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Induction Of Epithelialization And Suppression Of Tumorigenicity In An Aggressive Carcinoma By Protein Zero, A Nervous System IgCAM

Lisa Beth Spiryda

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

Induction Of Epithelialization And Suppression Of Tumorigenicity In An Aggressive Carcinoma By Protein Zero, A Nervous System IgCAM

By

Lisa Beth Spiryda

Advisor: David R. Colman, Ph.D.

HeLa is an aggressive cervical carcinoma that lacks the features of a normal epithelium. It proliferates in an anchorage-independent manner, secretes high amounts of matrix degrading enzymes, and is highly invasive and tumorigenic. In the peripheral nervous system, Protein Zero (P_0), a homophilic IgCAM, mediates self-adhesion of Schwann cell membranes as they enwrap axons and generate compact myelin. P_0 expression in HeLa led to ultrastructural rearrangements and biochemical changes consistent with epithelial junction formation suggesting that this carcinoma morphologically reverted to an epithelial-like phenotype.

My thesis was directly focused on determining the functional significance of these morphologic changes. I found that P_0 expression not only triggered the normal morphological features found in epithelia, but also re-engaged the characteristic physiology of epithelia. Constitutive P_0 expression in HeLa cells restores several important aspects of normal epithelial cell physiology, including functional tight junctions, cell polarity, contact inhibition, and adhesion-mediated growth control. Of greatest interest, HeLa cells constitutively expressing P_0 did not form tumors in athymic nude mice. This IgCAM elicits an inherent but dormant or "sluggish" intracellular pathways which, when activated, triggers epithelialization and the suppression of the tumorigenic and transformed properties of this cervical carcinoma cell line. Since N-cadherin, plakoglobin, α-catenin and β-catenin were significantly upregulated in the P_0-HeLa cells, it appeared that P_0 mediated

epithelialization in HeLa through activation of N-cadherin/catenin signaling systems. I showed that the expression of N-cadherin in HeLa also restored the morphological and physiologic characteristic of epithelia. Additionally, in this cervical carcinoma, E-cadherin expression could substitute for N-cadherin and elicited an identical sequence of events leading to complete reversion of tumorigenicity.

By all these criteria, P_0 expression appears to augment functional epithelial junction formation and efficiently suppress long term, the transformed state of this carcinoma cell line. It can be concluded that the forced expression of *bona fide* adhesion molecules, such as P_0, may serve as "upstream" inducers of an essentially dormant but undamaged cadherin-based adhesion program in carcinoma cells that ultimately triggers them to regain and maintain normal epithelial characteristics thereby suppressing tumorigenicity.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

Overview Of Adhesion Molecules

Cell adhesion molecules (CAMs) are found ubiquitously in multicellular organisms and are involved in almost every aspect of development and maintenance of tissue architecture. CAMs are usually divided into five different families according to structural and sequence similarity: the immunoglobulin gene superfamily (IgCAMs), integrins, cadherins, selectins, and tetraspan proteins. Although most cell to cell interactions mediated by CAMs operate by binding to partners within the same subfamily (homophilic interactions), there are some important exceptions where CAMs can bind heterophilically and interact with other members of their family as well as different families. For example, integrins can bind to certain IgCAMs and cadherins; selectins mediate adhesion by interacting with proteins and lectins modified by particular carbohydrates. Together, CAMs exert influences which guide complicated events, for example segregation of different cell layers in the embryo, compaction of myelin in the nervous system, and the maintenance and communication between epithelial cells within an organ. Improper expression and mutations of these molecules appear to hasten the development of many pathologies and in particular, neoplastic transformation.

Cadherin Superfamily

The cadherin superfamily is a large and diverse family consisting of three subgroups - the classic cadherins, desmosomal cadherins, and the protocadherins (Geiger and Ayalon, 1992; Sano et al., 1993; Koch and Franke, 1994; Suzuki, 1996b). These proteins exhibit calcium dependent homophilic cellular adhesion. All subgroups of the cadherin superfamily share one common feature -- an ectodomain consisting of multiple repeating units or cadherin motifs. The individual subgroups differ in the specific repeat features, in particular in sequence and number of repeating units which determines the adhesive activities specific to each cadherin. Cadherin subgroups show the most sequence

divergence in their intracellular domains and therefore associate with different cytoplasmic proteins and can engage different signaling pathways. Members of the cadherin superfamily are found in virtually every tissue type and are involved in a variety of physiologic processes (Takeichi, 1991; Geiger and Ayalon, 1992; Ranscht, 1994; Suzuki, 1996b; Redies, 1997).

Classic Cadherins

The classic cadherins were the first members to be described and were named for the cell type in which they were initially isolated (e.g. E-cadherin - epithelial cells, N-cadherin - neural tissue) (Takeichi et al., 1988). Now it is known that the expression pattern of individual cadherins is complex and can encompass multiple tissues. Several cadherins may be co-expressed within a single tissue, although not normally by the same cell. For example, E-cadherin is expressed in epithelial tissue and in neuronal tissue and N-cadherin is detected in muscle tissue, endothelial cells and neural cells. All family members exhibit high degrees of sequence homology amongst each other and across species (Grunwald, 1993).

The cadherins are Type I integral membrane surface glycoproteins (120-140 kD). The extracellular segment contains the intercellular adhesive component and is divided into five cadherin repeat domains (EC1-EC5) (Grunwald, 1993). High resolution crystal structure and NMR analyses of EC1 of N-cadherin or E-cadherin as well as the structures of EC1-EC2 of either of these proteins suggests that each of the cadherin subunits fold as an Ig-like loop with Ca^{++} binding sites located between the EC domains (Overduin et al., 1995; Shapiro et al., 1995a: Nagar et al., 1996; Tamura et al., 1998). Once the appropriate number of Ca^{++} ions articulate with their corresponding binding sites, rigidity is conferred to the ectodomain (Koch et al., 1997).

X-ray crystallography studies predict that cadherins emanate from cell surfaces as either monomers or strand dimers (Overduin et al., 1995; Shapiro et al., 1995a; Nagar et

al., 1996). It has been speculated that perhaps the monomeric cadherins are inactive in adhesion, but may be recruited to the active strand dimer form that confers strong adhesion by interacting with strand dimers from opposing plasma membranes to form a continuous adhesive zipper (Shapiro et al., 1995a; Shapiro et al., 1995b; Tamura et al., 1998). In support of this model, electron microscopic images of cadherin-based junctions show that their intercellular spaces contain electron dense material suggestive of interacting cadherin ectodomains (Farquhar and Palade, 1963; McNutt, 1970). Additionally, it has been shown that lateral clustering and dimerization of cadherin ectodomains leads to increased overall adhesion (Brieher et al., 1996; Yap et al., 1997) even in the absence of the cytoplasmic region.

The intracellular carboxyl region is the most conserved domain of the classic cadherins. It interacts with the actin network via the catenins, polypeptides that mediate intracellular signaling (Geiger and Ayalon, 1992; Grunwald, 1993) and regulate adhesive properties (Nagafuchi and Takeichi, 1988) as will be discussed later. If the intracellular domain is deleted, the cadherin is rendered non-functional and cannot mediate tight adhesion (Nagafuchi and Takeichi, 1988). Adhesion of a truncated cadherin can only be enhanced through clustering of the ectodomains. One member of this family, T(truncated)-cadherin is glycosyl-phophatidylinositol (GPI-) linked to the lipid bilayer. Although T-cadherin lacks a cytoplasmic tail, it is still capable of mediating strong cell to cell adhesion suggesting that it may associate with the cytoskeleton through auxiliary proteins within the lipid bilayer to help exert adhesion (Vestal and Ranscht, 1992).

Cadherins influence fundamental cellular functions essential for embryonic development. E-cadherin is the first cadherin expressed in the single cell and preimplantation stage mouse embryos and plays a critical role in early morula stage embryos during compaction, one of the first polarization events during embryonic development (Riethmacher et al., 1995). Embryos lacking E-cadherin cannot develop past this stage and die. Segregation and remodeling of embryonic tissue is also highly

dependent on the differential expression of cadherins. Initially, E-cadherin is expressed in all three germ layers, but is selectively lost from mesoderm as these cells gain mesenchymal characteristics in chick embryos (Hatta and Takeichi, 1986). E-cadherin expression is also lost from the dorsal ectodermal region as it is induced to form the neural plate and the cells begin to express N-cadherin (Hatta and Takeichi, 1986). These differences in cadherin expression allow for cell movement and further development of a particular tissue and organs in the fetus. Mouse embryos lacking N-cadherin die at nine days and exhibit severe defects in neurulation, somatogenesis, and development of the myocardium because N-cadherin is critical for the organization and differentiation of the nervous, muscular and cardiovascular systems (Cifuentes Diaz et al., 1994; Linask et al., 1997; Nakagawa and Takeichi, 1997).

The fully mature organism requires cadherins to maintain these distinct tissue types and the architecture of organs. In epithelia for example, E-cadherin complexes with catenins and forms adherens junctions which mediate adhesion between epithelial cells and anchor the cells to the actin cytoskeleton. E-cadherin induced adhesion is believed to be the initiator and mediator of cell aggregation which leads to the organization of intercellular junctions that ultimately define the polarized and adhesive phenotype of epithelial cells (Gumbiner et al., 1988; Nelson, 1989; Rodriguez-Boulan and Nelson, 1989; McNeill et al., 1990; Geiger and Ayalon, 1992; Wollner et al., 1992; Watabe et al., 1994; Amagai et al., 1995; Angres et al., 1996). It has been shown that E-cadherin expression regulates the formation of desmosomes and expression of integrins during stratification of keratinocytes (Lewis et al., 1994; Amagai et al., 1995) and is required for the organization of the cytoskeleton in epithelia (Nelson, 1992). Furthermore E-cadherin has been shown to mediate the formation of gap junctions and their subsequent conductance in liver cells (Jongen et al., 1991). If E-cadherin expression is disrupted, epithelial junctions and cell polarity will be disrupted (McNeill et al., 1990). In Schwann cells, E-cadherin is expressed exclusively in regions of non-compacted myelin in association with catenins

(Fannon et al., 1996). These complexes mediate adhesion by forming adherens-like junctions between plasma membranes elaborated by a single Schwann cell. Since these junctions mediate interactions between membranes of a single cell, they have been termed "autotypic" adherens-type junctions (Fannon et al., 1996). It has been proposed that E-cadherin may organize and target myelin proteins into their appropriate domains which may lead to the polarization of the Schwann cells (Fannon et al., 1996). More recently, N-cadherin has also been proposed to have a role in organizing junctions (Fannon and Colman, 1996; Uchida et al., 1996). Our laboratory has found that N-cadherin is frequently associated with synaptophysin within synaptic junctions throughout the CNS of adult mice (Fannon and Colman, 1996). Others have found that two cadherin-associated proteins, αN-catenin and β-catenin also co-localize with N-cadherin and synaptophysin (Uchida et al., 1996). This raises the intriguing possibility first proposed on the basis of electron microscopy (Ellisman, 1987; Ichimura and Hashimoto, 1988; Peters et al., 1991) that synaptic junctions may be homologous structures to the adherens junctions. N-cadherin may play a role in initiating and organizing synaptic junctions in the same way E-cadherin mediates junction formation in epithelial tissue and possibly in Schwann cells.

Aberrant expression of the classic cadherins, in particular E-cadherin, plays a leading role in the pathogenesis and metastasis of carcinoma cells (Geiger and Ayalon, 1992). It has been repeatedly shown that as tumors lose E-cadherin expression, they also lose features of normal epithelial organization. The role of cadherins in the transition from normal epithelium to carcinomatous phenotype will be discussed in greater detail below.

Desmosomal Cadherins

Desmosomes are disc-shaped cell adhesion junctions that bring cell membranes within 20 - 50 nm of each other. These junctions are found in several cell and tissue types including epithelial cells, myocardial cells and Purkinje fiber cells of the heart, and follicular dendritic cells of the lymph nodes. These junctions aid in cell coupling as well as

providing mechanical strength in cell and tissue architecture due by stabilizing associations with intermediate filaments. The adhesive components of the desmosomes are desmosomal cadherins - desmocollin and desmoglein - which form a subclass within the cadherin superfamily. There are at least three genotypically distinct forms of desmoglein (Dsg1, Dsg2, Dsg3) and desmocollin (Dsc1, Dsc2, Dsc3). The distribution of each can vary according to tissue, particular cell type and position within a stratified epithelium (Schmidt et al., 1994; Garrod, 1995; Garrod et al., 1996; Green and Jones, 1996). The extracellular domains of these transmembrane proteins are arranged into five cadherin-like subdomains with EC1 to EC4 showing the highest sequence similarity to the classic cadherins (Koch and Franke, 1994). The cytoplasmic domain is the most conserved region among the desmosomal proteins and it is the least similar to classic cadherins. This domain attaches to intermediate filaments via desmoplakin and other plaque proteins (e.g., plakoglobin) (Koch and Franke, 1994). In addition to this conserved domain, there are also some variations among the desmosomal cadherins. There are two splice isoforms of desmocollin differing in length of their cytoplasmic domain - spice variant a is 11 amino acids longer than variant b; it has been shown that these particular amino acids are absolutely required for desmosome assembly and have been shown to bind plakoglobin (Troyanovsky et al., 1993; Koch and Franke, 1994; Troyanovsky et al., 1994). The desmogleins do not have splice variants that differ in carboxyl terminal region; each desmoglein type has a varying number of repetitive sequence elements of 29 amino acids -- Dsg1 has five, Dsg2 has 6 and Dsg3 contains two of these motifs; the exact function of these motifs is not known (Koch and Franke, 1994).

It has been shown that when a full length clone of desmoglein or desmocollin are expressed separately or together in non-adhesive L-cells that are devoid of classic cadherins, they are not sufficient to confer cell adhesion (Amagai et al., 1994; Chidgey et al., 1996; Kowalczyk et al., 1996). The additional expression of classic cadherins is required to induce heterophilic interactions between desmocollin and desmoglein that

ultimately lead to desmosomal assembly and adhesion (Chitaev and Troyanovsky, 1997). If classic cadherin expression is disrupted (by blocking antibodies or expression of a dominant negative form), both adherens junctions and desmosomes will be abrogated (Lewis et al., 1994; Amagai et al., 1995) even though the desmosomes are not composed of classic cadherins. If E-cadherin is expressed in a retinal pigment cell line that is devoid of desmosomes, desmoglein mRNA and protein levels increase and desmosomes assemble. These studies provide evidence that the assembly of adherens junctions and desmosomes are tightly regulated and may be mediated by plakoglobin which is common both desmosomes and adherens junctions (Lewis et al., 1997). Additionally, it has been shown that co-expression of desmocollin, desmoglein, and plakoglobin in L cells also confers tight adhesion, most likely due to direct clustering of the desmosomal cadherins at the cell surface by plakoglobin (Marcozzi et al., 1998).

