Proteomic analysis of a neoplastic mouse lung epithelial cell line whose tumorigenicity has been abrogated by transfection with the gap junction structural gene for connexin 43, Gja1

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In order to examine how tumorigenicity is abrogated by gap junctional intercellular communication (GJIC), protein expression was analyzed in four related mouse lung epithelial cell lines that vary in their GJIC status and neoplastic potential. Since alterations in protein expression underlie neoplastic behavior, this proteomic analysis provides insights into the molecular pathogenesis of lung cancer. E10, an immortalized but non-tumorigenic cell line derived from alveolar type II pneumocytes, has functional GJIC. E9, a spontaneous transformant of E10, is GJIC-deficient and is tumorigenic upon injection into a syngeneic mouse. Stable transfection of E9 with Gja1, the gene for the gap junctional protein, connexin 43, re-established GJIC and rendered this line (designated E9-2) non-tumorigenic; the vector transfection control line, E9-41, remains tumorigenic. Proteins extracted from these cell lines were separated by two-dimensional electrophoresis (2DE) and visualized by Coomassie blue staining. We consistently observed differential expression of 27 proteins between E10 and E9 and identified 11 of these by peptide mass mapping. The functions of these proteins include stress response, cytoskeletal structure, signal transduction, apoptosis, immune response, pre-mRNA processing, and carbohydrate metabolism. Gja1 transfection affected the concentrations of four of these proteins, viz. PDI, α-enolase, aldolase A, and gelsolin-like protein. PDI concentration was most profoundly affected; E10 cells contain twice as much PDI as E9, and PDI was restored to E10-like levels in the E9-2 transfectant line while remaining at E9-like levels in the vector control E9-41 cells. An association between connexin 43 and PDI expression was also observed in a second set of independently derived type II cell lines. The PDI superfamily has multiple cellular roles including chaperoning assembled glycoproteins, regulating the activities of transcription factors, and regulating disulfide bond formation.

Introduction

During normal tissue homeostasis, cells respond to signals directing them to divide, differentiate or initiate programmed cell death; dysregulation of these processes may result in tumorigenesis (1). In addition to relaying diffusible signals that bind to membrane receptors, cells communicate directly via GJIC, in which small molecules pass from one cell to another without entering the extracellular space. Gap junctions consist of membrane-spanning connexin proteins that assemble into hexameric units called connexons. These generate a pore at the interface between two neighboring cells. Up to 20 mammalian connexin genes encode proteins that form homotypic or heterotypic connexons and are expressed in a cell-type specific manner (2). GJIC is regulated by dynamic changes in gap junctional permeability following alterations in the expression and post-translational modification of connexins, and by fluctuations in intracellular calcium ion concentrations and pH (3).

Among the cells of pulmonary adenocarcinoma origin are alveolar type II pneumocytes (4). When the fragile, non-dividing type I cells that line most of the alveolar surface are injured, type II cells undergo compensatory hyperplasia and differentiate into type I cells (5). Lung injury can therefore provide a growth stimulus that expands neoplastic type II cell populations. Type II cells express connexin proteins Cx26 and Cx43 in vivo, while type I cells contain Cx43 and Cx46 (6). With increasing time in culture, connexin expression in isolated rat type II cells shifts towards the type I connexin phenotype (6); cultured mouse lung type II cells contain only Cx43 (7).

Cell proliferation may require reduced GJIC, since this process decreases during normal cell cycle transit and is disrupted during neoplastic growth (8). Cx43 expression is decreased in poorly differentiated human pulmonary adenocarcinoma and squamous cell carcinoma (9), and malignant mouse lung tumors express less Cx43 than benign tumors (10). In a screen of 25 human and mouse lung epithelial cell lines, both Cx43 expression and the dye coupling used to measure GJIC function were inversely related to tumorigenic potential (11). An oxidative metabolite of BHT that is pneumotoxic (12) and has lung tumor promoting activity (13) decreased GJIC in cultured type II cells (14). In contrast, the growth-inhibitory agent, DBcAMP, increased GJIC in these cells (15).

