Junctional Adhesion Molecule-A Is Critical for the Formation of Pseudocanaliculi and Modulates E-cadherin Expression in Hepatic Cells

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Hepatocytes are polarized epithelial cells whose function depends upon their ability to distinguish between the apical and basolateral surfaces that are located at intercellular tight junctions. It has been proposed that the signaling cascades originating at these junctions influence cellular activity by controlling gene expression in the cell nucleus. To assess the validity of this proposal with regard to hepatocytes, we depleted expression of the tight junction protein junctional adhesion molecule-A (JAM-A) in the HepG2 human hepatocellular carcinoma cell line. Reduction of JAM-A resulted in a striking change in cell morphology, with cells forming sheets 1–2 cells thick instead of the normal multilayered clusters. In the absence of JAM-A, other tight junction proteins were mislocalized, and pseudocanaliculi, which form the apical face of the hepatocyte, were consequently absent. There was a strong transcriptional induction of the adherens junction protein E-cadherin in cells with reduced levels of JAM-A. This increase in E-cadherin was partially responsible for the observed alterations in cell morphology and mislocalization of tight junction proteins. We therefore propose the existence of a novel mechanism of cross-talk between specific components of tight and adherens junctions that can be utilized to regulate adhesion between hepatic cells.

Hepatocytes, the primary cell type within the liver parenchyma, are polarized epithelial cells. The apical surface of hepatocytes is defined as the region of the cell that generates bile canaliculi, which act as a conduit for bile secretion and therefore mediate the exocrine activities of the liver. The membrane at the basal surface of hepatocytes faces the sinusoidal endothelium and is actively involved in exchange of nutrients, toxins, and metabolites to and from the blood supply. The segregation of exocrine and endocrine functions via distinct hepatocyte membrane domains requires maintenance of cell polarity, which in turn is partly dependent upon the formation of tight junctions. Tight junctions are areas of localized contact found at the apical region of adjacent epithelial cells (1) and are the foremost junctional complexes abutting the edges of bile canaliculi in hepatocytes. The traditional view of tight junctions is that they serve as a paracellular seal that regulates the flow of small molecules and ions and inhibits the flow of lipids and membrane-enriched proteins (1, 2). In addition to their structural role, recent reports have highlighted the importance of tight junctions in regulating signaling pathways from the apical cell surface to the nucleus, as well as from the interior of the cell to the surface (3–5). However, the contribution of junctional complexes to the signaling cascades regulating the function of hepatocytes remains unknown.

To test whether a loss of cell junctions could affect hepatic gene expression, we utilized lentivirally expressed short hairpin RNAs (shRNAs)4 to deplete components of cell junctions in hepatoma cells. HepG2 cells are polarized human hepatocellular carcinoma cells, which contain “pseudocanaliculi” (6–10) and express several cell adhesion proteins that correctly localize to the apical cell surface (11–13). We began our studies by examining a major component of tight junctions, junctional adhesion molecule-A (JAM-A). JAM-A (also called JAM, JAM-1, or F11 receptor) is a member of the immunoglobulin superfamily that is localized to tight junctions of both epithelial and endothelial cells (14–17). Numerous studies have shown that JAM-A is linked to signaling cascades, both directly and indirectly, through its interactions with other tight junction proteins (4, 5, 18), making JAM-A a good candidate for investigation. We found that depletion of JAM-A from HepG2 cells by RNAi caused a dramatic change in cell morphology, mislocalization of other junction proteins, and a significant reduction in pseudocanaliculi formation. Gene array analyses revealed that expression of multiple genes was altered, including that of E-cadherin that was increased by >5-fold upon loss of JAM-A. The increase in E-cadherin expression occurred as a consequence of an elevated transcriptional response elicited by the E-cadherin proximal promoter (19–21). The increase in E-cadherin expression was partially responsible for the changes in cell morphology and tight junction protein mislocalization associated with JAM-A depletion. We conclude that signaling...
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pathways exist in hepatic cells to facilitate a compensatory increase in cell adhesiveness when cell junctions are disrupted. This may reflect a mechanism through which cell junctions are established between nascent hepatocytes following liver damage.

EXPERIMENTAL PROCEDURES

Animals—The Animal Care Committee at the Medical College of Wisconsin approved all animal procedures used in this study. Embryonic stem cells (KST235) were obtained from BayGenomics and used to generate chimeras by injection into C57BL/6J blastocysts following standard procedures. Chimeras were bred into C57BL/6J mice to yield C57BL/6J blastocysts following standard procedures. Chimeras were used to generate chimeras by injection into study. Embryonic stem cells (KST235) were obtained from BayGenomics and used to generate chimeras by injection into study. Embryonic stem cells (KST235) were obtained from BayGenomics and used to generate chimeras by injection into

