**Abstract**

*Purpose:* *Helicobacter pylori* is a major cause of gastric carcinoma. To investigate a possible link between bacterial infection and genetic instability of the host genome, we examined the effect of *H. pylori* infection on known cellular repair pathways *in vitro* and *in vivo*. Moreover, various types of genetic instabilities in the nuclear and mitochondrial DNA (mtDNA) were examined.

*Experimental Design:* We observed the effects of *H. pylori* infection on a gastric cell line (AGS), on C57Bl/6 mice, and on individuals with chronic gastritis. In AGS cells, the effect of *H. pylori* infection on base excision repair and mismatch repair (MMR) was analyzed by reverse transcription-PCR, Western blot, and activity assays. In mice, MMR expression was analyzed by reverse transcription-PCR and the CA repeat instabilities were examined by Mutation Detection Enhancement gel electrophoresis. Mutation spectra in AGS cells and chronic gastritis tissue were determined by PCR, single-stranded conformation polymorphism, and sequencing. *H. pylori vacA* and *cagA* genotyping was determined by multiplex PCR and reverse hybridization.

*Results:* Following *H. pylori* infection, the activity and expression of base excision repair and MMR are down-regulated both *in vitro* and *in vivo*. Moreover, *H. pylori* induces genomic instability in nuclear CA repeats in mice and in mtDNA of AGS cells and chronic gastritis tissue, and this effect in mtDNA is associated with bacterial virulence.

*Conclusions:* Our results suggest that *H. pylori* impairs central DNA repair mechanisms, inducing a transient mutator phenotype, rendering gastric epithelial cells vulnerable to the accumulation of genetic instability and thus contributing to gastric carcinogenesis in infected individuals.

The Gram-negative bacterium *Helicobacter pylori* colonizes the gastric mucosa of half of the world’s population (1). *H. pylori* induces chronic gastric inflammation, which can progress through chronic atrophic gastritis, intestinal metaplasia, and dysplasia toward gastric carcinoma. Only a small number of *H. pylori*-infected individuals develop tumors (1, 2) and complex interactions between bacterial and host factors may contribute to the development of gastric carcinoma (1, 3).

*H. pylori* virulence factors include the *cag* pathogenicity island (*cag PAI*), encoding a type IV secretion system, which translocates CagA into the host epithelial cells following bacterial attachment (1). The bacterial CagA effector protein interferes with host cell functions such as growth, adhesion, motility, and invasion (4–6). VacA cytotoxin is another virulence factor that is inserted in the host cell membrane inducing, among other cellular effects, cytoplasmic vacuolation (1). However, the activity of the cytotoxin differs considerably among strains because of sequence variations in the *vacA* gene (1). Combination of the signal sequence s1 with midregion m1 is considered the genotype most associated with disease (1). *H. pylori* strains containing a functional type IV secretion system, CagA-positive and VacA-cytotoxic, are frequently associated with increased risk for gastric carcinoma development, as well as for its precursor lesions (1, 7).

Experimental data suggest that *H. pylori* infection induces DNA damage and mutation and/or inhibits DNA repair (8–11). *H. pylori* also induces hypoxia-inducible factor-1α (12) that in turn inhibits mismatch repair (MMR) activity by decreasing both *hMSH2* and *hMSH6* expression (13).

Human MMR is initiated via the recognition of a mismatch by one of two protein complexes, *hMSH2/hMSH6* or...
**Translational Relevance**

Gastric cancer is the second most common cause of cancer-related death. Patients infected with *Helicobacter pylori* are at increased risk of gastric cancer. Our group has shown previously that gastric cancer seems to depend on the combined effects of bacterial pathogenicity and host susceptibility. It is currently suggested that *H. pylori* infection induces DNA damage and/or inhibits DNA repair. We believe that our article will be of interest to *Clinical Cancer Research* because it adds novelty to the field of *H. pylori* pathogenesis by showing that *H. pylori* infection, both *in vivo* and *in vitro*, induces a decrease in repair activity and a transient mutator phenotype, contributing to epithelial gastric genomic instability and to its neoplastic transformation.