Mice expressing a truncated form of desmoglein lacking 497 nucleotides of the extracellular domain exhibit many abnormal changes in the organization and differentiation of epidermal cells including marked edema, inflammation and degeneration of keratinocytes (Allen et al., 1996). Molecular analysis of the epidermis reveals that the desmosomes are vastly disrupted (Allen et al., 1996). These pathological and clinical signs resemble several human autoimmune epidermal blistering diseases, grouped together as pemphigus diseases (Amagai et al., 1992; Karpati et al., 1993; Stanley, 1993; Amagai, 1994). These patients have circulating antibodies to, or soluble forms of, the desmogleins which interfere with desmosomal assembly and cause epidermal blistering and other abnormalities (Amagai et al., 1992; Karpati et al., 1993; Stanley, 1993; Amagai, 1994).

Protocadherins

The most recently described subclass of the cadherin superfamily are the protocadherins. These proteins were initially isolated through polymerase chain reactions primed with degenerate oligonucleotides to the most conserved extracellular domains (EC3

and EC4) of the classic cadherins to generate several full length clones (Sano et al., 1993). The overall structure of these cadherins is very similar to the classic cadherins. They are type I glycoproteins containing greater than five cadherin repeats in their extracellular domains (Sano et al., 1993; Suzuki, 1996b; Suzuki. 1996a). The cytoplasmic domains are highly variable suggesting that this family is heterogeneous and may bind to different intracellular partners (Sano et al., 1993; Suzuki, 1996b; Suzuki, 1996a). Protocadherins are primarily expressed in the nervous system but have also been found in fibroblasts (Sano et al., 1993; Matsuyoshi and Imamura, 1997). Northern blot analysis reveals that there are higher levels of mRNA in adult brains than in fetal brains suggesting that these proteins are developmentally regulated (Sano et al., 1993). Protocadherins are localized at cellular contacts and elicit weak homophilic Ca^{++}-dependent adhesion (Sano et al., 1993; Obata et al., 1995). Unlike the classic cadherins, protocadherins do not interact with cytoskeletal proteins and can be easily extracted from the lipid bilayer which contributes to their labile cellular interactions (Obata et al., 1995). Their function *in situ* has remained elusive. Preliminary evidence shows that ectopic expression of protocadherin2 results in the disruption of retinal tissue structure (Suzuki, 1996a). Clearly more studies will be done in the future to fully understand the roles of this family in nervous system development and differentiation.

Integrins

Integrins predominantly mediate cell to substratum adhesion but also are involved with cell to cell adhesion in a Ca^{++}- or Mg^{++}-dependent manner (Hynes, 1992; Haas and Plow, 1994). Each integrin is composed of two transmembrane glycoproteins (α and β subunits) that are non-covalently linked, creating an α ß heterodimer. These subunits are members of their own multigene families. The ligand specificity of the integrins arises from the interaction of various paired combinations of α and β subunits (Hynes, 1992;

Haas and Plow, 1994). Integrin heterodimers participate in multiple signaling pathways that modulate many fundamental processes including embryological development and morphogenesis, wound healing and leukocyte migration and function to name a few. As ascertained from transgenic mouse studies, every tissue and cell type expresses distinct set of heterodimeric pairs each with its own specific and distinct function (Fasslar et al., 1996).

The extracellular portion of the integrin heterodimer recognizes peptide sequences such as the well-studied arginine-glycine-aspartate (RGD) sequence (D'Souza et al., 1988), that enables associations with extracellular matrix components, in particular fibronectin, but also type I collagen, and laminin (Ruoslahti and Pierschbacher, 1987). The cytoplasmic domains of the integrins is short, ranging from 13 amino acids (α_1) to 58 amino acids (β_7). The cytoplasmic domains aid in dimerization of the α and β subunits and in localization of the heterodimer to the appropriate membrane domains (e.g. β_4 cytoplasmic domain directs the localization of $\alpha_6\beta_4$ to hemidesmosomes; (Geiger and Salomon, 1992; Spinardi et al., 1993)). Additionally, the cytoplasmic regions interact with the actin cytoskeleton either directly (α1) or through actin binding proteins including talin, vinculin, and α-actinin. In general the cytoplasmic domains themselves, with a few exceptions, are not modified directly, but instead kinases and phosphatases accumulate at the sites of integrin mediated adhesive contacts. Furthermore, these domains link the integrin to multiple secondary messenger signaling pathways involving focal adhesion kinases, proteins kinases, and G-protein signaling (Ginsberg et al., 1992; Garratt and Humphries, 1995; Lafrenie and Yamada, 1996). Once the appropriate ligand is bound to the integrin receptor in the extracellular matrix, kinases and phosphorylases become either active or inactive and commence a cascade of signaling events within the cell. This process creates networks coordinating signals from the extracellular matrix with the inside of the cell (Hynes, 1992; Sastry and Horwitz, 1993; Haas and Plow, 1994; Lafrenie and Yamada, 1996).

One example of how extracellular integrin engagement activates and regulates internal signaling events occurs during cartilage development and homeostasis and involves interactions between fibronectin and its receptor, $\alpha5\beta1$ (Clancy et al., 1997). It was shown that once chondrocytes bind fibronectin, several internal events occur. At the sites of fibronectin-integrin complexes, actin monomers accumulate and polymerize, and several signaling proteins including focal adhesion kinase, (a tyrosine kinase) and rho A (a tyrosine phophorylated G protein regulated by ras) are activated. Additionally, once fibronectin and $\alpha5\beta1$ have made adhesive contacts, chondrocytes elaborate proteoglycans in an FGF dependent manner; the synthesis of proteoglycans involves a highly complex signaling pathways that is not a direct result of fibronectin engagement with its receptor and may involve the MAP kinase signaling pathways (Clancy et al., 1997).

These multiple signaling events are disrupted in several diseases that cause a dysregulation of cartilage homeostasis including osteoarthritis and inflammatory arthritis where abnormally high levels of nitric oxide (NO) pools within the diseased intraarticular cartilage. NO was shown to trigger actin depolymerization, cause translocation and inhibition of activity of key signaling proteins, FAK and rho in chondrocytes. Additionally, NO treated chondrocytes are unable to synthesize and secrete proteoglycans in the presence of FGF (Clancy et al., 1997). It has been shown that there is direct signaling between $\alpha5\beta1$ and nitric oxide through a cGMP signaling pathway. It can be concluded that NO may disrupt the normal signaling events associated with $\alpha5\beta1$. These studies underscore the importance of integrin involvement in multiple signaling pathways (tyrosine kinases, G proteins, MAP kinases, cGMP protein kinases) which together mediate cartilage homeostasis and can be applied to other systems as well.

Another integrin, $\alpha6\beta4$, unlike most other integrin heterodimers, does not link to the actin microfilaments. Instead it associates with intermediate filaments at cell-matrix attachments to form hemidesmosomes and mediates attachment to laminin in the basement membrane. $\alpha6\beta4$ is also known to be linked to several signaling pathways (Green and

Jones, 1996). Additionally, this integrin has been found in myelinating Schwann cells where it is believed to assist in axon ensheathment (Einheber et al., 1993; Feltri et al., 1994; Niessen et al., 1994). As will be discussed in a later section, $\alpha 6\beta 4$ is also directly involved with multiple signal transduction pathways that leads the progression to carcinomatous transformation of breast cells (Shaw et al., 1997).

Integrins are also capable of mediating heterophilic adhesion with members of the IgCAMs and cadherins. One example is $\alpha 4\beta 1$, (VLA-4 integrin), found on leukocytes which binds to V-CAM, an endothelial cell IgCAM (Elices et al., 1990). More recently it has been demonstrated that $\alpha E\beta 7$ on mucosal lymphocytes can adhere to the ectodomain of E-cadherin in epithelial cells in a specific and regulatable manner which is independent of Ca^{++} (Higgins et al., 1998). It is not known what is the physiological relevance of this interaction, but it can be speculated that it may provide a mechanism of antigen presentation and lymphocyte activation within the epithelium.

It now appears that specific integrin subunit expression can be regulated by classic cadherins. In differentiating keratinocytes it has been shown that E-cadherin, and to a lesser degree P-cadherin, can downregulate specific integrins (Hodivala and Watt, 1994). It can be hypothesized that these interactions will be essential for normal epithelial cell behavior. If the expression of E-cadherin is lost or decreased, as is observed in carcinomas, there is a dissolution of all epithelial junctions including the integrin containing the focal contacts. It has also been shown that during neural crest cell migration several integrin subunits, $\beta 1$ and $\beta 3$ are able to regulate N-cadherin localization and activity through signaling events involving transmembrane fluxes of Ca^{++} and activation of several phophatases and kinases (Monier-Gavelle and Duband, 1997). These studies suggest an interplay between integrins/ECM interactions and the cadherins/catenin complexes involved with cell to cell adhesion. These two adhesions systems may integrate several signaling pathways through the actin microfilament network (Potard et al., 1997; Wu et al., 1998).

Selectins

The selectin family has three known members -- L(leukocyte)-selectin, P(platelet)-selectin, and E(endothelial)-selectin -- that are all encoded by closely linked genes on chromosome 1 (Bevilacqua et al., 1991; Rosen and Bertozzi, 1994). These proteins share sequence and structural homology and probably arose through gene duplication. Each has an extracellular segment containing a carbohydrate recognition domain, a single epidermal growth factor-like motif and multiple complement-like repeats followed by a hydrophobic transmembrane domain. The short (35 amino acids) cytoplasmic region has no known intracellular partners, but is essential for function, suggesting it may interact with cytoskeletal proteins to maintain ectodomain conformation or to regulate binding specificity. (Bevilacqua et al., 1991; Rosen and Bertozzi, 1994). The carbohydrate recognition domain in the selectin ectodomain heterophilically interacts with specific sialylated and fucosylated carbohydrates (e.g. sialyl $Lewis_x$ and $Lewis_a$) found on proteins and lectins. These modified proteins and lectins are usually present on adjacent endothelial cells, leukocytes, or platelets (von Andrian et al., 1993; Rosen and Bertozzi, 1994). The selectins are primarily involved in the inflammatory process where they mediate leukocytic rolling and extravasation across vascular endothelium and thrombosis (Rosen and Bertozzi, 1994). Animal models provide evidence that this family also contributes to the pathological processes in chronic inflammatory diseases including allergen-induced airway obstruction.

Tetraspan Proteins

The tetraspan superfamily are a heterogeneous group that have the unique feature of four hydrophobic domains suggesting that they span the lipid bilayer four times; most of the sequence homology is limited to the four stretches of hydrophobic domains (Maecker et al., 1997). Representative classic members of this family include: CD9, a surface antigen expressed on hematopoietic and epithelial cells, CD37, an antigen found on mature B cells,

CD81, expressed on numerous cell types, ME491/CD63, an antigen expressed on activated platelets, uroplakin 1a and 1b, found on urinary bladder epithelium where it is thought to strengthen the apical surface, and peripherin, expressed in the ocular system (Wu et al., 1994; Yu et al., 1994; Maecker et al., 1997). Although some tetraspans may be adhesive, these proteins are not traditionally viewed as having pronounced adhesive properties. Proposed members of this family are occludin and the myelin proteolipid proteins that subserve at least in part an adhesive function. Occludin is a transmembrane component of the epithelial tight junctions and is believed to be its main functional unit maintaining the paracellular permeability barrier and cell polarity (Furuse et al., 1993; McCarthy et al., 1996; Wong and Gumbiner, 1997). In cultured fibroblasts, occludin elicits calcium independent adhesion; it co-localizes with E-cadherin and ZO-1 at cell contacts (VanItallie and Anderson, 1997). It appears that occludin is unable to cluster at cell interfaces and thus cannot mediate adhesion in the absence of cadherins. If classic cadherin expression is abrogated, occludin can no longer confer adhesion in these L cells. Proteolipid proteins (PLP and DM-20) are found in major proportions in CNS myelin of terrestrial vertebrates. These proteins aid in the compaction of CNS myelin which results in the formation of the intraperiod line (Kirschner et al., 1989; Kitagawa et al., 1993; Yoshida and Colman, 1996).

Immunoglobulin Gene Superfamily

The first CAMs to be recognized were found to be related to immunoglobulins, and so were termed the immunoglobulin gene superfamily (IgCAMs) (Williams and Barclay, 1988; Elices et al., 1990). These proteins mediate homophilic and heterophilic adhesion independent of calcium. Representative family members include neural adhesion molecule (N-CAM), vascular cell adhesion molecule (V-CAM), carcinoembryonic antigen (CEA), and protein zero, (P_0). Additionally invertebrate multidomain IgCAMs have been isolated

in Drosophila (e. g., fasciclin II). The IgCAMs have in their extracellular domain one or more "immunoglobulin structural subunits", composed of 70 to 110 amino acids arranged in two parallel ß-sheets that are cross-linked and stabilized by disulfide bonds. IgCAMs vary in the number of Ig domains -- N-CAM has five, whereas P_0 only has one. All of the IgCAMs are encoded by single copy genes and unlike the secreted immunoglobulins and the T cell receptor, they do not undergo somatic recombination to create new binding specificities. Just proximal to the transmembrane domain or in some cases the GPI-linkage, there may be several tandem repeats of fibronectin type III motifs (Williams and Barclay, 1988; Elices et al., 1990). In the fibronectin molecule itself, these regions mediate the attachment of fibronectin to extracellular matrix components (Elices et al., 1990). It can be speculated that in IgCAMs they may serve an analogous role and serve to reinforce adhesive interactions. Additionally it has been shown that some IgCAMs, like L1 bind to integrins (Felding-Habermann et al., 1997). Taken together this implies cross talk between the extracellular matrix, IgCAMs and integrins. The cytoplasmic tail varies in length and is proposed to interact directly or indirectly with intracellular signaling pathways and possibly the cytoskeleton (Williams and Barclay, 1988; Wong and Filbin, 1994; Wong and Filbin, 1996). For example, L1 and N-CAM have been found to bind the cytoskeletal associated proteins ankyrin and spectrin respectively (Burden-Gulley et al., 1997; Dahlin-Huppe et al., 1997; Garver et al., 1997). N-CAM has also been found to be linked to intracellular pathways involving G proteins, inositol phosphate, and tyrosine kinases in particular during the process of neurite outgrowth (Doherty et al., 1991; Doherty and Walsh, 1992).