Decreased GJIC is therefore deemed important in lung tumorigenesis, but the growth inhibitory signals that pass through gap junctions have not been identified. We are using

Abbreviations: 2DE, two-dimensional electrophoresis; BHT, butylated hydroxytoluene; DBcAMP, dibutyryl cyclic adenosine monophosphate; DTT, dithiothreitol; EF-2, elongation factor 2; Gja1, gap junction membrane channel protein α1; GJIC, gap junctional intercellular communication; HIF1α, hypoxia inducible factor 1α; hnRNP A2/B1, heterogeneous nuclear ribonucleoprotein A2/B1; HSP90, heat shock protein 90α; IEF, isoelectric focusing; MALDI-ToF, matrix-assisted laser desorption/ionization time-of-flight; PMM, peptide mass mapping; PDI, protein disulfide isomerase.
related cell lines to investigate the role of GJIC in tumorigenesis. E10 cells, isolated as a clonal outgrowth of normal lung tissue (16, 17), contained lamellar bodies and expressed surfac-
tant apoproteins at early passage; and were assigned a type II cell origin. E9 cells, selected by their resistance to growth
inhibition by dexamethasone, are not contact inhibited, grow
in soft agar, proliferate at a faster rate than E10 cells, and are
tumorigenic (18). This sibling pair has been extensively studied
to examine the molecular basis of lung cell neoplasticity (19).
E10 has a high Cx43 content and displays GJIC (7), while E9
exhibits greatly reduced GJIC and expresses far less Cx43 than
E10 (7,20). Stable transfection of E9 with Gja1, the structural
gene encoding Cx43, restored GJIC and rendered the new cell
line, called E9-2, non-tumorigenic, while the vector transfec-
tion control, E9-41, remained tumorigenic (20). To understand
the molecular basis of these differences in neoplastic phenotype,
we previously compared the contents of several cell cycle proteins in E10, E9, E9-2 and E9-41 cells. Cyclin D1, a marker
of mouse lung epithelial cell proliferation (21), exhibited a high
concentration in E9 cells, low levels in E10, and was expressed
to an intermediate extent in E9-2 cells (22). Levels of the
kinase inhibitor, p27kip1, were high in E10 cells, low in E9,
and intermediate in E9-2 cells. E9-41 concentrations of these
proteins were equal to those in E9 cells (10,22).

Herein, we broaden the scope of these comparisons by using
a proteomic approach. Proteins from whole-cell lysates were
separated by 2DE and visualized by Coomassie blue staining.
The wide dynamic range of protein expression in eukaryotic
cells makes large-scale analysis of these molecules more
challenging than microarray studies. Cellular concentration varies
by at least five orders of magnitude between the most and least
abundant proteins (23–25). We analyzed whole cell lysates sepa-
rated on acrylamide gels and stained with colloidal Coomassie
blue, which detects proteins with nanogram sensitivity, and
therefore we examined proteins expressed at moderate to high
levels. Low abundance proteins, such as signaling molecules
and transcription factors, are not detectable under these condi-
tions. Approximately 500–1000 spots were visible on each 2D
gel. Of these, 21 spots were consistently upregulated in E9
relative to E10, and 6 were downregulated; 11 of these proteins
were identified by mass spectrometry. In this first proteomic
investigation of the role of GJIC in lung cancer, four of these
11 identified proteins returned to E10-like levels. GJIC-derived
signals in E9-2 cells were identified by mass spectrometry. In this first proteomic
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11 identified proteins returned to E10-like levels. GJIC-derived
signals in E9-2 cells were identified by mass spectrometry.
was performed with UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT). Differences in protein expression by western blot were determined by ANOVA followed by Student Newman–Keuls post hoc analysis. A P-value of < 0.05 was considered significant.

