E–N-cadherin heterodimers define novel adherens junctions connecting endoderm-derived cells

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Abbreviations used in this paper: AJ, adherens junction; BS3, bis(sulfosuccinimidyl)suberate; IF, intermediate-sized filament; IP, immunoprecipitation; PLC, primary liver carcinoma; RIPA, radioimmunoprecipitation assay.

Introduction

The ordered association of cells into tissues and their integration into functional organs is generally effected by the patterns of intercellular junctions formed by clusters of cell type–specific, cell–cell-connecting protein and glycoprotein complexes subsumed under the category of adherens junctions (AJs). Apparently, all the transmembrane glycoproteins involved belong to a large superfamily of Ca2+-binding molecules, the cadherins, the carboxy-terminal domains of which are anchored in dense cytoplasmic plaques containing so-called armadillo-type and certain other proteins that can provide anchorage of intracellular cytoskeletal filaments. This architectonic system linking the functional order of the cell interior to the specific intercellular organization of the tissue is already visible in the evolutionarily first metazoa (Halbleib and Nelson, 2006) and has developed into a diversity of epithelial, mesenchymal, muscular, and neural tissues (Takeichi, 1988, 1995; Gumbiner, 2005; Franke, 2009).

Current concepts of the development of cell–cell associations are based—in the first order—on principles of homophilic cell–cell adhesion of cell type–specific, densely clustered cadherins (e.g., Hatta and Takeichi, 1986; Wheelock and Johnson, 2003; Perez and Nelson, 2004; Foty and Steinberg, 2005; Gumbiner, 2005; Shapiro and Weis, 2009). For example, epithelial cells are generally connected by the epithelial-type cadherin (E-cadherin), neural cells are connected by AJs containing N-cadherin, and mesenchymal cells are connected by AJs containing N-cadherin and/or cadherin-11. It was therefore a finding of fundamental importance when it was reported that epithelial cells change from exclusively or predominantly E-cadherin AJs to an increased proportion of N-cadherin AJs when they turn into mesenchymal cells as, for example, in early embryogenesis.

Similar changes have been reported by many researchers when malignantly transformed cells evade the epithelial cell layer continuity and change their topology to migratory metastatic cells, processes that are often correlated with an increase and often dominance of N-cadherin–based AJs, the “E- to N-cadherin switch” (e.g., Behrens et al., 1989, 1993; Frixen et al., 1991; Halbleib and Nelson, 2006; Wheelock and Johnson, 2003; Perez and Nelson, 2004; Foty and Steinberg, 2005; Gumbiner, 2005; Shapiro and Weis, 2009).
have noted frequent, often predominant cell–cell contact structures positive for both E- and N-cadherin. As this appears to be in provocative contrast to the prevailing concept of a mutually exclusive occurrence of E- and N-cadherin in AJs of such tissues, specifically to the concept of antagonistic functions of these glycoproteins in tumorigenesis, carcinoma invasion, and metastasis, we have examined the AJs of such cells in normal and tumorous tissues as well as in cell cultures in detail.

Light microscopy of normal and tumor tissues

In cryostat sections of frozen tissues as well as in sections through formaldehyde-fixed, paraffin-embedded tissues microwave treated for antigen retrieval, we consistently noticed densely spaced E- as well as N-cadherin–positive reaction sites along the plasma membranes connecting hepatocytes (Fig. 1, A–A’), including the bile canalicular regions (Fig. 1, B–B’). In some places, these E- and N-cadherin–positive sites could be resolved as linear arrays of punctate structures indicative of AJs (Fig. 1 B). When these arrays of N-cadherin–positive structures were compared by double-label immunofluorescence microscopy, with reaction sites of antibodies specific for desmosomal molecules, they were clearly different, often appearing in linear alternating arrays of AJs and desmosomes along the canalicular plasma membrane (presenting a direct comparison of
N-cadherin with desmoplakin; similar results were also obtained for other desmosomal marker molecules, specifically desmoglein Dsg2 and plakophilin Pkp2; Fig. 1, A–A”). In contrast, in double-label immunofluorescence microscopy comparisons of N- with E-cadherin, far-reaching colocalization was observed (Fig. 1, B–B”), and this was also often seen in comparisons of N-cadherin with α-catenin, protein ZO-1, and the armadillo proteins β-catenin, p120, and p0071 (Fig. S1, A and B). Practically identical results were obtained for all five species examined.

