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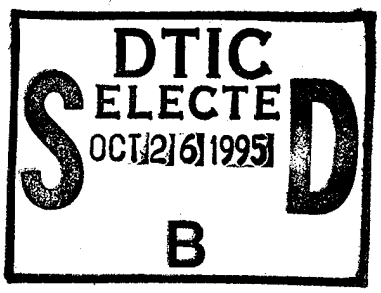

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During the report period we have shown that breast epithelial cells attach to connective tissue via specialized matrix connectors called hemidesmosomes. The latter are lost, as are many of their components, in invasive breast tumor cells. Primary breast epithelial cells assemble hemidesmosomes in vitro. The assembly of hemidesmosomes in such cells is enhanced when they are maintained on matrix secreted by the breast line MCF10a. Our results emphasize the importance of matrix components in assembly of hemidesmosomes.

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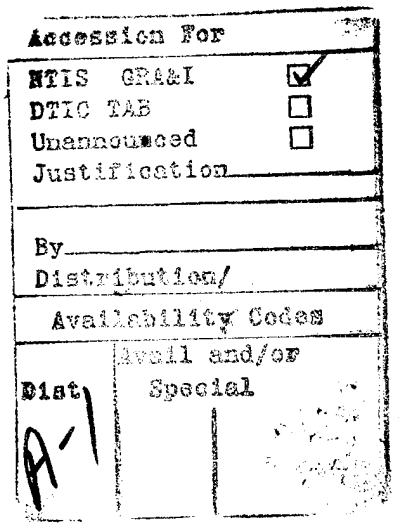

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(5) Introduction

Modulation in adhesion of tumor cells compared with their normal counterparts plays an important role in development of invasive potential. Our application, as funded, concerns the fate of certain epithelial cell-matrix connectors called hemidesmosomes which are involved in anchoring breast epithelial cells to underlying matrix (1,2). Loss of hemidesmosomes appears characteristic of more invasive epithelial tumor cells. Yet there have been very few studies on hemidesmosomes in either normal or tumor breast epithelial tissues (2). This is because of limited availability of cell and molecular probes for individual hemidesmosome components until recently (1). It is the goal of the studies that were detailed in our original application to analyze hemidesmosomes and hemidesmosomal components in a variety of breast tumor types. One major aspect of our work is to identify hemidesmosome-related markers which could be used in diagnostics. Our approach combines cell and molecular biological analyses of normal and tumor tissue as well as normal and tumor cells maintained in vitro.

(6) **Body**

i) Tissue Analyses

The results detailed here are a summary of those presented in Bergstraesser et al., (in revision, Am. J. Pathol) which is included in the Appendix. For our studies, normal breast tissues were obtained from reduction mammoplasties and tumor breast tissue biopsies were surplus to pathological examination. Tissues were processed either for electron or immunofluorescence microscopy.

Normal breast epithelial cells associate with the basement membrane zone (BMZ) via hemidesmosomes (2). These hemidesmosomes possess all the ultrastructural characteristics of classical hemidesmosomes (such as those in the epidermis) in that they have a cytoplasmic plaque to which keratin intermediate filaments bind as well as anchoring filaments and the so-called sub-basal dense plate in the laminin lucida region of the BMZ (1). Furthermore, normal breast epithelial cells express all the known hemidesmosomal proteins (including the two bullous pemphigoid antigens, $\alpha 6\beta 4$ integrin, laminin-5, IFAP300 and a 200kD plaque protein) as determined by immunofluorescence. All of the latter proteins are polarized, being localized to the region of cell-basement membrane attachment. In addition, the anchoring fibril protein (collagen VII) is present underlying each hemidesmosome, beneath the lamina densa of the BMZ.

In tumor tissues, cancer cells that have not invaded the connective tissue possess hemidesmosomes as visualized electron microscopically, provided that they are still in contact with a BMZ. In contrast all invasive epithelial tumor cells lack

hemidesmosomes as defined ultrastructurally. In general, in sections prepared for immunofluorescence, invasive cells are not stained by antibodies against both bullous pemphigoid antigens, α6 integrin and collagen VII. Thus such antibodies have potential value to distinguish "normal" from "tumor" cells. However, antibodies directed against β4 integrin and a 200kD hemidesmosome plaque component occasionally stain tumor cells and would thus be unsuitable for use in differentiating invasive from non-invasive cancer cells. We are continuing with these studies by processing more samples of tumors to more rigorously test the utility of some of our antibody probes in characterization of invasive carcinomas of the breast. In addition we are extending our analyses by investigating expression of the above hemidesmosome elements at the mRNA levels by undertaking in situ hybridization in tissue sections as briefly discussed in the original proposal.

ii) Analyses of Cultured Cells

Second and third passage normal and tumor breast epithelial cells have been maintained on uncoated substrates in culture for various times (1-14 days) using the culture system of Bergstraesser and Weitzmann (3). Cells were either processed for electron microscopy or for immunofluorescence using our panel of hemidesmosomal antibodies.

Between 1 and 7 days following plating, neither normal or tumor breast cells have obvious hemidesmosomes. At 14 days following plating epithelial cells derived from normal tissue have typical hemidesmosomes in regions of cell-substrate

interaction and also express all of the hemidesmosomal elements for which we possess antibody probes. This is also the case for cells derived from non-invasive tumor specimens. Cancer cells derived from some invasive tumors lack hemidesmosomes, consistent with our analysis of invasive cells in situ. However, we were surprised to observe hemidesmosomes at 14 days following plating in cells derived from certain invasive tissue specimens. We do not yet understand this anomaly. One explanation is that there is a reversion of the invasive cell phenotype in vitro, at least with regard to ability to assemble hemidesmosomes. However, another, more likely, possibility is that some of our invasive carcinoma specimens also contain a subpopulation of non-invasive cells, capable of producing hemidesmosomes. These cells may be selected for in some way in our culture system.

iii) Use of the MCF10a Line

In addition to using cells derived from donor breast tissues we have also screened numerous breast derived epithelial cell lines for expression of hemidesmosome components. Cells of the MCF7 cell line fail to express either of the bullous pemphigoid antigens nor $\alpha 6\beta 4$ integrin. In sharp contrast cells of the MCF10a line express all of the known hemidesmosome proteins and assemble hemidesmosomes readily in vitro even within 1 day after plating. This is quite distinct from normal and tumor breast cells derived from tissues which take a considerably longer time after plating (up to 14 days) to assemble hemidesmosomes (see above), even though these same cells express hemidesmosomal plaque and membrane

components in a polarized fashion as early as 1 day as assessed by both immunofluorescence and immunoblotting. Why is this? Recent studies from our lab. indicate that one potential limiting factor that negatively impacts hemidesmosome assembly in cells maintained in vitro relates to expression of matrix proteins (1,5). To test this in regard to breast epithelial cells, we have prepared MCF10a matrix according to Langhofer et al. (5). This involves growing MCF10a cells to confluence. The cells are removed using ammonium hydroxide while their matrix remains on the substrate. We then plate normal breast cells onto this material. Remarkably, under these conditions normal breast epithelial cells assemble hemidesmosomes at 1 day following plating (2). We are extending these observations by determining whether hemidesmosome assembly can be induced in tumor cells in vitro by plating them onto MCF10a matrix. Our studies involve electron microscopical, immunocytochemical and Northern blot analyses of cells.