Results

Protein expression in tumorigenic E9 and non-tumorigenic E10 mouse lung epithelial cell lines

The pattern on a 2DE gel of moderately to darkly stained proteins was consistent between several runs; more faintly stained spots exhibited greater variability. In assessing differential expression, we included only those proteins differentially expressed consistently upon repeated analysis. Approximately 40% of the protein spots analyzed by PMM were identified according to our criteria for acceptance of the MS-FIT protein assignment, and 97 protein isoforms expressed in both the E10 and E9 sibling cell lines were identified. This database is presented (Table I) as this tissue culture model is currently used in several laboratories. By 2DE, 27 protein spots were differentially expressed between E9 and E10; 21 were upregulated in E9 relative to E10 and 6 were downregulated. Of these proteins, 11 were identified (Table II). The differential expression between E10 and E9 cells of four of these proteins, EF-2, gelsolin, hnRNP A2/B1, and cytokeratin, is illustrated (Figure 1).

Expression of eight of these 11 proteins was further examined by immunoblotting, and densitometric analysis was performed (Table II). A western blot for cytokeratin was not performed because this protein exists in many forms, thereby making selection of the appropriate isoform-specific antibody difficult. Of the remaining proteins for which antibodies were commercially available, epitope-dependent western blots demonstrated an E10/E9 expression pattern identical to that found with 2DE for all proteins but EF-2. A train of four separate isoforms of EF-2 was identified on the 2DE gel, and one of these decreased in E9 relative to E10 (Figure 1). By immunoblotting, however, EF-2 runs as a single band of equal intensity among all cell lines (data not shown). The inability of one-dimensional analysis to discriminate between isoforms may explain this discrepancy between 2DE and western blot analysis of this protein, an example highlighting the greater resolution of 2DE over western blotting.

Proteins whose expression is affected by Gja1 transfection

Expression of Cx43 protein by western blot in the four cell lines used for proteomic analysis was consistent with previous reports (7,20). Four of the identified proteins whose expression differed between E9 and E10 cells were affected by Gja1 transfection: PDI, aldolase A, gelsolin-like protein, and α-enolase. Gja1 transfection most dramatically affected PDI expression. PDI levels were higher in E10 than E9; PDI concentration in E9-2 cells was at E10-like levels, while remaining at E9-like levels in E9-41 (Figure 2). By 2DE, gelsolin-like protein, aldolase A, and α-enolase decreased following Gja1 transfection (Figure 2A). Gelsolin-like protein is barely detectable in E10 cells, much more intense in E9 and E9-41 cells, and present at intermediate levels in E9-2; no western blot was performed because antibody against gelsolin-like protein was not commercially available. Aldolase A content in E9-2 cells appeared to be intermediate between that found in E10 cells and in E9 or E9-41 cells, and western blot results corroborated this finding (Figure 2B). By 2DE, α-enolase decreased to E10-like levels in E9-2 cells, but no effect of Gja1 transfection

### Table I. Proteins expressed in E10 and E9 mouse lung epithelial cells

<table>
<thead>
<tr>
<th>Protein identification by PMM</th>
<th>Protein identification by PMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate aminotransferase</td>
<td>GRP78</td>
</tr>
<tr>
<td>Annexin II</td>
<td>Aconitase</td>
</tr>
<tr>
<td>Annexin II</td>
<td>Aldolase A</td>
</tr>
<tr>
<td>Annexin III</td>
<td>Annexin IV</td>
</tr>
<tr>
<td>Annexin V</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP synthase α subunit</td>
<td>ATP synthase β subunit</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>t-complex polypeptide</td>
</tr>
<tr>
<td>Chaperonin, subunit 4 (β)a</td>
<td>Chaperonin-containing tcp-1 η subunit</td>
</tr>
<tr>
<td>Chaperonin-containing TCP-1 β subunit</td>
<td></td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>Cytofation 2</td>
</tr>
<tr>
<td>Elongation factor 2</td>
<td>Elongation factor gamma</td>
</tr>
<tr>
<td>α-Enolase d</td>
<td>Fascin</td>
</tr>
<tr>
<td>γ Actin</td>
<td>Gelsolin b</td>
</tr>
<tr>
<td>Gelsolin-like protein</td>
<td>Glyceroldehyde-3-phosphate</td>
</tr>
<tr>
<td>Glyceroldehyde-3-phosphate dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>HnRNP A2/b1</td>
<td>HnRNP Kα</td>
</tr>
<tr>
<td>Mitochondrial serine hydroxymethyl transferase</td>
<td></td>
</tr>
</tbody>
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### Table II. Relative concentrations of proteins differentially expressed between E9 and E10 as determined by immunoblot

