Muscle Segment Homeobox Genes Direct Embryonic Diapause by Limiting Inflammation in the Uterus*

Jeeyeon Cha†, Kristin E. Burnum-Johnson§, Amanda Bartos†, Yingju Li†, Erin S. Baker§, Susan C. Tilton§, Bobbie-Jo M. Webb-Robertson†, Paul D. Piehowski†, Matthew E. Monroe§, Anil G. Jegga†, Shigeo Murata***, Yasushi Hirota††, and Sudhansu K. Dey‡‡

From the †Division of Reproductive Sciences and §Division of Biomedical Informatics, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio 45229-3039, the ‡Biological Sciences Division, Pacific Northwest National Laboratory, Richland, Washington 99354, the Environmental and Molecular Toxicology, Oregon State University, Corvallis, Oregon 97331, the **Laboratory of Protein Metabolism, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 Japan, and the ‡‡Department of Obstetrics and Gynecology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655 Japan

Background: Embryonic diapause is a reproductive strategy that confers blastocyst dormancy and uterine quiescence without implantation until conditions become favorable.

Results: Mice devoid of uterine muscle segment homeobox genes (Msx) show heightened inflammatory signature with failure of diapause.

Conclusion: Msx coordinates various pathways limiting inflammation in the uterus for diapause.

Significance: This study identifies a previously unrecognized role of Msx in this unique phenomenon.

Embryonic diapause is a reproductive strategy widespread in the animal kingdom. This phenomenon is defined by a temporary arrest in blastocyst growth and metabolic activity within a quiescent uterus without implantation until the environmental and maternal milieu become favorable for pregnancy to progress. We found that uterine Msx expression persists during diapause across species; their inactivation in the mouse uterus results in termination of diapause with the development of implantation-like responses (“pseudoimplantation”) that ultimately succumbed to resorption. To understand the cause of this failure, we compared proteome profiles between floxed and Msx-deleted uteri. In deleted uteri, several functional networks, including transcription/translation, ubiquitin-proteasome, inflammation, and endoplasmic reticulum stress, were dysregulated. Computational modeling predicted intersection of these pathways on an enhanced inflammatory signature. Further studies showed that this signature was reflected in increased phosphorylated IkB levels and nuclear NFκB in deleted uteri. This was associated with enhanced proteasome activity and endoplasmic reticulum stress. Interestingly, treatment with anti-inflammatory glucocorticoid (dexamethasone) reduced the inflammatory signature with improvement of the diapause phenotype. These findings highlight an unexpected role of uterine Msx in limiting aberrant inflammatory responses to maintain embryonic diapause.

Implantation is a complex process orchestrated by reciprocal interactions between the embryo and uterus. The orderly execution of this process is critical for pregnancy success. Implantation is one of the earliest encounters between the mother and conceptus, and it culminates from embryo development to the blastocyst stage synchronized with the acquisition of uterine receptivity for implantation. Any aberration in the implantation process can lead to pregnancy failure soon thereafter or can be propagated as adverse ripple effects through the remainder of pregnancy, resulting in compromised pregnancy outcome (1). Thus, the quality of implantation can dictate the course and outcome of pregnancy.

Implantation occurs within a defined time frame (window of implantation) in most eutherian mammals for appropriate synchrony of uterine readiness and embryo maturation to the blastocyst stage. In all eutherian species studied to date, the uterus achieves this transient window of receptivity; in mice, this state lasts ~24 h and spontaneously transitions to a state refractory to implantation (2, 3). However, in many mammals, implantation can temporally disengage from parturition via embryonic diapause (delayed implantation), an evolutionarily conserved, natural pause in pregnancy prior to implantation. This phenomenon is defined by the temporary arrest of growth and metabolic activity of the embryo at the blastocyst stage in synchrony with uterine quiescence. This suspended state of pregnancy prevents blastocyst activation and implantation...
until environmental and maternal conditions are favorable for the survival and well-being of the mother and offspring.

Embryonic diapause has been identified in more than 100 species across seven orders and can last for days to months depending on the species (4, 5). Under favorable conditions, the uterus attains receptivity and the blastocyst is activated for implantation. Normally, delayed implantation can be induced during lactation after postpartum mating (facultative delay) or can occur in every gestation of a species (obligatory) influenced by photoperiod length and melatonin secretion.

Normally, delayed implantation in mice occurs naturally during lactation because of ovarian estrogen deficiency resulting from suckling stimulus; this stimulus increases prolactin release from the pituitary, decreasing ovarian estrogen secretion. Upon removal of suckling pups or estrogen administration, blastocyst activation occurs with initiation of implantation, resulting in successful pregnancy. Delayed implantation can also be induced by ovarioctomizing pregnant mice on the evening of day 4 before preimplantation ovarian estrogen secretion. This condition can be maintained for days to weeks by providing daily progesterone (P₄) in the absence of estrogen. With exposure to a small dose of estrogen in P₄-primed uteri of ovarioctomized mice, blastocysts undergo activation with the initiation of implantation. With continued steroid treatment successful pregnancy occurs with delivery of pups. Irrespective of the type of delay, embryonic diapause is under maternal endocrine regulation in all species studied to date (4). However, the molecular mechanism by which these processes are regulated is not clearly understood (4–8).

We recently found that muscle segment homeobox genes (Msx1 and Msx2), members of an ancient, evolutionarily conserved homeobox gene family, show persistent expression in mouse uteri during physiological or experimentally induced delayed implantation, suggesting that uterine Msx genes are critical for the initiation and maintenance of embryonic diapause (7). Indeed, mice with conditional uterine deletion of Msx genes (Msx1/Msx2/Msx2/d/d) fail to undergo true delay. This is evident from poor blastocyst survival and often inappropriate manifestation of implantation-like responses ("pseudoimplantation") at the site of the blastocyst, which are not sustained and ultimately undergo resorption in Msx1/Msx2/d/d mice (7). These findings suggested that Msx factors play a major role in the uterus to transiently confer uterine quiescence and blastocyst dormancy. We also observed persistent uterine expression of Msx1 or Msx2 in two other distantly related mammalian orders during diapause: Carnivora (American mink) and Diprotodontia (Australian tammar wallaby) (7). Taken together, these results suggest the presence of an evolutionarily conserved reproductive strategy across diverse mammalian species via uterine Msx (7).