One obvious question that is raised by the above experiment relates to the nature of the components of MCF10a matrix and identification of the "hemidesmosome" inducing component therein. An antibody that we have derived against rat laminin-5 recognizes several components of MCF10a matrix indicating that the matrix contains human laminin-5 (5). We are testing this further by assessing whether the GB3 monoclonal antibody, which is directed against human laminin-5, cross reacts with MCF10a matrix.

Is laminin-5 the active (hemidesmosome inducing) factor in MCF10a matrix? To assess this we are in the process of deriving a panel of monoclonal antibodies against various components in MCF10a matrix. Antibodies that we generate will be

tested for their ability to inhibit the rapid assembly of hemidesmosomes induced in normal breast epithelial cells by MCF10a matrix. This type of "adhesion assay" will indicate the active factor (laminin-5?) in the matrix. To further such studies we will also investigate expression of matrix components such as laminin-5 in tumor tissues. Overall, we are testing an hypothesis that down regulation of matrix may destabilize hemidesmosomes possibly allowing cells to loosen their contact with the BMZ prior to invasion.

(7) Conclusions

1. Breast epithelial cells utilize hemidesmosomes to attach to the BMZ in normal tissue.

2. Hemidesmosomes are lost in epithelial cells of invasive tumors of the breast.

3. Certain hemidesmosome antibody probes may be used to differentiate non-invasive from invasive carcinoma cells.

4. Normal and some tumor cells derived from breast tissue assemble hemidesmosomes in vitro.

5. MCF10a cells also assemble hemidesmosomes in vitro but much more rapidly than our normal cell populations.

6. Matrix elements secreted by MCF10a induce rapid assembly of hemidesmosomes in normal breast epithelial cells.

(8) **References**

1. Jones, J.C.R., Asmuth J., Baker, S.E., Langhofer, M., Roth S.I. and Hopkinson, S.B. (1994). Hemidesmosomes: extracellular matrix/intermediate filament connectors. Exp. Cell Res., 213:1-11.

2. Bergstraesser, L.M, Srinivasan, G., Jones, J.C.R. and Weitzman, S.A. Expression of hemidesmosomes and component proteins is lost by invasive breast cancer cells. In revision, Am. J. Path.

3. Bergstraesser, L.M. and Weitzman, S.A. (1993). Culture of normal and malignant primary mammary epithelial cells in a physiological manner simulates in vivo growth patterns and allows discrimination of cell type. Cancer Res., 53:2644-2654.

4. Riddelle, K.S, Green, K.J. and Jones, J.C.R. (1991). Formation of hemidesmosomes in vitro by a transformed rat bladder cell line. J. Cell Biol., 112:159-168.

5. Langhofer, M., Hopkinson, S.B. and Jones, J.C.R. (1993). The matrix secreted by 804G cells contains laminin-related components that participate in hemidesmosome assembly in vitro. J. Cell Sci., 105:753-764.

(9) **Appendix**

We include a draft copy of a paper entitled "Expression of hemidesmosomes and component proteins is lost by invasive breast cancer cells" (Bergstraesser et al.), currently in revision for the American Journal of Pathology, as part of our progress report.

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EXPRESSION OF HEMIDESMOSOMES AND COMPONENT PROTEINS IS LOST BY INVASIVE BREAST CANCER CELLS[1]

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Running Title: Hemidesmosomes and breast cancer.

Key Words: normal breast, breast cancer, hemidesmosome, adhesion molecule.

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[3]The abbreviation used is: HMEC, human mammary epithelial cells.

ABSTRACT

Hemidesmosomes are multiprotein structures that attach basal cells of stratified epithelia to basement membranes. Although normal human breast epithelia are not stratified, we observed hemidesmosomes by electron microscopy of tissue sections in breast epithelial and myoepithelial cells at the basal lamina. The hemidesmosomes appeared typical of those observed in other tissues, and the cells expressed the expected hemidesmosome protein components by immunofluorescence. Cultured primary normal human breast epithelial cells also contained electron dense hemidesmosomes and expressed component proteins. Invasive breast cancer cells did not express hemidesmosomes nor most of the component proteins in vivo. In carcinoma in situ, cells away from the basement membrane had no hemidesmosomes or hemidesmosome proteins, and even cells at the basement membrane exhibited abnormalities of hemidesmosome protein expression. Primary human malignant breast cells in culture exhibited a mix of hemidesmosome phenotypes. These data suggest that normal human mammary epithelial cells may use hemidesmosomes to maintain their position in mammary ducts, and malignant cells may eliminate hemidesmosomes to escape their usual tissue architectural restraints. In addition, these normal primary human cells expressing hemidesmosomes in culture and primary malignant cells without hemidesmosomes could be a useful model for studying normal and abnormal regulation of hemidesmosome formation.

INTRODUCTION

A body of literature has been accumulating suggesting that adhesion molecules - those proteins and other substances that cells use to adhere to their substrate - may play a role in the ability of cancer cells to invade and metastasize (1, 2). Data have suggested, on the one hand, that cancer cells may exhibit reduced adhesion molecule expression or function resulting in release of their substrate, and freeing cells to pile up or migrate. On the other hand, carcinoma cells may acquire the expression of new adhesion molecules to grip the basement membrane in order to traverse it, or to adhere to tissues at sites of metastasis (3, 4).

We and others have previously addressed this subject in the breast in studies of the integrin class of cell adhesion molecules, several of which are found to be present in normal breast tissue, but reduced or absent in carcinoma (for example 5-9). Hemidesmosomes are another adhesion structure to study in this context. These cell-connective tissue attachment structures are found only in epithelial cells (10), which usually remain in one place, but not in cells such as fibroblasts or macrophages which wander (for recent reviews see 11-13). Further, it has been suggested that hemidesmosomes, more than other adhesion structures, may mediate firm, relatively immobile attachment to the basement membrane (14, 15), thus preventing the cell movement characteristic of invading malignant cells.

Hemidesmosome loss has been seen in cutaneous basal cell carcinoma (16, 17). In addition, the $\alpha 6\beta 4$ integrin is a component of hemidesmosomes (18-20) and we and

others found that the $\alpha 6$ and $\beta 4$ integrin subunits were not expressed in some malignant mammary epithelial cells (5-7, 21, 22).

