Objectives: Mechanisms underpinning Gram-negative bacterial vaginosis-induced birth anomalies are obscure. Ethical issues limit such studies on peri-implantation-stage human embryos. Here we have used embryoid bodies (EBs) as an in vitro model to examine the effect of Gram-negative bacterial endotoxins/lipopolysaccharides (LPS) on the faithful induction of germ lineages during embryogenesis. The role of LPS-inducible cytokine and pluripotency-related DNA-binding protein HMGB1 was also studied in these EBs.

Methods: EBs derived from the human embryonic stem cell line HUES9 were exposed to 12.5 pg/ml of LPS for 48 h. The expression profile of the ectoderm, endoderm, mesoderm and trophectoderm lineage markers, such as 
\[
\beta^\text{III}-\text{tubulin}, \text{GATA4}, \text{BMP2}, \text{Brachury} \quad \text{and} \quad \beta^-h\text{CG},
\]
were studied, by RT-PCR and immunofluorescence. Inhibition of mesoderm induction was confirmed by RT-PCR analysis for hANP, cTnT, ABCG2, GATA2, BMP4 and HAND1. Osteoblast differentiation was induced in the EBs, and confirmed by von Kossa and Alizarin red staining. A comet assay was also carried out to assess the degree of apoptosis in these EBs.

Results and conclusions: We found that the LPS-treated EBs were selectively silenced for mesoderm markers and failed to differentiate into functional osteoblasts. HMGB1 expression was absent in the normal EBs and was found to be localized in the cytoplasm of the LPS-treated EBs. Overall, our data indicate that endotoxin-induced HMGB1 expression in the peri-implantation-stage embryos can bring about severe birth defects of, for example, the bone and heart. This study also indicates that HMGB1 could be involved in maintenance of pluripotency in the human embryonic stem cells by impeding their differentiation.

Human embryonic stem cells (hESCs) have been widely used to understand the molecular mechanisms underpinning human development. These pluripotent cells provide a reliable source for studying differentiation to all the germ layer lineages, namely ectoderm, endoderm, mesoderm and trophectoderm lineages [1,2]. HESCs have been successfully directed toward the formation of different tissues of various lineages [3]. These cells can also be used to produce preimplantation embryo- or blastocyst-like entities, known as embryoid bodies (EBs), which consist of a differentiated population of cells representing all the germ layers. These EBs, therefore, closely mimic a growing embryo, which consists of the placental precursors (trophectoderm) and the cells of the embryo proper (ectoderm, endoderm and mesoderm) [4]. It is known that ectoderm forms the skin and the nervous system, the mesoderm forms tissues such as the cardiomyocytes, bone and blood, and the endoderm forms, for example, the liver, lungs and intestine of the developing embryo [5].

Gram-negative bacterial infections of the maternal genital tract, known as bacterial vaginosis, can lead to the formation of poor-quality embryos, which fail to implant [6]. Subclinical or silent infections of Gram-negative bacteria such as Chlamydia trachomatis can also cause birth defects with poorly developed tissues and organs of the fetus [7]. Ethical issues limit studies on the molecular mechanisms underlying such pathogenesis in human embryos. Endotoxin lipopolysaccharides (LPS) is the main antigenic component of Gram-negative bacterial cell wall and is regularly shed in the surrounding environment. LPS is known to cause various perinatal complications [8]. In previous studies we have established the role of various proinflammatory and other LPS-inducible cytokines and growth factors, such as IL-1α, IL-1β, TNF-α and CSF1 during embryo implantation and in subsequent pregnancy loss [9–11]. However, the molecular events underlying poor fetal development and birth defects during silent infections are not known. We hypothesize that the presence of LPS in the...
The environment of the developing fetus may selectively inhibit the induction of one or more of the lineages during early pregnancy.

In this study we have used EBs as an in vitro model to examine the effect of LPS on the differentiation and faithful induction of the germ lineages during peri-implantation embryonic development. The expression of LPS-inducible and pluripotency-related gene high-mobility group box 1 (HMGB1) was studied to assess its possible involvement in the aberrant differentiation of the LPS-treated EBs [12,13]. HMGB1 is explicitly expressed by the cells of the inner cell mass and is absent in the trophectoderm cells of the blastocyst [14]. HMGB1 is also known as a DNA-binding protein that can regulate expression of genes [12]. Owing to its versatile roles both during development and in response to endotoxins, we hypothesized that HMGB1 may be a key player in mediating LPS-induced developmental defects.