Similar colocalization of E- and N-cadherin in AJ structures was seen in gall bladder epithelium, intra- and extrahepatic bile ducts (Fig. S1, C and D), and pancreatic ducts (Fig. S2), whereas the AJs of the surrounding mesothelium were positive only for E-cadherin (Fig. S1 E shows the absence of N-cadherin in mesothelial cells). Again, essentially identical results were obtained in all five species examined. Colocalization for E- and N-cadherin was also seen in AJ structures of various liver-, gall bladder-, or pancreatic duct–derived tumors as well as in human liver adenomas (Fig. 2), hepatocellular carcinomas, and ductal adenocarcinomas of the pancreas, both in primary and metastatic tumors.

Microscopy of cell cultures
When monolayer colonies of primary, secondary, and permanent human or rat hepatocyte cultures were examined by double- or triple-label immunofluorescence microscopy, we observed strongly N-cadherin–positive, punctate or even continuously linear-appearing reaction sites in cell–cell contact regions, frequently in perfect colocalization with E-cadherin (Fig. 3, A–B, examples of human cells). Generally, colocalizations of E- and/or N-cadherin were seen with α-catenin, protein ZO-1, and the armadillo proteins β-catenin, p120, and p0071. Colocalizations of E- and N-cadherin in AJs as well as with the aforementioned plaque proteins were also frequent in cultures of hepatocellular tumor cells, including permanently proliferative cell lines.
Figure 3. Identification and localization of E- and N-cadherin-containing AJ structures connecting cells of primary cultures of human hepatocytes and hepatocellular carcinoma cells of line PLC. (A–C′′′) Laser-scanning immunofluorescence microscopy of E–N-cadherin heterodimer–containing AJs in primary human hepatocytes (A and B) or hepatocellular carcinoma cells of the line PLC (C), freshly replated and allowed to reassociate in small colonies, after reaction with antibodies against N (A and A′′′, red; C and C′′′, green) and E (A′ and A′′′, green; C′ and C′′′, red)-cadherin, showing cell–cell contacts with extensive colocalization (yellow merge color; A′′′, B, and C′′′) in small punctate AJs or in extended linear structures. Note that some colonies show cell–cell contact regions positive for both cadherins and presenting extensive colocalization (A–B; the right hand four-cell colony in C–C′′′), whereas other—often directly adjacent—cell colonies present junctions that are positive only for one of the two cadherins (here, N-cadherin on the left of C–C′′′). Note also the additional occurrence of small whisker- or dot-shaped structures at the cell surface, which are N- or E-cadherin positive or show already a merged color (yellow). Bars: (A′′′ and B) 10 µm; (C′′′) 20 µm.
In addition, however, and more frequently in freshly trypsinized and replated cell cultures, we noticed small structures appearing as dots, short beaded chains, or whiskers at cell–cell contact sites, on free cell surfaces or on cytoplasmic vesicles, which could appear as positive for either only N-cadherin or E-cadherin or for both (Figs. 3, A–C and 4, A–D). Not infrequently, such small cadherin-positive structures were also positive for one of the aforementioned plaque proteins (unpublished data). And most startlingly, we repeatedly noted cell colonies in which all cell–cell contact regions were densely packed with AJs intensely stained for both E- and N-cadherin, whereas adjacent colonies were characterized by cell–cell contact structures positive for only one of the two cadherins (Fig. 3, C–C'). And such heterogeneities could also frequently be seen in the same colony, be it small or large (Fig. 4, A–D). The general impression was that in a given cell–cell contact region, one of the two cadherins started AJ formation, whereas the second cadherin appeared somewhat later to associate to heterocomplexes and -structures (Fig. 4, A–D).
these AJs showed rather small, electron-dense cytoplasmic plaques and were interspersed with mostly larger but less frequent typical desmosomes and small junction-free regions (Fig. 6, B–D). Although the plaques of the desmosomes were generally associated with laterally abutting bundles of keratin IFs, the plaques of the punctate AJs were only occasionally resolved to anchor microfilament bundle structures. In systematic evaluations of bovine liver cell structures, almost half (43%) of the lateral contact areas between hepatocytes was occupied by AJs.

Immunoelectron microscopy revealed that by far most, if not all, of these numerous punctate AJs contained not only E-cadherin (Fig. 6 C) but also N-cadherin (Fig. 6 D). Moreover, specific antibodies and preparation techniques allowed us to localize the glycosylated extracellular domains of these cadherins in the intercellular bridges connecting these AJs (Fig. 6 C). These immunolocalization results corresponded with reactions of the specific cytoplasmic plaques positive for the armadillo.
proteins β-catenin, p120, and p0071. Immunoelectron microscopic control reactions also showed the specific immunolabeling of the adjacent desmosomes with antibodies to desmoplakin, plakophilin Pkp2, and desmoglein Dsg2 (unpublished data).