N-CAM is the most extensively studied member of this family. It is primarily expressed in the nervous system where it has been implicated in a variety of developmental processes including neural crest migration, neurite outgrowth and fasciculation, neuromuscular junction formation, neuron to glia interactions, tectal innervation and formation of cell layers in the cerebellum and retina (Edelman and Crossin, 1991; Rutishauser, 1993). Additionally, N-CAM is also expressed in neuroectodermal and

neuroendodermal derivatives, cardiac cells and basolateral surfaces of the colonic epithelial cells (Roesler et al., 1997). N-CAM binds homophilically as well as heterophilically with many different ligands such as integrins and the proteoglycans heparin sulfate and chondroitin sulfate (Kallapur and Akeson, 1992; Grumet et al., 1993).

N-CAM consists of five tandemly arranged Ig-domains and two fibronectin Type III repeats. The primary transcript can be alternatively spliced creating many different isoforms of N-CAM (Edelman and Crossin, 1991; Rutishauser, 1993; Tomasiewicz et al., 1993). The three major isoforms are N-CAM120, N-CAM140, and N-CAM180 which differ in the length of their cytoplasmic tail. N-CAM120 is GPI-anchored to the plasma membrane and therefore lacks an intracellular domain and N-CAM180 has the longest tail and has been implicated to interact with cytoskeletal elements directly; the cytoplasmic domain of N-CAM140 is intermediate in length. Each has its own distribution and role in nervous system development. The N-CAM 120 and 140 forms are widely distributed in both neurons and glial cells. N-CAM 180 is primarily expressed in neuronal cells and is specifically enriched at neuromuscular synaptic junctions, olfactory bulb, and specific layers in the retina.

N-CAM can also be alternatively spliced in its ectodomain. The muscle form of N-CAM has a novel 37 amino acid sequence juxtaposed to the transmembrane domain which may dictate interactions specific for muscle homeostasis. The ectodomain of N-CAM, as with most IgCAMs, is modified post-translationally by sulfation and glycosylation. One unique addition to N-CAM is the 2, 8-sialic acid homopolymer or polysialic acid (PSA) which appears to reduce the binding avidity of N-CAM and gives this protein anti-adhesive properties (Rutishauser and Jessell, 1988; Edelman and Crossin, 1991; Rutishauser, 1993). Interestingly, the PSA form of N-CAM is found more frequently in embryonic tissue where cell migration is occurring. In adult tissue this form is specifically found in tissues that undergo continual remodeling such as olfactory neuron precursors (Rutishauser, 1996).

Transgenic mice null for either N-CAM 180 or all N-CAM isoforms are viable and fertile. These mice do have structural abnormalities in the nervous system as well as behavior disturbances in efficiency of learning and breeding. N-CAM 180 null mice have gross deformities in olfactory bulb formation due to defective migration of cell precursors into this region (Tomasiewicz et al., 1993). There are also subtle abnormalities in cell migration in the cerebellum, retina, and hippocampus (Tomasiewicz et al., 1993). These mutant mice underscore the importance of each N-CAM isoform during development.

Protein Zero (P_0), the major protein of PNS compact myelin is an IgCAM

P_0 is another member of the immunoglobulin gene superfamily (IgCAM) that constitutes well over 50% of the total peripheral myelin protein. Myelinated vertebrates express high levels of P_0 in central (fish and amphibia) and peripheral (all species) myelin. This glycoprotein is the major adhesive and structural element of peripheral myelin where it mediates self-adhesion of the Schwann cell plasma membrane (Everly et al., 1973; Greenfield et al., 1973; Kirschner and Ganser, 1980). Although the expression of P_0 is naturally limited to Schwann cells, the molecular mechanisms of P_0-mediated adhesion can be considered general and "obligatory", since when expressed in a variety of cell lines, P_0 induces strong intracellular adhesion (D'Urso et al., 1990; Filbin et al., 1990; Schneider Schaulies et al., 1990). Modeling studies, x-ray crystallographic analysis, and experimental site-directed mutagenesis have provided excellent working models for understanding how P_0 mediates adhesion at the atomic level (Wong and Filbin, 1994; Zhang and Filbin, 1994; Shapiro et al., 1996; Wong and Filbin, 1996; Zhang et al., 1996). These models remain to be experimentally tested. However, in humans, certain mutations in P_0 yield dysmyelinating disease, possibly due to disruption in the predicted P_0 lattice (Shapiro et al., 1996; Warner et al., 1996).

Evolutionary Considerations of P_0

As stated above, members of the IgCAM family have a variable number of Ig-like repeats in their extracellular domain. The ectodomain of P_0 contains only one Ig-like motif. The individual Ig domains either may be encoded by a single exon that is uninterrupted by introns, like the secreted immunoglobulins or alternatively, it may be encoded by two exons, as has been observed with P_0 and N-CAM (Owens et al., 1987; Lemke et al., 1988; Salzer and Colman, 1989). In the latter case, the intervening intron divides the single Ig domains into two symmetric half domains that are similar in both sequence and length. Therefore, it can be speculated that the contemporary Ig domain form arose from the duplication and subsequent joining of ancestral half domains to create an immunoglobulin-like homology unit (Lemke, 1988; Lemke et al., 1988; Salzer and Colman, 1989). Following this train of thought, it has been suggested that P_0 or another IgCAM with a single Ig-domain, like perhaps GPI-linked Thy-1, may most closely resemble the primordial IgCAM which gave rise to all the contemporary members of the IgCAM superfamily (Lai et al., 1987; Lemke et al., 1988; Williams and Barclay, 1988). Although P_0 may resemble the prototypic IgCAM in terms of single domain structure, this is not to say that P_0 itself is highly conserved in evolution. In fact, P_0 is likely to be a relatively new feature, unique to vertebrate myelin (Colman et al., 1996) .

Cartilaginous fish which arose about 440 million years before present, were likely to have been the first myelinated organisms. In modern cartilaginous fish (their contemporary descendants) P_0 is the major adhesive and structural element of compact myelin in both the CNS and PNS (Kirschner et al., 1989; Saavedra et al., 1989; Yoshida and Colman, 1996) . This is also true in modern amphibia - anurans, caecilians, and salamanders - all of whom are believed to be members of the same monophyletic group. This latter notion is supported by the expression and distribution patterns of P_0 and the proteolipid gene family products within amphibian species (Yoshida and Colman, 1996). In postamphibian vertebrates, P_0 is expressed exclusively in peripheral myelin (Franz et al., 1981; Yoshida

and Colman, 1996). In the CNS of these organisms, P_0 is "replaced" by analogous (in terms of abundance and distribution in myelin), but biogenetically unrelated proteins, DM-20 and proteolipid protein (PLP), which are the major integral membrane proteins of CNS compact myelin. It has been speculated that while P_0 and DM-20/PLP play adhesive roles in myelin compaction, PLP may also may function as an "adhesive" pore in the CNS myelin sheath (Kitagawa et al., 1993).

Molecular and Structural Characterization of P_0

Early experimental studies revealed that there were distinct differences in histochemical reactivity, solubility, and amino acid and lipid composition between highly purified PNS and CNS myelin (Horrocks, 1967; O'Brien et al., 1967; Wolfgram and Kotorii, 1968; Mehl and Wolfgram, 1969; Morris et al., 1971; Greenfield et al., 1973). With the advent of SDS gel electrophoresis, it was confirmed that the actual protein composition of myelin extracts from rat brain, spinal cord, and sciatic nerve were strikingly different (Greenfield et al., 1973) and P_0 was first isolated (Kitamura et al., 1976; Roomi et al., 1978). It was observed that P_0 was the major glycoprotein that constituted over 50% of PNS myelin (Everly et al., 1973; Greenfield et al., 1973; Kitamura et al., 1976; Roomi et al., 1978).

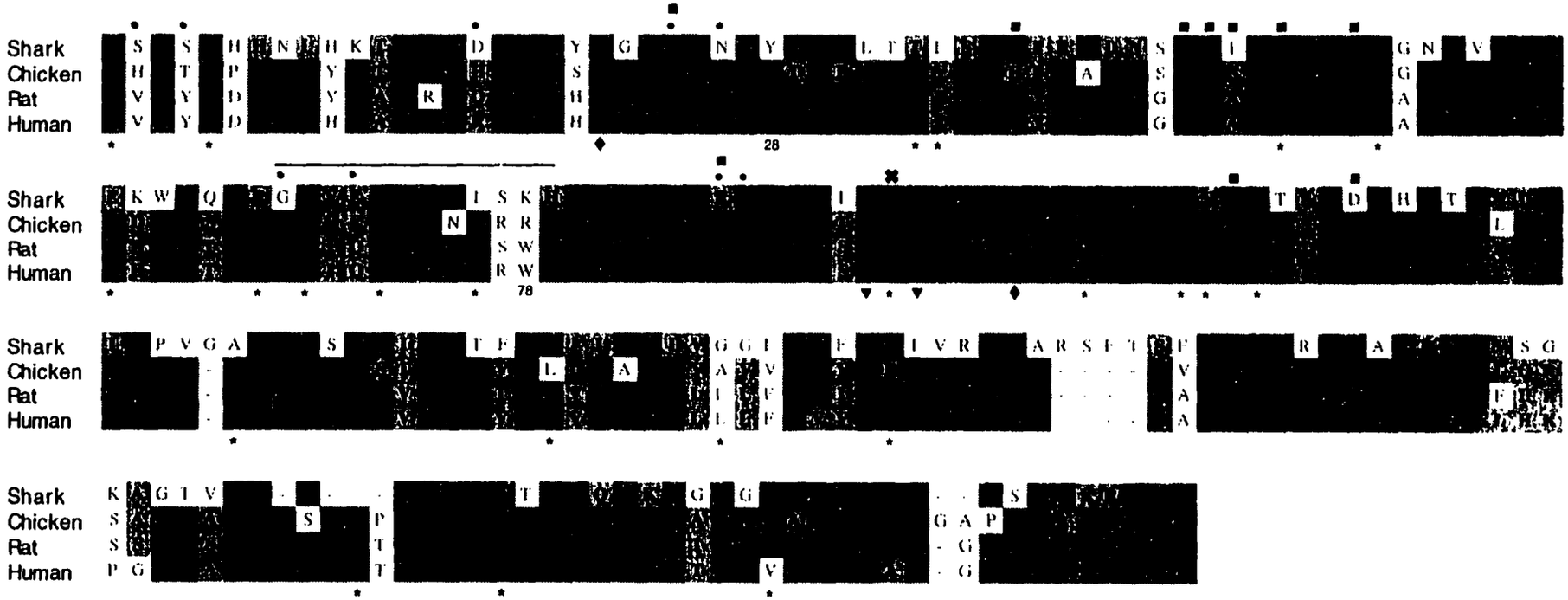
The partial amino acid sequence of P_0 was initially determined by peptide mapping of tryptic peptides of the purified glycoprotein (Ishaque et al., 1980). The complete amino acid sequence was determined directly from a cDNA clone of P_0 that was isolated by exploiting the differences in the mRNA (and protein) composition of the CNS and PNS myelin through differential screening and hybridization of rat sciatic nerve and brain libraries (Lemke and Axel, 1985). Following this, the bovine P_0 sequence was deduced by directly sequencing amino acids of the purified protein (Sakamoto et al., 1987). Subsequently, the P_0 gene was cloned from many species, including mouse (Lemke et al., 1988), trout (Schliess and Stoffel, 1991; Stratmann and Jeserich, 1995), shark (Saavedra

et al., 1989), Xenopus laevis (Schliess and Stoffel, 1991; Karthigasan et al., 1992), chicken (Barbu, 1990) and human (Hayasaka et al., 1993b). It should be noted that the P_0 molecule is not highly conserved in primary sequence across species. Comparison of the amino acid sequence of P_0s from different species reveals that the shark sequence has only 50% sequence identity when compared to avian and mammalian sequences (Figure 1.1); chicken P_0 is over 75% identical to rat and human sequences, which are themselves 94% identical. Residues that are most conserved, in general, are predicted to be essential for P_0 adhesive function (Figure 1.1; and see (Shapiro et al., 1996)). The 7 kb human gene maps to chromosome position 1q22-23 and is split into six exons by five introns (Hayasaka et al., 1993b; Pham Dinh et al., 1993). It has been predicted from the primary sequence and has been experimentally verified (D'Urso et al., 1990) that P_0 is a single pass integral membrane glycoprotein (Everly et al., 1973), synthesized as a polypeptide precursor with a cleavable amino-terminus signal sequence. The mature form of this 28 kD protein contains a hydrophobic ectodomain resembling an Ig variable-like domain, a single membrane spanning domain, and a highly basic intracellular domain (Lemke and Axel, 1985; Lemke et al., 1988). P_0 is therefore synthesized in the rough endoplasmic reticulum, where the signal peptide is cleaved. Further post-translational modifications (glycosylation, acylation, and phosphorylation) are performed as the polypeptide is transported through the endoplasmic reticulum, Golgi apparatus and finally to the cell surface (Everly et al., 1973; Wood and Dawson, 1973; Matthieu et al., 1975; Kitamura et al., 1976; Wiggins and Morell, 1980; D'Urso et al., 1990). Glycosylation with complex carbohydrates at Asn93 is a particularly important post-translational modification. It has been shown that cells expressing only the high-mannose form of P_0 were less adhesive than cells expressing this glycoprotein with complex carbohydrates (Filbin and Tennekoon, 1991) revealing that glycosylation with complex carbohydrates is essential for the strong adhesion elicited by P_0.

Figure 1.1 Alignment of P_0 amino acid sequences

Comparison and alignment of P_0 amino acid sequence of 4 species - shark (Saavedra et al., 1989), chicken (Barbu, 1990), rat (Lemke et al., 1988), and human (Hayasaka et al., 1993b) - reveals that this protein is highly conserved. Shark - chicken is 55% identical; shark - rat, human is 55%; chicken - rat, human 76% identical and rat to human is 94% identical. The regions of identity are greatest in the ectodomain and the transmembrane domain. Mutations in P_0 that lead to human demyelinating diseases (CMT, DSS and CH) occur at residues that are absolutely conserved throughout evolution (*). Additionally, residues involved with P_0 tetramer formation (•) as well as residues with in the adhesive interface (■) are also highly conserved. The hydrophobic side chains of Trp28 and Trp78 intercalate with opposing lipid bilayers. Site of glycosylation (✖). Cysteines involved with disulfide bond formation (▼; (Zhang and Filbin, 1994)). Mutations in these residues are associated with loss of adhesion in COS cells (♦; (Zhang et al., 1996)). Darkly shaded boxes indicate identity; lightly shaded represent similarity among residues.