In previous studies we have shown that LPS exposure can render the preimplantation embryo or 5-day-old blastocyst inefficient for implantation [15]. We therefore used early-stage 5-day-old EBs to closely mimic the peri-implantation stage of embryonic development (day 4–5). We found that LPS exposure for 48 h inhibited functional mesoderm formation in these EBs. LPS-induced HMGB1 expression in these EBs also indicates its possible role in silencing mesoderm induction. These findings indicate for the first time that the presence of endotoxins in the maternal environment can lead to predictable mesoderm tissue-specific birth defects, such as malformation of bones. This study also indicates that HMGB1 is related to pluripotency in hESCs and that its expression silences mesoderm-specific genes and differentiation.

Material & methods
Culture of hESCs & production of EBs
hESC line HUES-9 was obtained from Harvard University (MA, USA) and was used after institutional ethics committee approval. It was maintained on mouse embryonic feeder (MEF) cells. HUES-9 was maintained in embryonic stem cell medium (ES medium) consisting of 80% KnockOut Dulbecco's Modified Eagle's Medium (DMEM) and 20% KnockOut serum replacement (KSR), supplemented with 2 mM L-glutamine, 1% nonessential amino acid solution, 0.1 mM β-mercaptoethanol, 4 ng/ml human recombinant basic fibroblast growth factor (bFGF), and penicillin-streptomycin 50 U/ml (all from Invitrogen, CA, USA). For induction of EB formation, the hESCs were seeded on a low-adherent 60-mm plate (BD Biosciences, CA, USA) containing ES media without FGF2. Human ESCs from three confluent 35-mm dishes were collected after trypsinization and used for inducing EBs in each 160-mm low-adherent dish. The EBs formed were screened for their typical round morphology under a binocular microscope (Nikon). On day 2.5 they were collected and washed three times in the culture media and finally transferred to fresh media. This step was carried out to dispose of the dying MEFs and also to select the healthy-looking EBs.

Selection of time points & dose of LPS
The induction of all the germ lineages in the EBs and effect of LPS was tested at two different time points (day 4.5 and 9.5) and by using different doses of LPS. Early EBs at day 2.5 were washed in the culture media and were divided into groups of approximately 30 each. Each group was then exposed to a dose of 5, 10, 15 or 20 pg/ml LPS. A control group of EBs unexposed to LPS was also taken. Following 48 h of incubation the EBs were harvested on day 4.5 or 9.5. The RNA was isolated from each group and RT-PCR-based screening was carried out for the expression of the ectoderm, endoderm, mesoderm and trophectoderm lineage markers. All the doses (ranging from 5–20 pg LPS/ml) and both the time points studied exerted similar effects on the expression pattern of the lineage markers. The median-dose 12.5 pg LPS/ml and the earlier time point of complete lineage induction, for example, day 4.5 was chosen as a representative dose and time for this study. All subsequent studies and analyses were carried out using this dose and time point.

Exposure of EBs to LPS
EBs at day 2.5 were exposed to 12.5 pg/ml of endotoxin/LPS (Sigma) for 48 h supplemented in culture medium. The normal and the endotoxin-treated EBs were harvested on day 4.5. Postexposure, the control and endotoxin-treated EBs were divided into two groups. One group (n = 30) was lysed in TRIzol for RNA isolation and the other group (n = 90) was fixed in 4% paraformaldehyde for immunofluorescence. The expression profile of the ectoderm, endoderm, mesoderm and trophectoderm lineage markers, such as βIII-tubulin, GATA4,
BM-P2, Brachury and β-hCG, were studied by RT-PCR and immunofluorescence. The expression of the LPS-inducible and pluripotency-related DNA-binding protein HMGB1 was also studied in both the control and treated EBs.

RT-PCR
Total RNA from cells was isolated using TRI-ZOL-LS reagent (Invitrogen) as per the manufacturer's protocol. Complementary DNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen) as per the manufacturer's instructions. PCR was carried out using 1U Taq DNA polymerase (Sigma) and MgCl₂ to a final concentration of 1.5 mM in a total volume of 25 µl/reaction. GAPDH was used as the housekeeping control. PCR cycles consisted of an initial denaturation at 95°C for 5 min followed by 35 amplification cycles of denaturation at 94°C for 45 s, annealing for 45 s and extension at 72°C for 45 s and final extension at 72°C for 10 min. The RT-PCR primers, amplicon sizes and their annealing temperatures are given in Table 1.