Plasmid Construction—To generate shRNAs, annealed oligonucleotides were ligated into pL3.7 (22) at Xhol and Hpal sites. The following oligonucleotides were utilized: JAM-Ai1, 5'-TGGCATTGGCGCATTTACATTCGAAGAGATGTAAACACTGCCAATGCTTTTGGAACAC-3' and 5'-TCGAGTTTCCACTGCCAATATGCAAGAGAC-3'; JAM-Ai2, 5'-TGTCGAGAGGAAAATTACATTTCAAGAGAGGAAACTGTTGTGTTCAAGAGACGGAGAAAATTACATTTCAAGAGAGGAAACTGTTGTGTTCAAGAGACGGAGAAAATTACATTTCAAGAGAGGAAACTGTTGTGTTCAAGAGACGGAGAAAATTACATTTCAAGAGAGGAAACTGTTGTGTTCAAGAGACGGAGAAAATTACATTTCAAGAGAGGAAACTGTTGTGTTCAAGAGACGGAGAAAATTACATTTCAAGAGAGGAAACTGTTGTGTTCAAGAGACGGAGAAAATTACATTTCAAGAGAGGAAACTGTTGTGTTCAAGAGACGGAGAAAATTACATTTCAAGAGAGGAAACTGTTGTGTTCAAGAGACGGAGAAAATTACATTTCAAGAGAGGAAACTGTTGTGTTCAAGAGACGGAGAAAATTACATTTCAAGAGAGGAAACTGTTGTGTTCAAGAGAC

Immunofluorescence—HepG2 cells were washed in HBSS (Invitrogen) and fixed in 4% paraformaldehyde, 0.1% Triton X-100 in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature, and blocked in 3% BSA in PBS for 1 h at room temperature. Cells were then incubated with primary antibodies (diluted 1:50 in PBS) for 1 h at room temperature, followed by incubation with secondary antibodies (diluted 1:500 in PBS) for 1 h at room temperature. Images were acquired using a Leica confocal microscope and analyzed using ImageJ software.

Immunoblotting—Cell lysates were prepared by scraping cells into lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 2× Complete protease inhibitor cocktail, 1× PhosSTOP phosphatase inhibitor cocktail, 1 mM dithiothreitol, 3 mM β-mercaptoethanol) and sonicating briefly. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% nonfat dry milk in TBST for 1 h at room temperature, probed with primary antibodies (diluted according to manufacturer's specifications) overnight at 4 °C, and washed extensively with TBST. membranes were incubated with secondary antibodies conjugated to HRP and detected with chemiluminescent substrate. Images were acquired using a ChemiDoc XRS+ system (Bio-Rad) and analyzed using ImageJ software.

Electron Microscopy—Cells were grown on tissue culture plastic or Thermax® plastic coverslips, fixed in 2.5% glutaraldehyde, and processed for electron microscopy as described in the Methods section. Images were acquired using a Zeiss 902 electron microscope and analyzed using ImageJ software.
glutaraldehyde in 0.1 M sodium cacodylate buffer or in buffer supplemented with either 1% lanthanum nitrate or 0.05% ruthenium red (kept in the dark) for 3 h (24–26), and fixed in 1% osmium tetroxide containing the same concentration of the tracers. The coverslips were then embedded in epoxy resin and sectioned both in the transverse and lateral planes and viewed in a JEOL 2100 TEM.

**RT-PCR**—Total RNA was extracted from cells using an RNeasy kit (Qiagen). RT-PCR was performed as described (27) using the primer pairs listed in supplemental Table 1. Fold changes were determined using a PhosphorImager (GE Healthcare) scanner and normalized to POLR2A expression.

**Luciferase Assays**—HepG2 cells were transfected with 0.5 μg of reporter construct that expressed *Photinus pyralis* (firefly) luciferase and 0.025 μg of *Renilla* luciferase plasmid (pRL-EF) using FuGENE (Roche Applied Science) according to the manufacturer’s instructions. Forty eight hours after transfection, cells were lysed and analyzed using the dual luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Assays were performed three independent times, with each transfection duplicated and firefly luciferase values normalized to expression of *Renilla* luciferase.

**Oligonucleotide Array Analyses**—RNA (15 μg) from four independent vector-transduced samples and five independent JAM-Ai1 samples was used to generate biotinylated cRNA that was subsequently hybridized to human oligonucleotide arrays (U133A 2.0, Affymetrix). Fold changes were calculated using a 2.5-fold cutoff and a p value ≤0.05 using dChip software (28). A call of “present” was required for genes in all JAM-Ai1 samples for up-regulated genes, and a call of present was required for genes in all vector samples for down-regulated genes, as determined by the Gene Chip Operating System software (Affymetrix). Gene expression data have been deposited into NCBI Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) and are accessible through GEO series accession number GSE6080. Genes with significant fold changes of 1.5 or greater were analyzed using Ingenuity Pathway Analysis software (Ingenuity® Systems).

**RESULTS**

**HepG2 Cell Morphology Is Altered by Depletion of JAM-A**—JAM-A is a component of tight junctions that is expressed in the mouse liver (29–33). Previous work has demonstrated that administration of an anti-JAM-A monoclonal antibody or depletion of JAM-A by small interfering RNA (siRNA) treatment in a colonic epithelial cell line leads to a reduction in transepithelial resistance, suggesting that JAM-A is critical for maintaining the permeability and polarity of these cells (34, 35). Therefore, we hypothesized that JAM-A might also be critical to maintain the integrity of hepatocyte junctions. Two different lentiviral shRNAs were generated to trigger siRNA-mediated depletion of JAM-A (JAM-Ai1 and JAM-Ai2; Fig. 1A) in HepG2 cells. These lentiviruses also contained a gene encoding puromycin resistance allowing for selection of stably transduced cells. Polyclonal stable cell lines were created by infecting HepG2 cells with JAM-Ai1 or JAM-Ai2 viruses, or control virus generated from the lentiviral vector without a hairpin (Vector). Immunoblot analyses using an antibody against JAM-A revealed that cells transduced with either JAM-Ai1 or JAM-Ai2 shRNAs exhibited a 90% reduction in the level of JAM-A protein compared with control cells (Fig. 1B).