Biochemical analyses

In gel electrophoretic analyses of the proteins of total cells, of cytoskeletal residues, or of plasma membrane fractions enriched in bile canalicular structures (for electron microscopy of such fractions see Franke et al., 1979), N-cadherin was a major protein in liver tissues as well as in cultures of hepatocyte-derived, normal, or malignantly transformed cells (Fig. 7 A presents a series of results). As expected, N-cadherin was also a major protein in cell culture lines of mesenchymal origin, including human hepatic stellate cells of line LI-90 or human glioma cells of line hG-U333 but was totally absent in diverse other epithelial tissues and carcinomas, including several endoderm-derived cell lines, such as human colon carcinoma CaCo-2 cells.

Figure 6. Electron microscopy of ultrathin sections through bovine liver tissue, showing cell–cell contact regions with high densities of small AJ's of the puncta adhaerentia type that contain E- as well as N-cadherin. (A and B) Electron micrographs of ultrathin sections showing lateral contact regions of hepatocytes with small but frequent—and for the most part closely spaced—puncta adhaerentia (denoted by arrows), in comparison with desmosomes (Des) and gap junctions (GJ). G, glycogen aggregate. (C and D) Immunoelectron microscopy of ultrathin sections obtained from cryostat sections and reacted with antibodies specific for E (C) or N (D)-cadherin shows that most, if not all, puncta adhaerentia contain both E-cadherin (denoted by arrows; here, the specific epitope is located on the extracellular portion of the molecule) as well as N-cadherin (denoted by arrows; the inset in D also shows a desmosome on the left and an intensely labeled punctum adherens on the right). Bars, 0.5 µm.
fractions, enriched cell particles, or total cytoskeletal residues to various protocols of chemical cross-linking with a wide range of bond bridge distances. Even with the shortest bond distance examined (2.05 Å), i.e., in a reaction with Cu2+-phenanthroline to allow the oxidative formation of disulfide bridge bonds, remarkable proportions of both E- and N-cadherin were recovered in covalently linked heterodimer complexes of E- and N-cadherin (Fig. 8, A and B). By exposure to reducing agents, usually DTT or mercaptoethanol, these disulfide bonds could be completely cleaved. Similar covalent heterodimeric complexes were also obtained with other disulfide cross-linkers or with reagents connecting other amino acid residues, including bis(sulfosuccinimidyl)suberate (BS3) as the longest distance (>11 Å) cross-linker applied, which in addition, allowed to obtain even larger cross-link products, up to at least the size of double heterodimers (Fig. 8 C).

To examine the stability and the dynamics of the E–N heterodimer complexes, we exposed monolayer cultures of cells derived from human hepatocytes and hepatocellular carcinomas (Fig. 7 A). Liver epithelial cells contained comparable amounts of E-cadherin, which was also found as a major component in CaCo-2 and other colon and colon carcinoma cells but was absent from LI-90 and hG-U333 cell cultures (Fig. 7 A). Remarkably, in most of the total cell lysates or cytoskeletal protein preparations from hepatocyte origin, the silver staining and immunoblot reactions indicated near-isostoichiometric amounts of N- and E-cadherin (Fig. 7 A, lanes 2, 4, 5, and 7–10).

When particles, proteins, and protein complexes obtained from liver tissue or from cultured hepatocyte-derived cells after mild nondenaturing detergent treatments were analyzed by gel filtration chromatography or sucrose gradient centrifugations, the majority of both E- and N-cadherin was recovered in particles of ~11.5 S and with apparent mean molecular masses of 220–240 kD, which is indicative of heterodimers (Fig. 7 B). When these fractions were subjected to immunoprecipitations (IPs) with antibodies specific for E- or N-cadherin, the peak fractions indeed contained both cadherins in major amounts suggestive of an isostoichiometric ratio (Fig. 7 C).

