uterine biopsies [40]. Studies using breast cancer cells have shown that HDACs, particularly HDAC1, regulate the *ESR1* gene, which encodes ERα, by binding to the *ESR1* promoter [41, 42]. By inference, this suggests that HDAC1 may also be involved in the ER-dependent regulation of the *PR* gene. Furthermore, low expression of HDAC1 was associated with increased expression of *PR-A* in myometrium during labor, and HDAC1 down-regulated *PR-A* expression by binding to the promoter region of *PR-A* [43]. These results implicate HDAC1 in regulating the levels of both ER and PR-A in the context of functional progesterone withdrawal and estrogen activation. Additionally, in laboring

human myometrium, Chai *et al.* [44] found significantly higher levels of histone acetylation at the *PR-A* promoter compared to the *PR-B* promoter, which is consistent with progesterone sensitivity being 'switched off' as a result of epigenetic activation of *PR-A* expression. An epigenetic modifier that prevents *PR-A* promoter activation may therefore preserve the progesterone-sensitive phenotype of the myometrium and potentially other progesterone-sensitive gestational tissues. The observation by Ilicic *et al.* [35] that TSA suppressed *PR-A* expression in pregnant human myometrium *in vitro* raises the possibility that HDACi could prove useful for maintaining or potentially even restoring myometrial progesterone responsiveness during pregnancy.

In 2021, Zierden *et al*. [45] examined the vaginal delivery of HDACis via mucoinert nanosuspensions for the prevention of lipopolysaccharide (LPS)-induced preterm birth in mice. They found that systemic administration of the clinically used 17-α-hydroxyprogesterone caproate did not prevent LPS-induced preterm birth. Vaginal administration of progesterone alone, either as the gel Crinone or in mucoinert nanosuspensions was also ineffective [45]. Furthermore, when Crinone was given to the vehicle (saline) control group, it worsened preterm birth outcomes [45]. However, vaginal administration of progesterone combined with either of the HDACis TSA or SAHA in nanosuspensions, prevented preterm birth, resulted in higher litter viability, and offspring that exhibited neurotypical development (brain weight, behavior studies) [45]. Pharmacokinetic studies for the vaginally administered progesterone/TSA nanosuspension showed that both progesterone and TSA reached the cervix and myometrium with sustained exposure for up to 8 hours [45]. The progesterone/TSA nanosuspension combination also led to decreased endotoxin content in the amniotic fluid and had anti-inflammatory effects in myometrial tissue, decreasing the myometrial expression of *Il6*, *Nfkb1*, *Ptgs2*, *Gja1*, *Ccl3*, and *Oxtr*. Additional studies in an immortalized human myometrial cell line, hTERT-HM$^{A/B}$, stably transfected with PR-A and PR-B transgenes such that each can be experimentally controlled using independent inducers, revealed that the progesterone/TSA nanosuspension combination promoted the anti-inflammatory action of progesterone by decreasing the PR-A/PR-B ratio and increasing PR-B stability. The nanoparticles suppressed contractility in PHM1-41 cells at the drug concentrations measured in the mouse myometrial tissue [45]. These findings demonstrate that vaginally delivered HDACis can block LPS-

induced preterm birth by down-regulating the myometrial expression of inflammatory genes and reducing myometrial cell contractility. Importantly, live, neurotypical offspring have been delivered after the intervention. These findings also highlight that the administration of HDACis via vaginally administered nanosuspensions [45, 46] or uterine-targeted nanoparticles [38] is a therapeutic strategy with the potential to prevent preterm birth.

Cyclic AMP signaling

Placentally derived human chorionic gonadotropin (hCG) is crucial for the maintenance of myometrial quiescence [47]. The action of hCG is mediated by the chorionic gonadotropin (CG)/luteinizing hormone (LH) receptors, which are coupled to adenyl cyclase by the stimulatory G protein, $G\alpha_s$, resulting in the formation of cAMP. Genomic cAMP signaling has both pro-relaxatory and pro-contractile roles (reviewed by Butler *at al*. [48]). Even though cAMP signals predominantly through protein kinase A (PKA) to reduce contractility [49], prolonged exposure to cAMP can activate pro-contractile mitogen-activated protein kinase (MAPK) signaling [50] and upregulate pro-contractile genes [51]. Decreased levels of hCG/LH receptors in the myometrium are associated with term and preterm labor [52], which suggests that maintaining high levels of hCG/LH receptor can help prevent early labor onset. The expression of the hCG/LH receptor gene (*LHCGR*) in myometrial cells was found to be regulated by specificity protein-1 to -4 (Sp1-4) [53]. Pull-down assays using double-stranded, biotinylated oligonucleotides, electro-mobility shift assays and luciferase reporter experiments demonstrated the association and functional involvement of Sp1/Sp3, Sp4, Sp-like factor(s), and HDAC1/2 complexes in *LHCGR* promoter regulation in primary cultures of human myometrial cells [53]. Treatment of the myometrial cells with TSA increased hCG/LH receptor mRNA expression and protein levels, suggesting that HDACis function to regulate *LHCGR* gene activity by controlling acetylated histone and/or non-histone protein levels at critical regulatory elements [53]. These findings further corroborate the notion that TSA is a tocolytic (contraction blocking) agent.

As mentioned, the relaxing effects of cAMP are mediated through PKA, which is a heterotetrameric protein complex that consists of two regulatory (R) and two catalytic (C) subunits. Recent data shows that the level of the PKA regulatory subunit RIIα increases throughout pregnancy and then decreases during parturition in the myometrium [54]. Karolczak-Bayatti *et al.* [55]

investigated the mechanism by which the RIIα gene was regulated in the uterine muscle. They showed that all three SpI-III (GC) binding domains within the RIIα promoter are occupied by Sp1 and Sp3, which then recruit HDAC1/2 and the histone binding protein RbAp48 to regulate gene expression [55]. Inhibition of HDAC1/2 activity by TSA resulted in significantly increased mRNA expression and protein levels of RIIα [55]. This increase in mRNA expression was proceeded by an increase of aH3 and binding of Sp3, HDAC2, and RNA-polymerase II large subunit RPB1 to the three SpI-III (GC) *cis*-elements within the RIIα promoter [55]. The results imply that the RIIα gene in human myometrial smooth muscle cells is regulated by epigenetic mechanisms that involve histone acetylation, Sp3 acetylation, and recruitment of HDAC1/2 to multiple GC *cis*-acting elements within the proximal promoter of the gene. Furthermore, TSA reduced contractions of myometrial strips under conditions when RIIα expression and (total) aH3 were increased indicating that HDACi could be useful to maintain RIIα levels and myometrial quiescence throughout pregnancy.

G-proteins are key components of the signaling pathways that mediate the actions of the activators and inhibitors of myometrial smooth muscle activity [56]. As discussed, the binding of hCG to its receptor activates $G\alpha_s$, which in turn stimulates adenylate cyclase and results in cAMP accumulation increasing PKA activity [57]. The binding of oxytocin to its receptor activates the G-proteins $G\alpha_{q/11}$ and $G\alpha_{i/o}$, which stimulate phospholipase C, thus hydrolyzing phosphatidylinositol 4,5-bisphosphate to inositol triphosphate and diacylglycerol [57]. Signaling through $G\alpha_{i/o}$ leads to decreased adenylate cyclase activity and reduced intracellular cAMP level, whilst signaling through $G\alpha_{q/11}$ leads to an increase in the intracellular concentration of calcium ions (Ca^{2+}) [58-60]. Increased intracellular Ca^{2+} can activate myosin light-chain kinase to phosphorylate myosin light chains and instigate the contraction of the myocytes [61]. The balance between the various Gα proteins that control relaxatory and contractile pathways is crucial for the regulation of myometrial activity. Sp-like transcription factors binding to GC boxes within the promoter region stimulated expression of the $G\alpha_s$ gene (located in the *GNAS complex locus*) through a PKA-dependent mechanism [62]. Karolczak-Bayatti *et al*. [63] explored the role of Sp1-4 proteins and their transcriptional and epigenetic co-regulators in $G\alpha_s$ gene regulation in primary cultures of human myometrial cells. Transcriptional complexes consisting of Sp1-4, HDAC1/2, RbAp48, and mSin3A were found at four

out of six Sp1-4 sites within the proximal promoter region of the $G\alpha_s$ gene. Given that HDAC1/2 were recruited to the $G\alpha_s$ promoter, the effect of TSA, a pan class I/II HDACi, was determined on $G\alpha_s$ expression [55]. Treatment with TSA led to increased $G\alpha_s$ protein levels; however, there was no increase in $G\alpha_s$ mRNA expression. The proteasome inhibitor MG132 increased $G\alpha_s$ protein levels similarly to TSA, and treatment with both drugs resulted in the accumulation of high molecular weight polyubiquitinated proteins indicating the suppression of proteasome activity. Thus, the increase in $G\alpha_s$ protein level in response to TSA may have occurred due to the reduction of protein degradation, demonstrating that HDACi have post-translational and non-genomic actions as discussed in more detail below.

The above evidence indicates that HDACis promote myometrial quiescence via cAMP signaling; however, it is important to note that cAMP signaling has both pro-relaxatory and pro-contractile roles and that prolonged exposure to cAMP can promote myometrial contractility. As such, further research will be necessary to determine the role of HDACis in cAMP signalling.

Inflammatory signaling

Webster *et al*. [64] reported that TSA-induced myometrial relaxation was inhibited by TNFα via a NF-κB-dependent mechanism, but TNFα did not stimulate contractions on its own. Previous data by the same group indicated that TNFα repressed $G\alpha_s$ through the NF-κB RelA subunit in myometrial cells in a non-DNA binding fashion by competing for limiting amounts of the key transcription co-factor CBP [65], which is a HAT. They had also demonstrated that the -837 to -618 region of the $G\alpha_s$ promoter was occupied by the transcription factors, CREB, Egr-1, Sp1, and CBP [64]. Furthermore, the CBP-containing transcriptional complexes formed within this region increased $G\alpha_s$ expression [64]. In the presence of TNFα, there were no changes in the levels of CREB, Egr-1, and Sp1, while CBP levels were significantly reduced in agreement with competition for a limiting amount of CBP by RelA [64]. This was associated with increased levels of HDAC1 as well as increased H4K8ac, but no change of H3K9ac levels at the promoter and no change of protein acetylation globally [64]. At the same time, overexpression of HDAC1 did repress the activity of a $G\alpha_s$-luc construct, while CBP overexpression and TSA induced $G\alpha_s$-luc promoter construct activity [64]. These results further demonstrate that the involvement of protein/histone acetylation in

regulating genes that influence myometrial contractility occurs by complex and genomic locus-specific mechanisms with an overall effect of promoting relaxation.