hMSH2/hMSH3 (14), which subsequently recruit the hMLH1/ hPMS2 complex (14). Defects in human DNA MMR result in genetic instability and predisposition to several types of epithelial cancer (15). Microsatellite instability (MSI) is a hallmark of MMR-deficient cells and is frequently encountered in colorectal and gastric adenocarcinomas (14, 16). Besides the role of nuclear DNA mutations, mutations in the mitochondrial DNA (mtDNA) are common in a broad range of cancers, including gastric carcinoma (17, 18). Mutations in the mtDNA may have severe effects on the mitochondrial oxidative phosphorylation pathway (17). Repair of mtDNA has not been fully characterized but is known to involve multiple pathways (19). The base excision repair (BER) is initiated by DNA glycosylases, such as hOGG1, which recognize and remove the damaged base leaving an abasic (AP) site. hAPE1 follows and cleaves the AP site, giving way to the other enzymes that will insert and ligate new nucleotides to the DNA chain (20).

To clarify the relation between *H. pylori* infection and genomic instability, we analyzed the status of major DNA repair activities in infected human cells and in mice. We show that nuclear MMR activity is down-regulated on *H. pylori* infection. In addition, we show that *H. pylori* promotes genomic instability both *in vitro* and *in vivo*, and this mutator phenotype in mtDNA correlates with *H. pylori* virulence. The clinical relevance of these results is further supported by analogous results obtained from *H. pylori*-infected patients. We conclude that *H. pylori* infection induces genomic instability that may contribute to gastric carcinogenesis.

**Materials and Methods**

**Cell culture, *H. pylori* strains, and growth conditions**

Human AGS gastric adenocarcinoma cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 200 μg/mL streptomycin, and 200 IU/mL penicillin (Invitrogen) at 37°C under 5% CO2 humidified atmosphere. Infections were done with *H. pylori* strains 26695 (ATCC 700392, cag PAI+, vacA s1/m1) and 60190 (ATCC 49503, cag PAI+, vacA s1/m1), and *H. pylori* insertion mutants with inactivation of the cagA (60190cagA-), cagE (60190cagE-), or vacA (60190vacA-) genes. The SS1 strain, able to chronically colonize the mouse stomach, was used for *in vivo* experiments. Bacteria were grown in trypticase soy agar supplemented with 5% sheep blood (BioMérieux) and incubated for 48 h at 37°C under microaerophilic atmosphere.

**Infection of gastric cells**

For the 24 h infection, 80% confluent AGS cells were washed three times in PBS and incubated in antibiotic-free medium. Growth colonies (48 h) of *H. pylori* were collected and the bacterial cells were added to the monolayer at a multiplicity of infection (MOI) of 100 bacteria per cell. Cultures were maintained at 37°C under a 5% CO2 humidified atmosphere.

For the 5-day infection, 30% confluent AGS cells were treated similarly as for the 24 h infection. Every 24 h, cells were washed three times with PBS and fresh medium and bacteria were added at a MOI of 10. In parallel experiments, 6 × 106 mol/L H2O2 was added to AGS cells every 24 h. Control cells were processed similarly in the absence of bacteria.

**Infection of mice**

Six-week-old specific pathogen-free C57BL/6 male mice (Charles River Laboratories) were infected oro-gastrically once with 106 colony-forming units of *H. pylori* strain SS1 (n = 15). Control groups of uninfected mice (n = 15) were given peptone trypsin broth alone. After 3, 6, and 12 months, 5 uninfected mice and 5 *H. pylori* SS1-infected mice were sacrificed. The serologic *H. pylori* status was controlled for each mouse and stomach was isolated as already described (11).

**Patients with chronic gastritis**

In total, 99 *H. pylori*-infected individuals with chronic gastritis (94 males and 5 females; age, 43.6 ± 6.2 years; range, 30-62 years) were studied. Individuals were recruited among shipyard workers who had undergone standard gastroscopy as part of a screening program for premalignant lesions of the gastric mucosa. Individuals with evidence of past or present peptic ulcer disease were excluded from this study. All procedures were in accordance with the institutional ethical standards. All samples were delinked and unidentified from their donors. All individuals provided written informed consent.