To explore the mechanism by which Msx transcription factors regulate quiescence and maintain a uterine environment conducive to embryo survival, we performed proteomics analyses of littermate floxed (Msx1/Msx2/d/d) and Msx1/Msx2/d/d mice under delayed implanting conditions experimentally induced by ovarioctomy and P₄ treatment (6–8). These analyses identified several classes of signaling pathways enriched in transcription, translation, chromatin remodeling, inflammation, proteasome activity, ubiquitination, chaperone-mediated protein folding, oxidative, and ER stress responses in Msx1/Msx2/d/d uteri. These findings were reflected in higher uterine levels of proteasome subunits, polyubiquitinated proteins, and ER stress consistent with proteotoxic stress/burden. Computational modeling predicted that these diverse pathways intersected on an enhanced inflammatory signature in Msx1/Msx2/d/d uteri. These findings, coupled with our observations of failure of embryonic diapause with formation of pseudoimplantation sites in Msx1/Msx2/d/d uteri, increased the signature of inflammation reflected in higher phosphorylated IkB (pIkB, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor) levels and nuclear NFκB (nuclear factor κ-light-chain-enhancer of activated B cells) expression, and evidence of proteotoxic stress, are consistent with reported interactions between heightened proteasome activity with inflammation (9, 10).

Aggravation of proteotoxicity with bortezomib resulted in an increased rate of pseudoimplantation in Msx1/Msx2/d/d uteri, whereas inhibition of inflammatory response by dexamethasone substantially reduced the incidence and size of pseudoimplantation sites. In conclusion, this study identifies an unexpected role for Msx genes in limiting uterine stress-mediated inflammatory responses during embryonic diapause.

Experimental Procedures

*Mice*—Mice with uterine deletion of Msx1 and Msx2 (Msx1/Msx2/d/d) and control littermates (Msx1/Msx2/d/d) were generated as previously described (11). All protocols for the present study were reviewed and approved by the Cincinnati Children’s Research Foundation Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines. Adult Msx1/Msx2/d/d and Msx1/Msx2/d/d female mice were mated with fertile males to induce pregnancy (day 1 = vaginal plug).

**Experimental Delay**—To induce delayed implantation, plug-positive mice were ovarioctomized on the morning of day 4 (0800–0900 h) and subcutaneously given P₄ in sesame oil daily (2 mg/100 µl/dose) on days 5–7 or 5–9 for tissue collection on day 8 or 10, respectively (7). For mass spectrometry analysis, uteri were flash-frozen and stored at −80 °C after gently flushing the horns with cold PBS containing protease and phosphatase inhibitors (Sigma) to recover embryos and to confirm pregnancy.

**Pharmacological Treatments**—The proteasome inhibitor bortezomib (0.2 mg/kg of body weight/day) was suspended in vehicle (5% v/v PEG 400 and 5% v/v Tween 80 dissolved in water) and was given as a single oral gavage on days 5, 7, and 9 of pregnancy. A selective Cox2 inhibitor celecoxib (10 mg/kg of body weight/day) was suspended in vehicle (0.5% w/v methylcellulose and 0.1% v/v polysorbate 80 dissolved in water) and was given as a single oral gavage on days 5, 7, and 9 of pregnancy. Dexamethasone (20 µg/100 µl/dose) was injected subcutaneously on days 5–9 of pregnancy. Respective control groups received vehicle alone.

---

*The abbreviations used are: P₄, progesterone; plkB, phosphorylated IkB; IMS, ion mobility spectrometry.*
Mass Spectrometry Sample Preparation—Mass spectrometry was performed in the Environmental Molecular Sciences Laboratory, a U.S. Department of Energy Office of Biological and Environmental Research. Frozen tissue samples were plunged in LN₂ and immediately pulverized using a cryoPREP impactor (Covaris, Inc). Proteins were extracted with 500 μl of 8 M urea and 10 mM DTT in 100 mM ammonium bicarbonate at 37 °C for 1 h. Samples were centrifuged at 15,000 rpm to collect the supernatant. Protein extract (125 μg) was digested with sequencing grade trypsin (Promega) overnight with 1:50 enzyme:substrate ratio. The resulting peptides were desalted by C18 SPE (Supelco) and rehydrated in mobile phase A. Peptide concentration was measured by BCA assay (Thermo Scientific) and normalized to 0.5 μg/μl for LC-MS analysis.

LC-MS/MS and LC-MS Analyses—Analysis of the digested peptide mixtures from uterine tissues was performed on both a Thermo Fisher Scientific LTQ Orbitrap Velos mass spectrometer (San Jose, CA) operated in tandem MS (MS/MS) mode and an instrument built in-house that couples a 1-m ion mobility separation (12, 13) with an Agilent 6224 TOF MS that was upgraded to have a 1.5-meter flight tube for resolution of ~25,000. Two different LC systems were used for this analysis. The same fully automated two-column HPLC system built in-house (14) equipped with packed capillary columns was used for both instruments with mobile phase A consisting of 0.1% (v/v) formic acid in water and B comprised of 0.1% (v/v) formic acid in acetonitrile. A 100-min LC separation was performed on the Velos MS (using 60-cm-long columns having an outer diameter of 360 μm, an inner diameter of 75 μm, and 3-μm C₁₈ packing material), whereas only a 60-min gradient with shorter columns (30-cm-long columns with the same dimensions and packing) was used with the ion mobility-MS (IMS-MS) because the additional IMS separation helps address detector suppression. Both gradients were linear increasing mobile phase B from 0 to 60% until the final 2 min of the run when B was purged at 95%. Each sample (5 μl) was injected for both analyses, and the HPLC was operated under a constant flow rate of 0.4 μl/min for the 100-min gradient and 1 μl/min for the 60-min gradient. The Velos MS data were collected from 400–2000 m/z at a resolution of 60,000 (automatic gain control target: 1 × 10⁶) followed by data-dependent ion trap MS/MS spectra (automatic gain control target: 1 × 10⁶) of the 12 most abundant ions using a collision energy setting of 35%. A dynamic exclusion time of 60 s was used to discriminate against previously analyzed ions. IMS-TOF MS data were collected from 100–3200 m/z.