It is well established that labor is an inflammatory process associated with the increased production of pro-inflammatory mediators [66]. NF-κB is a key orchestrator of inflammatory responses and has been suggested to play a crucial role in labor [67]. The NF-κB subunit RelA is subject to acetylation at multiple sites with major effects on its function [68], and work by Condon *et al*. [26] has indicated that there might be a therapeutic use for HDACis in the management of preterm birth. Thus, it was crucial to investigate the impact of HDACis on NF-κB activity in the myometrium. Lindstrom *et al*. [69] showed that short-term (1-2 h) treatment with TSA increased interleukin (IL)-1β-induced NF-κB DNA binding and nuclear localization in cultured primary myometrial cells. In contrast, long-term treatment (24 h) inhibited IL-1β-induced NF-κB DNA binding [69]. The short-term increase of NF-κB nuclear localization was due to the delayed recovery of I-κBα, the inhibitory subunit of the complex, in TSA plus IL-1β treated cells due to an increase of I-κB kinase activity by the drug, which enhanced I-κBα degradation. Remarkably, TSA increased both the short- and long-term IL-1β-induced NF-κB transcriptional activity when assayed with a transfected promoter construct despite the drop of DNA binding at 24 h. This suggested that TSA acted on the transcriptional activity of NF-κB independently of its effect on DNA binding. Moreover, both short- and long-term exposure to TSA plus IL-1β inhibited the expression of the pro-labor genes, *PTGS2*, *CXCL8* (IL-8), *IL6* (IL-6), and *CCL5* (RANTES), which was associated with the reduced expression of the pro-inflammatory transcription factor, c-Jun [69]. These results indicate that pan-HDACis, like TSA, might act on various acetylated histone and non-histone protein targets affecting multiple regulatory pathways. The pro-inflammatory genes examined by Lindstrom *et al*. [69] might be suppressed by HDACis through c-Jun and not the NF-κB system in myometrial cells; however, a full gene expression profile may reveal sets of pro- and anti-inflammatory genes that are responsive to HDACis via modulating NF-κB. HDACi exposure favors uterine quiescence, but the administration of a HDACi in combination with an NF-κB inhibitor might be an approach that reduces potential undesirable effects of HDACis, such as the induction of pro-inflammatory pathways.

Ion channels

The quiescent and contractile states of the myometrium are mediated through mechanisms that involve ion channels. Previous research has shown that MaxiK (large-conductance Ca^{2+}- and voltage-activated potassium ion (K^+) channel) is one of the major pro-quiescence K^+ channels and L-type Ca^{2+} channels (LTCC) are the predominant pro-contractile Ca^{2+} channels in the myometrium. A dynamic balance between these is likely to be crucial in the switch from electrical quiescence to activation and phasic contractions. MaxiK channel activity has been reported to decrease in the myometrium of late pregnant rats [70]. Waite *et al*. [71] demonstrated that both MaxiK splice variants (M1, M3, and M4) and LTCC splice variants (exons 8, 30-34, 40-43) were expressed in non-laboring term pregnant myometrium. They further showed that TSA was able to significantly reduce MaxiK expression as well as the expression of MaxiK splice variants M1, M3, and M4 in primary myometrial cell cultures [71]. TSA exhibited a dual effect on LTCC splice variants in primary myometrial cell culture [71]. TSA significantly induced expression of the exon 8 variant, whilst also significantly reducing expression of other LTCC splice variants including that of encoding exons 30-34 and exons 40-43 [67]. It is known that MaxiK and LTCC are responsive to $G\alpha_s$ dependent cAMP signaling, which results in the enhanced efflux of K^+ via MaxiK (reviewed in [71]) and may contribute to the relaxing effect of TSA, which increases $G\alpha_s$ expression (see above). The reduction of LTCC and MaxiK expression and alterations of splice variants distribution by TSA demonstrate additional facets of the many-sided relaxatory actions of this HDACi, supporting its potential tocolytic utility.

Non-genomic effects of HDACis

In addition to their genomic effects, there is evidence that HDACis exert non-genomic effects on human myometrium, which are also tocolytic. Moynihan *et al.* [72] used human myometrial tissue strips to analyze the effects of three HDACis, TSA, VPA, and SBHA, on human uterine contractility. TSA and SBHA each dose-dependently inhibited both spontaneous and oxytocin-induced contractions in the human myometrium with SBHA being the more potent [72]. VPA, an anti-seizure agent, also inhibited both spontaneous and oxytocin-induced contractions but was the least effective of the three HDACis [72]. VPA is a human teratogen that affects fetal neural development [73], and thus would not be appropriate for use in pregnancy [73]. The short timeframe in which the HDACis

blocked uterine contractility (20 – 60 min) suggested that contractions were blocked through non-genomic mechanisms.

HDAC8, a class I HDAC, is expressed by human smooth muscle cells *in vivo*, including vascular and visceral smooth muscle cells, myoepithelial cells, and myofibroblasts [74]. Remarkably, the enzyme is localized mostly in the cytoplasm of smooth muscle cells both *in vivo* and *in vitro*, rather than in the nuclear compartment [74]. Furthermore, HDAC8 displays a striking stress fiber-like pattern of distribution and is co-expressed with two major constituents of the smooth muscle contractile apparatus, smooth muscle α-actin (α-SMA) and smooth muscle myosin heavy chain. *In vitro* and *in vivo* studies have found that HDAC8 associates with α-SMA but not with β-actin [75]. In addition, inhibition of HDAC8 by RNA interference results in a markedly reduced ability of human smooth muscle cells to contract collagen lattices [75]. These results indicate that HDAC8 might be involved in the regulation of smooth muscle cytoskeleton dynamics [75]. Based on these results, the highly selective linkerless hydroxamic acid HDAC8 inhibitor, termed Compound 2 [76], was utilized to determine the interaction of HDAC8 with α-SMA and myosin heavy chain [77]. Compound 2 inhibited both spontaneous and oxytocin-induced contractions in human myometrium *in vitro* [77]. The inhibitory effects of Compound 2 were not associated with an increase in aH3 nor marked global gene expression changes [77]. TSA, however, exerted an inhibitory effect on both spontaneous and oxytocin-induced contractions in human myometrium *in vitro*, [72] as well as significantly increased aH3 and acetylated α-tubulin levels, as expected of a pan-class I/II HDACi [77]. These results suggest that the relaxatory effects of Compound 2 are likely through non-epigenetic pathways, and as such require further investigation.

As previously discussed, Karolczak-Bayatii *et al*. [55] showed an increase of mRNA expression and protein levels for the PKA regulatory subunit, RIIα, in human myometrial cells following TSA treatment. In addition, treatment with TSA caused a significant decrease in oxytocin-induced contractions of myometrial strips, measured as isometric tension changes [55], within a short time frame (90 minutes), consistent with a previous study by Moynihan *et al.* [72]. Decreased contractility was associated with an increase in RIIα protein expression as well as an increase in aH3 levels in the strips. These results demonstrate that HDAC1/2 inhibitors, such as TSA, have

concomitant fast actions on gene expression, chromatin structure, and cytoplasmic mechanisms of contraction, which could result in expeditious, clinically useful, tocolysis.

The ability of HDACis to block myometrial contractility [55, 72, 77] raised interest in determining the conditions under which the tocolytic action occurs. Webster *et al*. [64] showed that TSA alone caused dose-dependent relaxation of spontaneous myometrial contractions; however, this relaxatory effect was overcome by higher doses of TNF that induced NF-κB activity in primary myometrial myocytes. TNF on its own did not induce myometrial contractions [64]; however, data reviewed by Lindstrom and Bennett [67] suggest that NF-κB plays a crucial role overall in myometrial regulation at labor. Moreover, the NF-κB inhibitor N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine, which inhibits endogenous TNF synthesis, exerted dose-dependent relaxation of spontaneous myometrial contractions and significantly attenuated the action of TNF on TSA-mediated relaxation [64]. The results indicate that TNF's uterotonic actions have an NF-κB-regulated element and that TNF's uterotonic effects may only occur once other factors, that are yet to be identified, have been acetylated. These findings also highlight that more work is required to delineate the conditions under which HDACis maintain myometrial quiescence.

A Closer Look at Resveratrol

Resveratrol has attracted attention due to its extensive pharmacological actions. The compound is a stilbenoid, which is a natural polyphenol produced by more than 70 plants. Resveratrol is reported to have potential medical applications related to cancer, cardiovascular disease, diabetes, aging, and lifespan extension [78]. One intriguing aspect of resveratrol is that evidence indicates that it is an inhibitor of class I, II, and IV HDACs, but an activator of class III HDACs [79].

Resveratrol promotes smooth muscle relaxation, including blood vessels [80-83], gallbladder [84], and the rat uterus [85, 86]. In the non-pregnant rat uterus, Hsia *et al*. [85] demonstrated that resveratrol inhibits contractions induced by $PGF_{2\alpha}$, oxytocin, acetylcholine, carbachol, high K^+ concentrations, and a Ca^{2+} channel activator. Furthermore, they found that resveratrol inhibited $PGF_{2\alpha}$-induced increases in Ca^{2+} in human primary uterine smooth muscle cells and mimicked Ca^{2+}

channel blockers to block Ca^{2+} influx via voltage-operated Ca^{2+} channels in the plasma membrane [85]. These results indicate that resveratrol inhibits uterine contractions by blocking external Ca^{2+} influx, causing decreased intracellular Ca^{2+} concentrations [85]. Novakovic *et al*. [86] reported that resveratrol treatment inhibits spontaneous rhythmic contractions, as well as both phasic and tonic oxytocin-induced contractions in the non-pregnant rat uterus. Using different K^+-channel blockers, they determined that the inhibitory effect of resveratrol on spontaneous rhythmic contractions and phasic contractions involves various K^+-channels [86]. Moreover, high concentrations of resveratrol had further mechanisms of action that were independent of K^+-channels [86]. In 2015, Novakovic *et al*. [87] found that resveratrol induced concentration-dependent relaxation of oxytocin-induced contractions in human term pregnant myometrium. Consistent with the studies performed in rat myometrium, it appears that resveratrol exerts its effects on induced contractions by regulating K^+-channels in human myometrium [87]. Although the ability of resveratrol to relax myometrium and other smooth muscles has now been established, these studies did not examine the involvement of altered protein/histone acetylation in its contraction-blocking effects. A serious caveat is that resveratrol is an established pan-assay interference compound (PAINS) and exhibits untargeted cell membrane perturbing properties potentially explaining many of its multifarious actions [88, 89]. Additional research, perhaps using derivatives that do not modify membranes, is required to determine whether resveratrol's contraction-blocking actions are mediated by impacting on protein acetylation, and, in particular, to determine whether this may be through inhibiting classical HDACs (class I, II, and IV), or through activating class III HDACs.