To test for a possible close neighborhood of the E- and N-cadherin molecules in the cell cultures and tissues positive for both E- and N-cadherin, we exposed preparations of cell fractions, enriched cell particles, or total cytoskeletal residues to various protocols of chemical cross-linking with a wide range of bond bridge distances. Even with the shortest bond distance examined (2.05 Å), i.e., in a reaction with Cu2+-phenanthroline to allow the oxidative formation of disulfide bridge bonds, remarkable proportions of both E- and N-cadherin were recovered in covalently linked heterodimer complexes of E- and N-cadherin (Fig. 8, A and B). By exposure to reducing agents, usually DTT or mercaptoethanol, these disulfide bonds could be completely cleaved. Similar covalent heterodimeric complexes were also obtained with other disulfide cross-linkers or with reagents connecting other amino acid residues, including bis(sulfosuccinimidyl)suberate (BS3) as the longest distance (>11 Å) cross-linker applied, which in addition, allowed to obtain even larger cross-link products, up to at least the size of double heterodimers (Fig. 8 C).
to conditions in which the one or the other or both of the two cadherins were drastically reduced or even depleted. In siRNA experiments, Fig. S3, for example, presents the situation at day 3 of exposure of hepatocellular carcinoma cells of line PLC to siRNAs interfering with E-cadherin, N-cadherin, or with both. When the level of E-cadherin was reduced, the level of N-cadherin was only slightly affected, and the reverse was found in experiments interfering with mRNA encoding N-cadherin. In striking contrast, interfering with mRNAs for both cadherins (Fig. S3, lane 3) led to very dramatic reductions of both E- and N-cadherin. Interestingly, in such experiments, the levels of the armadillo proteins (Fig. S3, β-catenin) were only moderately reduced. When the times of exposure to media containing these siRNAs were prolonged for a further 1 d, only very minor residues of the specific cadherins were detected.

In such mRNA interference experiments using siRNA for E- or N-cadherin or mixtures of both, the state of cell–cell adhesion was also routinely examined by immunofluorescence microscopy in combination with phase or interference contrast optics (Fig. 9 presents results obtained at the end of day 3). When one of the two cadherins was markedly reduced or even appeared completely depleted, the monolayer organization remained—by and large—in close cell–cell contact mediated by “normal” homodimer cadherin complexes (Fig. 9, A–H). Control experiments revealed the presence of desmosomes, tight junction, and gap junction connections in such cell colonies.

Drastic changes, however, were noted in the cell culture colonies treated with both siRNAs, i.e., after drastic reduction of both E- and N-cadherin. Here, only some residual, irregularly shaped cell colonies were seen, which presented the AJ plaque proteins still in continuous zonula adherens–like structures extending along the remaining cell–cell contact regions (Fig. 9, I and J) or—in a more advanced state of AJ disintegration—as long curvilinear bands containing the plaque proteins, which were translocated deeper into the cytoplasm (Fig. 9, J–M, protein ZO-1). As these structures of detached plaques resembled, in both light and electron microscopy, very much the cytoplasmic AJ belt retraction aggregates known from exposures of epithelial cell cultures to media of reduced Ca2+ contents (e.g., Kartenbeck et al., 1982), we also performed such experiments with low Ca2+ media or with 2 mM EGTA and obtained very similar results ( unpublished data). Remarkably, in such cell colonies, the appearance of AJ plaque retraction belts was accompanied by both the retention of some E- or N-cadherin at the plasma membrane amino acids as well as the formation of cadherin-containing structures in the cytoplasm (Fig. 9, J–M), which on electron microscopic examination, were revealed as clusters of vesicles (compare Kartenbeck et al., 1982; Le et al., 1999; Akhtar and Hotchin, 2001; de Beco et al., 2009; Hong et al., 2010).

Heterotypic trans-connections between E–N-cadherin heterodimer and N-cadherin homodimer AJ

When we examined the possibility that E–N-cadherin–based complexes of epithelial cell AJJs might also stably attach to other kinds of cadherin AJJs in cell culture experiments, we found that the E–N-cadherin AJJs of hepatocyte-derived cell cultures were able to form direct heterotypic junctions with the pure N-cadherin AJJs of various mesenchymally derived cells. For example, we show a mixed culture of hepatocellular PLC cells confronted with transformed fibroblasts of line SV80 (known to form only N-cadherin AJJs; Rickelt et al., 2009). Clearly, such epithelial cells are able to establish numerous new junctions connecting the epithelial E–N heterodimer AJJs with the purely N-cadherin–containing AJJs of the fibroblasts (Fig. 10, B and C). Similar results were obtained in confronting cultures of other hepatocyte-derived normal and tumorous cells with diverse fibroblastoid and myoid cell types. It is also not unlikely that
Our results demonstrate the presence of large amounts of both E- and N-cadherin in diverse endoderm-derived epithelial cells, including hepatocytes, i.e., probably the biochemically most studied mammalian cell type, as well as bile, pancreatic duct epithelia, and gall bladder epithelium. Large, but often regionally

Discussion

the heterotypic coupling of the AJs of cultured chick hepatocytes to cultured chicken eye lens cells reported by Volk et al. (1987), interpreted as E- to N-cadherin adhesion, might have been based on the coupling of hepatic E-N-cadherin dimers to lens N-cadherin. Heterophilic binding of E- to N-cadherin with remarkable adhesion strength has more recently also been demonstrated in transfected CHO cell cultures (Katsamba et al., 2009).
varying, amounts of both cadherins are also found in the corresponding pathologically altered epithelial systems of adenomas and carcinomas and also in diverse kinds of cultured cells derived from such tissues and tumors. Moreover, in most of the cell types examined, these two classical cadherins occur in near-isostoichiometric proportions, and this appears to be readily explained by our finding that a very large proportion of these molecules occurs in the form of E–N heterodimers clustered into AJs connecting such cells.