Proteomics Data Processing and Statistical Analysis—Identification and quantification of the detected peptide peaks were performed using the accurate mass and time tag approach (15, 16). Peptide database generation utilized Velos tandem MS/MS data (17, 18). Because of the greater sensitivity and dynamic range of measurements (16), relative quantitation of peptide peaks utilized our IMS LC-MS data. Multiple bioinformatics tools developed in-house (19, 20) were used to process the IMS LC-MS data and correlate the resulting LC-MS features to our accurate mass and time tag database that contained IMS drift time, accurate mass, and LC separation elution time information for each peptide tag. Data processing of our 16 data sets (two uterine horns for each of four Msx1/Msx2<sup>t</sup>/t</sup> and four Msx1/Msx2<sup>d/d</sup> mice) included averaging peptide abundance data from the two uterine horns for each mouse. Further processing (21, 22) decreased our peptide value from 14,394 to 10,232 (i.e. peptides with inadequate data for statistics were removed). Normalization was performed using a rank invariant peptide subset that was in common between the two data sets (23). This rank invariant peptide subset was used for median centering of the data. Processing of the peptide data found 3,710 significant peptides at p < 0.05. These significant markers were used to generate signatures where each peptide increased, decreased, or had no statistical difference in the Msx1/Msx2<sup>d/d</sup> versus Msx1/Msx2<sup>t</sup>/t</sup> uteri. We then used the BP-Quant quantification (24) approach to estimate abundance at the protein level. There were a total of 1,019 significant proteins at p < 0.05. Of these significant proteins, 448 were identified by ≥2 peptides (supplemental Data Set S1, worksheets 1–4).

MetaCore Statistical Interactome—To identify major hubs or regulators of the significant proteins in Msx1/Msx2<sup>d/d</sup> uteri on day 8 of delayed implantation in comparison with control delayed uteri, the Statistical Interactome tool (MetaCore) was used to measure the interconnectedness of proteins in the experimental data set relative to all known interactions among all proteins measured in the study by hypergeometric distribution. Networks were constructed for experimental data and predicted hubs based on direct interactions from the Metacore knowledgebase and visualized in Cytoscape (25) (supplemental Data Set S1, worksheets 8 and 9).

Immunohistochemistry—Immunostaining was performed in formalin-fixed paraffin-embedded sections using antibodies to p1kB (rabbit, Cell Signaling, 9426), Rpn13 (rat, laboratory-generated), and Cox2 (rabbit, lab generated) as previously described (26).

Immunofluorescence—Frozen sections of Msx1/Msx2<sup>d/d</sup> uteri and control uteri were taken from snap frozen tissues at 12-μm thickness. Immunofluorescence was performed using NFkB (rabbit, sc-372), GRP78/Bip (goat, sc-1050), CD45 (rat, BioLegend, 103102), and F4/80 (mouse, Serotec, MCA497RT) antibodies after cold methanol or paraformaldehyde fixation. Cy3-conjugated donkey antibodies (Jackson ImmunoResearch Laboratories) were used for secondary antibodies. Nuclear staining was performed with Hoescht 33342. Immunofluorescence was visualized with confocal microscopy (Nikon Eclipse TE2000).

Immunoblotting—Protein extraction and Western blotting were performed as previously described (14). Antibodies to phosphorylated 1kB (mouse, Cell Signaling Technology, 9246), total 1kB (rabbit, Cell Signaling Technology, 9242), 20S proteasome α₁, 2, 3, 5, 6, 7 subunits (mouse, Enzo, MCP231), α6 (rabbit, laboratory-generated), Rpt6 (mouse, laboratory-generated), Rpn8 (rabbit, laboratory-generated), HSPA5/Bip (goat, sc-1050), phosphorylated eIF2α (rabbit, Cell Signaling, 3957S), total eIF2α (rabbit, sc-11386), ubiquitin lysine 48 (rabbit, Millipore, 05-1307), and ubiquitin lysine 63 (rabbit, Millipore, 05-1308) were used as previously described (27). Actin (goat, sc-1615) served as a loading control.

Statistical Analysis—Statistical analyses of proteomic data were performed using two-tailed Student’s t test for quantita-
Msx Genes Limit Uterine Inflammation for Diapause

tive comparisons and a g-test for qualitative comparisons as appropriate (22). Values of $p < 0.05$ were considered statistically significant.

Results

Msx1/Msx2<sup>d/d</sup> Uteri Fail to Undergo True Delay under Experimental Conditions—In mice, delayed implantation induced experimentally by ovariectomy on the morning of day 4 prior to preimplantation estrogen secretion can be maintained for days to weeks with continued P<sub>4</sub> administration (Fig. 1A) (7). Msx1/Msx2<sup>d/d</sup> females showed reduced blastocyst recovery and often inappropriate manifestation of some implantation-like responses (“pseudoimplantation”) at the site of the blastocyst predominantly by day 10 of delayed implantation (6 days of dormancy) in the absence of estrogen; these pseudoimplantation sites underwent resorption (Fig. 1, B and C). Blastocyst recovery showed unhealthy embryos with some cells adherent to the abembryonic pole. These pseudoimplantation sites showed signs of increased vascular permeability by blue dye assay (3, 29), albeit at different intensities, with swellings of various sizes. In contrast, no such swellings or blue reaction was noted in littermate floxed females under similar conditions.