**Histology**

Biopsy specimens from antral and corpus mucosa and surgical specimens were formalin-fixed, embedded in paraffin, and stained with H&E, Alcian blue-periodic acid Schiff, and modified Giemsa. Only cases with adequately sized biopsy specimens of both antral and corpus mucosa were accepted for histologic assessment according to the updated Sydney system (21). Histologic slides were examined by an experienced pathologist (F.C.) blinded for the clinical information of the patients.

**Analysis of gene expression**

RNA was extracted using either the TriPure Isolation Reagent (Roche Diagnostics) or the TRIzol extraction (Invitrogen) and purified on RNeasy mini-columns (Qiagen). cDNA was synthesized using random hexamers and the Advantage RT-for-PCR Kit (Clontech). Gene expression was analyzed by reverse transcription-PCR using primers shown in Supplementary Table S1 as well as described elsewhere (22, 23). Amplified fragments were visualized by electrophoresis, quantified with the Quantity One Software (Bio-Rad), and normalized to GAPDH expression.

**Analysis of protein expression**

Protein levels were analyzed by Western blots. Proteins were detected with polyclonal antibodies against hMLH1 (Calbiochem), hMSH2 (Calbiochem), hMSH6 (Santa Cruz Biotechnology), or actin (Neomarkers). Proteins were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).
Proliferation and apoptosis assay

The proliferative indexes were determined as a function of 5-bromodeoxyuridine incorporation (24). The TUNEL assay (In situ Cell Death Detection Kit; Roche Diagnostics) was used to detect apoptotic cell death. The percentages of 5-bromodeoxyuridine-positive cells and apoptotic cells were determined using a Leica DM IRE2 fluorescence microscope.

Analysis of MMR activity

MMR activity in extracts of AGS cells uninfected or infected with H. pylori for 5 days was assayed using a 2-bp loop heteroduplex substrate. Repair efficiency was determined with the following: repair efficiency (%): (mixed plaques in extract-treated sample)/(mixed plaques in extract-untreated sample).

Analysis of MMR expression level in AGS cells infected with H. pylori

H. pylori (MOI of 100) was added to AGS cells at a MOI of 10. Left, gel from Western blot using anti-MLH1, anti-MSH2, and anti-MSH6 antibodies; actin was used as a loading control; right, quantification of protein expression levels. D, mRNA levels in AGS cells infected for 5 d with strain 60190 and its cagE, cagA, and vacA mutants at a MOI of 10 each. Mean ± SD. Representative of three independent experiments.

Analysis of genomic instability

Analysis of nuclear MSI in vitro. After 5 days of infection, DNA was extracted using the SDS/proteinase K method. Nuclear MSI status was determined using the HNPCC MSI Test kit (Roche Diagnostics) according to the manufacturer’s instructions. For each sample, 100 ng genomic DNA was used as a template to amplify the microsatellite loci DSS346 (APC), BAT25, BAT26, D17S250 (Mfd15CA), and D2S123 by PCR. The PCR products were analyzed using an ABI 377 sequence analyzer and GeneScan 3.0 (Perkin-Elmer) software.

Analysis of mtDNA instability in vitro. After 5 days of infection, DNA was extracted using the SDS/proteinase K method. Nuclear MSI status was determined using the HNPCC MSI Test kit (Roche Diagnostics) according to the manufacturer’s instructions. For each sample, 100 ng genomic DNA was used as a template to amplify the microsatellite loci DSS346 (APC), BAT25, BAT26, D17S250 (Mfd15CA), and D2S123 by PCR. The PCR products were analyzed using an ABI 377 sequence analyzer and GeneScan 3.0 (Perkin-Elmer) software.

Analysis of mutational instability in CA repeats loci

DNA was isolated from stomach of mice infected and noninfected and sacrificed after 6 and 12 months as described previously (11). Instabilities at the D6Mit59, D7Mit91, and D19Mit36 loci were determined using the ABI Prism BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer) and an ABI Prism 377 DNA Sequencer (Perkin-Elmer). To determine mismatches, the sequences were used as queries against a mitochondrial database.