<table>
<thead>
<tr>
<th>Protein</th>
<th>E9/E10</th>
<th>Standard error</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDI</td>
<td>0.37</td>
<td>0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HSP90α</td>
<td>2.66</td>
<td>0.11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EF-2b</td>
<td>0.88</td>
<td>0.04</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>hNRP A2/B1</td>
<td>4.66</td>
<td>0.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α-Enolase</td>
<td>3.58</td>
<td>0.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gelsolin</td>
<td>1.53</td>
<td>0.12</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>1.33</td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>β-Tubulin</td>
<td>2.4</td>
<td>0.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gelsolin-like protein</td>
<td>0/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>0/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin III</td>
<td>0/a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Average of immunoblots from three plates per cell line that were assayed independently.

*Proteins differentially expressed by 2DE, but western blots did not detect differential expression, as discussed in the text.

*n/a, not applicable. An antibody was not available for these proteins.
was observed by western blot. An explanation similar to that presented above for EF-2 may resolve this discrepancy. α-Enolase appears by 2DE as a cluster of five spots that may represent post-translationally modified species, at least three of which are more abundant in E9 than in E10. Gja1 transfection reduced the contents of two minor species to levels observed in E10, but the remaining species are unaffected (Figure 2A). By western blot with a pan-enolase antibody, enolase appears as a single band (Figure 2B), presumably representing the composite of post-translationally modified forms observed by 2DE analysis. In this case, the unchanged species probably mask differential expression of the two minor species. Additionally, the antibody may have only recognized one isoform.

To extend this correlation between high PDI content and GJIC, we also performed western blot analysis of PDI and Cx43 in the C10 and A5 cell lines, which are type II cell derived clones with origins similar to E10 and E9; A5 is the spontaneous transformant of non-tumorigenic C10 cells (Figure 3). C10 and E10 cells express Cx43, while Cx43 was undetectable in A5 and E9. The Cx43 antibody reacts with two bands; treatment with alkaline phosphatase removes the upper band, consistent with one or more phosphorylated sites on Cx43 (data not shown). As was true in the E10/E9 pair, C10 cells expressed more PDI than A5 cells.

Discussion

To understand how GJIC regulates tumorigenesis, we previously investigated the expression of specific signaling molecules and transcription factors in the cell lines employed herein (10,22). We have now used 2DE/PMM, to compare
protein expression in non-tumorigenic E10 cells with their tumorigenic sibling E9 cells. The proteins identified include cytoskeletal proteins (cytokeratin, gelsolin, and β-tubulin), an anti-inflammatory protein (annexin III), proteins associated with stress response (PDI and heat shock protein 90α), glycolytic enzymes (α-enolase and aldolase A), and proteins involved in mRNA processing (hnRNPA2/B1) and translation (EF-2). Several of these proteins have previously been implicated in neoplastic transformation. For example, elevated hnRNPA2/B1 content in cells exfoliated into sputum is a biomarker for early detection of human lung cancer (32). Over-expression of hnRNPA2/B1 in the neoplastic E9 cell line illustrates the validity of this in vitro system as a tool for studying the molecular pathogenesis of pulmonary adenocarcinoma. Several of the proteins identified in E9 cells, including multiple heat shock and molecular chaperone proteins, L4-3-3ζ, PDI, tricose phosphate isomerase, annexins, calreticulin, actin, and t-complex polypeptide subunits (Table I) were also identified in the first large-scale proteomic analyses of human pulmonary adenocarcinoma and other lung cancers (33,34). We identified 11 proteins that are differentially expressed between the non-tumorigenic line, E10, and its spontaneous transformant, E9, and quantified their relative expression by western blot (Table II).