HepG2 cells characteristically grow as epithelial cell clusters or islands (7, 36). The morphology of HepG2 cells infected with control virus was indistinguishable from uninfected cells (Fig. 1D and data not shown). In contrast to control cells, HepG2 cells transduced with either JAM-Ai1 or JAM-Ai2 had a very different appearance (Fig. 1, E and F), with the cells displaying a flattened morphology and forming a 1–2-cell thick sheet across the entire culture dish. Although the altered cell morphology gave an appearance that the number of JAM-Ai cells per field had increased (Fig. 1, D–F), the rate of JAM-Ai1 and JAM-Ai2 cell proliferation was significantly less than that of control cells as determined by cell counting (supplemental Fig. 1) and phosphohistone H3 immunocytochemistry to detect cells in mitosis (data not shown). To demonstrate the specificity of the shRNA...
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treatment, we designed a virus that expressed GFP as well as a mutated JAM-A cDNA called JAM-Amut (Fig. 1A). The third nucleotide in five of six codons targeted by the JAM-A2 shRNA was mutated in the JAM-Amut cDNA such that the altered nucleotide sequence retained the capacity to encode a wild type JAM-A protein. JAM-A2 HepG2 cells were infected with the JAM-Amut-expressing virus, and infected cells were sorted for GFP expression and maintained under puromycin selection. Fig. 1C shows that expression of the JAM-Amut cDNA restored JAM-A expression in JAM-A1-transduced cells. Moreover, the expression of the JAM-Amut cDNA in JAM-A1-transduced cells re-established a normal HepG2 cell morphology (Fig. 1G), demonstrating that the phenotype associated with the JAM-Ai cells is a direct consequence of a reduction in JAM-A rather than nonspecific effects of the shRNA.

Depletion of JAM-A Leads to Misllocalization of Several Tight Junction Proteins—The change in morphology induced in HepG2 cells upon depletion of JAM-A raised the possibility that a disruption in tight junction formation had occurred. To address this possibility, we examined whether other tight junction proteins could localize to the apical boundary of JAM-A-depleted HepG2 cells. The pseudocanalculus of HepG2 cells can be visualized as a small circle formed between apposing membranes (6–10), and previous studies have demonstrated that ZO-1 (11–13) and occludin (11, 12) localize to this region. Immunocytochemistry detected occludin, claudin-1, and ZO-1 in such circular areas of control cells (Fig. 2, A, B, C, and E; arrows), consistent with localization of tight junction proteins to this domain of HepG2 cells. However, in JAM-Ai cells, these tight junction proteins failed to localize at the apical surface and instead were distributed as polygons giving a chicken wire appearance surrounding the plasma membrane (Fig. 2, D and F; arrowheads). Staining with multiple antibodies revealed that the tight junction proteins examined co-localized in both control and mutant cells (data not shown). Quantification of the localization data found a significant reduction in the pseudocanalicular localization of claudin-1, occludin, and ZO-1 in JAM-Ai1 cells compared with controls (supplemental Fig. 2). In addition, quantification of the polygonal localization of junction proteins found this arrangement significantly increased in JAM-Ai1 cells compared with controls (supplemental Fig. 2). The localization of the tight junction proteins in the JAM-Ai cells was mainly above the plane of the nuclei (x-z confocal images Fig. 2, B, D and F), similar to the apical positioning of junctions in columnar epithelial cells such as MDCK cells (37). This suggests that loss of JAM-A reorients junction proteins from the typical hepatic orientation around the pseudocanalculus to a more columnar epithelium-like pattern. This abnormal localization was not a reflection of increased abundance of these proteins, because immunoblot analyses demonstrated that the total levels of ZO-1, occludin, and claudin-1 were unchanged in JAM-Ai cells compared with control cells (Fig. 2G).