Immunofluorescence & cell counting
HESCs were grown on coverslips coated with MEFs and then fixed with 4% paraformaldehyde (Sigma) followed by permeabilization in 0.2% Triton X100 (Sigma). The slides were then incubated with primary antibodies 1:500 dilution of SSEA4 (Chemicon, CA, USA), 5 µg/ml Nanog (Santa Cruz Biotechnology, CA, USA), 10 µg/ml Brachury (R&D Systems Inc., MN, USA), and 1.5 µg/ml HMGB1 (Sigma) overnight at 4°C. After washing three times with PBS, fluorescein isothiocyanate/Texas red-labeled secondary antibodies against the primary goat/rabbit/mouse were added as 1:500 dilutions and incubated for 2 h. DAPI (Sigma) was used for nuclear staining and then washed with PBS. The negative controls were done without primary antibodies. Slides were mounted with DABCC (Sigma) and images were acquired using Nikon Eclipse 90i microscope (Nikon Corporation, Japan) and Image-Pro Express software (Media Cybernetics, Inc., MD, USA). Similarly, the LPS-treated (n = 30) and control (n = 30) EBs were divided and checked from the immunolocalization of Brachury and HMGB1. Approximately ten EBs were checked for each antibody. The expression and localization of these proteins in the EBs were also checked on day 9.5 to see whether their expression was delayed or altered as a result of LPS treatment. The results were then compared with the control cells (non-LPS-treated EBs). To count the number of cells per EB, the number of DAPI-stained nuclei were counted in ten each of the control and LPS-treated EBs.

Osteoblast differentiation
To assess the differentiating potential of EBs toward tissues of mesoderm origin, EBs were produced and exposed to LPS until day 4.5, as described previously. The normal and LPS-treated EBs, 30 each, were then subjected to osteoblast differentiation from day 8 onward [16]. To stimulate differentiation into osteogenic cells, the EBs were plated on regular 35-mm tissue culture dishes, and ES medium containing 10-8 M dexamethasone, 50 µg/ml L-ascorbic acid and 5 mM sodium-β-glycophosphate was used. The medium was changed every 2-3 days and the differentiation was continued up to 15 days. The osteoblast differentiation was characterized by identifying mineralized areas using von Kossa and Alizarin red staining [17]. These were visualized and acquired using a Nikon Eclipse 90i microscope.

Comet assay
Detection of DNA damage in individual EBs was carried out with a slight modification of the method described by [18]. Comet tail length was calculated by measuring the streak of DNA comet tail between the edge of the EBs and the end of tail using Nikon Eclipse 90i microscope and Image-Pro Express software.

Results
Effect of time points & doses of LPS
Induction of all the germ lineages were studied in control or normal EBs on days 4.5 and 9.5. Complete lineage induction for the ectoderm, endoderm, mesoderm and trophectoderm was found as early as day 4.5 by positive expressions of the markers (βIII-tubulin, GATA4, BMP2, Brachury and β-hCG) detected by RT-PCR. The day-9.5 EBs were also positive for the expression of these germ lineage markers. All the doses of LPS tested (5-20 pg/ml) induced specific silencing of BMP2 and Brachury, the mesoderm markers, when tested in EBs at days 4.5 and 9.5. Since the induction of all the lineages could be seen on day 4.5 onward, we considered this as equivalent to a developing peri-implantation blastocyst, and used it for further studies. Similarly, since all
the tested doses specifically silenced mesoderm induction, we decided to use the median dose 12.5 pg/ml for further studies.

Effect of LPS on the expression of pluripotency, germ lineages markers & HMGB1 in the EBs

In this study we have used 5-day-old EBs as entities equivalent to peri-implantation-stage blastocysts. The effect of endotoxins/LPS on the development and induction of lineages in the EBs were examined. The hESC line HUES9 was grown and passaged every 5 days. Figure 1A shows normal phase contrast pictures of this cell line grown on supporting MEF cells. The pluripotency of these cells were checked by RT-PCR analysis for Oct4, SSEA4 and Nanog (Figure 2). We found positive mRNA expression for Oct4, Nanog, SSEA4 and HMGB1 in these normal hESCs at day 5. The localization and expression of SSEA4, Nanog and HMGB1 was confirmed by immunofluorescence (Figure 3A, B & C). SSEA4 was found to be surface localized, whereas Nanog and HMGB1 were localized in the nucleus of the normal HUES9 cells. The mRNA expression of ectoderm, endoderm, mesoderm and trophectoderm lineage markers, such as βIII-tubulin, GATA4, BMP2, Brachury and β-hCG, were found to be negative in the HUES9 cells, indicating their undifferentiated status.