Formations of cis-homodimers of either E- or N-cadherin have previously been reported in various epithelial systems (e.g., Adams et al., 1998; Chitaev and Troyanovsky, 1998; Yap et al., 1998; Wheelock and Johnson, 2003; Perez and Nelson, 2004; Gumbiner, 2005; Troyanovsky, 2005; Shapiro and Weis, 2009; Zhang et al., 2009). Cis-homodimer E-cadherin complexes have also been shown to assemble upon cotransfections of cDNA constructs into AJ-free nonepithelial cells (Takeda et al., 1999; Ozawa, 2002; Takeda, 2004), whereas heterodimer cadherin complexes have so far only been reported to form between N- and R-cadherin upon transfection of mouse fibroblastoid L cells with specific cDNA constructs (Shan et al., 2000). These authors have further emphasized that in their experiments, E-cadherin could not at all dimerize with N-cadherin, similar to the study of Johnson et al. (1993) that in cells containing E-cadherin together with P-cadherin, these two glycoproteins segregate into strictly separate homodimers. On the other hand, Duguay et al. (2003) and Foty and Steinberg (2005), also using L cell transfections, have described heterotypic cadherin adhesion complexes of various kinds controlling cell sorting but have not determined the specific biochemical nature of the complexes formed.

Surprisingly, the abundance of stable heterodimers of N- and E-cadherin in normal and malignantly transformed hepatocytes—both in tissues and in cell cultures—has hitherto escaped detection, probably because of methodological reasons. Obviously, it is difficult to relate our observations in hepatocytes and hepatocyte-derived tumor cells to those reported by previous authors who have either seen only E-cadherin (e.g., Ihara et al., 1996) or have mentioned the additional occurrence of a special cadherin, called hepatocyte N-related cadherin, at certain lateral plasma membranes but not at all at canalicular membranes or in bile duct epithelial cells (Kozyraki et al., 1996). Moreover, these authors have reported that the hepatocyte N-related cadherin occurs only in a portion of the hepatocellular carcinomas, whereas a much lower proportion of carcinomas contain only N-related cadherin but no E-cadherin. Nuruki et al. (1998) have reported immunostaining reactions for both E- and N-cadherin in human hepatocytes, whereas in many hepatocellular carcinomas, E-cadherin but not N-cadherin is markedly displaced. Again, differently, Doi et al. (2007) have described that in adult mouse liver, E- and N-cadherin do not show the same distribution but rather a mutually exclusive pattern in different zones of the liver lobules. According to Tsuchiya et al. (2006), E- and N-cadherin immunostaining can be seen along plasma membranes of hepatocytes and bile duct epithelium but not in pancreatic ducts. And recently, Govindarajan et al. (2010) have reported the absence of N-cadherin in early cultures of rat

Figure 10. Localization and characterization of N-cadherin-containing heterotypic AJ structures connecting human fibroblasts and hepatocellular carcinoma cells of line PLC. (A–C) Laser-scanning, double-label immunofluorescence microscopy (on a phase-contrast basis) of cocultures of freshly dispersed transformed human fibroblasts (shown here is line SV80) and hepatocellular carcinoma cells of line PLC in a mixed cluster cell colony grown to confluency, after reaction with antibodies against N-cadherin (red) and the IF protein vimentin (green) as a marker of the fibroblasts. Punctate AJs containing N-cadherin occur not only as homotypic junctions formed between SV80 fibroblasts but also as heterotypic structures connecting N-cadherin AJs of fibroblasts and E-N-cadherin heterodimers of AJs of adjacent PLC cells. In the latter, the AJs are also positive for E-cadherin. Shown here are an overview (A) and details presenting special clarity at higher magnification (B and C), the latter allowing the resolution of an array of at least eleven variously sized AJ structures (red dot structures) connecting a fibroblastoid with a hepatocyte-derived cell. The yellow-stained structures are N-cadherin AJs located on the bottom side of the cells. Bars: (A) 20 µm; (B) 10 µm; (C) 5 µm.
Liver epithelial cells, but they have also noted its appearance after several passages. In these studies, distinct molecular structures have not been identified as colocalization sites of E- and N-cadherin.