Msx1/Msx2<sup>d/d</sup> Uteri Show a Unique Proteome Signature under Delayed Conditions That Intersects on Inflammation—To identify molecular targets of Msx transcription factors, we compared proteomics profiles between Msx1/Msx2<sup>f/f</sup> and Msx1/Msx2<sup>d/d</sup> uteri on day 8 of delayed implantation (4 days of dormancy). Our analysis identified 448 proteins by ≥2 peptides ($p < 0.05$) (Fig. 2A and supplemental Data Set S1, worksheets 1–4) (24). We performed comparisons of enriched gene ontology and pathway designations within the up-regulated and down-regulated proteins using the programs ToppGene Suite (30) and ToppCluster (31) ($p < 0.05$ with FDR correction) and resolved the most significantly enriched gene ontology biological processes and pathways (supplemental Data Set S1, worksheets 5 and 6). Computational modeling was performed with networks containing select enriched terms to visualize relevant functional relationships using Cytoscape in a spring embedded layout function (25). We identified multiple interactions between pathways, particularly for inflammatory response, NFκB-mediated inflammation, kinases of NFκB inhibitor IκB (IKKB and IKKe), ubiquitin-proteasome system, ER stress, chaperone-mediated protein folding, oxidative stress, and antioxidant activity (Fig. 2B and supplemental Data Set S1, worksheet 7).

To identify a common hub among all dysregulated pathways in the context of inflammation, we utilized the Statistical Interaction tool (MetaCore, Thomson Reuters, Philadelphia, PA). This tool allowed us to assess the interconnectedness of proteins in the experimental data set relative to all known interactions among all proteins measured in the study by hypergeometric distribution to delineate major hubs or regulators of significantly altered proteins in Msx1/Msx2<sup>d/d</sup> uteri (Fig. 3 and supplemental Data Set S1, worksheet 8). Networks were constructed for experimental data and predicted hubs based on direct interactions from the Metacore knowledgebase. Interestingly, significant hubs for the proteins altered in Msx1/Msx2<sup>d/d</sup> uteri under delayed conditions included NFκB subunits RelA/p65 and c-Rel, IKK-ε (IKKE), IKK-β (IKKB), SAM68, NF-X1, TGM2, and Fetuin-A (supplemental Data Set S1, worksheet 9), identifying enriched interconnectedness of the proteins within the data set for the NFκB signaling pathway and inflammation.

Inflammatory Responses Are Up-regulated in Msx1/Msx2<sup>d/d</sup> Uteri under Delayed Conditions—Considering our phenotype of pseudoimplantation sites in Msx1/Msx2<sup>d/d</sup> uteri under
delayed conditions (7), we were intrigued by the changes in regulatory pathways of inflammation identified by our proteomics and computational analysis. We found up-regulation of several proteins involved in inflammation and acute inflammatory responses in Msx1/Msx2<sup>d/d</sup> uteri, such as α2-macroglobulin, LSP1 (lymphocyte-specific protein 1), KNG1 (kininogen), complement components (C1qbp and C3), Park 7 (Parkinson protein 7), IL enhancer binding factor 2, and importin α-subunit 1 (KPNB1) (supplemental Data Set S1, worksheets 4–6). Notably, we previously identified up-regulation of epithelial C3 expression in Msx1/Msx2<sup>d/d</sup> uteri (11). In contrast, interferon regulatory factor 7y, monocyte inhibitory factor, and Ig chains were down-regulated (supplemental Data Set S1, worksheets 4–6).

To evaluate the status of NFκB-mediated inflammation, we examined the status of uterine levels of pIκB. Phosphorylation of IκB by IKK allows for ubiquitination and degradation of IκB, permitting NFκB dissociation and translocation to the nucleus. Indeed, Western blotting results showed enhanced IκB phosphorylation on day 8 in Msx1/Msx2<sup>d/d</sup> uteri (Fig. 4A). This was reflected in increased uterine immunolocalization of pIκB on days 8 and 10 along with increased nuclear NFκB localization on day 10, particularly in the epithelium and subepithelial stroma of Msx1/Msx2<sup>d/d</sup> mice (Fig. 4, B and C). Notably, there were insignificant changes in the distribution of leukocytes and macrophages between floxed and Msx1/Msx2<sup>d/d</sup> uteri (Fig. 4D). Our previous study showed Ptgs2 expression (encoding Cox2) in the luminal epithelium at the pseudoimplantation sites of Msx1/Msx2<sup>d/d</sup> mice with embryos entrapped within the intact luminal epithelium (7). Here we show by immunohistochemistry that Cox2 is also expressed in the luminal epithelium of Msx1/Msx2<sup>d/d</sup> uteri away from the site of blastocyst on day 10 of pregnancy (day 6 of dormancy); no such expression was noted in floxed uteri under similar conditions (Fig. 4E). In addition, pseudoimplantation sites showed modest decidualization on day 10 in the absence of luminal epithelial breaching, suggesting that the embryo transmitted some signals through Msx-deleted luminal epithelium to the subepithelial stroma (Fig. 4F). However, these responses were unsustainable, leading to resorption (7). Taken together, these
results suggest that inflammatory responses seen in Msx1/Msx2\textsuperscript{d/d} uteri under delayed conditions are mediated by increased NF\textsubscript{KB} signaling and are consistent with aberrant Cox2 expression, as observed in other systems (32–36).

The Ubiquitin-Proteasome System Is Enriched in Msx1/Msx2\textsuperscript{d/d} Uteri under Delayed Conditions—We next asked what contributes to the enhanced inflammatory responses in Msx1/Msx2\textsuperscript{d/d} uteri under delayed conditions. In addition to several proteins implicated in translation and secretory machinery, we found that numerous proteasome core and regulatory components were up-regulated in Msx-deleted uteri (supplemental Data Set S1, worksheets 4–6). The proteasome system encompasses a highly conserved and regulated network of proteins that normally degrade damaged and/or misfolded proteins to maintain protein homeostasis. They also participate in cell cycle regulation, protein turnover, gene expression, immune response, and responses to oxidative stresses (37–40). There is evidence that proteasome activity is correlated with heightened inflammation secondary to increased intracellular protein turnover and antigen processing (9, 10, 41). Furthermore, accumulation of misfolded proteins can lead to harmful effects collectively known as proteotoxicity (42).