The HUES9 cells were harvested on day 5 and used for induction of EBs. The control EBs were collected on day 4.5 of culture (Figure 1B). The LPS-treated EBs were also collected on day 4.5 and were compared with the normal EBs for morphological changes under the microscope (Figure 1C). The normal and LPS-treated EBs did not show any visible morphological differences in terms of

### Table 1. List of genes and RT-PCR primers used.

<table>
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<th>Sequence</th>
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<th>Product size (bp)</th>
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<td>572</td>
</tr>
<tr>
<td></td>
<td>CCCCCGTCCCCCATCCCTA</td>
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<tr>
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<td>GGGGCCATCCACGTCTTCTG</td>
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Role of HMGB1 in pluripotency and infection – RESEARCH ARTICLE

Effect of LPS on differentiation of EBs to osteoblasts
We found that the normal EBs could be successfully differentiated to osteoblast cells, which were characterized by mineral depositions confirmed by Alizarin Red and von Kossa staining at the end of 15 days of differentiation. The normal EBs could be successfully differentiated as evidenced by positive staining for Alizarin Red and von Kossa (Figure 3H & J). The LPS-treated EBs (8-day old) failed to differentiate into functional osteoblasts, as indicated by the absence of mineral depositions with no positive signals for Alizarin Red and von Kossa (Figure 3L & K).

Cell numbers & DNA fragmentation
For a count of the average number of cells per EB, the 4',6-diamidino-2-phenylindol (DAPI)-stained nuclei were counted in individual control and LPS-treated EBs under epifluorescence. The average number of cells/EB (as mean ± standard deviation) in the control were 142.33 ± 48.41 cells/EB, and in the LPS-treated group were 175 ± 75.47 cells/EB. These values did not differ significantly (p = 0.57), as analyzed by a Students t-test. However, the LPS-treated EBs showed significantly (p = 0.028) more DNA tailing or fragmentation (28.64 ± 14.36 µm average) compared with the control EBs, with an average tailing of 2.48 ± 1.0701 µm (Figure 4).

Discussion
Genital tract infection is a predominant cause of birth anomalies, both in cases of normal conception or after assisted reproductive techniques [19]. Several of these infections are caused by Gram-negative bacteria, such as C. trachomatis, which are asymptomatic and cause chronic upper tract infections [20]. In 70% of birth defect cases the underlying causes are unknown. Here we have studied the effect of Gram-negative bacterial vaginosis on aberrant fetal development using an embryonic stem cell model. During such injections the preimplantation embryos are exposed to bacterial endotoxins/LPS in the environment [8,11]. The effect of LPS on preimplantation embryonic development and subsequent failure of implantation has been widely studied in animal and rodent models [10,11]. Studies on the underpinning mechanisms leading to developmental abnormalities in human embryos are not possible owing to ethical limitations in the use of human embryos.

Figure 1. Phase contrast pictures of HUES-9 colonies and embryoid bodies.

Panel (A) shows morphology of undifferentiated HUES9 colonies growing on mouse embryonic feeder. Panels (B) and (C) show morphologies of normal and lipopolysaccharides-treated day 5 embryoid bodies, respectively. Pictures were acquired at 10× magnifications.
We started with characterization of the day 4.5- and 9.5-old EBs for the presence of all the germ layer lineages. The positive expression of βIII-tubulin, GATA4, BMP2, Brachury and β-hCG indicated the presence of all the germ layer lineages (the endo-, ecto-, meso- and trophectoderm) in these EBs on both days. This also established the fact that the day-4.5-old EBs were equivalent to developing peri-implantation-stage embryos or blastocysts in terms of their constituent cells representing all the germ lineages. These similarities between developing embryos and EBs derived from hESCs have been established by previous workers [4]. In the present study, LPS was supplemented in the culture media at a concentration of 12.5 pg/ml. This dose is more than twice that of a report, which showed that as low as 2–5 pg/ml LPS was sufficient to cause alterations in the proliferation of hematopoetic precursor cells in culture [21].

The effect of LPS on the induction of the germ lineages in the EBs was studied by RT-PCR and immunofluorescence analyses of the lineage-specific markers. We found a specific silencing of all the eight mesoderm markers, namely Brachury, BMP2, hANP, cTnT, ABCG2, GATA2, BM4 and HAND1. We found that the mesoderm markers were silenced in the day 4.5 and 9.5 EBs after the initial 48-h LPS exposure. This indicates that the expression of the mesoderm markers in the EBs were really silenced for a long time and not merely delayed by the exposure to LPS. The failure to induce osteoblast differentiation after 15 days of osteo-induction in these EBs further strengthens the suggestion that the effect was long term. The mesoderm lineage is known as a precursor for tissues such as the osteogenic cells and hematopoietic precursor cells [22]. Birth defects in bone and muscles are very common and their underlying causes largely remain unknown. This study provides for the first time an in vitro human model for such studies and indicates a role of LPS in such abnormalities.