Conclusions

Here we have reviewed currently available information about HDACis' pro-quiescence and anti-inflammatory effects in the pregnant uterus, including pregnant human myometrium. The data strongly support that HDACis act through both genomic and non-genomic mechanisms resulting in a 'dual action'; in the short-term, they are potent tocolytics inhibiting both spontaneous and oxytocin-induced uterine contractions, while in the long-term, HDACis regulate transcription factor function and chromatin structure, promoting a myometrial gene expression profile that favors progesterone sensitivity and uterine relaxation. HDACis may therefore represent a new therapeutic strategy to

inhibit uterine activity and thus inhibit preterm labor. That said, the deployment of agents during pregnancy that operate through regulating gene expression and chromatin structure raises concerns for potential placental transfer and detrimental effects on fetal development. This may seriously undermine the clinical utility of HDACis for the prevention of preterm birth, but even in that case, HDACis will remain a valuable tool to elucidate key regulatory mechanisms of human parturition. However, it is important to point out that multiple studies have now deployed TSA during mouse pregnancy with no obvious impacts on litter size or fetal viability [26] while leading to the birth of neurotypical offspring [45]. Moreover, the development of uterine-targeted nanoliposomes [38] and mucus penetrating nanoparticles for local administration [45, 46], may lead to HDACis deployed effectively and selectively to the pregnant myometrium blocking preterm labor while avoiding any undesired off-target maternal or fetal side-effects. Progress in these directions is highly desirable and expected considering the intractable health problem of premature birth worldwide.

List of abbreviations

aH3	histone H3 acetylation
Ca^{2+}	calcium ions
CBP	CREB-binding protein
CCL5	C-C motif chemokine ligand 5
CG	chorionic gonadotropin
CREB	cAMP-response element-binding protein
CXCL8	C-X-C motif chemokine ligand 8
DNMT1/3a	DNA Methyltransferase 1/3a
Egr-1	early growth response 1
ERα	estrogen receptor alpha
ESR1	estrogen receptor 1
$G\alpha_s$	G_s alpha subunit
GJA1	Gap Junction Protein alpha 1
HATs	histone acetyltransferases
hCG	human chorionic gonadotropin

Hda1	protein encoded by HDA1
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
IL	interleukin
K^+	potassium ion
LH	luteinizing hormone
LHCGR	luteinizing hormone/choriogonadotropin receptor
LPS	lipopolysaccharide
LTCC	L-type Ca^{2+} channels
NATs	N-terminal acetyltransferases
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAINS	pan-assay interference compound
PKA	protein kinase A
PR	progesterone receptor
PTGS2	prostaglandin-endoperoxide synthase 2
Rpd3p	protein encoded by RPD3
SAHA	suberoylanilide hydroxamic acid
SBHA	suberic bis-hydroxamate
Sir2	protein encoded by SIR2
Sirt6	sirtuin 6
Sp1-4	specificity protein-1 to -4
α-SMA	smooth muscle α-actin
SRC	steroid receptor coactivators
TSA	trichostatin A
VPA	valproic acid

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Tables

Table 1. Classes of HDACs.

CLASS	MEMBERS	COMMENT
I	HDACs 1 – 3, 8	Exhibit similarity to the yeast transcriptional regulator and deacetylase, Rpd3p.
IIa	HDACs 4, 5, 7, 9	Exhibit similarity to the yeast the deacetylase, Hda1, and continually shuttle between the nucleus and cytoplasm.
IIb	HDACs 6, 10	Exhibit similarity to the yeast deacetylase, Hda1, but localized to cytoplasm and have two catalytic sites.
III	Sirtuins 1 – 7	Exhibit similarity to the yeast silencing protein, Sir2.
IV	HDAC 11	Exhibits similarity to both Class I and Class II.

Abbreviations: HDACs, histone deacetylases; Rpd3p, protein encoded by RPD3; Hda1, protein encoded by HDA1; Sir2, protein encoded by SIR2.

Table 2. HDACis that have been used to investigate parturition.

HDACi Name	HDAC Classes Inhibited
TSA	Class I, II [90] and Sirt6 (Class III) [30]
SAHA	Class I, IIa, IIb, IV [91]
VPA	Class I, IIa [92]
SBHA	Class I, III [93]
Compound 2	Class I [76]
Resveratrol	Class I, II, IV [79]

Abbreviations: HDACi, histone deacetylase inhibitor; HDACs, histone deacetylases; TSA, trichostatin A; Sirt6, sirtuin 6; SAHA, suberoylanilide hydroxamic acid; VPA, valproic acid; SBHA, suberic bis-hydroxamate.

Figure titles and legends

Figure 1. Regulation of protein structure and function by acetylation. The addition and removal of acetyl functional groups affects protein structure, which in turn regulates protein function.

Figure 2. Acetylation of histone tails regulates the condensation and decondensation of chromatin. HDACis prevent the removal of acetyl functional groups from histone tails, which helps to maintain chromatin in a decondensed state that permits access to the DNA for gene transcription.

Figure 3. Structure of HDACis used to investigate myometrial biology. TSA, SAHA, VPA, SBHA, Compound 2 and Resveratrol have each been used to gain insight myometrial biology through their structure permitting the inhibition of different classes of HDACs.

Figure 4. Repression of PR-A expression by Trichostatin A. TSA represses the expression of *PR-A* through yet to be confirmed mechanisms but does not repress the expression of *PR-B*, resulting in a low PR-A/PR-B ratio. The comparatively higher levels of PR-B, liganded with progesterone, then repress the expression of pro-labor genes, such as *GJA1*.