JAM-A Is Required for the Formation of Pseudocanalliculus in HepG2 Cells—The observation that tight junction proteins failed to localize to the normal apical domain of hepatoma cells depleted of JAM-A implied that cell polarity was disrupted and that the development of pseudocanalliculus was adversely affected. To determine whether this implication was correct, we first assessed whether loss of JAM-A influenced the polarity of HepG2 cells as a consequence of tight junction protein mislocalization. We evaluated whether the localization of a known marker of hepatic polarity, DPPIV (7), was changed upon loss of JAM-A. Consistent with the mislocalization of tight junction proteins, knockdown of JAM-A leads to a 60% reduction in DPPIV localization (supplemental Fig. 3). Next, we directly examined the presence of cell junctions and pseudocanalliculi.
by performing electron microscopy on sections of control and JAM-Ai cells. To ensure that the presence of junctions was not inadvertently overlooked because of the plane of section, electron microscopy was performed on both horizontal and vertical sections through cell layers grown on plastic coverslips. Control cells displayed a classic hepatic arrangement of pseudocanalicular containing numerous microvilli surrounded by triplicate junctions consisting of tight junctions, adherens junctions, and desmosomes (Fig. 3A) (1, 38–40). In contrast to control cells, loss of JAM-A resulted in the apparent absence of normal pseudocanaliculari; instead, JAM-Ai cells were found to contain small gaps between apposed membranes, which occasionally contained a few poorly developed microvilli (Fig. 3, B and C). Although electron dense regions between JAM-Ai cells could occasionally be identified, they appeared to have a rudimentary structure reminiscent of desmosomes or immature adherens junctions (Fig. 3, B and C, braces). This was in contrast to the clearly defined junction structures flanking the pseudocanalicular of control cells (Fig. 3A, brace). We also examined the integrity of cell-cell contacts in control and mutant cells by examining lanthanum nitrate and ruthenium red distribution (24–26). As expected, staining was restricted to the basolateral surface and excluded from the apical domain of control cells (27/40 cell-cell contacts examined; Fig. 3D), presumably because of the presence of intact tight junctions. In contrast, tracer dye labeling in JAM-A-depleted cells was widely distributed throughout the plasma membrane (36/38 JAM-Ai1 and 27/29 JAM-Ai2 cell-cell contacts examined; Fig. 3, E and F, and supplemental Fig. 4), suggesting a lack of functional tight junction barriers. Based on these cumulative data, we conclude that JAM-A is essential for the integrity of tight junctions and the formation of pseudocanalici in HepG2 cells.

Depletion of JAM-A Affects the Expression of a Subset of Genes in HepG2 Cells That Includes E-cadherin—The disruption to tight junction formation in JAM-Ai HepG2 cells allowed us to examine whether the loss of tight junctions affected hepatic gene expression. As mentioned above, several studies have implicated tight junctions in the regulation of signaling cascades from the apical surface of cells to their nuclei (4, 5, 18). Therefore, we performed oligonucleotide array analyses using Affymetrix U133A arrays, with cRNA from four independent control cell populations and five independent JAM-Ai1 cell populations. Using a 2.5-fold cutoff and a p value ≤0.05, comparison of JAM-Ai1 cells with control cells revealed four genes whose mRNA levels were significantly down-regulated and 26 genes significantly up-regulated in JAM-Ai1 cells (Table 1). The predicted changes in mRNA levels were confirmed by RT-PCR analyses (Table 1 and data not shown). Somewhat surprisingly, the level of JAM-A mRNA was only modestly reduced (4.7-fold by array and 1.2-fold by RT-PCR), suggesting that the observed reduction in JAM-A protein (∼10-fold; Fig. 1B) is predominantly a consequence of post-transcriptional events as has been described previously (41).

Although the expression of a diverse array of protein types was altered in JAM-Ai HepG2 cells, the mRNA found to have the most substantial increase (13-fold) was CDH1, which encodes E-cadherin. We confirmed the up-regulation of CDH1 at the mRNA level by conducting RT-PCR analyses. In these particular experiments, JAM-Ai1 and JAM-Ai2 cells had a 6.8- and 5.3-fold increase in CDH1 compared with control cells, respectively, whereas the level of CDH1 in JAM-Amut cells was similar to that found in control cells (Fig. 4, A and B). The increase in CDH1 mRNA levels was considered provocative because E-cadherin plays an integral role in initiating junction formation in other epithelial cell types (42–44).

To determine whether increased expression of E-cadherin in JAM-Ai cells was because of changes in transcription, we performed transient transfection assays using fragments of the E-cadherin proximal promoter that controlled expression of a firefly luciferase reporter gene (20, 21). All constructs tested had significantly higher activity in the JAM-Ai cells than in control cells (Fig. 4C), confirming that the up-regulation of E-cadherin was a transcriptional response to depletion of JAM-A. We also noted that the fold difference between cell lines using the smallest promoter fragment (−108 to +125) was similar to the fold change using larger fragments (−1359 to
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TABLE 1