The key enzyme responsible for mammalian proteolysis is the 26S proteasome, which is comprised of the barrel-shaped 20S core particle consisting of unique outer \(\alpha\)- and inner \(\beta\)-subunits and two 19S regulatory particles. The 19S particle is further subdivided into a 9-subunit base that directly interacts with the \(\alpha\)-ring of the 20S core particle and a 10-subunit lid. These components confer substrate specificity and other minor enzymatic activities for proteolysis. To confirm the up-regulation of proteasome components in Msx1/Msx2\textsuperscript{d/d} uteri, we examined the \(\alpha\)-subunits of the 20S core identified in our proteomics analysis by Western blotting and found their up-regulation along with increased levels of the specific core \(\alpha\)-subunit 6 (\(\alpha6\)), lid subunit Rpn8, and base subunit Rpt6 in Msx1/Msx2\textsuperscript{d/d} uteri on day 10 (Fig. 5, A and B, and supplemental Data Set S1, worksheets 4–6).

The ubiquitin–proteasome system targets proteins for degradation by polyubiquitination (43). We next assessed whether our proteomics analysis showed dysregulation of ubiquitination pathway components in Msx1/Msx2\textsuperscript{d/d} uteri. We found elevated protein levels of ubiquitin regulators (supplemental Data Set S1, worksheets 4–6). Ubiquitin chains can be formed by lysine linkages either via Lys-48 or Lys-63, and these linkages

FIGURE 3. MetaCore network analysis of proteins changed in Msx1/Msx2\textsuperscript{d/d} mice showed increased connectivity with the inflammatory NF\textsubscript{KB} pathway. Red, increased in Msx1/Msx2\textsuperscript{d/d} mice compared with Msx1/Msx2\textsuperscript{f/f} mice; blue, decreased in Msx1/Msx2\textsuperscript{d/d} mice compared with Msx1/Msx2\textsuperscript{f/f} mice; purple, significant trends in both directions.
appear to be enhanced for either proteasome degradation or signaling, respectively (38). Interestingly, enhanced polyubiquitination via linker Lys-48 in Msx1/Msx2<sup>d/d</sup> uteri under delayed conditions was identified by Western blotting; Lys-63 linkages were undetectable (Fig. 5B). Furthermore, immunohistochemistry in Msx1/Msx2<sup>d/d</sup> uteri on day 10 showed higher levels of Rpn13/ADRM1, a proteasome regulatory subunit and ubiquitin receptor (Fig. 5C) (44). This expression was pronounced in the luminal and glandular epithelia, the site of Msx expression during delayed implantation in floxed uteri. Overall, these results suggest that Msx1/Msx2<sup>d/d</sup> uteri have enhanced proteasome expression and accumulation of ubiquitinated proteins targeted for proteasome degradation compared with Msx1/Msx2<sup>f/f</sup> uteri under similar experimental conditions.

**Chaperone and Co-chaperone Proteins Are Up-regulated in the Msx1/Msx2<sup>d/d</sup> Uterus under Delayed Conditions**—Molecular chaperones and co-chaperones are critical for distinguishing normal versus misfolded proteins; they facilitate correct folding or ubiquitin-mediated proteasomal degradation of aberrant proteins (45, 46). Accumulation of misfolded proteins can lead to proteotoxic stress and inflammation (47–49). Proteotoxicity in the cytosol can initiate the unfolded protein response, resulting in ER stress. This response results in decreased translation and increased heat shock protein response to help protein refolding or degradation (42). In this respect, heat shock proteins HSP10, HSP70, HSP105, HSPA5 (Bip), GRP75, chaperonin 60, T-complex protein 1 (TCP1, multiple subunits), and immunophilin FKBP4 (FKBP52) were all up-regulated in Msx1/Msx2<sup>d/d</sup> uteri (supplemental Data Set S1, worksheets 4–6). Interestingly, mice missing FKBP4 (FKBP52) show pregnancy failure because of diminished P<sub>4</sub> sensitivity, which is reversed by exogenous P<sub>4</sub> administration (50, 51). In addition, Bip is a marker of ER stress involved in the unfolded protein response and is also expressed in the mouse uterus (52, 53). We indeed found increased levels and expression of Bip at the apical surface of the epithelium and in interspersed subepithelial stromal cells in Msx1/Msx2<sup>d/d</sup> uteri (Figs. 5D and 6A), suggesting heightened ER stress.

Under normal conditions, Bip binds to the luminal domains of ER transmembrane proteins IRE1 (inositol requiring 1), PRK-like ER kinase (54), and ATF6 (activating transcription factor 6). During conditions of ER stress, Bip dissociates from these sensors for activation of the unfolded protein response to restore protein homeostasis (55). Our proteomics results had identified increased levels of elf2α in Msx1/Msx2<sup>d/d</sup> uterus under delayed conditions. Phosphorylation of elf2α is a surro-
Msx genes are critical for uterine receptivity and implantation (11), and they have indispensable roles in the onset and maintenance of delayed implantation by conferring blastocyst dormancy and uterine quiescence (7). Wild-type uteri under delayed conditions show persistent Msx1 expression and remain quiescent without any sign of implantation or endometrial vascular permeability at the site of blastocyst apposition (7, 11, 61). In contrast, mice with conditional uterine inactivation of Msx1 and Msx2 fail to undergo true delay and manifest unique phenotypes—continued cell proliferation in the blastocyst and formation of pseudoimplantation sites in the uterus at the site of the blastocyst without trophoderm invasion through the luminal epithelium. Although implantation can be initiated in delayed implanting floxed mice if estrogen is given after P4 priming, our observation of pseudoimplantation in Msx1/Msx2<sup>d/d</sup> uteri in the absence of estrogen suggests that a different mechanism is operative to terminate delayed implantation. Thus, although termination of diapause in the absence of Msx is not physiological and leads to resorption with pregnancy failure, termination of diapause in P<sub>4</sub>-treated floxed mice after estrogen exposure is physiological and results in a live birth.
FIGURE 6. ER stress is evident in Msx1/Msx2
d/d uteri under delayed conditions and is aggravated by bortezomib treatment. A, representative results of immunofluorescence showing apical epithelial expression of HSPA5/Bip in Msx1/Msx2

 uteri, with increased intensity and dispersed Bip-positive stromal cells in Msx1/Msx2
d/d uteri. Stromal Bip expression is widespread at the pseudoimplantation site (Pseudo-IS). Arrowheads, dispersed Bip-positive cells. Bar, 250 μm.