Figure 1.1



- Associated with mutations in human demyelinating diseases
- Essential for tetramer formation
- Residues of proposed adhesive interface
- Region associated with neurite outgrowth properties
- Cysteines involved with disulfide bond formation
- If mutated, will lose adhesive properties
- Site of glycosylation

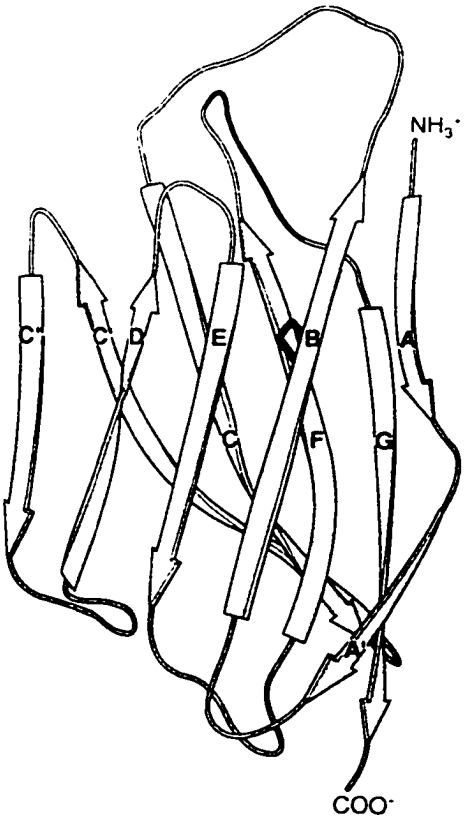
X-ray crystallography data confirmed the idea (Lai et al., 1987; Lemke et al., 1988; Karthigasan et al., 1992) that the extracellular domain of P_0 folds as an Ig-like domain with 10 antiparallel beta strands organized as two β sheets (Shapiro et al., 1996). By convention, D, E, B, A make up one sheet and the other β sheet comprises of A', G, F, C, C', and C" (Figure 1.2). It has been confirmed that these two sheets are held together by disulfide bonds (Zhang and Filbin, 1994). As with other Ig-like domains, the A strand forms hydrogen bonds with the B strand and A' hydrogen bonds to G. Once folded, the C'-C" loop resembles the immunoglobulin variable heavy chain (Wells et al., 1993). As predicted from the P_0 crystal structure analysis, ultracentrifugation studies reveal that P_0 ectodomain monomers oligomerize to form dimers, tetramers, and oligomers under physiologic conditions, and, at high protein concentration, the ectodomains can reversibly form a gel from aqueous solution (Shapiro et al., 1996). Taken together, all of the structural data suggests that individual P_0 molecules emanate from the membrane as tetramers surrounding a large central hole whose base is the myelin lipid bilayer (Shapiro et al., 1996). The B-C loop of one P_0 molecule interacts with the C"-D and E-F loops of the adjacent molecule for tetramer formation.

Different types of homophilic interactions are predicted to be formed between P_0 ectodomains from opposing membranes. It has been predicted that one adhesive interface is due to intermolecular hydrogen bonds that form between residues in the C' strands of P_0 ectodomains allowing protomer:protomer interactions. A second adhesive interaction may be mediated by tryptophans (Trp28, Trp78) exposed at the apex of the B-C loop that make van der Waals contacts with residues on the B-C loop of the facing P_0 ectodomain. Through their hydrophobic side chains, these tryptophans may also intercalate into the hydrophobic phase of the opposing plasma membrane lipid bilayer (Wells et al., 1993; Shapiro et al., 1996). Therefore, it can be postulated from this model that two independent mechanisms of adhesion (the tetramer: tetramer interactions and tryptophanal associations with the lipid bilayer) act together to form an adhesive lattice which by electron microscopy

Figure 1.2 Structural representation of P_0 ectodomains

X-ray crystallographic analysis reveals that P_0 ectodomains fold as an immunoglobulin-like domains consisting of 10 antiparallel β sheets (A). The A strand forms hydrogen bonds with the B strand and the A' strand hydrogen bonds to the G strand.

Figure 1.2



is seen as the intraperiod line (Figure 1.3). It is important to note that residues essential for tetramer formation and for the interlocking tetramer:tetramer interactions of opposing molecules are highly conserved throughout evolution (Figure 1.1).

The intracellular domain of P_0 is highly basic and may mediate the adhesion of intracellular leaflets of compact myelin through electrostatic interactions between this domain and bilayer phospholipids, leading to the formation of the major dense line (Figure 1.3; (Lemke and Axel, 1985; Ding and Brunden, 1994; Hilmi et al., 1995; Eichberg and Iyer, 1996)). In support of this, it has been shown that a synthetic peptide derived from this region can bind and aggregate negatively charged phospholipid vesicles (Ding and Brunden, 1994). Furthermore, posttranslational phosphorylation (i.e. neutralization of positive charge) of this region appears to modulate P_0 affinity for the phospholipids (Ding and Brunden, 1994; Hilmi et al., 1995; Eichberg and Iyer, 1996). Additionally, it is of interest that in some *in vitro* studies an intact intracellular domain is absolutely required for extracellular adhesion (Wong and Filbin, 1994; Wong and Filbin, 1996). This suggests that the carboxyl terminal region may affect the conformation transbilayer of the ectodomain, thereby modulating adhesion strength during maturation of compact myelin. This "inside out" signaling has been observed in other adhesion systems (e.g., the integrins; see (Ginsberg et al., 1992; Garratt and Humphries, 1995))

Functions of P_0

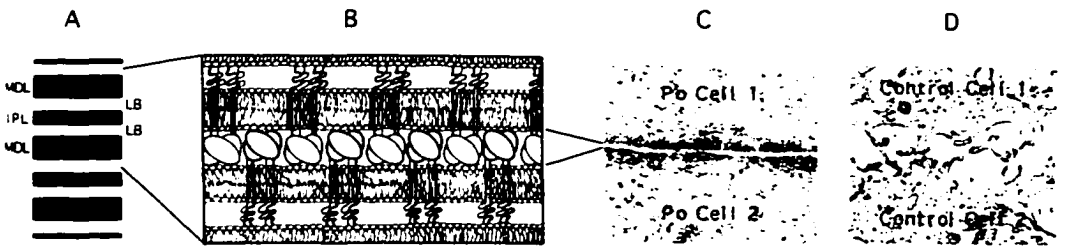
Compaction of Myelin

In the peripheral nervous system, axons are ensheathed by Schwann cells which are neural crest cell derivatives. There are several defined stages of Schwann cell differentiation. Initially, Schwann cell precursors are migratory and therefore do not elaborate a basal lamina nor do they have specific associations with axons. A few days later, Schwann cells stop migrating and their processes envelope axonal bundles and begin to form basal lamina. Myelination commences after Schwann cells have made one to one

Figure 1.3 P_0 is an "obligatory" adhesion molecule

Electron microscopic analysis of compacted PNS myelin reveals that there are alternating zones of compaction -- major dense line (MDL) and intraperiod line (IPL) which are diagrammatically represented in A. P_0 ectodomains emanate from membranes as tetramers and interact with tetramers from opposing lipid bilayers (LP) and bring Schwann cell membranes very close together (3-5 nm) to form the IPL. When carcinoma cells (HeLa) devoid of epithelial junctions are engineered to express P_0 (C, D), the membranes are brought within close proximity (approximately 5 nm) of one another and form a structure analogous to the intraperiod line (C).

Figure 1.3



associations with axons. The inner tongue of the Schwann cell membrane begins to spiral around the axon; after several wraps, cytoplasm is somehow removed and plasma membrane wraps appose each other to create compact myelin.

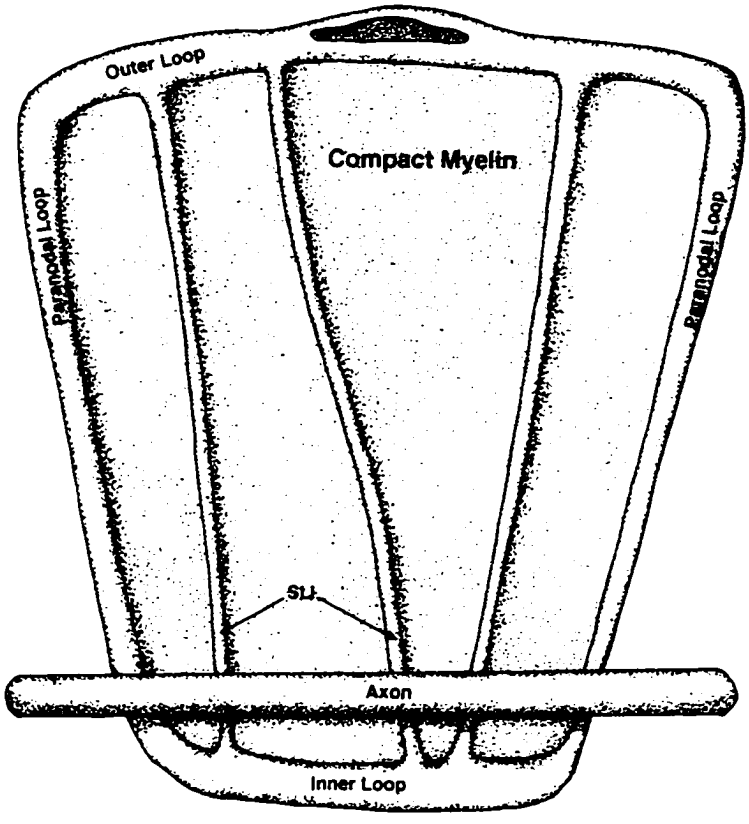
In development, the expression of P_0 mRNA occurs prior to the appearence of any protein and before the commencement of myelin. P_0 mRNA is detected in neural crest precursors of myelinating Schwann cells, as well as progenitors of non-myelinating Schwann cells and satellite cells of the olfactory nerve in both mammals and aves as early as E14 in rat and E4 in chicken (Bhattacharyya et al., 1991; Zhang et al., 1995). P_0 protein is first expressed in Schwann cells several days before myelination commences as Schwann cells contact the axons of the sciatic nerves and spinal roots (Barbu, 1990; Baron et al., 1994a; Baron et al., 1994b). Initially, P_0 expression is highest in Schwann cells in contact with larger motor axons as well as at the periphery of nerves where myelination first proceeds (Baron et al., 1994a). Levels of expression peak postnatally at around day 14 when myelination and compaction rates are at their highest levels. Upon nerve maturation and the completion of myelin compaction, P_0 expression falls to a steady state level that is maintained throughout the life of the organism. In zones of compact mature myelin, P_0 is primarily glycosylated with complex sugar residues which has been shown to be the more adhesive form of glycosylation (Filbin and Tennekoon, 1991). If expression of P_0 is disrupted, the architecture of myelin is compromised. For example, when antisense P_0 mRNA is expressed in primary Schwann cell cultures, only a single layer of membrane is formed around axons, and the characteristic compact myelin spiral fails to develop (Owens and Boyd, 1991).

One curious feature of mature myelin is that there appears to be little flux of cytoplasm and membrane associated molecules to and from the large expansions of compact myelin membranes and the surrounding highly dynamic, cytoplasmic channels -- the Schmidt-Lanterman incisures, paranodal, inner, and outer loops (Figure 1.4; (Singer and Bryant, 1969)). It is possible that the extensive adhesive latticework created by

Figure 1.4 Schwann cell architecture

As Schwann cells enwrap and myelinate axons, regions of compact myelin are surrounded by cytoplasmic channels -- Schmidt-Lanterman incisures (SLI), paranodal, inner, and outer loops -- referred to as uncompacted myelin. P_0 is found exclusively the regions of compact myelin where the P_0 adhesive lattice may act to restricts the flux of cytoplasm and membrane associated molecules between the zones of compacted and uncompacted myelin.

Figure 1.4



homophilic interactions between P_0 molecules on opposing membranes (Shapiro et al., 1996) may create a partial intercellular barrier. The distance between P_0 compacted Schwann cell membranes in myelin is 3 - 5 nm, depending on species, which is larger than the juxtaposed membranes in tight junctions (estimated to be 0 nm), the intercellular barrier of epithelial cells (Farquhar and Palade, 1963; Raine, 1984; Shapiro et al., 1996), but is much smaller than the intermembrane distance in other epithelial junctions, such as adherens junctions (≈30 nm) which are cadherin-mediated structures (Farquhar and Palade, 1963; Geiger and Ayalon, 1992). We have shown that when P_0 is introduced into carcinoma cells devoid of tight junctions, cells acquire the ability to retard the flux of ions and macromolecules in a manner analogous to tight junctions (Spiryda and Colman, 1998). Conceivably, the P_0 adhesive lattice may act to restrict the flux of molecules and ions across regions of compact myelin in an analogous manner to tight junctions.

Neurite Extension

Two studies have provided evidence suggesting that P_0 may enhance neurite outgrowth. Co-culturing experiments show that P_0 expressing cells (CV-1 and glioma cells) can enhance the outgrowth of neurites from dorsal root ganglia and cortical neurons (Schneider Schaulies et al., 1990; Yazaki et al., 1994). Furthermore, when P_0 expressing glioma cells were implanted into transected spinal nerves in rat, there was significant axonal elongation into these areas as compared to control animals (Yazaki et al., 1994). Neurite outgrowth into P_0 expressing cells can be completely inhibited by a monoclonal antibody against Glu68 - Lys79 in the P_0 extracellular domain (Yazaki et al., 1994). This suggests that P_0 may promote neurite extension via heterotypic interactions with unidentified partners on the neuron (Yazaki et al., 1994). Alternatively, since this region overlaps with amino acids predicted from the crystallographic model (Figure 1.1; (Shapiro et al., 1996)) to be involved with tetramer formation (Glu68, Gln71) as well as with interactions with the opposing plasma membranes (Trp78), P_0 tetramers may form on the glial surface and serve

as a scaffold or matrix that may promote outgrowth in the absence of a cognate binding partner on the neurite surface.

Although there is some P_0 mRNA expressed early in a subset of neural crest precursors (Bhattacharyya et al., 1991; Zhang et al., 1995), the neurite promoting activity of P_0 is not likely to play a significant role in normal nervous system development. The majority of P_0 is not synthesized and expressed until axons have grown out and been engaged by Schwann cells. More likely, this activity may enhance regeneration and repair after axonal damage and degeneration. Interestingly, oligodendrocytes of CNS myelin of more primitive vertebrates express P_0 and CNS neurons have regenerative capabilities, whereas oligodendrocytes of post amphibian vertebrates do not express P_0, and their CNS neurons regenerate poorly once injured.