Although hemidesmosomes have been most commonly described in stratified epithelia, and the breast parenchyma is not normally stratified, ductal epithelia from various sources have been shown to contain hemidesmosomes (13). It is thought that the tissues which contain hemidesmosomes may be those in which the epithelium undergoes greater shear stress. The breast contains a ductal epithelium which must undergo great shear stress during lactation, and some electron microscope studies have suggested that at least breast myoepithelial cells, and perhaps luminal cells that contact the basement membrane, may have hemidesmosomes (23-28). It is not clear however whether these electron dense structures contain the same molecular components described in other well-studied hemidesmosomes.

In this paper we studied normal and malignant breast cells in tissue sections and in culture for the presence of hemidesmosomes and some of their constituent and associated proteins including the M_r 180 and 230 bullous pemphigoid antigens, a M_r 200 protein, and collagen VII. Normal breast epithelial cells had hemidesmosomes in vivo and in culture, and expressed the expected spectrum of hemidesmosome proteins, whereas invasive carcinoma cells lacked hemidesmosomes in vivo, and malignant cells in culture exhibited defects in hemidesmosome assembly.

MATERIALS AND METHODS

Tissues

Breast tissues were obtained from Northwestern Memorial Hospital, Evanston Hospital, or the Cooperative Human Tissue Network of the National Cancer Institute. Tissues used included infiltrating ductal carcinoma, normal breast tissue from such cancer patients, and normal tissue from reduction mammoplasties. In addition, one sample with benign fibrosis and one sample of normal lactating tissue were obtained as the "normal" tissue from two of the mastectomies above. Specimens were obtained fresh from surgery, and processed for electron microscopy, frozen sections, or tissue culture.

Cell Culture

Mammary epithelial cells were derived from eleven infiltrating ductal carcinomas, seven reduction mammoplasties, and sites distant from two of the carcinomas. Epithelial or carcinoma cells were culled and grown on plastic by the method of Stampfer (29) with revisions for the growth of tumor cells (30) in MCDB-170 medium (American Bioorganics Inc., Niagara Falls, NY) + serum-free supplements (29).

Cell strains were determined to be epithelial by their expression of desmosome proteins by immunofluorescence; and determined to be malignant by their ability to proliferate in the absence of certain growth factors, in the presence of transforming

growth factor β, and at high cell concentrations, and their inability to form three-dimensional structures on Matrigel basement membrane-like substance (30).

Immunofluorescence

Tissues fresh from surgery were snap frozen in liquid nitrogen and stored at -70^{0}C until use. Pieces of frozen tissue were embedded in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, IN), sectioned on a Tissue-Tek cryostat (Miles Laboratories) to a depth of approximately 8 μm, and placed on poly-L-lysine (Sigma Chemical Company, St. Louis, MO)-coated glass microscope slides (VWR, Media, PA). Sections were fixed for five minutes in -20°C acetone (Mallinckrodt, Paris, KY) and air dried. Tissues from seven infiltrating ductal carcinomas, six normal specimens, one sample with benign fibrosis, and one sample of normal lactating tissue from a cancerous breast were used in a total of three experiments.

Cultured cells were grown on glass coverslips (VWR) in six-well plates (Falcon, Lincoln Park, NJ) at 10^4 - 5 x 10^4 cells per well. Following three washes in phosphate buffered saline pH 7.4 containing 0.2 g/liter KCl-0.2 g/liter KH_2PO_4-8.0 g/liter NaCl-1.15 g/literNa_2HPO_4, cells on coverslips were fixed for three minutes in -20°C methanol (Fisher Scientific, Fair Lawn, NJ) and air dried. Cells from six carcinomas and four normal tissues (three reduction mammoplasties and one mastectomy) were used in five experiments.

Immunofluorescence was performed as previously described (5). Slides were observed and photographed using a Leitz Laborlux D fluorescence microscope and

TMAX 100 film (Eastman Kodak Co., Rochester, NY). All photographic exposures were for 1.5 min.

The following primary antibodies were used: serum from a bullous pemphigoid patient containing human autoantibodies reactive primarily with a M_r 230 plaque protein of the hemidesmosome (31), 180 mouse monoclonal (32) and J17 rabbit polyclonal (33) antibodies to a M_r 180 transmembrane hemidesmosome protein, 6A5 mouse monoclonal antibody to a M_r 200 hemidesmosome protein (34), 9C3 mouse monoclonal antibody, and EBA human autoantibody (35) to collagen VII anchoring fibril protein (which is also the epidermolysis aquisita antigen). Secondary antibodies included fluorescein-conjugated anti-mouse IgG + IgM, anti-human IgG + IgM and anti-human IgM; and rhodamine-conjugated anti-mouse IgG + IgM and anti-rabbit IgG (Kierkegaard and Perry, Gaithersburg, MD). Antibody concentrations were determined based on concentration curves.

Electron Microscopy

Electron microscopy was performed using standard methodology (36). Briefly, 1 mm^3 tissues fresh from surgery were fixed in 2.5% glutaraldehyde in 0.1 M Sorensens's phosphate buffer, postfixed in 1% OsO_4, stained in 2.5% uranyl acetate, dehydrated and infiltrated with propylene oxide and embedded in Spurr epoxy resin (all from Electron Microscopy Sciences, Fort Washington, PA). Thick sections were examined by a pathologist to confirm diagnoses and find regions of carcinoma. Twelve carcinomas and fourteen normal tissues (two from reduction mammoplasties and twelve from cancer patients) were used for electron microscopy.

Cultured cells were grown on glass coverslips in six-well plates at 10^4 - 5 x 10^4 cells per well, processed similarly to tissue samples and embedded in Epon-araldite resin 812 (Tousimis, Rockville, MD; or Fisher Scientific). Samples from five malignant cell strains and five normal cell strains (four reduction mammoplasty patients' cells and one cancer patient's normal cells) were processed.

Thin sections were cut on a Reichert Ultracut E microtome (Reichert Instruments, Buffalo, NY), mounted on copper grids, stained with uranyl acetate and lead citrate (Electron Microscopy Sciences) and viewed at 80 kV in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA).

RESULTS

In Vivo (Tissues)

Mammary epithelial cells in vivo contained electron dense hemidesmosomes at their basal plasma membranes where they were in contact with the basement membrane (Fig. 1). These triangular-shaped plaques were associated with intermediate filaments intracellularly, and anchoring filaments and anchoring fibrils extracellularly. Hemidesmosomes were seen in all cells where contact with the basement membrane could be shown. This included luminal cells that reached from the lumen to the basement membrane (Fig. 1D,E) and myoepithelial cells that did not appear to reach the lumen (Fig. 1B).