B, blastocyst recovery (top bar graph) from Msx1/Msx2
d/d after bortezomib treatment females was significantly lower with enhanced rate of pseudoimplantation sites (bottom bar graph) compared with floxed littermates on day 10 (means ± S.E.). Numbers in parentheses indicate number of females that produced blastocysts or pseudoimplantation sites, respectively, compared with the number of females assessed.

FIGURE 7. Aggravation of ER stress and inflammation by bortezomib treatment in Msx1/Msx2
d/d uteri under delayed conditions. A, Western blotting results showing increased levels of Bip and pIκB in Msx1/Msx2


 Mice After Bortezomib (BZB) Treatment. B, quantification of Western blotting results (means ± S.E.; *, p < 0.05). C, Immunofluorescence showing increased Bip expression in Msx1/Msx2


 uterine sections and pseudoimplantation sites (Pseudo-IS) under delayed conditions compared with floxed littermates after bortezomib treatment. D, Immunofluorescence showing increased nuclear localization of NFκB in Msx1/Msx2
d/d uterus and pseudoimplantation sites (Pseudo-IS) under delayed conditions compared with control littermates after bortezomib treatment. le, luminal epithelium; ge, glandular epithelium; s, stroma. Bars, 250 μm.
Using proteomics and computational modeling, we identified dysregulation of several pathways in Msx1/Msx2<sup>d/d</sup> uteri with a signature of heightened inflammatory responses promoting a uterine environment unfavorable for embryonic diapause (7). Indeed, an inflammatory signature with increased phosphorylation of IκB, NFκB nuclear localization, and Cox2 induction was evident in Msx1/Msx2<sup>d/d</sup> uteri under delayed conditions. This is consistent with our findings of heightened proteasome activity facilitating enhanced destruction of target proteins, such as pIκB, by the proteasome. These findings suggest that Msx transcription factors play an important role in tempering inflammatory responses in the uterus.

The signature of enhanced inflammation and dysregulated proteasome activity seen in Msx1/Msx2<sup>d/d</sup> uteri are similar to that of pathological processes such as multiple myeloma, neurodegenerative diseases, non-Hodgkin’s lymphoma, and autoimmune diseases (62–65). In addition, there is evidence that enhanced translation and accumulation of misfolded proteins incurs proteotoxic and ER stresses (10). These conditions engage chaperones and co-chaperones to direct refolding of misfolded proteins or targeting damaged proteins to the ubiquitin-proteasome machinery (45, 46, 52). Failure to relieve ER stresses results in harmful cellular responses and apoptosis (42). Therefore, the ubiquitin-proteasome system maintains protein homeostasis for normal functioning, and its dysregulation in Msx1/Msx2<sup>d/d</sup> uteri suggests a role for Msx in protein homeostasis during embryonic diapause. It is interesting to note that ubiquitin pathway modifiers have been identified in diapausing cysts of the crustacean Artemia sinica (66) and Caenorhabditis elegans in dauer diapause (67), suggesting a role in protein turnover and homeostasis in various forms of diapause. Intriguingly, Artemia cysts has also been shown to up-regulate a diapause-specific protein chaperone (68).

We speculate that the up-regulation of proteasome subunits and activity served to limit proteotoxic burden from higher levels of misfolded proteins and attenuate unwarranted inflammation. This is consistent with our results of increased incidence of pseudoimplantation sites and evidence of ER stress (higher Bip levels) in the Msx1/Msx2<sup>d/d</sup> uteri after treatment with proteasome inhibitor bortezomib consistent with its reported mechanism of action (64). Our findings of enhanced NFκB nuclear localization and heightened ER stress in Msx-deleted uteri corroborate reports of enhanced NFκB-mediated inflammation and ER stress (69). In contrast, reduced incidence and size of pseudoimplantation sites in uteri under similar conditions after dexamethasone treatment suggests that unchecked inflammation in the absence of Msx is a major cause of failure of delayed implantation.

Msx deficiency is considered to enhance tumorigenesis in human breast cancer (70). Because inflammation is a hallmark of cancer progression, it is possible that dysregulation of Msx promotes the cancer phenotype via enhanced inflammatory signature in affected tissue types. In this regard, we believe that our study is the first to show the relationship between Msx activity and inflammation.

Proteins that incur antioxidant properties were also up-regulated in Msx1/Msx2<sup>d/d</sup> uteri under delayed conditions (supplemental Data Set S1, worksheets 4–6). Their up-regulation is perhaps an attempt to limit inflammation and promotion of anti-inflammatory mechanisms. However, we cannot
Msx Genes Limit Uterine Inflammation for Diapause

- Deletion of uterine Msx genes resulted in aberrant inflammation arising from increased transcription/translation, proteotoxic stress from protein burden, and ER stress. Inflammatory responses were reflected in higher NFκB signaling with premature termination of embryonic diapause and formation of pseudoimplantation sites caused by loss of synchrony between the blastocyst and uterus. Although proteasome components, antioxidants, and a pro-resolving molecule were up-regulated perhaps in an attempt to quell aberrant inflammation, these changes could not mitigate uterine inflammatory responses (smaller font and dashed lines). However, treatment with a long acting glucocorticoid dexamethasone (Dex) improved the embryonic diapause phenotype.

- We believe that our model of aberrant inflammation in the uterus devoid of Msx genes would suggest that inflammation is tightly regulated during natural pregnancy and embryonic diapause (Fig. 9). Under normal conditions, exit from diapause induces blastocyst activation synchronized with uterine receptivity after implantation-inducing stimuli. This is in contrast to mice with uterine deletion of Msx genes that show poor embryo recovery and survival and formation of pseudoimplantation sites, which fail to produce a live birth. Taken together, these results propose a new concept that Msx genes have a major role in coordinating various pathways to limit inflammation in the mammalian uterus for embryonic diapause.