Regulation of P_0 Gene Expression

Expression of myelin genes, and in particular P_0, is a precisely controlled event during development of the nervous system and Schwann cell differentiation. Once a one to one relationship between axons and Schwann cells is established, the transcriptional activity of the Schwann cells shifts from "premyelinating" genes (N-CAM, L1) to "myelinating" gene products like P_0. There is substantial evidence that signals from the axolemma (possibly growth factors), trigger a wave of cAMP which in turn modulates various transcription factors leading to P_0 (as well as other myelin genes) transcription and its subsequent translation (Lemke and Chao, 1988; Morgan et al., 1991; LeBlanc et al., 1992). It is not fully understood how cAMP elicits P_0 gene expression, since the P_0 promoter does not have any known cAMP binding sites or response elements (CREs). Perhaps cAMP may regulate the activity of transcription factors that interact with P_0 promoter region during Schwann cell differentiation.

Analysis of the P_0 promoter reveals that the 350 bp region proximal to the transcription start site is absolutely conserved amongst mammals (Brown and Lemke,

1997). The core promoter contains several motifs including a G/C rich region and two CAAT boxes that are known to bind transcription factors. In vitro DNA:protein binding assays reveal that NF-Y and Sp1 can bind the G/C rich region and CAAT boxes respectively (Brown and Lemke, 1997). It is speculated that these factors in conjunction with other specific regulatory proteins may activate P_0 transcription.

One factor that has been shown to *repress* P_0 expression is SCIP/Oct-6/Tst-1 (Monuki et al., 1990; He et al., 1991; Monuki et al., 1993; Jaegle et al., 1996). SCIP/Oct-6/Tst-1 is a POU domain protein that is dramatically upregulated in Schwann cells just prior to myelination, but its levels are decreased as these cells begin to myelinate (Monuki et al., 1989; Monuki et al., 1990; Collarini et al., 1992). The POU domain proteins are a family of transcription factors that were grouped together following the observation that the products of three mammalian genes (*Pit-1*, *Oct-1*, *Oct-2*) and one product of a *Ceaenorhabditis elegans* gene (*unc-86*) shared a domain of sequence homology termed a POU domain. The POU domain is a bipartite DNA binding domain consisting of two highly conserved regions linked together by a variable linker domain (Ryan and Rosenfeld, 1997). SCIP/Oct-6/Tst-1 appears to inhibit the progression of Schwann cell maturation and myelination by repressing the myelin genes, like P_0 (Collarini et al., 1992; Jaegle et al., 1996). Transgenic mice expressing a dominant negative form of SCIP/Oct-6/Tst-1 which can bind DNA, but cannot regulate transcriptional activity, exhibit hypermyelination of axons resulting in a severe peripheral neuropathy due to the overexpression of late myelin genes like P_0 (Weinstein et al., 1995). In vitro systems show that SCIP/Oct-6/Tst-1 can specifically bind the distal promoter of P_0 via its amino terminus and POU domain leading to the inhibition of P_0 gene transcription (He et al., 1991; Monuki et al., 1993). This interaction is probably facilitated and strengthened by other nuclear proteins, possibly SOX10, a member of the SOX family which may be accessory proteins for the POU domain proteins (Kuhlbrodt et al., 1998).

It can be speculated that during early stages of development prior to contact with axons, Schwann cells are highly proliferative and SCIP/Oct-6/Tst-1 is not expressed. Once Schwann cells interact with axons, high amounts of SCIP/Oct-6/Tst-1 are expressed and bind to the P_0 promoter in conjunction with other regulatory proteins, which leads to the suppression P_0 gene transcription. As Schwann cells establish a one to one relationship with axons, a signal from the axon triggers an influx of cAMP which inactivates SCIP/Oct-6/Tst-1 and activates other nuclear factors (perhaps, Sp1 and NF-Y) leading to P_0 expression and myelination (Zorick and Lemke, 1996; Scherer, 1997). Transgenic mice lacking SCIP/Oct-6/Tst-1 expression have Schwann cells that make one to one contact with axons but never proceed towards myelination revealing that the role of SCIP/Oct-6/Tst-1 is more complex than merely as a repressor of Schwann cell differentiation. This factor may also stimulate or prime Schwann cells for myelination (Bermingham et al., 1996; Jaegle et al., 1996). Isolating the proteins responsible for P_0 repression and induction will help discern what triggers myelin formation and compaction. Furthermore, understanding how to trigger P_0 expression may provide a mechanism to activate this system in demyelinating diseases and nerve regeneration.

Human Demyelinating Diseases Caused by Mutations in P_0

Mutations in many myelin genes lead to several phenotypically related hereditary motor and sensory peripheral neuropathies in humans. Mutations in P_0 are associated with a spectrum of human demyelinating diseases including Charcot-Marie-Tooth (CMT), Dejerine-Sottas Syndrome (DSS), and Congenital Hypomyelination (CH) that disrupt myelin architecture to varying degrees (Warner et al., 1996). These diseases have similar clinical and pathologic presentation, but differ in severity, with CMT being the least severe and DSS and CH being more severe. Clinical symptoms are characterized by peripheral nerve demyelination which leads to progressive distal muscle atrophy and decreased motor

nerve conduction velocities and in the most severe cases of DSS and congenital diseases, delay of neurologic milestones due to lack of functional myelin.

There are no known naturally occurring P_0 mutations in mice. P_0 heterozygote and homozygote knockout mice were created through transgenic techniques to provide models for human diseases as well as to further elucidate the role of P_0 in normal human myelin maturation and architecture and in demyelinating diseases (Giese et al., 1992; Martini et al., 1995). As might be expected, heterozygote mice, with only one null copy of P_0, have less severe pathology and disease than mice lacking two copies. Initially, in heterozygote mice, myelination proceeds normally between 4 and 10 weeks. However, as early as four months, demyelination occurs leading to moderate electrophysiologic conduction abnormalities. It appears that half the dose of P_0 is sufficient for myelination to commence, but is inadequate for its maintenance. Symptoms in P_0 null mice develop at a much earlier age and are much more severe. By three weeks, these mice exhibit tremors and occasional convulsions due to abnormal conduction velocities. Pathologic analysis reveals that the one to one relationship of the Schwann cell to axon is not disrupted, but myelination and compaction do not proceed further and many myelin-like figures arrest at this stage (Giese et al., 1992). In some instances membranes begin to loop around axons but myelin remains thin and never becomes compacted. Furthermore, axons that are enveloped by these uncompacted membranes begin to degenerate in conjunction with the surrounding Schwann cell membranes (Giese et al., 1992). These studies indicate that the dosage of P_0 is important and it is absolutely required for the elaboration, compaction, and maintenance of myelin in the peripheral nervous system.

Based on the proposed structural model, it can be predicted that certain mutations in P_0 would be the most disruptive and lead to the most severe pathology and clinical symptoms (Figure 1.1; (Shapiro et al., 1996; Warner et al., 1996)). In one mild form of CMT, there is an allelic deletion in the middle of a β strand at position 34 (Kulkens et al., 1993; Shapiro et al., 1996; Warner et al., 1996). This would be predicted to disrupt the

alternating hydrophobic-hydrophilic configuration of this domain and the synthesized protein would possibly misfold, and so be unstable. It might not reach the cell surface and instead be targeted for degradation, leaving only one non-mutant allele of P_0 in myelin. According to this scenario, the non-mutant allele commences myelination but the reduced amount of P_0 is unable to maintain the myelin wraps because of the half dosage of P_0 leading to mild symptoms, as was observed for the heterozygote P_0 mice (Giese et al., 1992; Martini et al., 1995). Alternatively, if the serine at position 34 in one allele of P_0 is mutated to a cysteine, a severe form of DSS manifests (Hayasaka et al., 1993a; Shapiro et al., 1996; Warner et al., 1996). The outwardly pointing thiol group of the cysteine at this position may cause the formation of disulfide aggregates of the mutated P_0 allele in the extracellular spaces. This might disrupt any P_0 interactions or lattices that would have formed with the non-mutant P_0 allele, and so lead to the absence of compact myelin. Similar pathology occurs in DSS patients who inherit two mutant alleles of P_0 from parents, each of whom carry only one mutant P_0 and therefore have milder symptoms (Warner et al., 1996) .

Many mutations in P_0 that would not be predicted to overtly disrupt its primary structure or P_0 lattice assembly are known to cause pathology and symptoms in patients. It seems clear that the structural models that attempt to define the molecular basis for adhesion may need to be re-evaluated. Also, several mutations in the intracellular domain are associated with disease (Figure 1.1). One form of congenital hypomyelination occurs when a glutamine at position 186 is mutated to a stop codon. The resulting protein is truncated and should be targeted for degradation. Some of the truncated protein escapes degradation and instead is transported to the cell surface. Here it may interfere with the formation of P_0 lattices leading to complete absence of myelination. Other mutations in the intracellular domain of P_0 are frameshifts that generate slightly larger than normal proteins. The resulting protein has an extended cytoplasmic tail that may cause abnormal formation and compaction of the major dense line resulting in severely abnormal neurologic

phenotypes (Warner et al., 1996). In vitro studies have shown that the intracellular domain of P_0 is absolutely required for extracellular adhesion (Wong and Filbin, 1994; Wong and Filbin, 1996). Cells expressing P_0 lacking a cytoplasmic tail are not as self-adhesive as are cells expressing wild type P_0. It can be surmised that perhaps the changes of the cytoplasmic region of P_0 results in abnormal signaling events within the Schwann cell as myelination begins. The pathology and clinical presentation of each of these demyelinating diseases depend on the nature of the specific mutation in P_0 and how it may affect individual tetramer formation, P_0 lattice formation and its intracellular associations.

Mutations can either result in loss of function of P_0 due the degradation of a severely misfolded and/or truncated protein, or as a dominant negative where the aberrant protein is not degraded, but disrupts the homophilic interactions and possibly tetramer formation of the non-mutant allele of P_0 (Shapiro et al., 1996; Warner et al., 1996). Site directed mutagenesis studies in the future of P_0 and subsequent biochemical analysis of the resulting proteins will determine the exact effects each mutation associated with demyelinating diseases has on the structure, processing, and homophilic interactions on P_0. Additionally, amino acids responsible for either cis tetramer formation or trans dimer formation should be mutagenized and tested to ascertain if this disrupts the adhesive properties of P_0, in order to confirm the ultracentrifugation and x-ray crystallography data, and the proposed model of P_0 adhesion. This will help discern which domains are necessary for myelin formation from the initial stages before compaction to intraperiod and major dense line formation, and how may pathology arise.

In conclusion, understanding the different aspects of P_0 behavior and regulation may ultimately lead to therapeutic mechanisms that can enhance myelin formation and maintain axonal connections in degenerative diseases and injuries to the myelin sheath in both the PNS and CNS. Additionally, determining the precise molecular basis for the adhesive nature of P_0 will contribute to current knowledge on general mechanisms underlying adhesive behavior between membranes and cells.

The above overview highlights the diverse roles that cell adhesion molecules have in all aspects of cell development and differentiation. Loss of expression or misexpression of CAMs may result in abnormalities of cell morphology and physiology that can cause or be symptomatic of a variety of diseases, including the pathogenesis of cancers.

Carcinoma Pathogenesis

More than 85% of tumors in humans are carcinomas; that is to say they are derived from epithelia. Epithelia have certain fundamental features that distinguish them from other cell types. Typically, these cells assemble tight junctions, adherens junctions, and desmosomes at lateral cell borders. They grow in a contact-inhibited manner, and require matrix attachment for cell division. Epithelial cells lack invasive capabilities and normally express low or null levels of matrix metalloproteinases (MMP's) which in metastatic tumor cells, compromise the integrity of the extracellular matrix, thereby facilitating invasion. Carcinomas to one extent or another lose the typical structural and functional characteristics of the epithelia from which they are derived. Cell to cell adhesion is disrupted, and the stereotypic array of junctions becomes disorganized and may disappear (Birchmeier and Behrens, 1994). Additionally, they do not exhibit contact inhibition typical of normal epithelia and may grow uncontrollably. Carcinomas do not require attachment to the basement membrane for continuous cell proliferation and thus, typically grow in an anchorage-independent manner (Freedman and Shin, 1974). As some carcinoma cells become more aggressive, they secrete matrix degrading enzymes (Khokha and Denhardt, 1989) and acquire the ability to seed tumors at distant sites after they break through basement membranes.

These properties are not exhibited as uniform characteristics of all carcinomas, nor are they necessarily immutable; instead, carcinomas may display a spectrum of these

features. Thus, there is a transitional relationship between the intact epithelium and carcinomatous transformation. Many factors, soluble or membrane-associated, can drive the equilibrium to either direction. For example, P19 mouse embryonal carcinoma cells are pluripotent and can differentiate into neuroectodermal, mesodermal or endodermal cell types depending upon treatment with retinoic acid or activation/inactivation of specific transcription factors (Oulad Abdelghani et al., 1996; Suzuki et al., 1996).

There is now substantial evidence that the levels of surface membrane expression of certain adhesion molecules influence the degree of aggressiveness or metastatic potential of a carcinoma. Normally, a complex interplay between CAMs mediates the initial adhesive steps necessary to trigger a cascade of adhesive and molecular events that ultimately leads to the differentiated phenotype characteristic of the epithelial cell. When these systems go awry either through loss of expression or in some cases overexpression of certain proteins, this causes disruptions in normal signaling pathways and leads to the activation of alternate molecular pathways that lead to changes in cell architecture and loss of substratum attachment which may ultimately trigger tumor development and subsequent invasion through the extracellular matrix and passage through the vasculature leading to metastasis to distant sites.

Selectins, Integrins, And IgCAMs In Neoplastic Transformation

Selectins on endothelium facilitate the movement of leukocytes and platelets by interacting with carbohydrate ligands on their surface. One such ligand, the sialyl $Lewis_x$ antigen, is also highly expressed on the surface of numerous metastatic carcinoma cells including colorectal tumors (Nakamori et al., 1993), small cell lung carcinomas (Stone and Wagner, 1993), neuroblastomas (Stone and Wagner, 1993), breast carcinomas (Renkonen et al., 1997), and melanomas (Ravindranath et al., 1997). This epitope is correlated with a poor patient prognosis (Nakamori et al., 1993; Pignatelli and Vessey, 1994). It has been

postulated that selectins expressed on the endothelium of blood vessels serve as receptors for these carcinoma cells thereby facilitating the movement of the cancer cells to the endothelium, in a similar manner to the integrin-IgCAM interaction described below, promoting metastasis to distant sites (Pignatelli and Vessey, 1994; Rosen and Bertozzi, 1994).