Figure 1

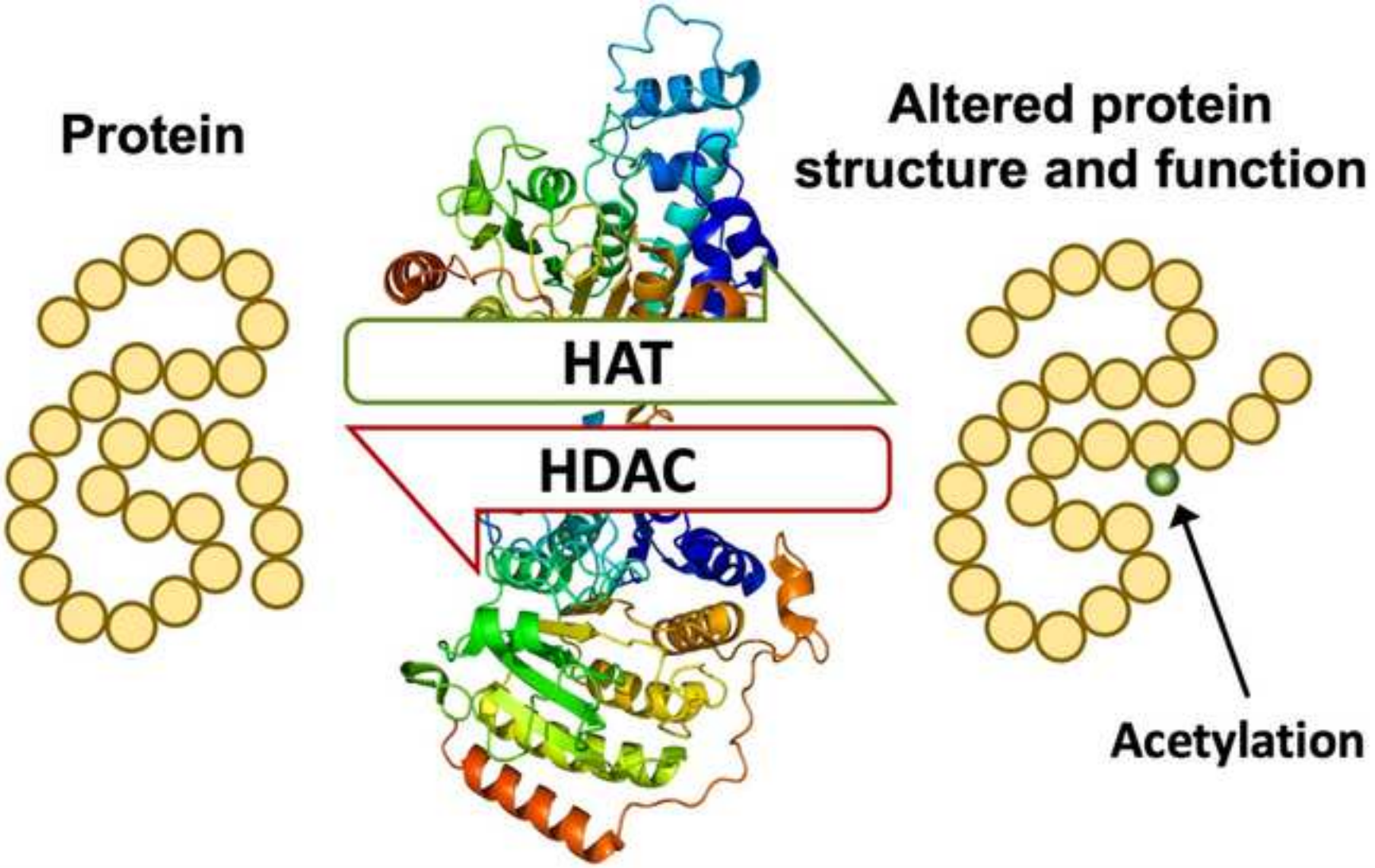


Figure 2

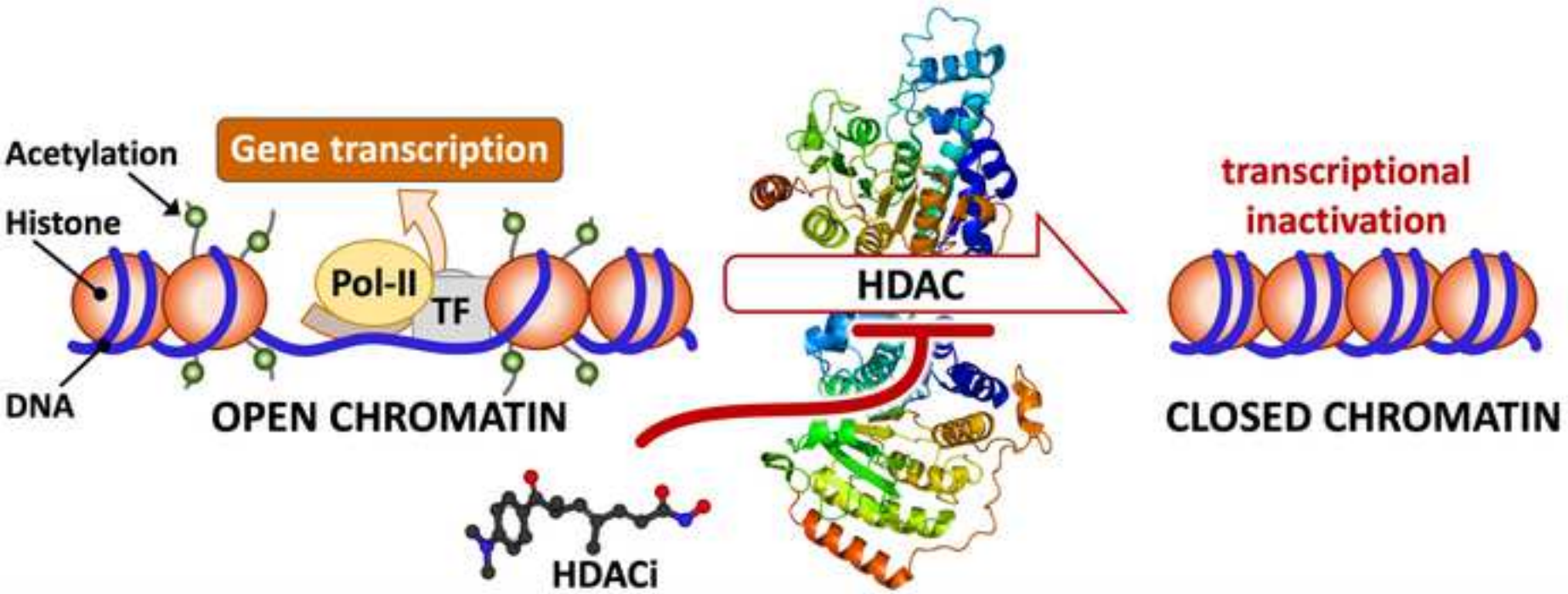
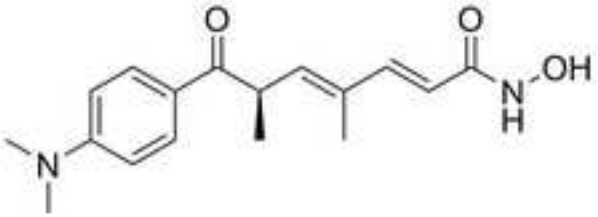


Figure 3

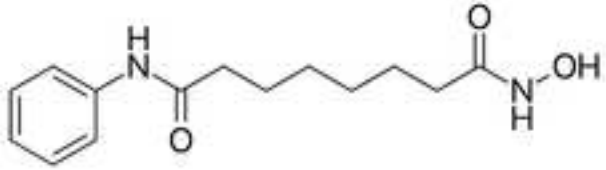
A. Trichostatin A (TSA)

Inhibits: Class I, II HDACs & Sirt6 (Class III)



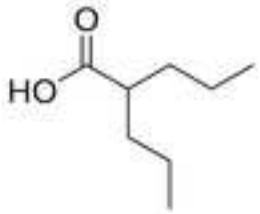
B. Suberoylanilide hydroxamic acid (SAHA)

Inhibits: Class I, II & IV HDACs



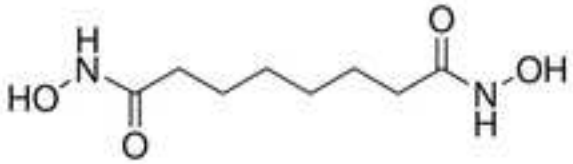
C. Valproic acid (VPA)

Inhibits: Class I & IIa HDACs



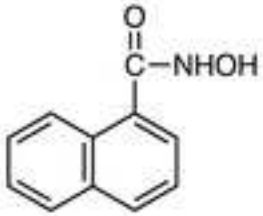
D. Suberic bis-hydroxamate (SBHA)

Inhibits: Class I & III HDACs



E. Compound 2

Inhibits: Class I HDACs



E. Resveratrol

Inhibits: Class I, II & IV HDACs

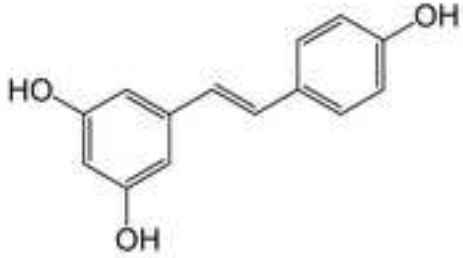
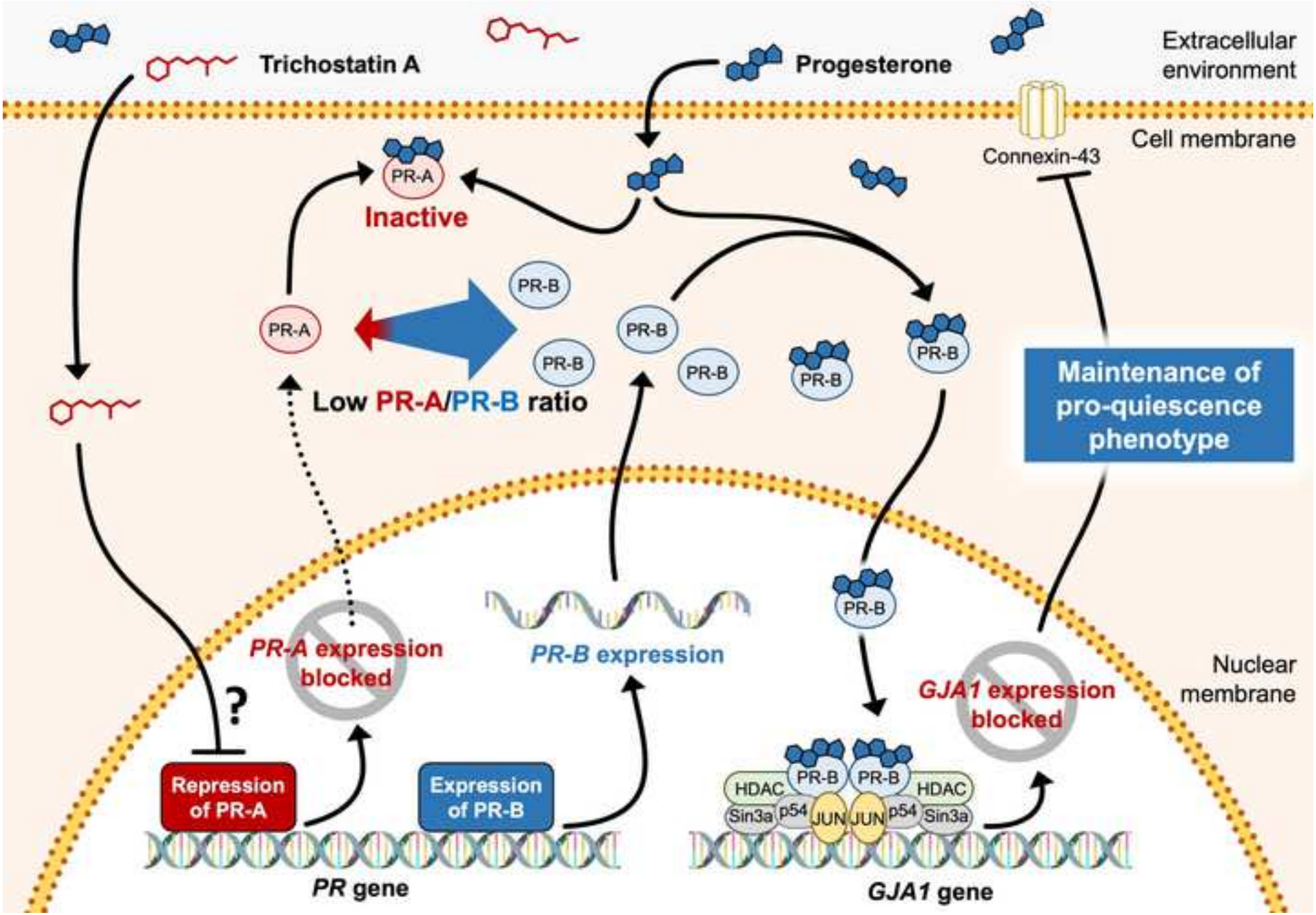


Figure 4



IRB Approval Statement:

Institutional Review Board approval is not applicable as the article submitted for publication is a review, meaning no human tissues or patients were used/involved in the production of this review manuscript.

Response to Reviewer #1

This review comprehensively describes the literature on the use of histone deacetylase inhibitors in parturition research. The manuscript is informative and well-written. It is a unique addition to the literature written by the experts. My only comment for the authors to consider is that the figures, while all new, are relatively simple. Did the authors consider including some selected figures from the literature described herein? I have no comments on the text itself, well done.

We thank the Reviewer for the positive feedback. We have added a more complex mechanistic figure that illustrates the selective repression of PR-A, but not PR-B, by Trichostatin A to maintain a pro-quiescence phenotype (Figure 4).

Response to Reviewer #2

This is a good review that will benefit the myometrial and tocolytic research community. Several minor issues need to be addressed, with the most substantive of these regarding cAMP/PKA in myometrium (see below).

General Criticism:

It should also be noted that throughout the manuscript there is inconsistency on the level of detail presented for discussed experiments. The amount of detail seems arbitrary at times, with the information not always providing useful information to the reader. The authors should thoroughly scrub the manuscript and determine where additional experimental detail is needed to push the narrative, and where it can be omitted.

We thank the Reviewer for the feedback. We have made changes aimed at addressing the reviewer's concerns. Please see below for more details.

Specific Criticisms:

Page 4 Line 58: sentence that ends on this line requires a reference.

Reference has been added to this sentence.

Page 5 Lines 1-28: While I understand that these paragraphs are highlighting background information, there is not a single reference to source literature.

This paragraph has been referenced.

Page 6 Lines 11-15: There is newly published data concerning Cx43 expression/PTMs in PTL myometrium that would be pertinent here.

We thank the Reviewer for the suggestion. Nadeem et al. study published in 2018 has now been included in the review.

Page 7 Line 55: I recommend avoiding the term "recently," as this word becomes meaningless, and even confusing, as time progresses. Instead use calendar years. For instance, "In 2017, Ilicic et al. found that…."