**References**

1. Cha, J., Sun, X., and Dey, S. K. (2012) Mechanisms of implantation: strategies for successful pregnancy. *Nat. Med.* 18, 1754–1767
2. Ma, W. G., Song, H., Das, S. K., Paria, B. C., and Dey, S. K. (2003) Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2963–2968
3. Psychouyas, A. (1973) Endocrine control of egg implantation. In *Handbook of Physiology* (R. O. Greep, E. G. Astwood, and S. R. Geiger, eds) pp. 187–215, American Physiology Society, Washington, D. C.
4. Renfree, M. B., and Shaw, G. (2000) Diapause *Annu. Rev. Physiol.* 62, 353–375
5. Lopes, F. L., Desmarais, J. A., and Murphy, B. D. (2004) Embryonic diapause and its regulation. *Reproduction* 128, 669–678
6. Yoshinaga, K. (1961) Effect of local application of ovarian hormones on the delay in implantation in lactating rats. *J. Reprod. Fertil.* 2, 35–41
7. Cha, J., Sun, X., Bartos, A., Fenelon, J., Lefèvre, P., Daikoku, T., Shaw, G., Maxson, R., Murphy, B. D., Renfree, M. B., and Dey, S. K. (2013) A new role for muscle segment homeobox genes in mammalian embryonic diapause. *Open Biol.* 3, 130035
8. McLaren, A. (1968) A study of blastocysts during delay and subsequent implantation in lactating mice. *J. Endocrinol.* 42, 453–463
9. Skaug, B., Jiang, X., and Chen, Z. J. (2009) The role of ubiquitin in NF-κB regulatory pathways. *Annu. Rev. Biochem.* 78, 769–796
10. Wertz, I. E., and Dixit, V. M. (2010) Signaling to NF-κB: regulation by...
11. Daikoku, T., Cha, J., Sun, X., Tranguch, S., Xie, H., Fujita, T., Hirota, Y., Lydon, J., DeMayo, F., Maxson, R., and Dey, S. K. (2011) Conditional deletion of Msx homeobox genes in the uterus inhibits blastocyst implantation by altering uterine receptivity. *Dev. Cell* 21, 1014–1025
12. Baker, E. S., Clowers, B. H., Li, F., Tang, K., Tolmachev, A. V., Prior, D. C., Belov, M. E., and Smith, R. D. (2006) Spectral filtering of MS/MS database search results. *J. Proteome Res.* 5, 997–1006
13. Baker, E. S., Livesay, E. A., Orton, D. J., Moore, R. J., Danielson, W. F., 3rd, Livesay, E. A., Tang, K., Taylor, B. K., Buschbach, M. A., Hopkins, D. F., Prior, D. C., Ibrahim, Y. M., LaMarche, B. L., Zhao, R., Shen, Y., Orton, D. J., Moore, R. J., Kelly, R. T., Udseth, H. R., and Smith, R. D. (2008) Fully automated four-column capillary LC-MS system for maximizing throughput in proteomic analyses. *Anal. Chem.* 80, 294–302
14. Kaneko, T., Hamazaki, J., Iemura, S., Sasaki, K., Furuyama, K., Natsume, T., Tanaka, K., and Murata, S. (2009) Assembly pathway of the mammalian proteasome base subcomplex is mediated by multiple specific chaperones. *Cell* 137, 914–925
15. Wang, H., and Dey, S. K. (2006) Roadmap to embryo implantation: clues from mouse models. *Nat. Rev. Genet.* 7, 185–199
16. Chen, J., Barde, E. S., Aronow, B. J., and Jegga, A. G. (2009) ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Res.* 37, W305–W311
17. Ahn, K. S., and Aggarwal, B. B. (2005) Transcription factor NF-kB: a sensor for smoke and stress signals. *Ann. N.Y. Acad. Sci.* 1056, 218–233
18. Tegeder, I., Pleilshifer, J., and Geisslinger, G. (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J.* 15, 2057–2072
19. Glickman, M. H., and Ciechanover, A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* 82, 373–428
20. Thyagarajan, B., and Hochstrasser, M. (2008) Diversity of degradation signals in the ubiquitin-proteasome system. *Nat. Rev. Mol. Cell Biol.* 9, 679–690
21. Schwartz, A. L., and Ciechanover, A. (2009) Targeting proteins for destruction by the ubiquitin system: implications for human pathobiology. *Annu. Rev. Pharmacol. Toxicol.* 49, 73–96
22. Murata, S., Yashiroda, H., and Tanaka, K. (2009) Molecular mechanisms of proteasome assembly. *Nat. Rev. Mol. Cell Biol.* 10, 104–115
23. Förster, F., Unverdorben, P., Sledz, P., and Baumeister, W. (2013) Unveiling the long-held secrets of the 26S proteasome. *Structure* 21, 1551–1562
24. Sherman, M. Y. (2011) Proteotoxic stress targeted therapy (PSTT). *Onco-target* 2, 356–357
25. Weissman, A. M. (2001) Themes and variations on ubiquitylation. *Nat. Rev. Mol. Cell Biol.* 2, 169–178
26. Husnjak, K., Elsasser, S., Zhang, N., Chen, X., Randles, L., Shi, Y., Hofmann, K., Walters, K. J., Finley, D., and Dikic, I. (2008) Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature* 453, 481–488
27. Sontag, E. M., Vonk, W. I., and Frydman, J. (2014) Sorting out the trash: the spatial nature of eukaryotic protein quality control. *Curr. Opin. Cell Biol.* 26, 139–146
28. Clausen, T., Kaiser, M., Huber, R., and Ehrmann, M. (2011) HTRA proteases: regulated proteolysis in protein quality control. *Nat. Rev. Mol. Cell Biol.* 12, 152–162
29. Esser, C., Alberti, S., and Hohfeld, J. (2004) Cooperation of molecular chaperones with the ubiquitin/proteasome system. *Biochem. Biophys. Acta* 1695, 171–188
30. Banfalvi, J. M., Broadley, S. A., Schaffar, G., and Hartl, F. U. (2004) Roles of molecular chaperones in protein misfolding diseases. *Semin. Cell. Dev. Biol.* 15, 17–29
31. Kaste, M., and Grune, T. (2012) Interactions of the proteasomal system with chaperones: protein triage and protein quality control. *Proc. Mol. Biol. Transl. Sci.* 109, 113–160
32. Tranguch, S., Cheung-Flynn, J., Daikoku, T., Prapapanich, V., Cox, M. B., Xie, H., Wang, H., Das, S. K., Smith, D. F., and Dey, S. K. (2005) Cochaperone immunophenol FKBP52 is critical to uterine receptivity for embryo implantation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14326–14331
33. Tranguch, S., Wang, H., Daikoku, T., Xie, H., Smith, D. F., and Dey, S. K. (2007) FKBP52 deficiency-conferred uterine progesterone resistance is
Msx Genes Limit Uterine Inflammation for Diapause