Changes in mRNA levels measured by oligonucleotide array analyses

| Gene Name                  | Gene symbol | Gene ID | -Fold change dChip | -Fold change RT-PCR |
|----------------------------|-------------|---------|--------------------|---------------------|
| Decreased                  |             |         |                    |                     |
| Junctional adhesion molecule-A | JAM-A       | 50848   | -4.7               | -1.2                |
| Insulin-like growth factor 2 | IGF2        | 3481    | -3.2               | -4.2                |
| Chromosome 1 open reading frame 121 | Clorf121    | 51029   | -3                | -2.1                |
| Homeobox D1                | HOXD1       | 3231    | -2.9               | -1.4                |
| Increased                  |             |         |                    |                     |
| Cadherin 1, type 1, E-cadherin | CDH1        | 999     | 13.3               | 17                  |
| Annexin A3                 | ANXA3       | 306     | 7.9                | 3.2                 |
| Sodium channel, nonvoltage-gated 1α | SCNNA1     | 6337    | 6.6                | 8.1                 |
| Insulin-like growth factor-binding protein 7 | IGBP7      | 3490    | 6.5                | -50                 |
| KIAA0746 protein           | KIAA0746    | 23231   | 6.3                | 11.9                |
| Ankyrin repeat domain 1 (cardiac muscle) | ANKRBD1     | 27063   | 5.2                | 2.6                 |
| Transgelin                 | TAGLN       | 6876    | 4.7                | 2.0                 |
| Ubiquitin D                | UBID        | 10537   | 3.6                | 3.7                 |
| G protein-co coupled receptor 56 | GP56       | 9289    | 3.6                | 3.7                 |
| S100 calcium-binding protein A10 | SI00A10    | 6281    | 3.5                | 1.4                 |
| Sulfotransferase family 1E, estrogen-prefering, memb1 | SULT1E1 | 6783    | 3.4                | 1.8                 |
| Guanylate-binding protein 1 | GBP1        | 2633    | 3.4                | 3.2                 |
| Palladin, cytoskeletal associated protein | PALLD | 23022   | 3.3                | 1.2                 |
| 2’,5’-Oligoadenylate synthetase 1 | OAS1     | 4938    | 3.0                | 5.6                 |
| Lectin, galactoside-binding, soluble, 3 | LGALS3     | 3958    | 2.8                | 2.0                 |
| CDP-diacylglycerol synthase 1 | CDS1       | 1040    | 2.8                | 3.5                 |
| Placenta-specific 8        | PLAC8       | 51316   | 2.7                | 1.8                 |
| WW, C2 and coiled-coil domain containing 1 | WWCI    | 23286   | 2.6                | 1.2                 |
| Adenose deaminase          | ADA         | 100     | 2.6                | 6.1                 |
| Protein phosphatase 1, regulatory (inh) subunit 13 like | PPP1R13L   | 10848   | 2.6                | 1.7                 |
| Hypothetical protein FLJ20489 | FLJ20489 | 55652   | 2.5                | 1.7                 |
| Discoidin receptor family, member 1 | DDR1    | 780     | 2.5                | 1.1                 |
| Protein-tyrosine phosphatase, receptor type, M | PTPRM | 5797    | 2.5                | 2.3                 |
| Histone 2, H2be            | HIST2H2BE   | 319190  | 2.5                | 1.4                 |
| Zinc finger protein 165    | ZNF165      | 7718    | 2.5                | 1.5                 |
| N-Acetyltransferase 8 (carnello-like) | NAT8     | 9027    | 2.5                | 3.8                 |

+125 and −368 to +125). This suggested that the element regulating the increase in E-cadherin transcription in JAM-A-depleted HepG2 cells was present within the −108 to +125 promoter region. Next, we investigated whether this change in transcriptional activity correlated with a significant change in E-cadherin at the protein level. HepG2 cells express very low levels of E-cadherin under normal conditions, which is similar to that found in invasive dedifferentiated hepatocellular carcinoma cell lines (36, 45, 46). Immunoblot analyses demonstrated that the levels of E-cadherin protein were indeed increased in JAM-Ai1 and JAM-Ai2 cells (Fig. 4D). Quantification of the immunoblots revealed an 8-fold increase in E-cadherin in JAM-Ai1 cells compared with control cells (Fig. 4E), which closely correlates with the −7-fold increase in CDH1 mRNA levels assessed by RT-PCR analyses (Fig. 4B). The increase in E-cadherin expression in JAM-Ai1 cells compared with control cells was confirmed using immunocytochemistry (Fig. 4, F and G). Moreover, these immunostaining studies revealed that E-cadherin was distributed in a polygonal pattern of JAM-A-depleted cells instead of being localized to pseudocanaliculi. Based on these data, we conclude that the predominant change in gene expression found in HepG2 cells upon depletion of JAM-A is the up-regulation of E-cadherin, which occurs primarily through increased transcription of the CDH1 gene.

To examine whether an increase in E-cadherin levels also occurred upon loss of JAM-A in vivo, we generated a mouse line (JAM-A<sup>−/−</sup>) from embryonic stem cells (BayGenomics) containing a gene trap in intron 4 of the gene Flt1 that encodes JAM-A, as has been described by others (33). Disruption of JAM-A expression had no effect on viability, which is consistent with previous reports (33, 47–49), and adult livers appeared normal (data not shown). However, examination of fetal livers isolated from control and JAM-A<sup>−/−</sup> mice at embryonic day 15.5, during which time the hepatic epithelium is being actively assembled, revealed an average 3-fold increase in Cdh1 mRNA levels (data not shown) and a 2.6-fold increase in E-cadherin protein expression (Fig. 4, H and I) in livers lacking JAM-A. Although we noted variation in E-cadherin levels among embryos, quantification of E-cadherin in the livers of six JAM-A<sup>−/−</sup> embryos compared with five heterozygote embryos demonstrated a consistent up-regulation of E-cadherin in 5/6 livers, which approached significance (Fig. 4I; Student’s t test, p = 0.06).

Microarray Data Pathway Analysis Uncovers Regulators of E-cadherin—in an effort to identify factors that may contribute toward increases in E-cadherin levels following loss of JAM-A, genes whose expression was altered by ≥1.5-fold in JAM-A null cells (supplemental Table 2) were analyzed using Ingenuity Pathway Analysis software. This software uses published data to identify functional relationships among genes whose expression is altered in array data sets.