For about two decades, relative frequencies and immunohistochemical reaction intensities of N- versus E-cadherin have been widely used as predictive criteria in pathology, liver tumor pathology included (e.g., Wei et al., 2002; Inayoshi et al., 2003; Cho et al., 2008). Generally, the increase of N-over E-cadherin in the so-called epithelial–mesenchymal transition cadherin switch is generally taken as a diagnostic indicator of the invasive and metastatic potential of a given carcinoma (e.g., Nieman et al., 1999; Ochiai et al., 1999; Hazan et al., 2000; Kim et al., 2000; Cavallaro and Christofori, 2004; Wheelock et al., 2008; Kalluri and Weinberg, 2009; Thiery et al., 2009). We now propose to use the identification of E-N-cadherin–positive AJs and, in particular, E-N-cadherin heterodimers as a diagnostic cell-type marker for the origin and nature of a given kind of tumor, which might be of special value in cases of distant metastases of carcinomas derived from liver, bile tract, or pancreatic duct.

Finally, our discovery of heterodimeric E–N-cadherin complexes in the AJIs of a special category of endoderm-derived cells should also enforce a general reconsideration of the cadherin switch hypothesis of carcinogenesis. Apparently, a remarkable level of promiscuity of different cadherins is tolerated in some of the endoderm-derived cell types and functions (e.g., Niessen and Gumbiner, 2002; Capaldo and Macara, 2007; Kan et al., 2007). On the one hand, the lack of E-cadherin, for example in hepatocytes and intestinal cells, or its complete exchange by N-cadherin has been found to interfere with specific functions and morphogenetic processes and may even result in pathogenic developments (e.g., Théard et al., 2007, 2008; Libusova et al., 2010). Our study now suggests that the formations, maintenance, and functions of E–N heterodimer AJs may define a special epithelial differentiation pathway of normal development as well as in tumor formation and progression. In particular, we think that our observations of E–N heterodimer–mediated coupling of epithelial and carcinoma cells with diverse kinds of mesenchymal cells should lead to general studies of cell–cell interactions in development and tumor growth, notably metastasis, as well as to the design of special therapeutic anticarcinoma cytotoxic peptide or other drugs of the N-cadherin interference category (Blaschuk and Deveney, 2009). And finally, our alarming finding that carcinoma cells equipped with E-N heterodimer AJIs can directly and stably attach to N-cadherin AJs of stromal cells will certainly lead to a reconsideration of the cell biological basis of tumor–stroma relationships.

Materials and methods

Tissues and cultured cells
Liver and pancreas tissues of bovine and porcine origin were obtained freshly in a local slaughterhouse, and murine (mouse and rat) tissue samples were obtained from the animal houses of the German Cancer Research Center, the National Center for Tumor Diseases Heidelberg, and Hannover Medical School. Cryopreserved human tissue samples, provided by T. Longerich and E. Herpel (National Center for Tumor Diseases, Heidelberg, Germany), included nontumorous liver tissues of partial hepatectomies from liver metastases of colorectal cancer as well as normal pancreas and gall bladder. Tumor samples were cryopreserved or formaldehyde fixed and paraffin embedded (e.g., Straub et al., 2008). For biochemical experiments, tissue samples were either directly used or frozen in liquid nitrogen and kept at –80°C until use. In parallel, human and rat hepatocytes were isolated and prepared as suspensions of cells or grown in cell culture.

Human adult hepatocytes were isolated as previously described (e.g., Alexandrova et al., 2005) using a modified three-step collagenase perfusion from surgical resectates (obtained from patients with informed consent). Primary adult murine (rats and C57BL/6 mice) hepatocytes were isolated by a two-step collagenase perfusion method originally described by Seglen (1979) with minor modifications. Perfusion solutions were introduced into the tissue through catheters placed into the portal or hepatic vein branches. After the digestion phase, the liver tissue was manually disrupted with sterile scissors and scalpels. To separate undigested tissue pieces, the suspended hepatocytes were passed through 750- and 500-μm filters into 50-ml tubes (Falcon; BD).