The word "recently" has been removed from the review and replaced with the publication year.

Page 8: When discussing results from corresponding author's personal manuscripts, additional detail, such as precise dosing of drugs, is used, where it is not in other parts of this review. Recommend greater consistency when discussing results through the manuscript.

We thank the Reviewer for the feedback. We have removed precise dosing of drugs to keep it consistent with the rest of the review.

Page 9 Line 53: Recommend defining this cell line hTERT-HMA/B.

The cell line hTERT-HMA/B has been defined.

Pages 10-11: While cAMP signaling may play a partial role in myometrial quiescence, beta activation is not an effective tocolytic strategy (ritodrine). It has also been shown that prolong exposure to cAMP agonist does not maintain relaxation (Lai et al., 2016). Regardless of HDAC's role in cAMP/PKA expression, the authors should address the limitations of cAMP signaling in myometrial quiescence and tocolysis.

Pro-relaxatory and pro-contractile roles of cAMP as well as HDACi's role in cAMP/PKA expression has been added under the "Cyclic AMP signaling" sub-heading.

Figure 1: This figure is acceptable but would benefit from a more mechanistic portrayal of acetylation as a protein PTM. It is unlikely that the audience for this manuscript will find such a simplistic portrayal of this pathway particularly useful.

We thank the Reviewer for the feedback. We have added a more complex mechanistic figure that illustrates the selective repression of PR-A, but not PR-B, by Trichostatin A to maintain a pro-quiescence phenotype (Figure 4).

Article Title: Histone deacetylase inhibitors: Providing new insights and therapeutic avenues for unlocking human birth

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Persons

Not applicable

Declarations

Competing interests

The authors declare that they have no competing interests

Availability of data and material

Not applicable

Code availability

Not applicable

Authors' contributions

All authors contributed to the manuscript development. MI and JWP wrote the manuscript. TZ, AG, WMH, and RS edited the manuscript. Figures by JWP. All authors read and approved the final manuscript.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Abstract (244 words)

The pregnant uterus remains relaxed throughout fetal gestation before transforming to a contractile phenotype at term to facilitate birth. Despite ongoing progress, the precise mechanisms that regulate this phenotypic transformation are not yet understood. This knowledge gap limits our understanding of how dysregulation of uterine smooth muscle biology contributes to ~~life~~ life-threatening obstetric complications, including preterm birth, and hampers our ability to develop effective therapeutic intervention strategies.

Protein acetylation plays a vital role in regulating protein structure, function, and subcellular localization, as well as gene transcription availability through regulating chromatin condensation. Histone deacetylase inhibitors (HDACis) are a class of compounds that block the removal of acetyl functional groups from proteins, and as such, have profound effects on important cellular events, including phenotypic transformation. A large body of data now demonstrates that HDACis have profound effects on pregnant human myometrium. Studies to date show that HDACis operate through both genomic and non-genomic mechanisms to affect myometrial function and phenotype. Interestingly, the effects of HDACis on pregnant myometrium are largely 'pro-relaxation', including the direct inhibition of contractile machinery as well as repression of pro-labor genes.

The 'dual action' effects of HDACis make them a powerful tool for unlocking the regulatory processes that underpin myometrial phenotypic transformation and raises prospects of their therapeutic applications. Here we review the new insights into human myometrial biology that have garnered through the application of HDACis and explore their potential therapeutic application toward the development of novel preterm birth prevention strategies.

Keywords (6 keywords)

Parturition, progesterone, epigenetics, histone deacetylase, histone deacetylase inhibitors, Trichostatin A.

Background

Despite continued research during recent decades, we still do not understand fully the process of human birth. Particularly, we have no clear understanding of the regulatory processes, biochemical signaling pathways, and associated physiological changes that ultimately culminate in the initiation of contractions within the smooth muscle layer of the pregnant uterus. This knowledge gap limits our understanding of how dysregulation of myometrial cell function leads to life-threatening obstetric complications, such as preterm birth, which is the most common cause of death among newborns. There remains a pressing need to elucidate key elements of the regulatory processes that underpin the transition of the pregnant uterus from quiescence to generating forceful, coordinated contractions necessary for normal birth.

Research to understand human birth is subject to ethical considerations that tightly constrain experimental intervention. Researchers rely heavily on the use of *in vitro* and *ex vivo* models ~~[1]~~ such as pregnant human myometrial cell lines, uterine tissue biopsies, and animal models of pregnancy complications [1]. Within these models, researchers routinely treat cells with different classes of chemical compounds to observe their effects on gene expression, phenotype, and in the case of uterine myocytes, contractile properties. One class of compounds that have led to significant insights into myometrial biology are histone deacetylase (HDAC) inhibitors (HDACis).

Acetylation is the addition of an acetyl functional group (CH_3CO) to a compound, which is catalyzed by histone acetyltransferases (HATs), while deacetylation is the process of removing an acetyl functional group, which is catalyzed by HDACs. The classification of HDACs is outlined in Table 1.

While lipids and carbohydrates can be acetylated, it is the acetylation of proteins that has received the most attention, as acetylation is an important post-translational modification impacting protein structure and function (Figure 1) [2]. Indeed, approximately 85% of human proteins are acetylated on their N-terminus, which plays an important role in regulating the synthesis, stability, and localization of the protein [3]~~{Polevoda, 2000 #62}~~. N-terminal amino acids that may be

acetylated by N-terminal acetyltransferases (NATs) include glycine, alanine, serine, methionine, and aspartic acid [4].

Within eukaryotic cell nuclei, ε~~e~~-acetylation of lysines on histone proteins by HATs plays a vital role in regulating gene expression [5]~~[5][5]~~. That is, through reducing the positive charge of histones, acetylation decreases the interaction between histones and the negatively charged phosphate backbone of DNA, which promotes the decondensation of chromatin, which in turn enables greater levels of gene transcription [5]. Lysine ε~~e~~-acetylation is therefore primarily associated with gene activation while deacetylation is primarily associated with gene silencing (Figure 2)[6].

Given that the transition from not-in-labor to in-labor is associated with protein post-translational events and differential gene expression, HDACis have been used to explore the role of protein acetylation in myometrial biology. The HDACis examined throughout these investigations include Trichostatin A (TSA), Suberoylanilide hydroxamic acid (SAHA), Valproic acid (VPA), Suberic bis-hydroxamate (SBHA), Compound 2, and Resveratrol (Table 2). The structure of these agents are shown in Figure 3. Studies utilizing these agents have led to significant findings, which we review here to obtain an up-to-date picture of the non-genomic and genomic effects of HDACis in the context of parturition.

New Insights into Myometrial Biology

Genomic effects of HDACis

Progesterone signaling

The steroid hormone progesterone plays a central role in maintaining pregnancy by promoting myometrial quiescence and relaxation [7-9]. The withdrawal of progesterone action signals the end of pregnancy, and in most non-primate mammals labor is initiated by a fall in maternal circulating levels of progesterone [10-14]. In humans and higher primates, however, maternal, fetal, and amniotic concentrations of progesterone remain elevated up to and during labor [15-17]. Nonetheless, blocking the actions of progesterone at any stage of pregnancy (e.g., by the

progesterone antagonist RU486) induces labor and stimulates cervical ripening [18, 19]. This indicates that progesterone action is essential in maintaining human pregnancy~~,~~ and that the onset of human labor involves a decrease of progesterone responsiveness ("functional progesterone withdrawal"), rather than a decline in circulating progesterone levels.

In 2016, Nadeem *et al.* [20] ~~recently~~ published important evidence of the molecular mechanism contributing to functional progesterone withdrawal in human myometrium. They found that found that during pregnancy, progesterone-liganded PR-B associates with Jun/Jun homodimers and the repressor complex P54nrb/Sin3a~~A~~/HDAC to repress the transcription of *GJA1*, which encodes the key contraction-associated protein, connexin-43 [20]. In contrast, during labor PR-A, a truncated PR isoform, dissociates from progesterone, and in this unliganded state interacts with Fos/Jun heterodimers to activate the transcription of *GJA1*, ~~when bound to progesterone, the full-length nuclear progesterone receptor (PR) isoform, PR-B, forms a complex that represses the transcription of *GJA1*, which encodes the key contraction-associated protein, connexin-43 [20, 21]. ~~thereby promoting term labor [20]. In 2018, Nadeem *et al.* [21] used mouse models of preterm labor to show that a similar myometrial AP-1 protein composition, from Jun/Jun homodimer during most of gestation to Fos/Jun heterodimer during labor, occurs during preterm labor, thus suggesting that similar molecular machinery is involved in the induction of preterm and term labor.

~~activates the expression of *GJA1*, thereby promoting labor [20]. Interestingly, PR-A was translocated to the nucleus to activate *GJA1* expression when unbound by progesterone [20]. ~~This finding builds upon a vast body of previous work demonstrating that PR-B is the principal mediator of progesterone's relaxatory actions through repressing the expression of genes that promote uterine contractility [22]. PR-A represses the transcriptional activity of PR-B, and, in doing so, decreases progesterone responsiveness [7, 8, 19, 23]. The amount of PR-A relative to PR-B in myometrial cells is therefore a key determinant of progesterone responsiveness. A low PR-A/PR-B ratio (a PR-B dominant state) is consistent with responsiveness to progesterone's relaxatory effects, while a high PR-A/PR-B ratio (a PR-A dominant state) is consistent with a decrease of progesterone responsiveness [7, 8, 19, 23]. In accordance with this, the PR-A/PR-B protein ratio in pregnant human myometrium was 0.5 (a PR-B dominant state) at 30-weeks gestation, which increased to 1.0

at term prior to the onset of ~~labor, and~~labor and increased further to 3.0 (a PR-A dominant state) at the time of labor [24]. Furthermore, the *PR-A/PR-B* mRNA ratio has been directly correlated with estrogen receptor α (*ERα*) mRNA levels, which is indicative of a functional link between the *PR-A/PR-B* ratio and the action of estrogens, which drive the expression of contraction-associated proteins [25].