The gene network containing the greatest percentage of genes affected by loss of JAM-A centered on CDH1. This network included many genes that encoded factors with roles in cell morphology and actin cytoskeleton signaling. Moreover, the network included genes encoding proteins that are known regulators of E-cadherin expression and activity (Fig. 5). For example, the level of mRNAs encoding transcription factor 8 (TCF8, also known as ZEB1), a transcription factor previously shown to negatively regulate CDH1 transcription at its proximal promoter (50–52), was reduced by 1.8-fold, insulin-like growth factor 2 (somatomedin A) (IGF2), which has been asso-
associated with degradation of E-cadherin (53), was reduced by 3.2-fold, and protein-tyrosine phosphatase, receptor type M (PTPRM), a positive regulator of E-cadherin activity (54), was increased by 2.5-fold upon JAM-A loss. The observed changes in TCF8, IGF2, and PTPRM mRNAs were confirmed by RT-PCR analysis (data not shown). These data suggest that the combinatorial effects of decreased TCF8 and IGF2 along with increased PTPRM could result in an increase in E-cadherin levels upon JAM-A loss.

Depletion of E-cadherin Partially Rescues the Phenotype Associated with a Reduction in JAM-A—Studies in MDCK and mouse L-cells have demonstrated that cell-cell adhesion contributes to the establishment of cell polarity in a process that may in part be mediated by E-cadherin (55–58). To test whether the increase in E-cadherin levels was primarily responsible for the changes in cell morphology and localization of tight junction proteins upon JAM-A depletion, we attempted to repress E-cadherin expression in JAM-Ai cells. To achieve this, we created a lentivirus that expressed a shRNA (E-cadherin-i1) that efficiently reduced E-cadherin protein levels. Depletion of E-cadherin in HepG2 cells did not have any effect on cell morphology or tight junction formation and protein localization (data not shown). HepG2 cells depleted of both JAM-A and E-cadherin (JAM-Ai1/E-cadherin-i1) were generated by first

FIGURE 4. Reduced JAM-A affects E-cadherin expression. A, RT-PCR for CDH1 (E-cadherin), JAM-A, or POLR2A (RNA polymerase II) using RNA from HepG2 stable cell lines demonstrates a reduction in JAM-A mRNA levels in either cell line transduced with a JAM-A shRNA and a concomitant increase in CDH1 mRNA levels. B, quantification of CDH1 mRNA levels normalized to POLR2A levels shows a significant increase in both JAM-Ai1 and JAM-Ai2 cell lines compared with vector or JAM-Amut cells. Normalized values represent the mean ± S.E. (*, analysis of variance, n = 4, p < 0.05). C, luciferase assays in stable HepG2 cell lines using human E-cadherin proximal promoter fragments. Activation of all constructs was significantly increased in the JAM-Ai1 cells compared with vector cells. Luciferase amounts were normalized to Renilla values and represent the mean ± S.E. (*, analysis of variance, n = 3, p < 0.005). D, Immunoblotting for E-cadherin reveals a corresponding increase in the amount of E-cadherin protein levels when JAM-A is depleted. Equal protein loading is demonstrated by ACTB levels. E, quantification of E-cadherin protein levels normalized to β-actin levels shows a significant increase in E-cadherin with JAM-Ai1. Normalized values represent the mean ± S.E. (*, Student’s t test, n = 5, p = 0.05). F and G, Immunocytochemistry to detect E-cadherin in vector (F) and JAM-Ai1 (G) cells shows an increased expression of E-cadherin in the JAM-Ai1 cells. x-y images show a compressed z-series, and scale bars are 40 μm. x-z sections are to the right of each image, and the orientation of these sections is indicated with B representing the bottom or surface of the coverslip and T representing the top. H, representative livers of embryonic day 15.5 mice that were heterozygotic (JAM-A+/gt) or homozygotic (JAM-Agt/gt) for a JAM-A gene trap were subjected to immunoblotting. I, quantification of protein expression by immunoblot from five JAM-A+/gt mice and six JAM-Agt/gt mice revealed a significant 70% reduction in JAM-A levels in JAM-Agt/gt mice as well as a 2.6-fold increase in E-cadherin, which approached significance (Student’s t test, p = 0.06). Error bars represent the mean ± S.E. (Student’s t test, n = 5 or 6; ***, p < 0.0001).
infecting with the JAM-Ai1 lentivirus and selecting with puromycin, and next infecting with the lentivirus expressing E-cadherin-i1 and sorting for GFP expression.

The presence of the shRNA against E-cadherin prevented the up-regulation of E-cadherin in JAM-Ai1 cells, as revealed by immunoblotting (Fig. 6A). The cell morphology of the JAM-Ai1/E-cadherin-i1 cells was more similar to that of control cells or E-cadherin-i1 cells than to that of JAM-Ai1 cells (Fig. 6, B–E), although some regions of abnormal morphology remained. In addition, the localization of tight junction proteins, such as ZO-1, was apical in JAM-Ai1/E-cadherin-i1 cells, again similar to control cells and E-cadherin-i1 cells, and in contrast to the polygonal localization pattern found in JAM-Ai1 cells (Fig. 6, F–I). The expression patterns of occludin and claudin-1 mirrored that of ZO-1 (data not shown). Quantification of these data revealed that E-cadherin-i1, JAM-Ai1/E-cadherin-i1, and control cells all had a similar number of areas containing claudin-1, occludin, or ZO-1 localized to pseudocanalicular and few polygonal areas, which was in contrast to JAM-Ai1 cells that displayed a significant increase in the number of polygons and a decrease in the number of pseudocanalicular (supplemental Fig. 2). Based on these data, we conclude that repression of E-cadherin in JAM-Ai cells can restore a relatively normal phenotype, and we propose that the abnormal localization of tight junction proteins in JAM-Ai cells is partially attributable to an increase in E-cadherin expression.