Analysis of nuclear DNA instability in CA repeats loci in vivo. Genomic DNA was isolated from stomach of mice infected and noninfected and sacrificed after 6 and 12 months as described previously (11). Instabilities at the D6Mit59, D7Mit91, and D19Mit36 loci were evaluated using a PCR-based assay (26) using primer sequences determined from the Mouse Genome Informatics database. The PCR products were separated on a Mutation Detection Enhancement gel analysis in CA repeats loci, DNA was isolated from the stomach of 5 mice infected or noninfected and sacrificed at 6 and 12 months as described previously (11). For each analyzed condition, a pool of DNA containing DNA from each of the 5 mice in equal proportion was done. Each lane of the gels reported in the Fig. 2D corresponds to one pool of DNA.

Analysis of mtDNA instability in vivo. DNA was extracted from gastric antral biopsy specimens using the method described by Boom et al. (27). Briefly, biopsy specimens were homogenized in guanidinium isothiocyanate with a sterile micropestle. DNA was captured onto silica particles, washed, and then eluted in 100 μL of 10 mmol/L Tris-HCl (pH 8.3). DNA from blood samples was isolated using a standard proteinase K digestion and phenol/chloroform extraction procedure.
Six mitochondrial genes encoding ND1, ND3, ND4, ND5, COI, and ATPase6, as well as two regions of the D-loop, D310 [C6] and D514 [CA5], were analyzed by the PCR single-stranded conformation polymorphism method as described previously (18). All cases showing band shifts were submitted to a second analysis (new PCR amplifications and single-stranded conformation polymorphism analysis using the initial DNA samples), and only reproducible bands were considered. Only cases with mtDNA alterations in the biopsy specimen and not in blood DNA were considered to harbor somatic mutations.

Abnormal bands, as well as the corresponding normal bands, detected by single-stranded conformation polymorphism were recovered from Mutation Detection Enhancement gels and submitted to PCR reamplification with the original set of primers. Reamplification products were purified and sequenced as described previously. Sequencing was done in both strands using the original primers.

**H. pylori vacA and cagA genotyping**

*H. pylori* vacA and cagA genotyping was done by multiplex PCR and reverse hybridization on a line probe assay as described earlier (28). Strains were grouped as the highest (*vacA* s1m1 *cagA*+) or lowest (*vacA* s2m2 *cagA−*) virulence-associated genotype. Cases infected with multiple *H. pylori* strains (*n* = 20) or infected with genotypes that could not be assigned to the highest or low virulence group (*n* = 6) were not included in the analysis.

**Statistical analysis**

Associations between *H. pylori* infection and the presence and type of mtDNA mutations as well as the frequencies of mtDNA mutations were assessed by the χ² and Fisher’s exact test. MMR, hypoxia-inducible factor-1α, and BER levels, MMR activity, and proliferation and apoptosis assay results were expressed as mean ± SD of at least two independent experiments and analyzed by Student’s t test. The differences between data sets were considered significant at *P* values < 0.05.

**Results**

**Analysis of MMR gene expression in vitro.** Previous reports using cells infected at high MOI have suggested that *H. pylori* affect MMR gene expression and cause increased cell death as well as growth inhibition (8, 10). Expression of MMR proteins are affected by cell growth (29) and bacterial-induced growth inhibition could lead to decreased MMR expression. To minimize the effect of *H. pylori*-induced cell death, we infected human gastric carcinoma AGS cells with the virulent *H. pylori* strains 60190 and 26695 for 24 h at a MOI of 100 or for 5 days at a MOI of 10. After 24 h of infection, there was a small, if any, decrease in MMR mRNA levels compared with control cells, particularly for *hMSH2* and *hMSH6* genes (Fig. 1A). In cells infected for 5 days with strain 60190, the decrease in MMR levels was more evident (Fig. 1B); *hPMS1*, *hPMS2*, *hMSH2*, *hMSH3*, and *hMSH6* mRNA levels were reduced 1.5- to 3-fold. Western blot analysis confirmed that *hMSH2* and *hMSH6* levels were affected by *H. pylori* infection (Fig. 1C). To investigate the role of *H. pylori* virulence factors in MMR expression, cells were infected with *H. pylori* strains lacking a functional type IV secretion system (*cagE* mutant), *cagA*, or *vacA* genes. No significant differences were observed between the parental strain and the mutants, with the exception of *hMSH6* (Fig. 1D). In cells treated with H2O2, we observed a similar decrease in...
MMR levels (Fig. 1B), suggesting that *H. pylori* infection affects expression of MMR genes comparable with an oxidative stress agent. We examined if the decrease in MMR expression in infected cells was caused by *H. pylori*-induced regulation of hypoxia-inducible factor-1α transcription factor. Only minor changes in hypoxia-inducible factor-1α levels were observed (Supplementary Fig. S1) and there were no major effects of the type IV secretion system, *cag*A, and *vacA* virulence factors, indicating that hypoxia-inducible factor-1α expression is not significantly affected by *H. pylori* infection.