The PRs interact with coactivators and corepressors to increase and decrease their transcriptional activities, respectively. The importance of these coregulators in the control of transcriptional activity of PRs was investigated by Condon *et al*. [26]. They found that the expression of PR coactivators cAMP-response element-binding protein (CREB)-binding protein (CBP) and steroid receptor coactivators (SRC) 2 and 3 was decreased in human fundal myometrium as well as in mouse uterus during labor [26]. CBP and SRCs possess HAT activity that may contribute to PR actions at progesterone responsive genes [27, 28]. Consistent with this, Condon *et al*. [26] detected decreased histone H3 acetylation (aH3) in myometrium obtained from women in labor, and also in pregnant mouse uteri at term [26]. This indicates that a decrease in coactivator levels within the uterus leads to a decline in histone acetylation, thus closing the chromatin and inhibiting general transcription factor interaction with the target genes that maintain uterine quiescence. To determine the functional significance of the decline in coactivator levels and histone acetylation, Condon *et al*. [26] investigated the effect of TSA, a potent, reversible Class I and II HDACi (and potentially Sirt6 (Class III) [29, 30], in pregnant mice. Daily administration of TSA to pregnant mice caused hyperacetylation of uterine histone H3 on gestation day 19. Parturition was delayed by 24 – 48 h in TSA-treated mice that were allowed to deliver spontaneously [26]. TSA administration did not cause any evident toxicity to the mother and did not change maternal serum progesterone levels when compared to untreated control mice [26]. Moreover, TSA-treated mice delivered viable pups of normal litter size when compared to control mice [26]. Previous studies have shown that the administration of TSA during the crucial stages of embryonic organogenesis caused teratogenic effects [31] and neural tube defects in mouse embryos *in vitro* [32]. Nervi *et al.* [33] demonstrated that TSA administrated to pregnant mice at post-implantation was not toxic to the pregnant mice, and did not cause any obvious malformation during somitogenesis or at later stages of development.

Comparable to Condon *et al*. [26], treated pups were born at a similar frequency and were of normal litter size when compared to control pups [33]. Moreover, pups developed to normal adult size and were fertile [33]. The inconsistency between these studies may be attributable to different TSA concentrations ~~analysed~~ analyzed as well as different experimental methods used. These studies suggest that the overall decrease in histone acetylation within the pregnant uterus at term, associated with significantly reduced levels of steroid receptor coactivators with intrinsic HAT activity, might contribute to loss of PR function leading to birth. An important implication of these results is that HDACis, which maintain histone acetylation, may represent an avenue for preventing preterm birth.

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In 2017, Ilicic *et al.* [34] ~~recently~~ found that placing non-laboring pregnant human myometrium in culture (as explants) causes the tissue to undergo a phenotypic transition toward a labor-like phenotype. During this transition, the expression of genes encoding ERα (*ESR1)*, prostaglandin-endoperoxide synthase 2 *(PTGS2),* and *PR-A* were significantly up-regulated [34, 35]. These outcomes suggest that freshly biopsied non-laboring human myometrium undergoes a transition from a pro-gestation phenotype, with a low *PR-A/PR-B* ratio, to a phenotype with a high *PR-A/PR-B* ratio, consistent with reduced progesterone sensitivity [35]. Significant elevation of the *PR-A/PR-B* ratio was observed after 6 hours of culture [35]. These changes are similar to gene regulatory events that occur during the onset of labor and are consistent with observations that strips of non-laboring human myometrium spontaneously develop rhythmic contractions *in vitro* following a period of equilibration that may take several hours [36-39]. Using this model of myometrial transformation, Ilicic *et al.* [35] investigated factors that regulate PR isoform expression in human myometrium. They showed that up-regulated *PR-A* expression and *PR-A/PR-B* ratio during myometrial transformation can be completely blocked by incubating myometrial tissue with TSA, consistent with maintaining progesterone responsiveness [35] (Figure 4). ~~In these studies, term non-laboring human myometrial explants were incubated for 48 hours in the presence of 0.5, 1.0, 2.5, or 5.0 μM TSA [30]. In the absence of TSA, the myometrial tissue transitioned from a low to high *PR-A/PR-B* ratio [30]. TSA treatment dose-dependently blocked the spontaneous rise of *PR-A* expression without affecting *PR-B* [30]. At ≥2.5 μM concentration, TSA preserved the low *PR-A/PR-B* ratio of the fresh non-laboring~~

tissue suggesting maintenance of progesterone responsiveness [30]. These results indicate that stabilizing histone and/or non-histone protein acetylation is critical for maintaining the progesterone-dependent quiescent phenotype of human myometrium in culture.

Consistent with these data, Li *et al*. [40] reported that *PR-A* expression was regulated by epigenetic mechanisms in term human myometrium. They found that increased *PR-A* expression was associated with decreased DNA methylation in its promoter and decreased expression of the DNA methyltransferase mRNAs encoding DNMT1 and DNMT3a (but not of *DNMT3b* mRNA) in uterine biopsies [40]. Studies using breast cancer cells have shown that HDACs, particularly HDAC1, regulate the *ESR1* gene, which encodes ERα, by binding to the *ESR1* promoter [41, 42]. By inference, this suggests that HDAC1 may also be involved in the ER-dependent regulation of the *PR* gene. Furthermore, low expression of HDAC1 was associated with increased expression of *PR-A* in myometrium during labor, and HDAC1 down-regulated *PR-A* expression by binding to the promoter region of *PR-A* [43]. These results implicate HDAC1 in regulating the levels of both ER and PR-A in the context of functional progesterone withdrawal and estrogen activation. Additionally, in laboring human myometrium, Chai *et al.* [44] found significantly higher levels of histone acetylation at the *PR-A* promoter compared to the *PR-B* promoter, which is consistent with progesterone sensitivity being 'switched off' as a result of epigenetic activation of *PR-A* expression. An epigenetic modifier that prevents *PR-A* promoter activation may therefore preserve the progesterone-sensitive phenotype of the myometrium and potentially other progesterone-sensitive gestational tissues. The observation by Ilicic *et al.* [35] that TSA suppressed *PR-A* expression in pregnant human myometrium *in vitro* raises the possibility that HDACi could prove useful for maintaining or potentially even restoring myometrial progesterone responsiveness during pregnancy.

RecentlyIn 2021, Zierden *et al*. [45] examined the vaginal delivery of HDACis via mucoinert nanosuspensions for the prevention of lipopolysaccharide (LPS)-induced preterm birth in mice. They found that systemic administration of the clinically used 17-α-hydroxyprogesterone caproate did not prevent LPS-induced preterm birth. Vaginal administration of progesterone alone, either as the gel Crinone or in mucoinert nanosuspensions was also ineffective [45]. Furthermore, when Crinone was given to the vehicle (saline) control group, it worsened preterm birth outcomes [45]. However, vaginal

administration of progesterone combined with either of the HDACis TSA or SAHA in nanosuspensions, prevented preterm birth, resulted in higher litter viability, and offspring that exhibited neurotypical development (brain weight, behavior studies) [45]. Pharmacokinetic studies for the vaginally administered progesterone/TSA nanosuspension showed that both progesterone and TSA reached the cervix and myometrium with sustained exposure for up to 8 hours [45]. The progesterone/TSA nanosuspension combination also led to decreased endotoxin content in the amniotic fluid and had anti-inflammatory effects in myometrial tissue, decreasing the myometrial expression of *Il6*, *Nfkb1*, *Ptgs2*, *Gja1*, *Ccl3*, and *Oxtr*. Additional studies in an immortalized human myometrial cell line, hTERT-HM$^{A/B}$, stably transfected with PR-A and PR-B transgenes such that each can be experimentally controlled using independent inducers, ~~human myometrial cells~~ revealed that the progesterone/TSA nanosuspension combination promoted the anti-inflammatory action of progesterone ~~in hTERT-HM$^{A/B}$ cells~~ by decreasing the PR-A/PR-B ratio and increasing PR-B stability. The nanoparticles suppressed contractility in PHM1-41 cells at the drug concentrations measured in the mouse myometrial tissue [45]. These findings demonstrate that vaginally delivered HDACis can block LPS-induced preterm birth by down-regulating the myometrial expression of inflammatory genes and reducing myometrial cell contractility. Importantly, live, neurotypical offspring have been delivered after the intervention. These findings also highlight that the administration of HDACis via vaginally administered nanosuspensions [45, 46] or uterine-targeted nanoparticles [38] is a therapeutic strategy with the potential to prevent preterm birth.

Cyclic AMP signaling

Placentally derived human chorionic gonadotropin (hCG) is crucial for the maintenance of myometrial quiescence [47]. The action of hCG is mediated by the chorionic gonadotropin (CG)/luteinizing hormone (LH) receptors, which are coupled to adenyl cyclase by the stimulatory G protein, $G\alpha_s$, resulting in the formation of cAMP. Genomic cAMP signaling has both pro-relaxatory and pro-contractile roles (reviewed by Butler *at al.* [48]). Even though cAMP ~~Cyclic AMP~~ signals predominantly through protein kinase A (PKA) to reduce contractility [49], ~~[49].~~ prolonged exposure to cAMP can activate pro-contractile mitogen-activated protein kinase (MAPK) signaling [50] and upregulate pro-contractile genes [51]. ~~[50]~~ Decreased levels of hCG/LH receptors in the myometrium

are associated with term and preterm labor [52], which suggests that maintaining high levels of hCG/LH receptor can help prevent early labor onset. The expression of the hCG/LH receptor gene (*LHCGR*) in myometrial cells was found to be regulated by specificity protein-1 to -4 (Sp1-4) [53]. Pull-down assays using double-stranded, biotinylated oligonucleotides, electro-mobility shift assays and luciferase reporter experiments demonstrated the association and functional involvement of Sp1/Sp3, Sp4, Sp-like factor(s), and HDAC1/2 complexes in *LHCGR* promoter regulation in primary cultures of human myometrial cells [53]. Treatment of the myometrial cells with TSA increased hCG/LH receptor mRNA expression and protein levels, suggesting that HDACis function to regulate *LHCGR* gene activity by controlling acetylated histone and/or non-histone protein levels at critical regulatory elements [53]. These findings further corroborate the notion that TSA is a tocolytic (contraction blocking) agent.

As mentioned, the relaxing effects of cAMP are mediated through PKA, which is a heterotetrameric protein complex that consists of two regulatory (R) and two catalytic (C) subunits. Recent data shows that the level of the PKA regulatory subunit RIIα increases throughout pregnancy and then decreases during parturition in the myometrium [54]. –Karolczak-Bayatti *et al.* [55] investigated the mechanism by which the RIIα gene was regulated in the uterine muscle. They showed that all three SpI-III (GC) binding domains within the RIIα promoter are occupied by Sp1 and Sp3, which then recruit HDAC1/2 and the histone binding protein RbAp48 to regulate gene expression [55]. Inhibition of HDAC1/2 activity by TSA resulted in significantly increased mRNA expression and protein levels of RIIα [55]. This increase in mRNA expression was proceeded by an increase of aH3 and binding of Sp3, HDAC2, and RNA-polymerase II large subunit RPB1 to the three SpI-III (GC) *cis*-elements within the RIIα promoter [55]. The results imply that the RIIα gene in human myometrial smooth muscle cells is regulated by epigenetic mechanisms that involve histone acetylation, Sp3 acetylation, and recruitment of HDAC1/2 to multiple GC *cis*-acting elements within the proximal promoter of the gene. Furthermore, TSA reduced contractions of myometrial strips under conditions when RIIα expression and (total) aH3 were increased indicating that HDACi could be useful to maintain RIIα levels and myometrial quiescence throughout pregnancy.