In all intraductal regions of malignant tumors, cells in contact with the basement membrane also exhibited apparently normal hemidesmosomes (Fig. 2). All cells in contact with the basement membrane had hemidesmosomes, and cells not at the basement membrane contained no hemidesmosomes, although numerous desmosomes and some adherens junctions were seen.

In all invasive regions examined, however, no hemidesmosomes were seen in any cells (Fig. 3) whether single cells, in small groups, or invading en masse and attached to one another with desmosomes.

Because hemidesmosomes have not been well studied in the breast, we looked by immunofluorescence microscopy at expression of several of the protein components of hemidesmosomes previously described in skin. The anchoring fibril protein collagen VII (Fig. 4C), M_r 180 (Fig. 5A) and M_r 230 bullous pemphigoid antigens, M_r 200 protein

(Fig. 6C), and the α6 and β4 integrin subunits (5) were all present in normal ducts at the basal aspects of cells (β4 and the M_r 200 protein exhibited a basolateral distribution).

We next wished to determine whether abnormalities of hemidesmosome protein expression would be seen in breast carcinoma. Two patterns emerged. The first pattern, seen with collagen VII, the M_r 180 and 230 proteins and the α6 integrin subunit (5) is illustrated for collagen VII in figure 4. Staining for the hemidesmosome proteins was seen at the basement membrane, here counterstained with laminin, in normal ducts and intraductal carcinoma. But cells piled up within cancerous ducts, and invasive cells exhibited no staining. A slight variation of this pattern was seen with the M_r 180 protein from which staining in all but one patient exhibited the aforementioned pattern, whereas the malignant cells from one patient exhibited no staining for the M_r 180 protein, even in regions of intraductal carcinoma (Fig. 5C,E).

The second pattern, seen with the M_r 200 protein and the β4 integrin subunit (5) is illustrated in figure 6. Staining was seen at the basement membrane in normal ducts. In both carcinoma in situ and invasive carcinoma, regions of staining were seen and regions devoid of staining were also observed. For any one patient there were regions of intraductal carcinoma outlined by staining, and intraductal carcinoma without staining, and invasive cells that stained or did not stain.

Cultured Cells

Because we saw abnormalities of hemidesmosome and hemidesmosome protein expression in vivo, we decided to study cultured HMEC[3] as a prelude to use of these cells to determine the molecular basis of hemidesmosomal changes and for experimental

manipulations.

Normal HMEC in culture exhibited electron dense, apparently normal, hemidesmosomes by two weeks in culture (Fig. 7). Malignant cell strains varied in their phenotype. For three patients, no cells could be found with hemidesmosomes; for one patient all cells had abundant, apparently normal hemidesmosomes; and the cells of one patient were a mix of cells with no hemidesmosomes, abundant hemidesmosomes, and a few hemidesmosomes.

We next stained cultured HMEC to see if the electron dense hemidesmosomes seen by electron microscopy might also contain the expected protein components. Normal HMEC expressed the anchoring fibril protein collagen VII in a basal secreted pattern (Fig. 8), and the M_r 200, 230 and 180 proteins in rows of basal tick mark-shaped plaques (Figs 9, 10). The $\alpha 6$ and $\beta 4$ integrins are also expressed in this manner and co-localize with one another (5).

Malignant HMEC also expressed all of the hemidesmosome-associated proteins tested for. However, several abnormalities of expression were consistently noted. When normal cells were plated on coverslips, it took five to seven days to see a basal secreted pattern of collagen VII staining, whereas malignant cells did not show this pattern for two to three weeks. In time course experiments (Fig. 8) we found that within one day normal HMEC produced antibody-detectable perinuclear intracellular collagen VII, whereas malignant cells had no immunostaining. At about seven days, malignant cells began to show perinuclear intracellular collagen VII staining while normal cells were already producing basal collagen VII in a secreted pattern.

The M_r 200 protein, on the other hand, continued to be expressed by malignant

cells in only a dotted cytosolic pattern at incubations as long as 30 days (Fig. 9). The protein was never expressed basally by malignant cells. The M_r 230 and 180 proteins exhibited lines of basal tick-marks in malignant cells just as in normal cells and with the same time course (Fig. 10). As reported previously, the β4 integrin subunit was also expressed identically in normal and malignant cells, whereas the α6 subunit was seen in only about 30% of malignant cells in culture (5).

DISCUSSION

In previous detailed electron microscope studies of the breast, hemidesmosomes were noted at the basement membrane in basal cells (23-28). This information seems to have gone relatively unnoticed in the hemidesmosome literature, where hemidesmosomes continued largely to be discussed as characteristic of stratified epithelia. In addition, it was not clear from past studies whether hemidesmosomes were unique to breast myoepithelial cells or are used by all breast epithelial cells to attach to the basement membrane.

In this study we clearly demonstrate that hemidesmosomes are found where all breast cells, whether myoepithelial or luminal, are in apposition to the basement membrane. This strengthens the data adding breast epithelial cells to the expanding list of cell types that express hemidesmosomes, which now includes stratified epithelia such as skin, cornea, mucosa and esophagus (10, 37); complex epithelia such as urinary bladder, trachea, and thymus (10); glandular epithelia such as apocrine and salivary glands (26, 10); and even the simple epithelium of the amnion (38).

We would like to emphasize that we noted that luminal cells as well as myoepithelial cells contained hemidesmosomes. Since breast carcinoma cells tend to differentiate toward a luminal phenotype in, for example, expression of cytokeratins and actins, it is important to note that the absence of hemidesmosomes from invading breast carcinoma cells does not merely represent the loss of surrounding myoepithelial cells, but a clear downregulation of hemidesmosome expression. Recently Clermont et al. (39)

studied the expression of collagen VII anchoring fibrils in the rat breast and also emphasized that both luminal and myoepithelial cells contain abundant hemidesmosomes in vivo.

We also determined that normal breast epithelium expressed at least six of the known hemidesmosome protein components, suggesting that hemidesmosomes have a similar structure and play a similar role in breast as they do in skin and other stratified tissues. This is not true in some other tissues such as arachnoidal cells of the meninges or Purkinje fiber cells of the heart which contain morphologically similar electron dense structures but do not express some of the expected hemidesmosome proteins (13).

The lack of hemidesmosomes in invasive breast carcinoma seen in this study may be contributory to the invasive phenotype by allowing such cells to become less adherent to the basement membrane. In fact during the branching morphogenesis that occurs in the breast during embryogenesis, cells in the penetrating endbud lose expression of hemidesmosomes to invade the stroma and branch into new ducts. (27, 40).