To functionally characterize the effect of *H. pylori* infection on MMR, we measured MMR activity (25). Whereas 46.6% of actively proliferating cells after infection by *H. pylori* strains SS1 for 3 months (Fig. 2B). In contrast, after a longer period of infection, there was no significant alteration in MMR levels (Fig. 2B) and mutagenicity (11), consistent with the absence of genetic instabilities in loci carrying (CA)n repeats (Fig. 2D, right). These data correlate with the induction of point mutations (11) and with the decreased level of MMR components during *H. pylori* infection for 3 months (Fig. 2B). To determine whether *H. pylori* induces mtDNA instability in vitro, we measured mutations in the D-loop region containing the mononucleotide D310 [C6] and dinucleotide D514 [CA5] repeats. The overall frequency of mutational events was higher in AGS cells infected with *H. pylori* strain 60190 (27.4%) than in noninfected control cells (11.2%; *P* = 0.020; Fig. 3A). Furthermore, we observed differences regarding the spectrum of mtDNA mutations (Supplementary Table S2). Cells infected with *H. pylori* 60190 showed a significantly higher number of transitions (17.8%) than uninfected control cells (5.6%; *P* = 0.036). The two transition events GC->AT and AT->GC were also observed in H2O2-treated AGS cells. Interestingly, insertions were only detected in *H. pylori* 60190-infected cells (4.1% Supplementary Table S2).

To further clarify the role of *H. pylori* virulence factors in the induction of mtDNA mutations, we infected AGS cells with *H. pylori* strains lacking *cag*E, *cag*A, or *vacA* and found that these bacterial mutants induced significantly lower overall mutation frequencies (10.7%, 11.2%, and 12.0%, respectively) than cells infected with the parental strain (27.4%; *P* = 0.012, 0.014, and
Table 1. Relationship between *H. pylori* genotypes and mtDNA mutations among individuals with chronic gastritis (P < 0.05, Fisher’s exact test)

<table>
<thead>
<tr>
<th><em>H. pylori</em> genotype*</th>
<th>Mutations in mtDNA</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Negative (%)</td>
<td>Positive (%)</td>
</tr>
<tr>
<td>vacA s1m1 / cagA+</td>
<td>29 (50.0)</td>
<td>12 (80.0)</td>
</tr>
<tr>
<td>vacA s2m2 / cagA-</td>
<td>29 (50.0)</td>
<td>3 (20.0)</td>
</tr>
</tbody>
</table>

*Of the 99 cases with chronic gastritis, 20 were infected with multiple strains, four were infected with vacA s1m2 genotype, and two were infected with vacA s1m1 cagA+ genotypes, and those were excluded from the analysis.

Discussion

Although a causal relationship exists between *H. pylori* infection and the development of gastric carcinoma (31), the molecular mechanisms underlying this observation have remained elusive. Because impairment of genomic integrity often leads to carcinogenesis, we focused on the response of the host DNA repair pathways to *H. pylori* infection.

*H. pylori* infection leads to down-regulation of MMR gene expression in AGS cells in a manner similar to that occurring in cells treated with H2O2. In mice chronically infected with...
H. pylori, we observed a similar decrease in MMR expression. After 12 months, however, the expression of MMR genes was comparable with uninfected cells, indicating that MMR mRNA levels recover after long-term exposure of mice to H. pylori. This could be explained by elimination of cells with high level of DNA lesions due to catastrophic cell death. In mice, infection causes active gastritis with metaplastic changes and an infiltration of inflammatory cells in the gastric mucosa (11) that could promote oxidative DNA damage. It is known that H. pylori infection leads to DNA damage of the epithelial cells due to production of ROS (1). DNA damage caused by H. pylori may likely result from both a direct DNA-damaging effect by oxygen radicals and an ineffective repair. Accordingly, in the absence of an efficient MMR system, DNA lesions can accumulate, acting as early events culminating in malignancy.