G-proteins are key components of the signaling pathways that mediate the actions of the activators and inhibitors of myometrial smooth muscle activity [56]. As discussed, the binding of hCG to its receptor activates $G\alpha_s$, which in turn stimulates adenylate cyclase and results in cAMP accumulation increasing PKA activity [57]. The binding of oxytocin to its receptor activates the G-proteins $G\alpha_{q/11}$ and $G\alpha_{i/o}$, which stimulate phospholipase C, thus hydrolyzing phosphatidylinositol 4,5-bisphosphate to inositol triphosphate and diacylglycerol [57]. Signaling through $G\alpha_{i/o}$ leads to decreased adenylate cyclase activity and reduced intracellular cAMP level, whilst signaling through $G\alpha_{q/11}$ leads to an increase in the intracellular concentration of calcium ions (Ca^{2+}) [58-60]. Increased intracellular Ca^{2+} can activate myosin light-chain kinase to phosphorylate myosin light chains and instigate the contraction of the myocytes [61]. The balance between the various Gα proteins that control relaxatory and contractile pathways is crucial for the regulation of myometrial activity. Sp-like transcription factors binding to GC boxes within the promoter region stimulated expression of the $G\alpha_s$ gene (located in the *GNAS complex locus*) through a PKA-dependent mechanism [62]. Karolczak-Bayatti *et al*. [63] explored the role of Sp1-4 proteins and their transcriptional and epigenetic co-regulators in $G\alpha_s$ gene regulation in primary cultures of human myometrial cells. Transcriptional complexes consisting of Sp1-4, HDAC1/2, RbAp48, and mSin3A were found at four out of six Sp1-4 sites within the proximal promoter region of the $G\alpha_s$ gene. Given that HDAC1/2 were recruited to the $G\alpha_s$ promoter, the effect of TSA, a pan class I/II HDACi, was determined on $G\alpha_s$ expression [55]. Treatment with TSA led to increased $G\alpha_s$ protein levels; however, there was no increase in $G\alpha_s$ mRNA expression. The proteasome inhibitor MG132 increased $G\alpha_s$ protein levels similarly to TSA, and treatment with both drugs resulted in the accumulation of high molecular weight polyubiquitinated proteins indicating the suppression of proteasome activity. Thus, the increase in $G\alpha_s$ protein level in response to TSA may have occurred due to the reduction of protein degradation, demonstrating that HDACi have post-translational and non-genomic actions as discussed in more detail below.

~~All t~~The above evidence indicates that HDACis promote myometrial quiescence via cAMP signaling; however, it is important to note that cAMP signaling has both pro-relaxatory and pro-contractile roles and that prolonged exposure to cAMP ~~agonist~~ can ~~lead to~~promote myometrial

spontaneous contractility. As such, further research will be necessary to determine the role of HDACis in cAMP signalling.

Inflammatory signaling

Webster *et al*. [64] reported that TSA-induced myometrial relaxation was inhibited by TNFα via a NF-κB-dependent mechanism, but TNFα did not stimulate contractions on its own. Previous data by the same group indicated that TNFα repressed $G\alpha_s$ through the NF-κB RelA subunit in myometrial cells in a non-DNA binding fashion by competing for limiting amounts of the key transcription co-factor CBP [65], which is a HAT. They had also demonstrated that the -837 to -618 region of the $G\alpha_s$ promoter was occupied by the transcription factors, CREB, Egr-1, Sp1, and CBP [64]. Furthermore, the CBP-containing transcriptional complexes formed within this region increased $G\alpha_s$ expression [64]. In the presence of TNFα, there were no changes in the levels of CREB, Egr-1, and Sp1, while CBP levels were significantly reduced in agreement with competition for a limiting amount of CBP by RelA [64]. This was associated with increased levels of HDAC1 as well as increased H4K8ac, but no change of H3K9ac levels at the promoter and no change of protein acetylation globally [64]. At the same time, overexpression of HDAC1 did repress the activity of a $G\alpha_s$-luc construct, while CBP overexpression and TSA induced $G\alpha_s$-luc promoter construct activity [64]. These results further demonstrate that the involvement of protein/histone acetylation in regulating genes that influence myometrial contractility occurs by complex and genomic locus-specific mechanisms with an overall effect of promoting relaxation.

It is well established that labor is an inflammatory process associated with the increased production of pro-inflammatory mediators [66]. NF-κB is a key orchestrator of inflammatory responses and has been suggested to play a crucial role in labor [67]. The NF-κB subunit RelA is subject to acetylation at multiple sites with major effects on its function [68], and work by Condon *et al*. [26] has indicated that there might be a therapeutic use for HDACis in the management of preterm birth. Thus, it was crucial to investigate the impact of HDACis on NF-κB activity in the myometrium. Lindstrom *et al*. [69] showed that short-term (1-2 h) treatment with TSA increased interleukin (IL)-1β-induced NF-κB DNA binding and nuclear localization in cultured primary myometrial cells. In contrast, long-term treatment (24 h) inhibited IL-1β-induced NF-κB DNA binding [69]. The short-term

increase of NF-κB nuclear localization was due to the delayed recovery of I-κBα, the inhibitory subunit of the complex, in TSA plus IL-1β treated cells due to an increase of I-κB kinase activity by the drug, which enhanced I-κBα degradation. Remarkably, TSA increased both the short- and long-term IL-1β-induced NF-κB transcriptional activity when assayed with a transfected promoter construct despite the drop of DNA binding at 24 h. This suggested that TSA acted on the transcriptional activity of NF-κB independently of its effect on DNA binding. Moreover, both short- and long-term exposure to TSA plus IL-1β inhibited the expression of the pro-labor genes, *PTGS2*, *CXCL8* (IL-8), *IL6* (IL-6), and *CCL5* (RANTES), which was associated with the reduced expression of the pro-inflammatory transcription factor, c-Jun [69]. These results indicate that pan-HDACis, like TSA, might act on various acetylated histone and non-histone protein targets affecting multiple regulatory pathways. The pro-inflammatory genes examined by Lindstrom *et al*. [69] might be suppressed by HDACis through c-Jun and not the NF-κB system in myometrial cells; however, a full gene expression profile may reveal sets of pro- and anti-inflammatory genes that are responsive to HDACis via modulating NF-κB. HDACi exposure favors uterine quiescence, but the administration of a HDACi in combination with an NF-κB inhibitor might be an approach that reduces potential undesirable effects of HDACis, such as the induction of pro-inflammatory pathways.

Ion channels

The quiescent and contractile states of the myometrium are mediated through mechanisms that involve ion channels. Previous research has shown that MaxiK (large-conductance Ca^{2+}- and voltage-activated potassium ion (K^+) channel) is one of the major pro-quiescence K^+ channels and L-type Ca^{2+} channels (LTCC) are the predominant pro-contractile Ca^{2+} channels in the myometrium. A dynamic balance between these is likely to be crucial in the switch from electrical quiescence to activation and phasic contractions. MaxiK channel activity has been reported to decrease in the myometrium of late pregnant rats [70]. Waite *et al*. [71] demonstrated that both MaxiK splice variants (M1, M3, and M4) and LTCC splice variants (exons 8, 30-34, 40-43) were expressed in non-laboring term pregnant myometrium. They further showed that TSA was able to significantly reduce MaxiK expression as well as the expression of MaxiK splice variants M1, M3, and M4 in primary myometrial cell cultures [71]. TSA exhibited a dual effect on LTCC splice variants in primary myometrial cell

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culture [71]. TSA significantly induced expression of the exon 8 variant, whilst also significantly reducing expression of other LTCC splice variants including that of encoding exons 30-34 and exons 40-43 [67]. It is known that MaxiK and LTCC are responsive to $G\alpha_s$ dependent cAMP signaling, which results in the enhanced efflux of K^+ via MaxiK (reviewed in [71]) and may contribute to the relaxing effect of TSA, which increases $G\alpha_s$ expression (see above). The reduction of LTCC and MaxiK expression and alterations of splice variants distribution by TSA demonstrate additional facets of the many-sided relaxatory actions of this HDACi, supporting its potential tocolytic utility.

Non-genomic effects of HDACis

In addition to their genomic effects, there is evidence that HDACis exert non-genomic effects on human myometrium, which are also tocolytic. Moynihan *et al.* [72] used human myometrial tissue strips to analyze the effects of ~~3~~ three HDACis, TSA, VPA, and SBHA, on human uterine contractility. TSA and SBHA each dose-dependently inhibited both spontaneous and oxytocin-induced contractions in the human myometrium with SBHA being the more potent [72]. VPA, an anti-seizure agent, also inhibited both spontaneous and oxytocin-induced contractions but was the least effective of the three HDACis [72]. VPA is a human teratogen that affects fetal neural development [73], and thus would not be appropriate for use in pregnancy [73]. The short timeframe in which the HDACis blocked uterine contractility (20 – 60 min) suggested that contractions were blocked through non-genomic mechanisms.

HDAC8, a class I HDAC, is expressed by human smooth muscle cells *in vivo*, including vascular and visceral smooth muscle cells, myoepithelial cells, and myofibroblasts [74]. Remarkably, the enzyme is localized mostly in the cytoplasm of smooth muscle cells both *in vivo* and *in vitro*, rather than in the nuclear compartment [74]. Furthermore, HDAC8 displays a striking stress fiber-like pattern of distribution and is co-expressed with two major constituents of the smooth muscle contractile apparatus, smooth muscle α-actin (α-SMA) and smooth muscle myosin heavy chain. *In vitro* and *in vivo* studies have found that HDAC8 associates with α-SMA but not with β-actin [75]. In addition, inhibition of HDAC8 by RNA interference results in a markedly reduced ability of human smooth muscle cells to contract collagen lattices [75]. These results indicate that HDAC8 might be involved in the regulation of smooth muscle cytoskeleton dynamics [75]. Based on these results, the

highly selective linkerless hydroxamic acid HDAC8 inhibitor, termed Compound 2 [76], was utilized to determine the interaction of HDAC8 with α-SMA and myosin heavy chain [77]. Compound 2 inhibited both spontaneous and oxytocin-induced contractions in human myometrium *in vitro* [77]. The inhibitory effects of Compound 2 were not associated with an increase in aH3 nor marked global gene expression changes [77]. TSA, however, exerted an inhibitory effect on both spontaneous and oxytocin-induced contractions in human myometrium *in vitro*, [72] as well as significantly increased aH3 and acetylated α-tubulin levels, as expected of a pan-class I/II HDACi [77]. These results suggest that the relaxatory effects of Compound 2 are likely through non-epigenetic pathways, and as such require further investigation.