In this study we found that HMGB1 was not expressed in the normal EBs and its expression was induced in the EBs treated with LPS. However, it is also possible that the expression of HMGB1 is downregulated to undetectable levels in the normal/control EBs. This reduced sensitivity could be due to the RT-PCR conditions and the low number (n=30) of EBs that we screened per reaction. HMGB1 is also shown to be silenced in the trophectoderm cells of human blastocysts [14]. HMGB1 is a known LPS-inducible cytokine [12] and its cytoplasmic expression in the LPS-treated EBs indicates its possible role as a nonclassical proinflammatory cytokine in causing the mesodermal defects. Anti-HMGB1 antibodies can be used to treat lethal endotoxemia and sepsis [12]. Whether this intervention could be effective for protecting the developing fetus from the adverse effects of endotoxins is not known. At the same time, the observed nuclear localization and expression of HMGB1 in the pluripotent hESCs and its loss of expression in a differentiated population of cells in the EBs indicate its probable involvement in maintenance of stemness in the hESCs. This observation is in support of a previous study, which showed that HMGB1 is specifically expressed in the inner cell mass of the blastocyst [14]. Our data also indicate that nuclear or DNA-binding forms of HMGB1 may be instrumental in silencing
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Differentiation to the various lineages and thus maintaining pluripotency in the hESC lines. Further studies on establishing HMGB1 as a pluripotency marker are currently underway in our laboratory.

We found that the average number of cells per EB in both the LPS-treated and control EBs was not significantly different. This indicates that the dose of LPS used in this study did not interrupt the cell divisions or the process of formation of the EBs. Therefore, the specific silencing of mesodermal genes possibly indicates a reprogramming of genes involved in the differentiation and induction of germ lineages during development. The comet assay showed more DNA tailing or fragmentation in the LPS-treated EBs compared with the controls. This indicates that many of the cells in the EBs were already undergoing apoptosis as an effect of LPS. We also noticed that during the induction of osteogenic differentiation in the control and treated EBs, no differences were found in their efficiency for attachment and proliferation. However, the LPS-treated EBs failed to undergo osteoblast differentiation, as confirmed by the absence of mineral deposition staining, such as von Kossa and Alizarin Red. However, it is not clear whether the LPS-induced apoptosis in the EBs was exclusively selective toward the populations of cells that were of mesoderm origin. The molecular mechanism for the selective mesoderm silencing and the possible role of HMGB1 needs to be deciphered.
Conclusion
Our study demonstrates for the first time a correlation between Gram-negative bacterial LPS and birth defects related to formation of tissues of mesoderm origin, such as the bones, blood and/or heart muscles. We have also shown that early EBs could be effectively employed as a model system to study fetal abnormalities caused by maternal infections or due to new drugs. Expression and cytoplasmic localization of the DNA-binding cytokine HMGB1 in the EBs after LPS exposure indicates its probable involvement in the formation of developmentally compromised embryos during such infections. At the same time, our finding strongly indicates that nuclear localization of HMGB1 maintains pluripotency in hESCs by inhibiting the faithful induction of all the germ layer lineages.

Financial & competing interests disclosure
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Asymptomatic and silent Gram-negative genital tract infections can lead to birth defects, specifically of the bone or other tissues of mesoderm origin.

- Lipopolysaccharides (LPS), the most common antigen from Gram-negative bacterial cell wall, can cause such birth defects, even at very low concentrations.

- Embryoid bodies (EBs) derived from human embryonic stem cells (hESCs) closely mimic a developing embryo and can be successfully employed to study developmental toxicity of LPS or any other molecule/drug on differentiation or induction of the lineages.

- HMGB1, which is expressed by the inner cell mass of a human blastocyst, is also expressed by the pluripotent hESCs, is nuclear localized and is associated with maintenance of pluripotency.

- Induction of EBs from hESCs, which consist of a mixed population of differentiated cells of all the lineages, do not express, or have significantly down-regulated expression of HMGB1, indicating that HMGB1 can be involved in maintenance of pluripotency.

- LPS induces HMGB1 expression in the cells of the EBs, and its protein is localized in the cytoplasm of these cells.

- LPS-induced HMGB1 expression is involved in silencing of mesoderm induction.

- This study indicates that several of the unexplained birth defects during normal pregnancy and after in vitro fertilization can be caused by silent genital tract infections of Gram-negative bacteria.

Bibliography