That hemidesmosomes are in fact adhesive structures used by normal epithelia to adhere to basement membranes is suggested by wound healing studies in which re-epithelialized cornea can be easily lifted off until basement membrane and hemidesmosomes have formed (41). Further, the epithelium releases the stroma as a sheet in the genetic blistering diseases dystrophic epidermolysis bullosa, in which anchoring fibrils are congenitally absent (42, 43), and lethal junctional epidermolysis bullosa, in which hemidesmosomes are abnormal (44); and in the aquired diseases epidermolysis bullosa aquisita and bullous pemphigoid, in which autoantibodies to collagen VII and to the M_r 230 and 180 hemidesmosome components, respectively, are

found (45, 46). Also, experimental addition of blocking antibodies to hemidesmosome proteins either in vivo or in culture causes loss of epithelial adhesion (47).

Even more importantly, hemidesmosomes are probably involved in stable rather than motile adhesion. For example, $\alpha 6\beta 4$ was found only in nonmotile cells in culture (15), and hemidesmosomes are downregulated in epithelial cells that become motile to fill in a wound (for example 35, 35, 47).

Another interesting observation in this study regards hemidesmosome expression in intraductal carcinoma. We noted hemidesmosomes in the basal cells by electron microscopy; however, by immunofluorescence there were many regions in which the M_r 200 protein and $\beta 4$ integrin were not expressed by basal cells. This suggests that even in intraductal carcinoma, the apparently ultrastructurally normal hemidesmosomes may actually be abnormal, and could be functionally impaired.

In addition, cells piled up within ducts and away from the basement membrane had no hemidesmosomes or staining for any component proteins. Abnormal downregulation of hemidesmosomes may allow these malignant cells to leave the basement membrane. Alternately, if the basement membrane regulates hemidesmosome expression, cells that have left the basement membrane with hemidesmosomes intact may then downregulate hemidesmosome expression as the normal response to a loss of contact with the basement membrane. This brings up the cause and effect question of hemidesmosome and basement membrane expression: does the basement membrane regulate hemidesmosome expression or vice versa?

Most wound healing studies show that in epithelial cells that have migrated to fill in a wound, hemidesmosomes reform before or simultaneously with basement membrane

(35, 47-53). In fact, basement membrane initially reforms discontinuously beneath hemidesmosomes as if the hemidesmosomes nucleate basement membrane formation (42, 54, 55). On the other hand, in embryogenesis, expression of basement membrane precedes hemidesmosome expression (for example, 56-58).

It is not clear which of these models best reflects the situation in the normal breast or in breast cancer. In our study there appeared to be a correlation between localization of a cell at the basement membrane and its expression of hemidesmosomes. Therefore the basement membrane could be regulating hemidesmosome expression. However, some cells at the basement membrane did not express every hemidesmosome protein, and some cells away from the basement membrane did express some hemidesmosome proteins. We therefore feel that the fact that expression of hemidesmosome proteins did not absolutely follow expression of basement membrane proteins suggests that the lack of hemidesmosomes in invasive breast cancer was not merely a result of lack of basement membrane, but rather an important malignant phenomenon in its own right. In addition, Wetzels, et al. (59) found, as we did, that collagen VII was lost from almost all (94 of 97) invasive ductal carcinomas, but 13 of these retained staining for basement membrane proteins, suggesting that loss of this hemidesmosome protein was not a result of loss of basement membrane protein.

In addition to in vivo hemidesmosome expression, we noted expression of hemidesmosomes in human breast epithelial cells in culture. Most groups that have reported previously on cells cultured on plastic or glass have seen either no hemidesmosomes by electron microscopy, or "prehemidesmosomes" or "immature hemidesmosomes" (Reviewed in 60, 61). Recently, however, a few rat and bovine cell

lines able to make mature hemidesmosomes have been reported (for example 10, 61, 62). In this paper we report that our normal primary human cells form hemidesmosomes in culture containing the anticipated protein components. This presents these breast epithelial cell strains as good models for study of the regulation of hemidesmosome formation.

In addition, we observed that several malignant breast cell strains lacked hemidesmosomes by electron microscopy. These cells may be used in the future to study the aberrant regulation of hemidesmosome formation in cancer or to reveal clues to requirements for normal hemidesmosome assembly. Some malignant cells in culture, however, did express hemidesmosomes. This may reflect the heterogeneity of the tissues from which the cells were derived. For example, in cells grown from one tumor, some cells were seen with and others without hemidesmosomes by electron microscopy; these cells could represent intraductal and invasive cells respectively. In the one cell strain where we saw a normal complement of hemidesmosomes, only intraductal cells may have grown; and in the three cell strains with no hemidesmosomes, only invasive cells may be represented.

Likewise malignant cells in culture had a more normal array of hemidesmosome protein expression by immunofluorescence than did malignant cells in vivo. As mentioned above, it is possible that the cells grown in culture were not fully representative of those found in vivo. For example, if a majority of the cells in culture were derived from the intraductal portion of carcinomas, a more normal phenotype would be observed.

A more interesting explanation is that hemidesmosome proteins that are not

expressed in vivo can be reexpressed by such cells when placed in a different milieu in culture. This suggests that the ability to express hemidesmosome proteins may not necessarily be lost by malignant cells even in vivo, but rather, hemidesmosome proteins may be downregulated in vivo by a whole program of altered differentiation which is partially reexpressed in culture. This was also suggested by the fact that malignant cells in vivo had such a complete change in hemidesmosome protein expression, when a loss of only one protein might have been expected if the loss were due to a mutation in that gene. It is further supported by the fact that collagen VII, which is eventually expressed basally in malignant cells in culture as in normal cells, exhibits a delayed conversion to the normal phenotype from that of intracellular expression. However, the fact that the M_r 200 protein remained abnormal in culture suggests the possibility of a permanent change in expression of this particular protein. Therefore, both the regulation of hemidesmosome proteins and some of the proteins themselves may be abnormal in breast cancer. This hypothesis will have to be tested further in subsequent studies.

As mentioned above, malignant breast cells in culture did exhibit some abnormalities of hemidesmosome protein expression by immunofluorescence: the M_r 200 protein and collagen VII were seen intracellularly. This localization is reminiscent of wound healing, in which hemidesmosome proteins are also expressed intracellularly by epithelial cells migrating to close a wound (63). Cancer invasion has been compared to wound healing, and internalization of hemidesmosome components could be a common mechanism by which epithelial cells become migratory.

In conclusion, in this paper we note the presence of hemidesmosomes in normal human mammary epithelial cells both in vivo and in culture containing the expected

proteins, and multiple abnormalities of hemidesmosome expression in malignant cells. These suggest that normal HMEC may use hemidesmosomes to maintain their position in the mammary duct, and that malignant cells may use downregulation of these structures as a means to escape their usual tissue architectural restraints. In addition, strains of normal primary breast epithelial cells which express hemidesmosomes in culture and primary malignant breast cells without hemidesmosomes could be useful models for studying normal and abnormal regulation of hemidesmosome formation.