DISCUSSION

Signaling pathways that connect cell junctions to the regulatory transcriptional machinery in the nucleus have been proposed to function in generalized epithelial tissues (4, 18). However, our understanding of the contribution of such pathways to cell function and development of hepatocytes, especially those involving tight junctions, has been limited. In this study, we describe a novel feedback mechanism that controls expression and the subsequent localization of junction components in the HepG2 hepatocellular carcinoma cell line.

Identification of JAM-A as a critical regulator of epithelial polarization in an intestinal cell line (23, 34) led us to examine a possible role for JAM-A in the generation and maintenance of hepatic polarization. Here we show that depletion of JAM-A in HepG2 cells leads to a disruption of junction formation and an increase in E-cadherin expression. As a consequence of loss of JAM-A, we propose that some undefined sensor in the cell increases transcription of the E-cadherin gene in an attempt to maintain cell adhesiveness (Fig. 7). Although we have narrowed down the region of E-cadherin regulation to the CDH1 proximal promoter, the factors involved in E-cadherin transcriptional activation upon loss of JAM-A remain to be uncovered. We believe that this regulation could either reflect increased activity of a transcriptional activator, such as hepatocyte nuclear factor 4α, or decreased activity of a transcriptional repressor such as TCF8, both of which have been shown to influence E-cadherin expression (20, 31, 50–52).

Our microarray data have revealed changes in several known modulators of E-cadherin expression and/or activity upon JAM-A loss, including two negative regulators, TCF8 and IGF2, and a positive regulator, PTPRM. A combination of effects of these genes could lead to the complex regulation of E-cadherin expression at its proximal promoter. The finding that increased E-cadherin in JAM-Ai HepG2 cells reflected an increase in expression of E-cadherin mRNA implies that there exists a sig-
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FIGURE 6. Loss of E-cadherin reduces the impact of JAM-A depletion. A, immunoblotting against E-cadherin, JAM-A, and ACTB in HepG2 stable cell lines. E-cadherin and JAM-A levels are reduced in cells transduced with both lentiviruses containing shRNAs for JAM-A (JAM-Ai1) and E-cadherin (E-cadherin-i1). B–E, phase contrast microscopy of stable HepG2 cell lines. Vector (B) and E-cadherin-i1 (D) cells exhibit a compact, cluster-like growth pattern, whereas JAM-Ai1 cells (C) display a flattened monolayer growth pattern, and JAM-Ai1/E-cadherin-i1 cells (E) have a combination of the two growth patterns. Scale bars are 100 μm. F–I, immunocytochemistry to detect ZO-1 reveals an apical localization of ZO-1 in vector (F), E-cadherin-i1 (H), and JAM-Ai1/E-cadherin-i1 (I) cells (arrows), whereas ZO-1 is found in a polygonal pattern in JAM-Ai1 cells (G) (arrowhead). X-y images show a compressed z-series, and scale bars are 40 μm. X-z sections are to the right of each image, and the orientation of these sections is indicated with B representing the bottom or surface of the coverslip and T representing the top.

A major goal of this study was to determine the impact of loss of JAM-A on hepatic gene expression. Differential gene expression upon knockdown of JAM-A is evident from our microarray data (Table 1 and supplemental Table 2). In addition to the genes that potentially affect E-cadherin expression, Ingenuity Pathway Analyses revealed changes in pathways that could be responsible for specific aspects of the JAM-A depletion phenotype (Figs. 1 and 2). For example, several genes whose expression was increased upon JAM-A loss are associated with modifying tumor necrosis factor-1α or interleukin 1β activity. This is relevant because tumor necrosis factor-1α and interleukin 1β are known mediators of tight junction permeability (61–64). Such genes include ubiquitin D (UBD) (65, 66), 2',5'-oligoadenylate synthetase 1, 40/46 kDa (OAS1) (67, 68), sodium channel, nonvoltage-gated 1a (SCNN1A) (69), S100 calcium-binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11)) (S100A10) (70), and guanylate-binding protein 1, interferon-inducible, 67 kDa (GBP1) (67, 71, 72). In addition, the expression of many genes with roles in controlling cell morphology are altered, such as adenosine deaminase (ADA) (73), E-cadherin (CDH1) (74), insulin-like growth factor 2 (somatomedin A) (IGF2) (75), insulin-like growth factor-binding protein 7 (IGFBP7) (76), and lectin, galactoside-binding, soluble, 3 (galactin 3) (LGALS3) (77, 78).

Work from the Parkos laboratory has previously demonstrated that inhibition of JAM-A through the application of either monoclonal antibodies (15) or siRNAs (34) affects barrier function and permeability without a concomitant alteration in the localization of junction proteins such as E-cadherin. However, both the T84 (15) and SK-CO15 (34) cell lines used in

However, previous work has demonstrated that JAM-A is recruited to tight junctions before other associated molecules (60) and may augment the association of proteins such as ZO-1 and occludin at the junctions. The effect on localization of other tight junction-associated proteins upon depletion of JAM-A (Fig. 2, A–F) may therefore not only be due to increases in E-cadherin expression but may also involve the loss of active recruitment of other junction proteins to the complex by JAM-A directly.