In this study we employed a proteomic strategy to uncover novel mechanisms by which GJIC suppresses lung tumorigenesis. Stable transfection of E9 cells with Gja1 to create the non-tumorigenic E9-2 cell line altered the levels of PDI, aldolase A, α-enolase, and gelsolin-like protein contents from E9-like levels towards those in E10 (Figure 2). These alterations may contribute to the non-tumorigenic phenotype of E9-2 cells. Gja1-transfection reduced the concentration of gelsolin-like protein, aldolase A, and two post-translationally modified forms of α-enolase. The cellular role of gelsolin-like protein is unknown; it has ~50% amino acid sequence homology to the actin-binding protein, gelsolin. In the glycolytic pathway, aldolase catalyzes fructose-1,6-bisphosphate cleavage, and enolase catalyzes formation of phosphoenolpyruvate from 2-phosphoglycerate. These enzymes are induced by hypoxia through HIF-1α-mediated transcription (35), and may help tumors adapt to a hypoxic environment. Gja1 transfection could limit growth beyond the microadenoma stage by preventing substantial production of these key enzymes. The commonly observed over-expression of glycolytic enzymes in tumor cells relative to normal tissue (36) may reflect enhanced proliferative status rather than specifically relating to a neoplastic phenotype (37). Indeed, the aldolase A and α-enolase contents in E10 and E9-2 cells are consistent with their slower transit through the cell cycle (20). By 2DE, we observed five α-enolase spots, two of which were altered by restoration of GJIC (Figure 2A). This is the first report of altered post-translational modification of α-enolase in association with GJIC and cancer.

We are particularly intrigued by the changes in PDI expression observed in these studies. E10 cells express twice as much PDI as E9 cells, and the PDI content in E9-2 cells is restored to that observed in E10 by Gja1 transfection while remaining unaffected in the transfection control (Figure 2). The correlation between PDI and Cx43 expression was also observed in the C10 and A5 cell lines (Figure 3). Replication of this expression pattern in a similar sibling pair supports the hypothesis that reduction in PDI expression accompanies neoplastic transformation of alveolar type II cells. PDI has several cellular functions, most of which involve exchange of intramolecular disulfide bonds (38). Most relevant to an inverse association with neoplastic behavior are its roles as a molecular chaperone to exert

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**Fig. 2.** Effects of Gja1 transfection on protein expression. (A) Selected regions from 2DE gels of proteins in E10, E9, the non-tumorigenic stable Gja1-transfectant (E9-2) and the vector transfection control (E9-41) are shown. (B) Immunoblot analysis of PDI, enolase, and aldolase A expression in E10, E9, E9-2, and E9-41. Each lane represents lysate from a separate plate of cells; n = 3 per cell line. (C) Densitometric analysis of PDI expression, as determined by immunoblot. Bars represent means ± standard error. *Significantly different from E10, P < 0.05.
quality control over newly synthesized glycoproteins (39) and regulation of transcription factor activity (40). The cellular content of various PDI isoforms is regulated by glucose deprivation (41), oxidative stress (42), differentiation (43), and neoplastic phenotype (44,45). In light of its multiple cellular roles, manipulation of PDI expression may produce pleiotropic effects.

GJIC has been pharmacologically modulated in E10 and E9 cells. The tumor promoting agent, BHT, and its bioactive metabolite each reduce GJIC in E10 cells (14), while DBcAMP increases GJIC in both lines (15). However, in addition to their effects on GJIC, BHT causes cell death (46) and DBcAMP inhibits proliferation (15), and the contents of many proteins not strictly associated with GJIC will therefore also change with exposure to these agents. Stable transfection with Gjal affects GJIC specifically. It is not known how Cx43 expression is downregulated in E9 cells, but because DBcAMP application increases Cx43 expression in these cells (15), the G jal gene is intact.

We have employed a proteomic approach to address the question ‘what specific differences in gene expression delineate the neoplastic phenotype among closely-related cell lines?’ Proteomic analysis is an unbiased means of examining the molecular basis of neoplasia, and this work is the first study to correlate GJIC with enhanced PDI expression. The exact role of PDI in neoplasia remains to be defined. We are currently attempting to better define GJIC and PDI from the perspective of molecular targeting of cancer therapies and preventive measures.

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References


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