52. Määttänen, P., Gehring, K., Bergeron, J. J., and Thomas, D. Y. (2010) Pro-
derivation of liver fibrosis protein signatures using multiplexed ion mobility

53. Das, S. K., Tan, J., Raja, S., Paria, B. C., and Dey, S. K. (2000) Estrogen targets genes involved in protein processing, calcium homeostasis, and Wnt signaling in the mouse uterus independent of estrogen receptor-α and β. J. Biol. Chem. 275, 28834–28842

54. Baker, E. S., Burnum-Johnson, K. E., Jacobs, J. M., Diamond, D. L., Brown, R. N., Ibrahim, Y. M., Orton, D. J., Piehowski, P. D., Purdy, D. E., Moore, R. J., Danielson, W. F., 3rd, Monroe, M. E., Crowell, K. L., Slyz, G. W., Gritsenko, M. A., Sandoval, J. D., Lamarche, B. L., Matzke, M. M., Webb-Robertson, B. J., Simons, B. C., McMahon, B. J., Bhattacharya, R., Perkins, J. D., Carithers, R. L., Jr., Strom, S., Self, S. G., Katze, M. G., Anderson, G. A., and Smith, R. D. (2014) Advancing the high throughput identification of liver fibrosis protein signatures using multiplexed ion mobility spectrometry. Mol. Cell. Proteomics 13, 1119–1127

55. Oslowski, C. M., and Urano, F. (2011) Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods Enzymol. 490, 71–92

56. Kondratyev, M., Avezov, E., Shenkman, M., Groisman, B., and Lederkremer, G. Z. (2007) PERK-dependent compartmentalization of ERAD and unfolded protein response machineries during ER stress. Exp. Cell Res. 313, 3395–3407

57. Kawabata, T., Tanimura, S., Asai, K., Kawasaki, R., Matsumaru, Y., and Kohno, M. (2012) Up-regulation of pro-apoptotic protein Bim and down-regulation of anti-apoptotic protein Mcl-1 cooperatively mediate enhanced tumor cell death induced by the combination of ERK kinase (MEK) inhibitor and microtubule inhibitor. J. Biol. Chem. 287, 10289–10300

58. Ni, M., and Lee, A. S. (2007) ER chaperones in mammalian development and human diseases. FEBS Lett. 581, 3641–3651

59. Johnson, D. C., and Dey, S. K. (1980) Role of histamine in implantation: dexamethasone inhibits estradiol-induced implantation in the rat. Biol. Reprod. 22, 1136–1141

60. Bigsby, R. M., and Young, P. C. (1993) Progesterone and dexamethasone inhibition of uterine epithelial cell proliferation: studies with antiprogestosterone compounds in the neonatal mouse. J. Steroid Biochem. Mol. Biol. 46, 253–257

61. Daikoku, T., Song, H., Guo, Y., Riesewijk, A., Mosselman, S., Das, S. K., and Dey, S. K. (2004) Uterine Msx-1 and Wnt4 signaling becomes aberrant in mice with the loss of leukemia inhibitory factor or Hoxa-10: evidence for a novel cytokine-homeobox-Wnt signaling in implantation. Mol. Endocri.

62. Schwartz, A. L., and Ciechanover, A. (1999) The ubiquitin-proteasome pathway and pathogenesis of human diseases. Annu. Rev. Med. 50, 57–74

63. McNaught, K. S., Olanow, C. W., Halliwell, B., Isacson, O., and Jenner, P. (2001) Failure of the ubiquitin-proteasome system in Parkinson’s disease. Nat. Rev. Neurosci. 2, 589–594

64. Adams, J. (2004) The development of proteasome inhibitors as anticancer drugs. Cancer Cell 5, 417–421

65. Dawson, T. M., and Dawson, V. L. (2003) Molecular pathways of neurodegeneration in Parkinson’s disease. Science 302, 819–822

66. Yang, F., Jia, S. N., Yu, Y. Q., Ye, X., Liu, J., Qian, Y. Q., and Yang, W. J. (2012) Deubiquitinating enzyme BAP1 is involved in the formation and maintenance of the diapause embryos of Artemia. Cell Stress Chaperones 17, 577–587

67. Horn, M., Geisen, C., Cermak, L., Becker, B., Nakamura, S., Klein, C., Pagano, M., and Antebi, A. (2014) DRE-1/FBXO11-dependent degradation of BLMP-1/BLIMP-1 governs C. elegans developmental timing and maturation. Dev. Cell 28, 697–710

68. King, A. M., Toxopeus, J., and MacRae, T. H. (2014) Artemin, a diapause-specific chaperone, contributes to the stress tolerance of Artemia franciscana cysts and influences their release from females. J. Exp. Biol. 217, 1719–1724

69. Kitamura, M. (2011) Control of NF-κB and inflammation by the unfolded protein response. Int. Rev. Immunol. 30, 4–15

70. Lanigan, F., Gremel, G., Hughes, R., Brennan, D. J., Martin, F., Jrström, K., and Gallagher, W. M. (2010) Homeobox transcription factor muscle segment homeobox 2 (Msx2) correlates with good prognosis in breast cancer patients and induces apoptosis in vitro. Breast Cancer Res. 12, R59

71. Serhan, C. N. (2014) Pro-resolving lipid mediators are leads for resolution physiology. Nature 510, 92–101

72. Wang, J., Kumar, R. M., Biggs, V. J., Lee, H., Chen, Y., Kagey, M. H., Young, R. A., and Abate-Shen, C. (2011) The Msx1 homeoprotein recruits polycomb to the nuclear periphery during development. Dev. Cell 21, 575–588

73. Wang, J., and Abate-Shen, C. (2012) The MSX1 homeoprotein recruits G9a methyltransferase to repressed target genes in myoblast cells. PLoS One 7, e37647