Since the integrins appear to be essential for the normal migration and differentiation of cells (Hynes, 1992; Haas and Plow, 1994), it can be speculated that the abnormal expression or lack of expression of these proteins may be important in the transformation and metastasis of tumor cells due to the activation of abnormal intracellular events or inactivation of normal signaling paths. It has been shown that integrin expression is altered or reduced in some cancers including breast (Zutter et al., 1990) and prostate (Bonkhoff et al., 1993) cancers, but it also has been observed that certain integrins may actually be overexpressed in some aggressive and metastatic head/neck carcinomas (Albelda et al., 1990).

Experimental systems have provided evidence supporting the hypothesis that abnormal integrin expression play a role in altering internal signaling events that may lead to tumor formation as well as facilitating tumor cell metastasis. Several studies in various cell lines (CHO cells, rhabdosarcomas, osteosarcomas, breast carcinomas) have shown a correlation between overexpression of certain integrins or a particular subunit and loss of a differentiated phenotype and/or increases in tumorigenicity (Schreiner et al., 1991; Zutter et al., 1995; Varner and Cheresh, 1996; Vihinen et al., 1996). Furthermore, it has been repeatedly shown that if the expression or adhesive activity of these overexpressed integrins is blocked, normal differentiation pathways ensue. For example, when an aggressive breast carcinoma is treated with inhibiting ß1 antibodies, junctional proteins rearrange and assemble into epithelial junctions at cell to cell contacts and cell to matrix contacts; these treated cells are also less tumorigenic in nude mice (Weaver et al., 1997).

Even more interesting is that certain integrins like, $\alpha 6\beta 4$, play a role in normal epithelial cell biology, but when its expression persists in transformed cells it is associated with highly metastatic carcinomas. In normal epithelia, $\alpha 6\beta 4$ mediates adhesive contacts to the substratum by the formation of hemidesmosomes which link the extracellular matrix to the intermediate filaments as discussed earlier (see integrin section pp.11-12) However, the expression of this integrin persists in many highly metastatic carcinomas where there are no adhesive attachments to the extracellular matrix, and its expression appears to facilitate cell movement by associating with the actin cytoskeleton suggesting that this integrin may be able to activate several different molecular pathways. This theory was corroborated by a study examining the role of $\alpha 6\beta 4$ in breast carcinoma invasion and metastasis. It appeared that breast carcinoma cell lines expressing high levels of this integrin were more likely to be invasive (Shaw et al., 1997). It was determined that $\alpha 6\beta 4$ activated a pathway involving phosphoinositide-3 OH kinase and its downstream effector, rac, a GTP-binding protein which ultimately leads to the stabilization of filopodia and lamellae required for cell movement (Shaw et al., 1997). It is becoming increasingly clear that the pathogenesis of carcinomas is much more complex than simply overexpression or loss of expression of a particular subunit or integrin heterodimer. Multiple signaling pathways within the cell are involved, which together lead to the different stages of carcinomas, from the primary carcinogenic event to invasion and metastasis (Varner and Cheresh, 1996; Schwartz, 1997).

The IgCAMs including the different isoforms of N-CAM, are also involved in both invasion and metastasis in carcinoma development (Pignatelli and Vessey, 1994). The N-CAM180 isoform is normally expressed at the basolateral surface of the colonic epithelial cells of the villous tips and its expression is retained in tumors with a benign clinical course (Roesler et al., 1997). In contrast, several aggressive or metastatic colon carcinomas were found to lack N-CAM180 expression (Roesler et al., 1997). This suggests that there may be an inverse correlation between N-CAM180 expression and patient prognosis in colon

carcinoma, and perhaps N-CAM maintains pathways associated with tumor suppressive activity (Roesler et al., 1997). Studies done in irradiated fibroblasts or gliomas, showed that overexpression of N-CAM could actually decrease the synthesis of matrix degrading enzymes, whereas the expression of the GPI-linked form of N-CAM was unable to change the synthesis or secretion of these enzymes. These studies further support the idea that N-CAM may be able to elicit signaling pathways within cells that promotes normal physiology (Edvardsen et al., 1993; Edvardsen et al., 1994).

Alternatively, there are several studies which suggest a tumor *promoting* role for N-CAM. It has been reported that certain neuroendocrine tumors (e.g., pituitary and parathyroid adenomas, pheochromocytomas, insulinomas) express high amounts of N-CAM and may correlate with poor patient prognosis (Jin et al., 1991; Moller, 1993; Pujol et al., 1993). It can be speculated that perhaps N-CAM expressed on these tumors can interact with integrins expressed on the vasculature and with the extracellular matrix, thus hastening tumor cell metastasis. Another possibility is that in these cases the less adhesive PSA-N-CAM, or an alternatively spliced N-CAM form is expressed, promoting abnormal functions through multiple signaling events.

These examples provide evidence that many adhesions systems and their corresponding signaling pathways are disrupted in neoplastic transformation. The classic cadherins, specifically E-cadherin, are believed to exert control over many pathways that ultimately lead to carcinoma pathogenesis. It has been proposed that once E-cadherin adhesion is disrupted or its expression is lost, irrespective of the integrity of other adhesion molecules, multiple intracellular signaling events are disrupted. These molecular events trigger (or inhibit) specific gene transcription/translation that cause collapse of epithelial junctions and changes in growth parameters and physiology. Cells may ultimately display aspects of the carcinomatous phenotype due to these specific interactions of many signaling pathways (Behrens et al., 1989; Rodriguez-Boulan and Nelson, 1989; Birchmeier and

Behrens, 1994; Pignatelli and Vessey, 1994; Amagai et al., 1995; Bracke et al., 1996; Shiozaki et al., 1996).

Cadherin Expression In Carcinomas

Loss of E-cadherin expression has been correlated with increased tumor formation and subsequent metastasis in many types of human malignancies - breast (Gamallo et al., 1993; Moll et al., 1993; Siitonen et al., 1996), ovarian (Veatch et al., 1994), endometrial (Sakuragi et al., 1994), colorectal (Dorudi et al., 1993), and lung cancers (Bohm et al., 1994). Human cell lines derived from bladder, breast, lung, and pancreas carcinomas that have decreased E-cadherin expression (Frixen et al., 1991; Navarro et al., 1991; Sommers et al., 1991; Vleminckx et al., 1991; Hoffman et al., 1993; Watabe et al., 1994) are more likely to be harvested from aggressive, as opposed to indolent carcinomas. The more aggressive cell lines in general do not assemble junctions appropriately, proliferate in an anchorage-independent manner, and tend to be more invasive in *in vitro* systems. If E-cadherin is introduced back into these cell lines, they will gain some normal epithelial cell characteristics and lose the ability to invade collagen matrix in part due to decreased secretion of matrix degrading enzymes (Frixen et al., 1991; Vleminckx et al., 1991; Watabe et al., 1994; Miyaki et al., 1995). Conversely, when an antibody to the cadherin extracellular domain is added to the medium bathing a normal cell line, homophilic cadherin interactions are disrupted, causing the disassembly of epithelial junctions which in turn leads to the loss of cellular adhesion and communication, and so tumorigenicity is enhanced. It is most likely that the loss of cadherin engagement and of adhesion do not directly cause neoplastic transformation (Gumbiner et al., 1988; Behrens et al., 1989; Frixen et al., 1991; Mareel et al., 1991). More likely, as will be discussed below, these changes disrupt normal epithelial physiologic pathways and this ultimately causes cancer pathogenesis.

The role of other cadherins in carcinoma pathogenesis has not been conclusively established. It may be predicted that alterations in whatever cadherin is operative in a particular cell type may, by analogy, yield neoplastic transformation in that cell type. On the other hand, some squamous carcinoma cell lines with a more fibroblastic and scattered phenotype express low levels of E-cadherin and P-cadherin, but high levels of N-cadherin (Islam et al., 1996). If N-cadherin expression is disrupted through antisense techniques, E-cadherin (and P-cadherin) expression is increased and cells adapt the classic epithelial phenotype (Islam et al., 1996). In some breast carcinoma cell lines, it has been demonstrated that expression of N-cadherin correlates with the loss of all E-cadherin expression and this may correlate with its invasiveness and metastatic potential (Hazan et al., 1996). It can be suggested from these observations that high N-cadherin expression may be characteristic of more aggressive breast cell lines, which would allow these carcinoma cells to interact and adhere to the N-cadherin expressing stromal cells, thus promoting cell movement and invasion. Overexpression of N-cadherin in a transformed fibroblast cell line changes the morphology of the cells to be more epithelial-like, but it does not reduce their tumorigenicity (Simcha et al., 1996). Alternatively, our laboratory and others have shown that N-cadherin may be able to organize one type of junction -- the synapse (Fannon and Colman, 1996; Uchida et al., 1996). Also, we have shown that if N-cadherin expression is induced (indirectly by P_0) in a cervical carcinoma cell line, the cells revert to an epithelial phenotype and appropriately assemble junctions. These studies show that N-cadherin may be able to behave as a junction organizer and tumor suppresser in some tissue types in the same capacity as E-cadherin, and these findings should be extended in future studies.

These studies also suggest that the loss of cadherin expression may hasten tumor formation and metastasis. Abnormal expression or absence of cadherins can cause loss of cell contact which leads to the disruption of the cohesive epithelial network. This may allow cells to break away from each other due to the collapse of the junctional complex.

Furthermore, concurrent with these morphologic changes, there is an induction of *tumor promoting and invasion* genes. The focus of many studies has been to determine the mechanisms by which cadherins exert their effects on cell architecture and behavior by deciphering the intracellular signaling events downstream of cadherin-mediated adhesion.

Downstream mediators of cadherin adhesion -- the catenins

Catenins are a distinct set of cytoplasmic proteins that include α-catenin, β-catenin, and γ-catenin (plakoglobin) that interact with the cadherin intracellular domain (Gumbiner, 1993). These proteins help coordinate extracellular adhesive signals of the cadherin with intracellular signals (Gumbiner and McCrea, 1993; Ranscht, 1994; Watabe et al., 1994; Aberle et al., 1996; Bracke et al., 1996; Jiang, 1996). It is clear that these interactions are absolutely necessary for normal function of the classic cadherins. It has been found that many types of cancers have a reduction in any one of the catenins with or without a loss of E-cadherin expression (Shiozaki et al., 1994; Andrews et al., 1997; Hao et al., 1997; Hiscox and Jiang, 1997; Krishnadath et al., 1997; Richmond et al., 1997; Umbas et al., 1997; vanderWurff et al., 1997). In one epidermal carcinoma cell line, the overexpression of either E-cadherin or P-cadherin does not revert the spindle-like morphology or its tumorigenic behavior to be more epithelial-like (Navarro et al., 1991; Navarro et al., 1993) suggesting that a downstream mediator of the cadherins is missing or mutated. Further analysis revealed that α-catenin and β-catenin were present and form a complex with E-cadherin, but there were only trace amounts of plakoglobin (Navarro et al., 1993). Additionally, a highly invasive lung carcinoma cell line (PC9 cells), was found to express normal levels of E-cadherin, but lacked α-catenin. If either full length α-catenin, or a chimeric protein containing ectodomain of E-cadherin and the carboxyl region of α-catenin, were transfected into these cells, junctional complex proteins were redistributed to the appropriate position in the membranes, cell to cell adhesion was restored and cells were no longer invasive (Nagafuchi et al., 1994; Watabe et al., 1994). Furthermore, an ovarian

carcinoma cell line expressed normal E-cadherin and β-catenin but a mutant form of α-catenin and exhibited features typical of a carcinoma. Once wild type α-catenin was introduced into these cells, tumorigenicity was abrogated and cells regained regulated and controlled growth (Bullions et al., 1997). It has been shown that N-cadherin overexpression in a transformed fibroblast cell line, changed their morphology to an epithelioid phenotype, but was not sufficient to change their invasiveness or tumorigenicity (Simcha et al., 1996). Co-transfection with plakoglobin was required to change growth parameters and to suppress tumor formation capabilities of this transformed cell line (Simcha et al., 1996).

This studies demonstrate that catenins serve as downstream mediators of the extracellular adhesive activities of classic cadherins and link the cadherins to intracellular signaling pathways. Understanding what proteins are involved in relaying catenin signaling of cadherin mediated adhesion will determine how cadherins effect epithelial cell morphology and physiology. It has been shown that these pathways involve a cascade of kinases and phosphatases that ultimately mediate signals to the nucleus and effect gene transcription (Daniel and Reynolds, 1997).

Kinases and phosphatases as downstream mediators of the cadherin/catenin complex

Inhibition of phosphatases in normal cultured epithelial cells causes the phosphorylation of adherens junction components which leads to the subsequent deterioration of this junction. Conversely, if tyrosine kinases are inhibited, adherens junctions will reform (Volberg et al., 1992). While cadherins themselves appear to be poor substrates for kinases and phospatases, their intracellular partners, the catenins are good substrates for these proteins and probably mediate the kinase-induced changes observed above.

It has been shown that MDCK transformed with a temperature sensitive v-src ($pp60^{src}$) lose their characteristic epithelial features. β-catenin was phosphorylated which led to deterioration of epithelial junctions and loss of cell polarity and adhesion (Behrens et al., 1993). Another protein that is found in the cadherin/catenin complex is p120, which has high degree of homology to β-catenin and γ-catenin (Shibamoto et al., 1995). This protein is also a substrate of several receptor tyrosine kinases including $pp60^{src}$ and in its phosphorylated form, it is found in association with cadherin/catenin complexes in several carcinoma cell lines (Shibamoto et al., 1995) suggesting that it may play a role in the downstream events that malfunction in carcinoma formation. In chicken retinal tissue a PTP1B-like tyrosine phosphatase is found in association with N-cadherin and it maintains β-catenin in the dephosphorylated state. If its activity is inhibited, then β-catenin will become phosphorylated and dissociate from N-cadherin (Balsamo et al., 1995). Additionally, two other phosphatases, PTPμ and PTPκ are also enriched at cell to cell contacts and play a role in sustaining junctional proteins in a dephosphorylated state and epithelial phenotype is maintained (Brady-Kalnay et al., 1995; Brady-Kalnay and Tonks, 1995; Fuchs et al., 1996). Interestingly, it has been recently shown that when an integrin associated kinase was overexpressed in an intestinal epithelial cell line, E-cadherin was downregulated and tumorigenicity was increased (Wu et al., 1998). This underscores the interdependence between the cadherin/catenin complex and the integrins in normal tissue and in carcinogenesis.