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FIGURE LEGENDS

Fig. 1 The presence of hemidesmosomes in normal breast epithelium by electron microscopy.

A. A normal duct in cross section. B. Higher magnification of box in "A" shows hemidesmosomes (arrows) at the bases of ductal epithelial cells. C. Higher magnification of a hemidesmosome showing intermediate filaments (IF, curved arrows), anchoring filaments (arrow) and anchoring fibrils (arrowheads along the length). LL=lamina lucida, LD=lamina densa. D. A luminal cell reaching from lumen to basement membrane containing basal hemidesmosomes (arrows). E. Higher magnification of the box in "D" showing hemidesmosomes (arrows). L=lumen, E=epithelial cell, F=fibroblast, ECM=extracellular matrix.

Fig. 2 Presence of hemidesmosomes in breast carcinoma in situ by electron microscopy.

A. An intraductal carcinoma showing many layers of cells within a duct with very little lumen (L) but surrounded by a basement membrane (BM). B. Higher magnification of the box in "A" shows multiple hemidesmosomes (arrows). C=carcinoma cell, ECM=extracellular matrix, F=fibroblast.

Fig. 3 Absence of hemidesmosomes in invasive breast carcinoma.

A. A group of invasive cells is pictured. Note the absence of a basement membrane at the epithelial:extracellular matrix border (curved arrows), presence of desmosomes (D, straight arrows). A cell, apparently at the invasive front, is surrounded by extracellular

matrix on three sides (box). C=carcinoma cell, ECM=extracellular matrix, F=fibroblast, P=pseudolumen. B. Higher magnification of the box in "A" shows two invasive cells (1 and 2) abutting the extracellular matrix (ECM) without any hemidesmosomes. C. Higher magnification of box in "B" shows cells (1 and 2) with cell membranes in direct contact with collagen (arrows) without any intervening basement membrane or hemidesmosomes.

Fig. 4 Expression of the anchoring fibril protein, collagen VII in normal duct and carcinoma in situ, but not invasive carcinoma by immunofluorescence.

A-C. A normal duct in cross section (A) has a basement membrane as determined by laminin staining (B) and expresses basal collagen VII (C). D-F. An intraductal carcinoma (D) has a basement membrane around the entire group of cells (E) and exhibits collagen VII staining (F) only at the basement membrane (arrowheads) whereas cells piled up within the duct do not exhibit collagen VII staining (arrow, compare with D, arrow). G-I. Invasive carcinoma (G), outlined by arrowheads, does not have a basement membrane (H) or express collagen VII (I). Ln=laminin, C VII=collagen VII, L=lumen.

Fig. 5 Expression of the M_r 180 bullous pemphigoid antigen and hemidesmosome protein by immunofluorescence.

A. Normal ducts in cross section show basal M_r 180 staining. B. In regions of intraductal carcinoma, staining for the M_r 180 protein outlines ducts from most patients. C. In one patient, no M_r 180 protein staining is seen even in regions of carcinoma in situ. D, E. Phase contrast photographs of "B" and "C" respectively.

Fig. 6 Expression of the M_r 200 hemidesmosome protein does not follow the basement membrane by immunofluorescence.

A. Negative control for immunofluorescent staining. B, C. A normal duct in cross section has a basement membrane illustrated by collagen IV staining (B) and expresses basolateral M_r 200 protein (C). D-F. An intraductal carcinoma in longitudinal section (D) has a basement membrane (E, arrowheads), but no M_r 200 protein staining (F). G-I. Invasive carcinoma (G) exhibits intracellular basement membrane protein staining (H), and faint M_r 200 protein staining surroundidn each cell (I). C IV=collagen IV.

Fig. 7 Electron microscopy of normal and malignant human mammary epithelial cells in culture.

A. A normal cell has basal hemidesmosomes (arrows). B. A malignant cell has no hemidesmosomes. C. Higher magnification of box in "A". D. Higher magnification of box in "B".

Fig. 8 Delayed expression of the anchoring fibril protein, collagen VII, in cultured malignant human mammary epithelial cells by immunofluorescence.

A. Normal cultured human mammary epithelial cells at one day show an intracellular staining pattern for collagen VII. B. Malignant human mammary epithelial cells do not express collagen VII at one day. C. By seven days normal cells stain for collagen VII at the base of cells in a secreted pattern. D. At seven days malignant cells show an intracellular staining pattern for collagen VII.

Fig. 9 Aberrant expression of the M_r 200 hemidesmosome protein in cultured malignant human mammary epithelial cells by immunofluorescence.

A. Normal cells express the M_r 200 protein in rows of basal tick mark-shaped plaques.

B. Malignant cells show a dotted intracellular M_r 200 protein staining pattern.

Fig. 10 Identical expression of the M_r 180 hemidesmosome protein in normal and malignant cultured human mammary epithelial cells.

Both normal (A) and malignant (B) cells express the M_r 180 protein in rows of basal tick mark-shaped plaques.

REFERENCES

1. Schwartz, M. A. Signaling by integrins: implications for tumorigenesis. Cancer Res., *53*: 1503-1506, 1993.

2. McIntyre, B. W., Bednarczyk, J. L., Passini, C. A., Szabo, M. C., Udagawa, T., and Wygant, J. N. Integrins: cell adhesion receptors in health and disease. Cancer Bull. *43*: 51-57, 1991.

3. Brodt, P., Fallavollita, L., Sawka, R. J., Shibata, P., Nip, J., Kim, U., and Shibata, H. Tumor cell adhesion to frozen lymph node sections - a correlate of lymphatic metastasis in breast carcinoma models of human and rat origin. Breast Cancer Res. and Treatment *17*: 109-120, 1990.

4. Hand, P. H., Thor, A., Schlom, J., Rao, C. N., and Liotta, L.. Expression of laminin receptor in normal and carcinomatous human tissues as defined by a monoclonal antibody. Cancer Res.*45*: 2713-2719, 1985.

5. Bergstraesser, L. M., and Weitzman, S. A. Alterations in integrin and basement membrane protein expression by malignant breast cells. Int J. Oncology *4*: 915-930, 1994.

6. Pignatelli, M., Cardillo, M. R., Hanby, A., and Stamp, G. W. H. Integrins and their accessory adhesion molecules in mammary carcinomas: loss of polarization in poorly differentiated tumors. Hum Pathol. *23*: 1159-1166, 1992.

7. D'Ardenne, A. J., Richman, P. I., Horton, M. A., McAulay, A. E., and Jordan, S. Co-ordinate expression of the alpha-6 integrin laminin receptor sub-unit and laminin in breast cancer. J. Path. *165*: 213-220, 1991.