Primary and secondary cultures of normal human, rat, and mouse hepatocytes, permanently growing human hepatocellular tumor lines, such as PLC, HepG2, Hep3b (obtained from the American Type Culture Collection), and HuH7 (JTC-39; Japanese Collection of Research Bioresources Cell Bank, Okayama University), as well as primary cells and various lines of rat hepatocytes (e.g., CRL-1439; clone 9; American Type Culture Collection) and hepatoma cells were allowed to grow on glass coverslips coated with 0.01% collagen or 0.005% fibronectin essentially as previously described (e.g., Borenfreund et al., 1980; Franke et al., 1981b; Kern et al., 2002). Human hepatic stellate cells of line U-90 were grown in DMEM containing 10% fetal calf serum (Higashi et al., 2004). Primary murine hepatocytes growing in culture were prepared from healthy mice anesthetized. Here, the liver was perfused through the vena cava, first with liver perfusion medium for 15 min at 37°C and thereafter with a solution containing collagenase type IV (Roche) for 8 min at 37°C, before they were taken up and grown in Williams’ medium E. For comparison, we also used the human colon carcinoma lines CaCo-2, Colo320DM, and HT29Glu, pancreatic adenocarcinoma cells of the lines Panc-1 and Capan-1, and human glioma cells of line hG-U333CG/343MG.

For analyses of proteins of AJIs, mostly bovine and murine liver tissue vesicle or bile canalicular fractions were used (e.g., Franke et al., 1979, 1981a; see also Yamazaki et al., 2008) as well as cultures of various hepatocellular carcinoma cells grown in media containing different Ca2+ concentrations (compare Duden and Franke, 1988; Kartenbeck et al., 1991; Demel et al., 1995).

Antibodies and reagents
Primary antibodies used in this paper are listed in Table S1. Secondary antibodies used were Alexa Fluor 488– and 594–coupled mouse and rabbit antibodies (MoBiTec) as well as the specific Cy3- and Cy5-coupled antibodies used were Alexa Fluor 488– and 594–coupled mouse and rabbit antibodies (Dianova). For immunoblot analysis, horseradish peroxidase-conjugated secondary antibodies were applied (Dianova).

Immunocytochemistry
Tissue cryosections were air dried for ≥1 h and then fixed in acetone for 10 min at –20°C. Liver, gall bladder, and pancreas tissue specimens were, in most cases, preincubated with PBS containing 0.2% Triton X-100 for 5 min followed by exposure to primary antibodies in PBS for 30 min each, two washes in PBS for 5–10 min each, and exposed to the specific secondary antibodies for 30 min. After two 5–10-min washes in PBS, the cell preparations were rinsed in distilled water, fixed for 5 min in ethanol, and mounted in Fluoromount G (Biolab Diagnostica).

The cell suspensions were centrifuged at 50 g for 10 min, and the cell pellets were resuspended in ice-cold buffer. An aliquot of the cell preparations was separated for cell counts and viability analyses (phase-contrast microscopy and trypan blue exclusion test). For all experiments, suspensions with >85% of viable hepatocytes were used. The nonhepatocyte fraction, including leukocytes and nonparenchymal liver cells, in the cell suspension was <5% (microscopic analyses). Plating efficacy of the hepatocytes on collagen-coated cell-culture dish surfaces exceeded 80% in all preparations. Primary and secondary rat hepatocytes were isolated and grown on coverslips essentially as previously described for liver and other cell cultures (e.g., De Smet et al., 1998; Peters et al., 2010; Franke and Rickett, 2011).
Immunofluorescence microscopy
Immunofluorescence microscopy of cell cultures and of sections through formalin-fixed, paraffin-embedded tissues was performed as previously described [e.g., Straub et al., 2003, 2008; Rickelt et al., 2009]. Therefore, cells grown on glass coverslips were briefly rinsed in PBS and fixed for 5 min either with 2% formaldehyde in PBS at RT or in methanol (5 min) followed by acetone [20 s], both at −20°C. The samples were then briefly air-dried and rehydrated in PBS just before the immunostaining procedure. After permeabilization in 0.2% Triton X-100 for 5 min, cells were washed several times in PBS. Primary antibodies were applied for 1 h at RT followed by three washes in PBS (5 min each), an incubation with secondary antibodies [30 min at RT], washing with PBS (3× 5 min), a short rinse in distilled water, and finally, dehydration in 100% ethanol (1 min) and xylene. Subsequently, sections were mounted with Fluoromount G. Epifluorescence was documented with a photomicroscope (Axiohotop; Carl Zeiss) equipped with a camera (AxioCam HR; Carl Zeiss) as well as Plan Neofluar 63×/1.25 NA oil and Plan Neofluar 40×/1.30 NA oil objectives. For confocal laser-scanning immunofluorescence microscopy, a microscope (LSM 510 Meta; Carl Zeiss) equipped with Plan Apochromat 63×/1.40 NA oil and Plan-Neofluar 40×/1.30 NA oil objectives was used. AxioVision Release 6.4.3.0 and LSM Image browser 3.2.0.115 software (both obtained from Carl Zeiss) was used for image processing, and Photoshop CS3 (Adobe) was used for final figure preparation.