These studies show that both tyrosine kinases and phosphatases are directly involved in mediating downstream signals of cadherins through the catenins. If these phosphatases are inactivated, or kinases are overactive, this will lead to changes in the conformation, composition or integrity of the cadherin/catenin complex and multiple downstream signals will be disrupted. This will alter cell morphology and physiology through changes in specific gene transcription that may ultimately lead to carcinoma

pathogenesis. The mechanism by which these events are transduced is has not been fully elucidated in epithelial cells.

Upstream Activation Of The Cadherin/Catenin Complex

A potential mechanism to prevent tumor formation and metastasis may be to determine how to trigger cadherin expression, directly or indirectly, in such a way that the normal epithelial program is reengaged. It would be of therapeutic interest to determine if a secreted factor or forced cell adhesion by an obligatory adhesion molecule could recapitulate or trigger the epithelialization program in a carcinoma so that there is complete suppression of its transformed carcinogenic phenotype.

Induction of Cadherin Expression - Role of Steroid Hormones

It appears that estradiol influences the maturation of the ovary by regulating the expression of N-cadherin and E-cadherin which are believed to be the key modulators of pathways leading to follicular development (MacCalman et al., 1994; MacCalman et al., 1995). Injections of estradiol into immature mice increases the amount of N-cadherin and E-cadherin mRNA synthesized and transported to the follicular surface epithelium of the mouse ovary; this cannot be mimicked by progesterone or testosterone injections (MacCalman et al., 1994; MacCalman et al., 1995). It seems that hormonal regulation of cadherins may be complex and tissue specific.

Many gynecological carcinomas express abnormally high amounts of hormone receptors including, estrogen receptors in their surfaces. Thus, it can be speculated that hormones such as estrogen may be able to trigger signaling pathways that suppress the expression of normal proteins like cadherins and leads to carcinomatous properties. It has been shown that when estrogen was added to a well differentiated endometrial carcinoma, the expression of E-cadherin, β- and α-catenin were suppressed. These proteins and their

respective mRNAs were only upregulated after treatment with anti-estrogenic factors such as danazol, progesterone, and medroxyprogesterone (Fujimoto et al., 1996). Additionally, in breast carcinomas, tamoxifen, an anti-estrogen, activated the expression of E-cadherin which lead to increased cell aggregation and decreased invasive potential (Bracke et al., 1994). These studies show that estrogen may trigger abnormal protein function and synthesis which leads to abnormal growth properties and invasiveness of endometrial and breast carcinomas. Therefore, it raises the possibly that abnormal growth and metastasis of carcinomas can be prevented by anti-estrogenic compounds. It has been recently shown that tamoxifen may prevent the development of breast cancer in high risk patients.

Artificial Induction Of Cadherin Expression By P_0, An Obligatory Adhesion Molecule -- The Cervical Carcinoma As A Model System Of Cancer Pathogenesis

The transformed cervical epithelium has been a useful and informative model for studying morphological and behavioral properties of tumor cells. The glandular element of the cervix is normally composed of tall columnar secretory epithelial cells which express and assemble the full complement of epithelial junctions. HeLa cells are a poorly differentiated cervical carcinoma cell line isolated from a highly aggressive adenocarcinoma of the cervix found in a patient at the NIH in the early 1950s. HeLa cells lack the normal characteristics associated with columnar epithelial cells. Although epithelium-derived, these cells express only trace amounts of any classic cadherins, in this case N-cadherin, and the normal repertoire of adhesion associated-proteins is severely reduced or even undetectable. In summary, HeLa cells do not assemble epithelial junctions, are not self-adhesive, lack contact inhibition, and are invasive and tumorigenic (Arai et al., 1976; Vessey et al., 1995).

When P_0 is expressed in HeLa, it initially accumulates and concentrates at the lateral cell borders leading to increased adhesion and aggregation. Subsequently, N-cadherin, as well as other proteins associated with epithelial junctions and their respective mRNAs were upregulated. Electron microscopic analysis reveals that tight junctions, adherens junctions, and desmosomes appear at lateral cell borders. By morphologic criteria at least, it seemed that P_0 expressors became more epithelial-like (D'Urso et al., 1990; Doyle et al., 1995). These results show that P_0 can elicit an inherent but dormant or "sluggish" intracellular pathway which, when activated, triggers N-cadherin synthesis, but not E-cadherin, and subsequent epithelial junction formation. In this way, P_0 is able to act upstream of the cadherins, inducing these carcinoma cells to regain epithelial characteristics. Morphologic and biochemical evidence supports the idea that P_0 expression in this carcinoma can activate a dormant epithelialization program.

Clearly, normal adhesion, morphology, and behavior inherent to epithelia involves the integration of many intracellular signaling pathways. Any of these elements can go awry in carcinomas. It has remained a question in the literature whether carcinomas have a damaged epithelial differentiation and maintenance program or if their epithelialization program is essentially dormant.

In the long term, our laboratory has been interested in understanding the molecular mechanisms underlying myelin membrane self-adhesion. Since it is very difficult to assess the role of specific myelin proteins in their natural setting, we reasoned that we might be able to study adhesive properties of certain myelin proteins in a simpler system. We chose to transfect protein zero (P_0) into a non self-adherent cell line (HeLa) to see if we could recapitulate the adhesive events known to transpire in Schwann cells as they make myelin. In fact this is exactly what occurred; P_0 concentrated at lateral cell borders creating an analogous structure to the intraperiod line in compact myelin. But, most interestingly, morphologically this carcinoma cell line appeared to revert to an epithelial-like state.

My thesis was directly focused on determining the functional significance of these morphologic changes. Specifically, I addressed if P_0 expression in this carcinoma changes its functional properties so that the normal epithelial behavioral repertoire is established. Additionally I began to determine how P_0 mediates these changes.

The major conclusions from my work are:

1.) Contrary to what has been generally accepted, the epithelialization program in this cervical carcinoma is not damaged, but operates insufficiently or is dormant, unable to produce and maintain the normal state in these cells.

2.) P_0 expression not only restores the normal morphological features, but also recovers the physiology indicative of epithelia; these cells are no longer tumorigenic.

3.) The changes elicited by P_0 mediated adhesion are sustained and long-lasting. Once this carcinoma has established de novo these epithelial characteristics due to P_0 expression and adhesion, they are not lost even if cell to cell contacts are then disrupted.

4.) P_0 mediates epithelialization in HeLa through activation of N-cadherin/catenin adhesion systems.

5.) In this cervical carcinoma it appears that E-cadherin can substitute for N-cadherin and elicit an identical sequence of events leading to complete reversion of carcinogenesis.

My major conclusions lead to the hypothesis that cell adhesion itself, no matter how it is brought about initially, can trigger intracellular pathways that lead to the establishment of the epithelial phenotype and its associated physiological properties. It can be concluded that, in general, obligatory adhesion molecules, such as P_0, may serve as inducers of dormant intracellular events in carcinoma cells that ultimately triggers these cells to regain epithelial characteristics. Understanding how this myelin program augments epithelial junction formation and the suppression of the transformed state of carcinoma cells may provide clues towards developing therapeutic approaches to inhibit tumor cell metastases.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture

HeLa cells and breast carcinoma cell lines MDA-MB-231 and MDA-MB-453 (obtained from the American Type Culture Collection) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 7.5% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamate (GIBCO, Grand Island, NY). Cells permanently expressing both P_0-pECE and pSV2-neo or expressing only pSV2-neo, were generated in our laboratory as described (D'Urso et al., 1990; Doyle et al., 1995), and maintained in supplemented DMEM containing 400 µg/ml of Geneticin G418 (GIBCO). In each experiment, control and P_0 expressing cells were pre-treated with 5 mM sodium butyrate which has been shown to increase the transcription of recombinant plasmids in mammalian cells (Gorman et al., 1983). In control experiments, butyrate treatment of nontransfected HeLa cells and pSV2-neo transfected cells does not induce epithelialization (D'Urso et al., 1990; Staugaitis et al., 1990; Allinquant et al., 1991; Doyle et al., 1995).

To generate N-cadherin and E-cadherin HeLa permanent cell lines and their respective control cell lines, cells were transfected with N-cadherin - $PCXN_2$, empty $PCXN_2$ vector, E-cadherin-P_cDNA3, or P_cDNA3 by a liposome-mediated transfection system (DOTAP, Boehringer Mannheim Biochemical). One day after transfection, 800 µg/ml was added to media for selection of expressing cells to isolate single colonies. Once permanent clones were identified by immunocytochemistry, they were maintained in complete DMEM containing 400 µg/ml of G418.

Plasmid Construction

Full length mouse N-cadherin and E-cadherin (gifts from M. Takeichi; (Takeichi et al., 1988; Takeichi, 1991)) were directionally cloned into the $PCXN_2$ or P_cDNA3 expression vectors. $PCXN_2$ has a β-actin promoter and P_cDNA3 has a CMV promoter. *Escherichia coli* strain - XL-Blue - competent cells were transformed to generate clones

containing appropriate vector. Large-scale plasmid preparations of plasmid DNA were done on the positive colonies (PROMEGA).

Antibodies

Rabbit antibody to N-cadherin was generated against the EC1 domain of this cadherin and was characterized in our laboratory (Fannon and Colman, 1996). Affinity purified polyclonal rabbit P_0-anti serum was also generated and previously characterized in our laboratory (D'Urso et al., 1990). Rat anti-uvomorulin (DECMA-1), mouse anti-N-cadherin, rabbit anti- α- and b-catenin, plakoglobin and placental alkaline phosphatase antibodies were purchased from Sigma Chem. Co. (St. Louis, MO). Rabbit anti- ZO-1 and occludin were purchased from Zymed Inc. and mouse monoclonal cytokeratin 18 clone RGE, was purchased from ICN Immunochemicals. Transferrin receptor antibody was purchased from Chemicon Intl., Inc. Other antibodies used were: a mouse monoclonal antibody that recognizes desmoplakin I and II (Cowin et al., 1985), guinea pig anti-desmocollin (Mechanic et al., 1991), and rabbit anti-desmoglein (Schmelz et al., 1986).

Immunocytochemistry

For immunostaining of cultures, cells were seeded on poly-L-lysine coated coverslips. Immunocytochemical analysis of junctional proteins was performed as previously described (Doyle et al., 1995). For immunodetection for most antibodies, cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.05% Triton X-100 for ten minutes. For immunocytochemistry of junctional proteins (including desmoplakin, desmocollin, desmoglein, α- and β- catenin, ZO-1), cells were incubated with a buffer containing 140 mM NaCl, 1.5 M KCl, 10 mM Tris-HCl [pH=7.4], 5 mM EDTA, and 0.5% Triton X-100 to remove soluble proteins prior to methanol fixation for ten minutes. For immunocytochemical studies assessing the distribution of placental alkaline phosphatase cells were fixed with 95% ethanol:5% acetic acid for placental alkaline

phosphatase (Arreaza and Brown, 1995) Following fixation, all coverslips were blocked with 5% normal goat serum (Sigma Chem. Co.) in PBS and incubated with primary antibody (diluted in blocking solution) for 2 hours. After several washes, the samples were incubated with a fluorochrome-conjugated secondary antibody for one hour (e.g. fluorescein, rhodamine, Jackson ImmunoResearch Laboratories, Inc.). After several washes, coverslips were placed on slides with mounting solution (50 mM Tris, pH=8.6, 2.5% DABCO [1,4-diazabicyclo{2.2.2} octane]; Sigma Chem. Co.).

Immunohistochemical analysis was performed on fixed and frozen tissue sections obtained from nude mice injected with P_0-PECE or vector alone. Briefly, sections were placed in phosphate buffered saline (PBS), followed by 100% ice cold methanol for 10 minutes. Sections were rehydrated in PBS and then placed in blocking solution (5% normal goat serum in PBS) and then incubated for 2 hours with a monoclonal mouse cytokeratin 18 antibody, clone RGE or affinity purified polyclonal rabbit anti P_0 serum (D'Urso et al., 1990). After several washes, the samples were incubated with a fluorochrome-conjugated secondary antibody for one hour. Coverslips were placed on slides with mounting solution as above.

A VANOX light microscope (Olympus Corp., Tokyo, Japan) and a Leica TCS 4D confocal laser scanning microscope (CLSM) was used to assess immunofluorescence of cultured cells. For CLSM, scans were taken in .80 - 1.0 μm increments in the z plane. The optical sections were then compressed into a single image. In cultures, confocal microscopy was used to assess the intracellular distribution of placental alkaline phosphatase and transferrin receptor immunofluorescence. Scans were recorded in .35-.40 μm increments in the z plane. Image processing was through Scanware and Adobe Photoshop software.

Western Blotting

Cell lysates (Laemmli, 1970) or enriched fractions of cytoskeletal elements and membranes (Doyle et al., 1995) were separated on a 7.5% polyacrylamide gels and transferred to nitrocellulose paper. The nitrocellulose was blocked with 5% nonfat milk, incubated with primary antibody for one hour, washed, and incubated with peroxidase conjugated secondary antibodies (Sigma Chem. Co.) for 30 minutes. Following several washes, blots were developed by chemiluminescence (Amersham Corp.). To quantitate the amount of protein loaded on gels, a standard BCA protein assay (PIERCE) was performed standardize on all samples.

Assessment of Anchorage Independent Growth

The ability of cell lines to grow and form colonies in the absence of a matrix was measured by a standard agar assay (Hoffman et al., 1993; Rodriguez-Fernandez et al., 1993). Cells were harvested in 0.1% trypsin (GIBCO), resuspended, and 10^3 or 10^4 cells were placed in top agar (0.35% Bacto-agar in complete DMEM with G418). This suspension was placed on solidified bottom agar (0.60% Bacto-agar in DMEM). Cells were fed every 2-3 days with top agar containing FCS. Colony formation was assessed and counted (colonies ≥100 μm) after 2 and 4 weeks. Each experiment was done in triplicate. Data was expressed as cloning efficiency (colonies formed/cells plated x 100).

Secretion of Matrixmetalloproteinases

The enzymatic activity and expression of two matrixmetalloproteinases (MMP-2 and MMP-9) were analyzed by gelatin zymography (Heussen and Dowdle, 1980). Conditioned media was obtained from growing cells and was diluted in 4x sample buffer containing no reducing agents. The cells from which the conditioned media was harvested were counted to adjust for cell number when loading samples. The samples were loaded in a non-denaturing 7.5% SDS-Tris protein gel containing 0.2% gelatin. After the gel was resolved, it was incubated in 2.5% Triton X-100 (23°C, 30 minutes) to remove SDS, was

rinsed extensively with distilled water and then, placed in 50 mM Tris-HCl (pH=7.7), 5 mm $CaCl_2$, and 0.02% sodium azide (37° C, 40h) to allow enzymatic digestion to proceed. Gels were stained with Coomassie Blue and destained in a standard acetic acid/methanol solution.