8. Koukoulis, G. K., Virtanen, I., Korhonen, M., Laitinen, L., Quaranta, V., and Gould, V. E. Immunohistochemical localization of integrins in the normal, hyperplastic, and neoplastic breast. Am J. Pathol. *139*: 787-799, 1991.

9. Zutter, M. M., Mazoujian, G., and Santoro, S. A. Decreased expression of integrin adhesive protein receptors in adenocarcinoma of the breast. Am. J. Pathol. *137*: 863-870, 1990.

10. Owaribe, K., Kartenbeck, J., Stumpp, S., Magin, T. M., Krieg, T., Diaz, L., and Franke, W. W. The hemidesmosomal plaque. I. Characterization of a major constituent protein as a differentiation marker for certain forms of epithelia. Differentiation *45*: 207-220, 1990.

11. Jones, J. C. R., Asmuth, J., Baker, S. E., Langhofer, M., Roth, S. I., and Hopkinson, S. B. Hemidesmosomes: extracellular matrix/intermediate filament

connectors. Exp. Cell Res. *213*: 1-11, 1994.

12. Legan, P. K., Collins, J. E., and Garrod, D. R. The molecular biology of desmosomes and hemidesmosomes: 'what's in a name?' BioEssays *14*: 385-393, 1992.

13. Schwarz, M. A., Owaribe, K., Kartenbeck, J. and Franke, W. W.. Desmosomes and hemidesmosomes: constitutive molecular components. Annu. Rev. Cell Biol. *6*: 461-491, 1990.

14. Mutasim, D. F., Takahashi, Y., Labib, R. S., Anhalt, G. J., Patel, H. P., and Diaz, L. A. A pool of BP antigens is intracellular and associated with the basal-cell cytoskeleton-hemidesmosome complex. J. Invest. Dermatol. *84*: 47-53, 1985.

15. Carter, W. G., Kaur, P., Gil, S. F., Gahr, P. J., and Wayner, E. A. Distinct functions for integrins $\alpha 3\beta 1$ in focal adhesions and $\alpha 6\beta 4$/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. J. Cell Biol. *111*: 3141-3154, 1990.

16. McNutt, N. S.. Ultrastructural comparison of the interface between epithelium and stroma in basal cell carcinoma and control human skin. Lab. Invest. *35*: 132-142, 1976.

17. Jones, J. C. R., Steinman, H. K., and Goldsmith, B. A.. Hemidesmosomes, collagen VII, and intermediate filaments in basal cell carcinoma. J. Invest. Dermatol. *93*: 662-671,

1989.

18. Jones, J. C. R., Kurpakus, M. A., Cooper, H. M., and Quaranta, M. A function for the integrin alpha 6 beta 4 in the hemidesmosome. Cell Reg. *2*: 427-438, 1991.

19. Stepp, M. A., Spurr-Michaud, S., Tisdale, A., Elwell, J., and Gipson, I. K. $\alpha 6\beta 4$ integrin heterodimer is a component of hemidesmosomes. Proc. Natl. Acad. Sci. USA *87*: 8970-8974, 1990.

20. Sonnenberg, A., Calafat, J., Janssen, H., Daams, H., van der Raaij-Jelmer, L. M. H., Falcioni, R., Kennel, S. J., Aplin, J. D., Baker, J., Loizidou, M., and Garrod, D. Integrin $\alpha 6/\beta 4$ complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. J. Cell Biol. *113*: 907-917, 1991.

21. Natali, P. G., Nicotra, M. R., Botti, C., Mottolese, M., Bigotti, A., and Segatto, O. Changes in expression of $\alpha 6\beta 4$ integrin heterodimer in metastatic breast cancer. Br. J. Cancer *66*: 318-322, 1992.

22. Falcioni, R., Sacchi, A., Resau, J. and Kennel, S. J. Monoclonal antibody to human carcinoma-associated protein complex: quantitation in normal and tumor tissue. Cancer Res. *48*: 816-821, 1988.

23. Tannenbaum, M., Weiss, M., and Marx, A. J. Ultrastructure of the human mammary

ductule. Cancer *23*: 958-978, 1969.

24. Ozzello, L., and Sanpitak, P. Epithelial-stromal junction of intraductal carcinoma of the breast. Cancer *26*: 1186-1198, 1970.

25. Stirling, J., and Chandler, J. A. The fine structure of the normal, resting terminal ductal-lobular unit of the female breast. Virchows Arch. A. Path. Anat. and Histol.*372*: 205-226, 1976.

26. Franke, W. W., Schmid, E., Freudenstein, C., Appelhans, B., Osborn, M., Weber, K., and Keenan, T. W. Intermediate-sized filaments of the prekeratin type in myoepithelial cells. J. Cell Biol. *84*: 633-654, 1980.

27. Williams, J. M., and Daniel, C. W. Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. Devel. Biol. *97*: 274-290, 1983.

28. Watson, R. J., Eyden, B. P., Howell, A., and Sellwood, R. A. Ultrastructural observations on the basal lamina in the normal human breast. J. Anat. *156*: 1-10, 1988.

29. Stampfer, M. R. Isolation and growth of human mammary epithelial cells. J. Tissue Culture Meth. *9*: 107-115, 1985.

30. Bergstraesser, L. M., and Weitzman, S. A. Culture of normal and malignant primary human mammary epithelial cells in a physiological manner simulates in vivo growth patterns and allows discrimination of cell type. Cancer Res *53*: 2644-2654, 1993.

31. Klatte, D. H., Kurpakus, M. A., Grelling, K. A., and Jones, J. C. R. Immunochemical characterization of three components of the hemidesmosome and their expression in cultured epithelial cells. J. Cell Biol. *109*: 3377-3390, 1989.

32. Riddelle, K. S., Hopkinson, S. B., and Jones, J. C. R. Hemidesmosomes in the epithelial cell line 804G: their fate during wound closure, mitosis and drug induced reorganization of the cytoskeleton. J. Cell Sci. *103*: 475-490, 1992.

33. Hopkinson, S. B., Riddelle, K. S., and Jones, J. C. R. Cytoplasmic domain of the 180-kD bullous pemphigoid antigen, a hemidesmosomal component: molecular and cell biologic characterization. J. Invest. Dermatol. *99*: 264-270, 1992.

34. Kurpakus, M. A., and Jones, J. C. R. A novel hemidesmosomal plaque component: tissue distribution and incorporation into assembling hemidesmosomes in an *in vitro* model. Exp. Cell Res. *194*: 139-146, 1991.

35. Kurpakus, M. A., Stock, E. L., and Jones, J. C. R. Analysis of wound healing in an *in vitro* model: early appearance of lamininand a 125 x 10^3 M_r polypeptide during adhesion complex formation. J. Cell Sci. *96*: 651-660, 1990.