Electron and immunoelectron microscopy
For ultra thin section experiments, liver, gall bladder, or pancreas tissue pieces of human, bovine, porcine, or rodent origin were fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2, containing 50 mM KCl and 2.5 mM MgCl2, for 30 min, washed three times in the same buffer, postfixed with 2% OsO4 in 50 mM cacodylate buffer for 2 h, and repeatedly washed in this buffer. Thereafter, the tissue blocks were stained overnight with 0.5% uranyl acetate in water, washed, dehydrated, and embedded in Epon. Ultrathin sections were poststained with lead (Franke et al., 1979). For immunoelectron microscopy, ∼5-µm-thick cryostat sections were fixed with 2% formaldehyde in PBS for 10 min, rinsed three times for 5 min each with PBS containing 50 mM NH4Cl to quench residual-free aldehyde fixed with 2% formaldehyde in PBS for 10 min, rinsed three times for 5 min with 0.5% uranyl acetate in water, washed, dehydrated, and embedded in Epon. Ultrathin sections were poststained with lead (Franke et al., 1979).

siRNA transfections, incubations, and evaluations
The specific siRNA for human E-cadherin (cdh1; L-003877-00-00) and N-cadherin (cdh2; L-011605-00) as well as control siRNAs [nontargeting control siRNA, ON-TARGETplus SMARTpool; see Pierrehoff et al., 2008] and lamin A/C siRNA [specific for human, mouse, and rat lamin A/C; Pierrehoff et al., 2008] were purchased from Thermo Fisher Scientific. All siRNA transfections were essentially performed according to the manufacturer’s protocols using lipid-based transfection reagents (DharmaFECT no. 1 or 4, Thermo Fisher Scientific; for methods of evaluation see Pieperhoff et al., 2008).

Online supplemental material
Fig. S1 shows the extensive colocalization of E- and N-cadherin in normal murine (rat) and bovine liver tissue. Fig. S2 shows the near-complete localization of E- and N-cadherin in human pancreatic duct epithelium. Fig. S3 presents immunoblot detection of E- and N-cadherin upon siRNA-mediated interference in cultured hepatocellular carcinoma cells of line P1C. Table S1 lists the antibodies used in this study for protein detection and characterization. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201106023/DC1.

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Electrophoresis, IPs, and immunoblotting
SDS-PAGE was essentially as previously described [Achtstaetter et al., 1986; Schäfer et al., 1994; Rickelt et al., 2009]. For IP, liver tissue pieces or loose pellets of cultured cells were taken up in Triton X-100–containing IP buffer (Triton-IP buffer, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA or 0.5 mM CaCl2, 1% Triton X-100, and 1 mM DTT plus protease inhibitors), or in Empigen-containing IP buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA or 0.5 mM CaCl2, 0.1 or 0.5% Empigen, 1 mM DTT, and protease inhibitors) and centrifuged at 16,100 g in a laboratory centrifuge (5414; Eppendorf) for 15 min at 4°C. The supernatant obtained was then precleared with protein G- or A-coupled magnetic beads (Dynal Dynabeads; Invitrogen) for several hours. In parallel, protein A- and/or protein G-coated magnetic beads were reacted with the specific antibodies or with unrelated antibodies (mouse myeloma IgG1; Invitrogen) in 50 mM Tris-HCl, pH 7.5, at 4°C for several hours. The precleared supernatant was then reacted with the antibody-coupled beads overnight in 4°C and washed intensely. The pellets obtained were solubilized in 20–40 µl SDS sample buffer, and the immunoprecipitates were compared by SDS-PAGE with the proteins contained in pellets and the supernatants of the preclearing steps before and after IP.

Chemical cross-linking
Chemical cross-linking was performed using the Cu2+-phenanthroline reaction to obtain short distance (2.05 Å) cysteine-bridged polypeptide molecules [compare, e.g., Saedler et al., 1985; Kapprell et al., 1987] for longer crossbridges with 1–5 mM amine-reactive reagent BS5 (Perbio Science) in PBS, a noncleavable, membrane-impermeable, water-soluble lysine–lysine long distance (>11 Å) cross-linking agent (Sinz, 2003; Leitner et al., 2010). After the reaction, the cells were washed with PBS, and pellets and lysate supernatants were analyzed directly or submitted to IP as described in the preceding paragraph.
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