As previously discussed, Karolczak-Bayatii *et al*. [55] showed an increase of mRNA expression and protein levels for the PKA regulatory subunit, RIIα, in human myometrial cells following TSA treatment. In addition, treatment with TSA caused a significant decrease in oxytocin-induced contractions of myometrial strips, measured as isometric tension changes [55], within a short time frame (90 minutes), consistent with a previous study by Moynihan *et al.* [72]. ~~The d~~Decreased contractility was associated with an increase in RIIα protein expression as well as an increase in aH3 levels in the strips. These results demonstrate that HDAC1/2 inhibitors, such as TSA, have concomitant fast actions on gene expression, chromatin structure, and cytoplasmic mechanisms of contraction, which could result in expeditious, clinically useful, tocolysis.

The ability of HDACis to block myometrial contractility [55, 72, 77] raised interest in determining the conditions under which the tocolytic action occurs. Webster *et al*. [64] showed that TSA alone caused dose-dependent relaxation of spontaneous myometrial contractions; however, this relaxatory effect was overcome by higher doses of TNF that induced NF-κB activity in primary myometrial myocytes. TNF on its own did not induce myometrial contractions [64]; however, data reviewed by Lindstrom and Bennett [67] suggest that NF-κB plays a crucial role overall in myometrial regulation at labor. Moreover, the NF-κB inhibitor N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine, which inhibits endogenous TNF synthesis, exerted dose-dependent relaxation of spontaneous myometrial contractions and significantly attenuated the action of TNF on TSA-mediated relaxation [64]. The results indicate that TNF's uterotonic actions have an NF-κB-regulated

element and that TNF's uterotonic effects may only occur once other factors, that are yet to be identified, have been acetylated. These findings also highlight that more work is required to delineate the conditions under which HDACis maintain myometrial quiescence.

A Closer Look at Resveratrol

Resveratrol has attracted attention due to its extensive pharmacological actions. The compound is a stilbenoid, which is a natural polyphenol produced by more than 70 plants. Resveratrol is reported to have potential medical applications related to cancer, cardiovascular disease, diabetes, aging, and lifespan extension [78]. One intriguing aspect of resveratrol is that evidence indicates that it is an inhibitor of class I, II, and IV HDACs, but an activator of class III HDACs [79].

Resveratrol promotes smooth muscle relaxation, including blood vessels [80-83], gallbladder [84], and the rat uterus [85, 86]. In the non-pregnant rat uterus, Hsia *et al*. [85] demonstrated that resveratrol inhibits contractions induced by $PGF_{2\alpha}$, oxytocin, acetylcholine, carbachol, high K^+ concentrations, and a Ca^{2+} channel activator. Furthermore, they found that resveratrol inhibited $PGF_{2\alpha}$-induced increases in Ca^{2+} in human primary uterine smooth muscle cells and mimicked Ca^{2+} channel blockers to block Ca^{2+} influx via voltage-operated Ca^{2+} channels in the plasma membrane [85]. These results indicate that resveratrol inhibits uterine contractions by blocking external Ca^{2+} influx, causing decreased intracellular Ca^{2+} concentrations [85]. Novakovic *et al*. [86] reported that resveratrol treatment inhibits spontaneous rhythmic contractions, as well as both phasic and tonic oxytocin-induced contractions in the non-pregnant rat uterus. Using different K^+-channel blockers, they determined that the inhibitory effect of resveratrol on spontaneous rhythmic contractions and phasic contractions involves various K^+-channels [86]. Moreover, high concentrations of resveratrol had further mechanisms of action that were independent of K^+-channels [86]. ~~More recently~~In 2015, Novakovic *et al*. [87] found that resveratrol induced concentration-dependent relaxation of oxytocin-induced contractions in human term pregnant myometrium. Consistent with the studies performed in rat myometrium, it appears that resveratrol exerts its effects on induced contractions by regulating K^+-channels in human myometrium [87]. Although the ability of resveratrol to relax myometrium and other smooth muscles has now been established, these studies did not examine the involvement of

altered protein/histone acetylation in its contraction-blocking effects. A serious caveat is that resveratrol is an established pan-assay interference compound (PAINS) and exhibits untargeted cell membrane perturbing properties potentially explaining many of its multifarious actions [88, 89]. Additional research, perhaps using derivatives that do not modify membranes, is required to determine whether resveratrol's contraction-blocking actions are mediated by impacting on protein acetylation, and, in particular, to determine whether this may be through inhibiting classical HDACs (class I, II, and IV), or through activating class III HDACs.

Conclusions

Here we have reviewed currently available information about HDACis' pro-quiescence and anti-inflammatory effects in the pregnant uterus, including pregnant human myometrium. The data strongly support that HDACis act through both genomic and non-genomic mechanisms resulting in a 'dual action'; in the short-term, they are potent tocolytics inhibiting both spontaneous and oxytocin-induced uterine contractions, while in the long-term, HDACis regulate transcription factor function and chromatin structure, promoting a myometrial gene expression profile that favors progesterone sensitivity and uterine relaxation. HDACis may therefore represent a new therapeutic strategy to inhibit uterine activity and thus inhibit preterm labor. That said, the deployment of agents during pregnancy that operate through regulating gene expression and chromatin structure raises concerns for potential placental transfer and detrimental effects on fetal development. This may seriously undermine the clinical utility of HDACis for the prevention of preterm birth, but even in that case, HDACis will remain a valuable tool to elucidate key regulatory mechanisms of human parturition. However, it is important to point out that multiple studies have now deployed TSA during mouse pregnancy with no obvious impacts on litter size or fetal viability [26] while leading to the birth of neurotypical offspring [45]. Moreover, the development of uterine-targeted nanoliposomes [38] and mucus penetrating nanoparticles for local administration [45, 46], may lead to HDACis deployed effectively and selectively to the pregnant myometrium blocking preterm labor while avoiding any undesired off-target maternal or fetal side-effects. Progress in these directions is highly desirable and expected considering the intractable health problem of premature birth worldwide.

List of abbreviations

aH3	histone H3 acetylation
Ca^{2+}	calcium ions
CBP	CREB-binding protein
CCL5	C-C motif chemokine ligand 5
CG	chorionic gonadotropin
CREB	cAMP-response element-binding protein
CXCL8	C-X-C motif chemokine ligand 8
DNMT1/3a	DNA Methyltransferase 1/3a
Egr-1	early growth response 1
ERα	estrogen receptor alpha
ESR1	estrogen receptor 1
$G\alpha_s$	G_s alpha subunit
GJA1	Gap Junction Protein alpha 1
HATs	histone acetyltransferases
hCG	human chorionic gonadotropin
Hda1	protein encoded by HDA1
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
IL	interleukin
K^+	potassium ion
LH	luteinizing hormone
LHCGR	luteinizing hormone/choriogonadotropin receptor
LPS	lipopolysaccharide
LTCC	L-type Ca^{2+} channels
NATs	N-terminal acetyltransferases
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAINS	pan-assay interference compound
PKA	protein kinase A
PR	progesterone receptor
PTGS2	prostaglandin-endoperoxide synthase 2
Rpd3p	protein encoded by RPD3

SAHA	suberoylanilide hydroxamic acid
SBHA	suberic bis-hydroxamate
Sir2	protein encoded by SIR2
Sirt6	sirtuin 6
Sp1-4	specificity protein-1 to -4
α-SMA	smooth muscle α-actin
SRC	steroid receptor coactivators
TSA	trichostatin A
VPA	valproic acid

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Tables

Table 1. Classes of HDACs.

CLASS	MEMBERS	COMMENT
I	HDACs 1 – 3, 8	Exhibit similarity to the yeast transcriptional regulator and deacetylase, Rpd3p.
IIa	HDACs 4, 5, 7, 9	Exhibit similarity to the yeast the deacetylase, Hda1, and continually shuttle between the nucleus and cytoplasm.
IIb	HDACs 6, 10	Exhibit similarity to the yeast deacetylase, Hda1, but localized to cytoplasm and have two catalytic sites.
III	Sirtuins 1 – 7	Exhibit similarity to the yeast silencing protein, Sir2.

IV	HDAC 11	Exhibits similarity to both Class I and Class II.

Abbreviations: HDACs, histone deacetylases; Rpd3p, protein encoded by RPD3; Hda1, protein encoded by HDA1; Sir2, protein encoded by SIR2.

Table 2. HDACis that have been used to investigate parturition.

HDACi Name	**HDAC Classes Inhibited**
TSA	Class I, II [90] and Sirt6 (Class III) [30]
SAHA	Class I, IIa, IIb, IV [91]
VPA	Class I, IIa [92]
SBHA	Class I, III [93]
Compound 2	Class I [76]
Resveratrol	Class I, II, IV [79]

Abbreviations: HDACi, histone deacetylase inhibitor; HDACs, histone deacetylases; TSA, trichostatin A; Sirt6, sirtuin 6; SAHA, suberoylanilide hydroxamic acid; VPA, valproic acid; SBHA, suberic bis-hydroxamate.

Figure titles and legends

Figure 1. Regulation of protein structure and function by acetylation. The addition and removal of acetyl functional groups affects protein structure, which in turn regulates protein function.

Figure 2. Acetylation of histone tails regulates the condensation and decondensation of chromatin. HDACis prevent the removal of acetyl functional groups from histone tails, which helps to maintain chromatin in a decondensed state that permits access to the DNA for gene transcription.

Figure 3. Structure of HDACis used to investigate myometrial biology. TSA, SAHA, VPA, SBHA, Compound 2 and Resveratrol have each been used to gain insight myometrial biology through their structure permitting the inhibition of different classes of HDACs.

Figure 4. Repression of PR-A expression by Trichostatin A. TSA represses the expression of *PR-A* through yet to be confirmed mechanisms but does not repress the expression of *PR-B*, resulting in a low PR-A/PR-B ratio. The comparatively higher levels of PR-B, liganded with progesterone, then repress the expression of pro-labor genes, such as *GJA1*.

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