Defects in MMR not only generate point mutations but also lead to generalized genomic instability (32). In fact, in stomach, MSI is observed in ~20% of sporadic gastric carcinoma cases. We show the occurrence of MSI in DNA isolated from mouse stomach after 6 months of infection but not after 12 months. This is similar to that observed previously for point mutations (11) and in accordance with the decrease of MMR protein levels seen after shorter periods and not after prolonged infection. These findings may be explained by compensatory mechanisms possibly due to an increase in gastric epithelial cell proliferation or a change in the balance between apoptosis and cell proliferation after infection (11). Accordingly, induction of epithelial cell proliferation and apoptosis were observed by proliferating cell nuclear antigen and activated caspase-3 immunohistochemistry experiments on the gastric mucosa on H. pylori-infected mice for 12 months.8 Taken together, our data suggest that H. pylori generates a transient mutator state, which might be critical for cellular transformation (Fig. 5), for the survival of just one mutated cell may lead the path to malignancy.

Mutations in the mitochondrial genome have been detected in nearly every type of cancer investigated to date (17), including gastric preneoplastic lesions (33). Previous studies have found that transition mutations are the major mutational events in mtDNA of gastric carcinomas (18). In agreement with these results, we observed an increase in mutation frequencies in both the mitochondrial D-loop region and in several genes encoding subunits of the electron transport chain when we infected human cells with H. pylori 60190. These mutations were mostly transition mutations and preferentially affected complex I ND1 and complex IV COI genes. This is in agreement with the data obtained from chronic gastritis-infected patients. It was also evident in this population that the H. pylori genotype influences the number of mtDNA mutations, with the more virulent strains inducing a greater load of mutations.

Our data suggest that, by increasing the mutation frequency of specific coding genes in the mitochondrial genome, H. pylori may interfere with the activity of the electron transport chain and therefore impair oxidative phosphorylation. Oxidative phosphorylation impairment may increase ROS generation and lead to even greater mtDNA damage.

Transition mutations that are known to be caused by oxidative damage, dominated in H. pylori-infected cells. The BER system is known to repair oxidative DNA damage in mtDNA (19). We did not detect any significant change in hOGG1 expression, whereas hAPE1 was down-regulated in infected gastric cells. During BER, AP sites are generated by hOGG1 and then repaired by hAPE1. An imbalance between generation and repair of AP site has been shown to be mutagenic by generating excess AP sites that can be converted into mutation by DNA polymerases or other repair enzymes (34). Other studies have reported an increase in APE1 levels during H. pylori infection (35, 36) in experiments using high MOI and shorter infection time (35) or using H. pylori protein extracts instead of live bacteria (36). Supporting our data, Meira et al. (37) showed that H. pylori infection in mice deficient in BER alkyladenine DNA glycosylase (Aag) activity may lead to development of precancerous stages. These authors suggested a model where increased levels of cellular damage and death due to ROS led to increased inflammation that consequently led to production of more ROS and tumor-promoting cytokines (37).

The multistep model of gastric carcinoma development postulates that carcinogenesis is initiated by inflammation caused by H. pylori infection as well as by dietary exposure to salt and nitrate, which cause DNA damage (38). To investigate this further, we have identified three mechanisms by which H. pylori may provoke a loss of genomic integrity and promote transformation. These molecular mechanisms include (a) increase in ROS and decrease repair activity, (b) mutations in mtDNA, and (c) induction of a transient mutator phenotype, which may generate mutations in the nuclear genome on short-term infections with H. pylori. We suggest that H. pylori-induced carcinogenesis is the combinatorial result of at least these three linked mutagenic phenomena (Fig. 5).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Rachel Thomas, Richard Argent, and John Atherton (University of Nottingham) for the mutant H. pylori strains used in this study and Keshav K. Singh, Lennart Friis-Hansen, Maria José Oliveira, and Jorge Lima for critical reading of the article and helpful suggestions.
References

Helicobacter pylori Infection Induces Genetic Instability of Nuclear and Mitochondrial DNA in Gastric Cells