For reasons that remain unclear, the E-cadherin that was induced as a consequence of reducing JAM-A levels was ectopically distributed at the cell surface instead of being localized to apical junctions of the HepG2 cells. We propose that the abnormal localization of E-cadherin, at least in part, contributed to the mislocalization of other junction proteins at the cell surface. The observation that E-cadherin interacts with multiple diverse junction proteins and mediates the establishment of cell polarity in other cell types is consistent with this proposal (59). How-
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those studies are colonic epithelial cells, whereas the current studies utilized HepG2 hepatoma cells. An additional difference is that Mandell et al. (34) found a reduction in β1 integrin levels as a consequence of JAM-A depletion in the SK-CO15 cells, whereas we did not observe such a change in β1 integrin expression in JAM-A-depleted HepG2 cells (data not shown). Another recent study utilized a canine kidney epithelial cell line, MDCK II, to demonstrate a requirement for JAM-A in epithelial cell polarization (79). Interestingly, that report shows mislocalization of ZO-1 upon loss of JAM-A similar to our findings in HepG2 cells (see Fig. 2); however, the effect of loss of JAM-A on adherens junctions or E-cadherin expression was not addressed. Other studies investigating the role of α6β1 integrin in HepG2 cells have shown that inhibition of this molecule leads to a reversal of the adhesive and migratory phenotype of HepG2 cells (80–82). Although these studies have not linked E-cadherin to such processes, it is possible that the regulatory mechanisms converge into a common phenotype. Taken together, these data suggest that the signaling cascades regulating junction formation in epithelial cells differ between cell types and that the mechanisms that result in increased E-cadherin expression in JAM-Ai HepG2 cells may be specific to liver cells.

JAM-A knock-out mice have been described previously and do not show any gross defects in any organ, including the liver (32, 49). One possible reason for the absence of a liver phenotype in the adult JAM-A null mice could be that, unlike cells growing in tissue culture, the process of organogenesis may provide time to select for cells that compensate for the loss of JAM-A through mechanisms that facilitate formation of functional cell junctions. In this study, we demonstrate that the absence of JAM-A in the developing mouse liver does lead to a modest, but reproducible, increase in E-cadherin expression during mid-gestation stages of development (Fig. 4). These data suggest that the signaling pathways uncovered using a cell line such as HepG2 cells can provide insight into the dynamic processes that occur during embryogenesis.

The existence of a pathway through which loss of JAM-A results in increased expression of E-cadherin may reflect a mechanism through which parenchymal hepatic cells respond to changes in adhesion. During liver disease or hepatotoxicity, hepatocytes have the capacity to re-enter the cell cycle to replace the damaged tissue. This process has been studied intensely in rats by surgically removing two-thirds of the liver’s mass and observing the regeneration of the remaining hepatic remnant (83). Using the rat model of liver regeneration, it has been observed that 1–2 days after hepatectomy, tight junction structure becomes temporarily disordered; the bile canaliculi adopt a dilated and tortuous morphology, and increased levels of bile acids are detected in the serum (84, 85). Immunoblotting studies have revealed that the observed change in tight junction integrity is accompanied by a transient increase in the expression of a subset of adhesion proteins, particularly that of E-cadherin (84). At around 40 h post-hepatectomy, normal tight junction permeability and morphology are restored, and E-cadherin levels return to basal levels by day 3 of recovery. Together with our own studies, these observations are consistent with the proposal that loss of junction integrity, either by depletion of JAM-A or in response to partial hepatectomy, induces expression of E-cadherin in an effort to maintain cell adhesiveness.

The existence of a signaling mechanism between cell junctions and expression of CDH1 is also appealing from a mechanistic viewpoint, because intercellular interactions between E-cadherin molecules are an early event in the development of cell polarity (55–57, 59). Using a liver regeneration experimental paradigm to test whether E-cadherin contributes to junction formation should be feasible with the availability of

FIGURE 7. Model describing the role of JAM-A in controlling hepatic cell adhesion. A, under basal conditions, hepatic cells are polarized and contain bile canaliculi with adjacent tight junctions and adherens junctions. Components of tight junctions include JAM-A, occludin, claudin-1, and ZO-1, and adherens junctions contain E-cadherin. Loss of the tight junction protein JAM-A leads to disruption of junction formation through an increase in E-cadherin in one of two proposed mechanisms. Depletion of JAM-A activates a signaling cascade that either removes a transcriptional repressor of E-cadherin such as TCF8 (B) or induces an activator of E-cadherin transcription such as PTPRM (C), allowing for increased expression of E-cadherin. The additional E-cadherin targets other tight junction components such as occludin, claudin-1, and ZO-1 to abnormal locations in the cell. As a consequence of this mislocalization of junction proteins, neither tight junctions nor adherens junctions form correctly, and the cells fail to generate pseudocanaliculi. 

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mice in which E-cadherin is specifically disrupted in hepatocytes (31).

In summary, we have identified the existence of a regulatory pathway linking JAM-A at tight junctions to the expression of E-cadherin in HepG2 cells. The inability to form tight junctions upon loss of JAM-A leads to increased expression of E-cadherin. The precise mechanism through which loss of JAM-A and tight junctions leads to activation of E-cadherin remains to be determined; however, we believe that the data presented here contribute to our understanding of both the signaling mechanisms regulating formation of the hepatic epithelium and how cells maintain an equilibrium of junction complexes through feedback signaling pathways.

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