36. Hyat, M. A., Ed. *Basic Techniques for Transmission Electron Microscopy*. Academic Press Inc, 1986.

37. Owaribe, K., Mishizawa, Y., and Franke, W. W. Isolation and characterization of hemidesmosomes from bovine corneal epithelial cells. Exp Cell Res. *192*: 622-630, 1991.

38. Robinson, H. N., Anhalt, G. J., Patel, H. P., Takahashi, Y., Labib, R. S., and Diaz, L. A. Pemphigus and pemphigoid antigens are expressed in human amnion epithelium. J. Invest. Dermatol. *83*: 234-237, 1984.

39. Clermont, Y., Xia, L., Turner, J. D., and Hermo, L. Striated anchoring fibrils-anchoring plaque complexes and their relation to hemidesmosomes of myoepithelial and secretory cells in mammary glands of lactating rats. Anat. Record *237*: 318-325, 1993.

40. Ormerod, E. J., and Rudland, P. S. Regeneration of mammary glands *in vivo* from isolated mammary ducts. J. Embryol. Exp. Morph. *96*: 229-243, 1986.

41. Khodadoust, A. A., Silverstein, A. M., Kenyon, K. R., and Dowling, J. E. Adhesion of regenerating corneal epithelium. Am J. Opthalmol. *65*: 339-348, 1968.

42. Briggaman, R. A., and Wheeler, C. E. The epidermal-dermal junction. J. Invest. Dermatol *65*: 71-84, 1975.

43. Epstein, E. H. Molecular genetics of epidermolysis bullosa. Science *256*: 799-804, 1992.

44. Verrando, P., Blanchet-Bardon, C., Pisani, A., Thomas, L., Cambazard, F., Eady, R. A. J., Schofield, O., and Ortonne, J.-P. Monoclonal antibody GB3 defines a widespread defect of several basement membranes and a keratinocyte dysfunction in patients with lethal junctional epidermolysis bullosa. Lab. Invest. *64*: 85-92, 1991.

45. Woodley, D. T., Burgeson, R. E., Lunstrum, G., Bruckner-Tuderman, L., Reese, M. J., and Briggaman, R. A. Epidermolysis bullosa acquisita antigen is the globular carboxyl terminus of type VII procollagen. J. Clin. Invest. *81*: 683-687, 1988.

46. Stanley, J. R. Cell adhesion molecules as targets of autoantibodies in pemphigus and pemphigoid, bullous diseases due to defective epidermal cell adhesion. Advances in Immunol. *53*: 291-325, 1992.

47. Kurpakus, M. A., Quaranta, V., and Jones, J. C. R. Surface relocation of alpha6beta4 integrins and assembly of hemidesmosomes in an *in vitro* model of wound healing. J. Cell Biol. *115*: 1737-1750, 1991.

48. Croft, C. B., and Tarin, D. Ultrastructural studies of wound healing in mouse skin. I. Epithelial behaviour. J. Anat. *106*: 63-77, 1970.

49. Hintner, H., Fritsch, P. O., Fiodart, J.-M., Stingl, G., Schuler, G., and Katz, S. I. Expression of basement membrane zone antigens at the dermo-epibolic junction in organ cultures of human skin. J. Invest. Derm. *74*: 200-204, 1980.

50. Stanley, J. R., Alvarez, O. M., Bere, E. W., Eaglestein, W. H., and Katz, S. I. Detection of basement membrane zone antigens during epidermal wound healing in pigs. J. Invest. Dermatol. *77*: 240-243, 1981.

51. Woodley, D. T., O'Keefe, E. J., and Prunieras, M. Cutaneous wound healing: A model for cell-matrix interactions. J. Am. Acad. Dermatol. *12*: 420, 1985.

52. Sciubba, J. J. Regeneration of the basal lamina complex during epithelial wound healing. J. Periodont. Res. *12*: 204-217, 1977.

53. Gipson, I. K., Spurr-Michaud, S., Tisdale, A., and Keough, M. Reassembly of the anchoring structures of the corneal epithelium during wound repair in the rabbit. Invest. Opthalmol. Vis. Sci. *30*: 425-434, 1989.

54. Briggaman, R. A., Dalldorf, F. G., and Wheeler, C. E. Formation and origin of basal lamina and anchoring fibrils in adult human skin. J. Cell Biol. *51*: 384-395, 1971.

55. Akimoto, Y., Obinata, A., Endo, H., and Hirano, H. Reconstruction of basement membrane in recombinants of epidermis and dermis of chick embryonic skin in vitro:

an electron microscopic study. Anat. Record *231*: 375-382, 1991.

56. Fine, J.-D., Smith, L. T., Holbrook, K. A., and Katz, S. I. The appearance of four basement membrane zone antigens in developing human fetal skin. J. Invest. Derm. *83*: 66-69, 1984.

57. Gipson, I. K., Spurr-Michaud, S. J., and Tisdale, A. S. Hemidesmosomes and anchoring fibril collagen appear synchronously during development and wound healing. Devel. Biol. *126*: 253-262, 1988.

58. Smith, L. T., Sakai, L. Y., Burgeson, R. E., and Holbrook, K. A. Ontogeny of structural components at the dermal-epidermal junction in human embryonic and fetal skin: the appearance of anchoring fibrils and type VII collagen. J. Invest. Dermatol. *90*: 480-485, 1988.

59. Wetzels, R. H. W., Robben, H. C. M., Leigh, I. M., Schaafsma, H. E., Vooijs, G. P., and Ramaekers, F. C. S. Distribution patterns of type VII collagen in normal and malignant human tissues. Am J. Pathol. *139*: 451-459, 1991.

60. Thacher, S. M., Malone, K. L., Dave, K., and Zhao, S. Localization of the 230-kilodalton bullous pemphigoid antigen in cultured keratinocytes: formation of a prehemidesmosome. Exp. Cell Res. *194*: 238-247, 1991.

61. Riddelle, K. S., Green, K. J., and Jones, J. C. R. Formation of hemidesmosomes in vitro by a transformed rat bladder cell line. J. Cell Biol. *112*: 159-168, 1991.

62. Hieda, Y., Nishizawa, Y., Uematsu, J., and Owaribe, K. Identification of a new hemidesmosomal protein, HD1: a major, high molecular mass component of isolated hemidesmosomes. J. Cell Biol. *116*: 1497-1506, 1992.

63. Gipson, I. K., Spurr-Michaud, S., Tisdale, A., Elwell, J., and Stepp, M. A. Redistribution of the hemidesmosome components $\alpha 6\beta 4$ integrin and bullous pemphigoid antigens during epithelial wound healing. Exp. Cell Res. *207*: 86-98, 1993.