In Vitro Assessment of Invasion

A chemoinvasion assay was performed as described (Albini et al., 1987). Matrigel matrix (50 μg) was coated on 12 mm polycarbonate filter inserts (12 μm pore size) and placed into a 12 well plate creating a modified Boyden chamber apparatus. Conditioned medium, obtained by incubating NIH-3T3 cells in DMEM with added 0.1% bovine serum albumin (BSA) and ascorbic acid (50 μg/ml) for 24 hours, was placed in the bottom chamber to serve as a chemoattractant. Cells were harvested with 0.1% trypsin and then resuspended in DMEM containing 0.1% BSA. Suspensions containing 2.5 x 10^5 cells were placed in the upper chamber. The plates were incubated for 12 hours at 37°C in 5% CO_2. Cells remaining on the upper side of the filter were removed with a cotton swab. In order to better visualize the cells that had invaded to the bottom side of the filter, fixation with 95% ethanol and staining with hematoxylin and eosin were performed. Cells that had invaded the matrix were quantified by counting several fields under the 100x lens of an inverted light microscope (Olympus Corp., Tokyo, Japan).

In Vivo Assessment of Tumor Formation

Confluent monolayers of P_0 or control cells grown in 100 mm dishes were pretreated with 5 mm sodium butyrate for 16 hours to upregulate P_0 expression; this treatment does not alter other cellular properties (D'Urso et al., 1990; Doyle et al., 1995). Cells were harvested with .1% trypsin and resuspended in DMEM. Athymic nu/nu 5 week old nude mice were injected intraperitoneally with 10^6 cells; there were 14 mice in each group. The

growth rate of these cells was assessed by plating 10^6 cells in 100 mm dishes (without any further butyrate treatment) and then counting with a hemocytometer over two weeks.

Animals were perfused with 4% paraformaldehyde (PFA) at two and four weeks. Representative tissues (lung, liver, spleen, brain, and any grossly apparent tumors) were removed and placed in 10% sucrose in 4% PFA for 1.5 hours at 4° C, and then in 20% sucrose in 4% PFA overnight at 4°C. Tissue was embedded in Tissue-TEK O.C.T. compound (Miles Inc.) on dry ice and was sectioned on a cryostat in 10 μm sections, and stored at -80°C. Standard hematoxylin and eosin staining was performed on sections to visualize the parenchyma and overall architecture of the selected tissues.

Measurement of Transepithelial Electrical Resistance

Cells were grown on 12 mm polycarbonate filters in 12 well dishes until confluent monolayers were formed. Transepithelial resistance was read with a electrical resistance meter (Millicell ERS, Millipore). Baseline measurements were taken after the cell monolayers were placed in calcium free medium for 4 hours. Monolayers were placed for 4 hours in complete media with calcium to allow tight junction formation and then another resistance reading was taken (adapted from (Wong and Gumbiner, 1997)). The resistance-reading was normalized to the monolayer area. The background resistance of the blank filter was subtracted from all readings.

CHAPTER 3

SUPPRESSION OF TUMORIGENICITY OF AN AGGRESSIVE CERVICAL CARCINOMA INDUCED BY THE EXPRESSION OF P_0, A NERVOUS SYSTEM IGCAM

In this chapter, I describe experiments in which I assessed the physiologic properties of P_0:HeLa and found that constitutive P_0 expression in HeLa cells restores several important aspects of normal epithelial cell physiology, including: functional tight junctions, cell polarity, contact inhibition, and adhesion-mediated growth control. Of greatest interest, HeLa cells constitutively expressing P_0 do not form tumors in athymic nude mice. These studies show that artificial adhesion elicited by P_0 acts to override the tumorigenic state of these carcinoma cells.

Results:

P_0 expressing HeLa cells generate physiologically operative tight junctions

We have shown by electron microscopy that P_0 expressing HeLa cells assemble morphologically identifiable epithelial junctions (Doyle et al., 1995). One role of tight junctions is to maintain a transcellular and paracellular permeability barrier that regulates the flux of electrolytes and macromolecules, thereby assisting in the maintenance of a particular environment characteristic of each tissue type. Carcinoma cells like HeLa do not produce an extensive network of tight junctions and so cannot form the intracellular seal necessary to maintain this barrier. One way to assess this function in situ is to measure the transepithelial electrical resistance (TER) across cell monolayers. This resistance is an indirect, but highly reliable measurement of the relative "tightness" or "leakiness" of these junctions (Claude and Goodenough, 1973; Powell, 1981). Cells that have a high TER form a tighter barrier (e.g., endothelial cells of the blood-brain barrier). Conversely, cells that lack tight junctions have low resistance values and electrolytes and macromolecules can easily pass across the monolayer.

Occludin, a transmembrane component (Furuse et al., 1993), co-localizes with other known tight junction proteins, such as ZO-1 and cingulin, but significantly, occludin has been shown to be one of the functional elements of the tight junction (Balda et al., 1996; McCarthy et al., 1996; Wong and Gumbiner, 1997). Mutational analysis and peptide

inhibition studies have revealed that if occludin is not appropriately expressed at the cell borders, cells have low TER because they fail to form the tight intracellular seal that wild type junctions confer, even though the tight junction morphology remains intact (Wong and Gumbiner, 1997).

Immunofluorescence for occludin revealed that control cells expressed very low amounts of this polypeptide; it was only found intermittently and at cell borders as isolated puncta (Figure 3.1 A; arrows). In contrast, P_0 expressors appropriately targeted this tight junction protein. Confluent monolayers accumulated long arrays of occludin around the perimeter of adjacent cells (Figure 3.1 B; arrows). Western blot analysis of total cell lysates confirms that more occludin is expressed in the P_0 cells than control cells (Figure 3.1 C).

Since occludin expression appeared to be normal in the P_0 expressors, we compared the TER in P_0 expressors and control cells to assess the functionality of assembled tight junctions. Cells were grown on filters and TER was measured 16 hours after confluent monolayers were treated with butyrate. In the absence of Ca^{++}, the TER of pSV2-neo cells was 10.95 Ω cm^2, as cells devoid of tight junctions would behave, whereas P_0 monolayers had a baseline TER of 26.4 Ω cm^2 (Figure 3.2). Once Ca^{++} was added to the system however, the resistance of the P_0 expressing monolayers increased to 76.2 Ω cm^2 while the resistance of the control cells (pSV2-neo cells) did not change (10.5 Ω cm^2) indicating tight junctions were assembled and working appropriately in P_0-HeLa.

Tight junction formation leading to the generation of TER is Ca^{++}-dependent (Wong and Gumbiner, 1997), whereas P_0 adhesion is not (D'Urso et al., 1990; Doyle et al., 1995). It is interesting to note that in the absence of calcium, when tight junctions are presumably inoperable, P_0-HeLa have a higher TER (by 15.45 Ω) than controls. It is possible that the extensive adhesive latticework created by homophilic interactions between P_0 molecules on opposing membranes (Shapiro et al., 1996) may create a partial intercellular barrier. The distance between P_0 compacted Schwann cell membranes in

Figure 3.1 Distribution of the tight junction protein, occludin.

Occludin immunofluorescence in control cells (A) is intermittent and punctate, whereas in P_0 cells, it is organized in long parallel arrays about the cell perimeter (B). (Bar 10 μm) Western blot analysis of cytoskeletal-enriched fractions indicate that there is an increase in protein synthesis or stabilization of protein in P_0 expressors (C). (-B: no butyrate added; +B: butyrate added)

Figure 3.1

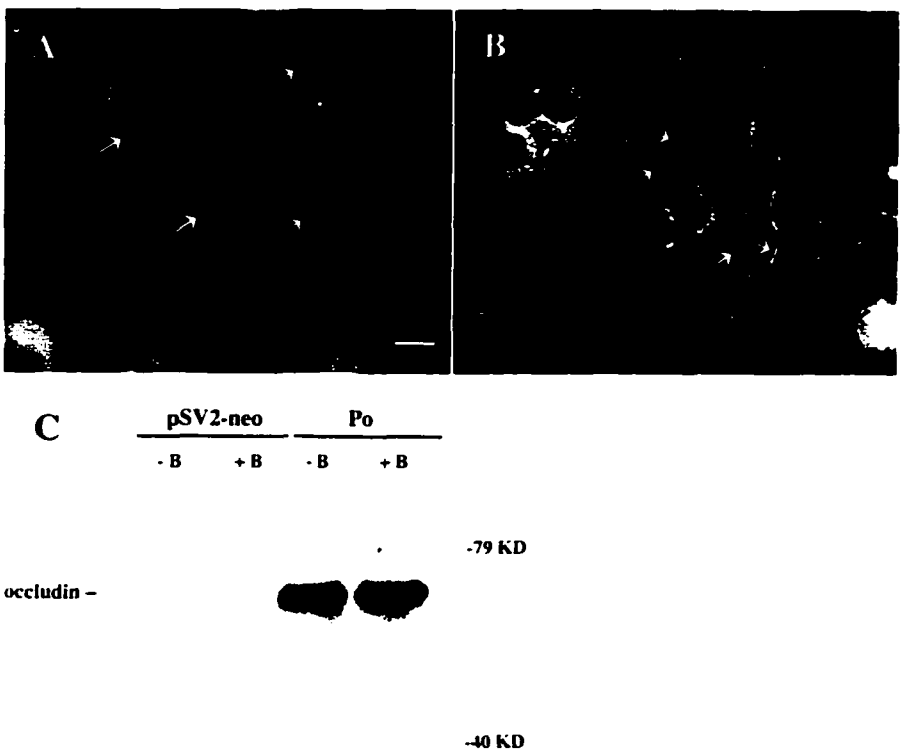
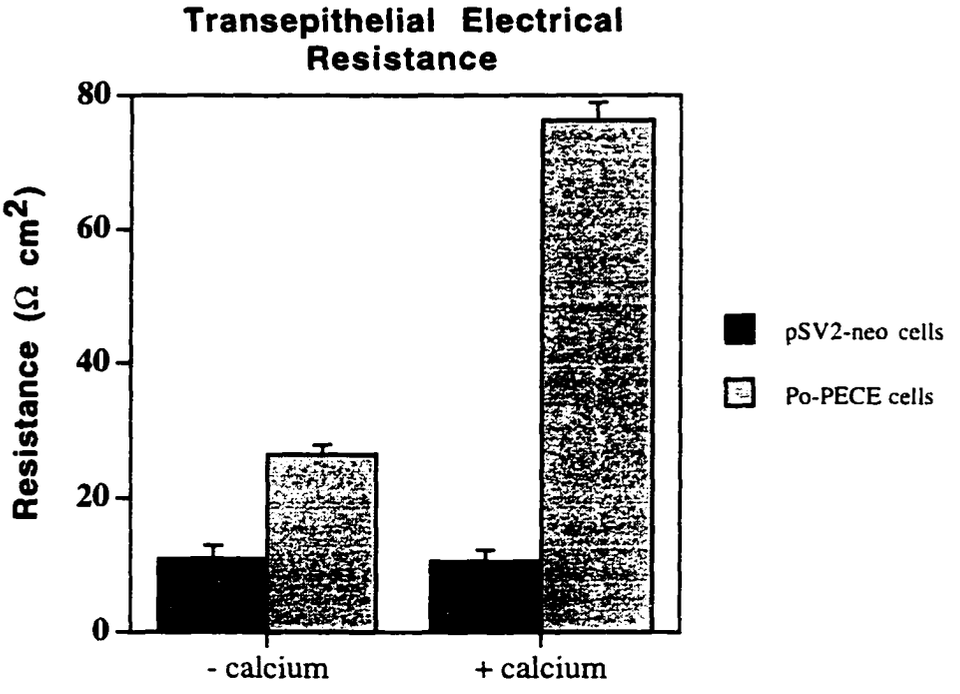


Figure 3.2 Comparison of transepithelial electrical resistance.

Cells were placed in calcium deficient media for 4 hours to obliterate tight junction resistance and baseline measurements were read. Cells were replenished with calcium to allow the assembly of occludin into tight junctions. P_0 expressors have a higher TER than control cells indicating that tight junctions are functional and form a tighter paracellular permeability barrier than control cells. Error bars indicate standard deviation.

Figure 3.2



myelin is 5.5 nm, which is larger than the juxtaposed membranes in tight junctions (0 nm) (Farquhar and Palade, 1963; Raine, 1984; Shapiro et al., 1996). Conceivably, the P_0 adhesive lattice may act as a type of junction which helps restrict the flux of molecules and ions across regions of compact myelin.

We can conclude from these experiments that P_0 expressors form a significant permeability barrier to electrolytes and macromolecules, in contrast to the control carcinoma. The increased TER correlates both with P_0 expression (Ca^{++} independent) and occludin incorporation into tight junctions (Ca^{++} dependent) and is compatible with the formation of functional tight junctions in the P_0 expressors. Additionally, P_0:P_0 interactions can generate TER and maintain intercellular permeability barriers in the absence of tight junctions (- Ca^{++})

P_0 expressors establish functional cell polarity

In epithelial cells, tight junctions not only form intracellular barriers to ion diffusion, but also act as the defining border between the apical and lateral plasma membranes, enabling cells to maintain specific proteins and lipids within these particular surface subdomains. In general, cells lacking functional tight junctions distribute proteins at random at the cell membrane. We examined optical sections through P_0 expressors and control cells to assess the distribution of placental alkaline phosphatase (PALP; Figure 3.3), which in normal epithelia, is an apically-directed protein, and the transferrin receptor (TR; Figure 3.4), a basolateral protein. In Figures 3.3 and 3.4, representative images are arranged from basal to apical. Placental alkaline phosphatase (Figure 3.3 A) and transferrin receptor (Figure 3.4 A) are randomly distributed in all subdomains of the plasma membrane in control pSV2-neo cells. In the P_0 expressors, placental alkaline phosphatase and transferrin receptor were appropriately sorted and targeted to the apical and basolateral domains, respectively (Figure 3.3 B, 3.4 B).

Figure 3.3 Distribution of apically-directed placental alkaline phosphatase.

PSV2-neo cells distribute placental alkaline phosphatase at random in all subdomains of the plasma membrane (A). P_0 cells appropriately target and maintain this protein exclusively in the apical domain which is the native domain for this protein (B). (Bar 10 μm) Arrows indicate regions of low cell to cell contact and asterisks point out areas with high amounts intercellular contact.

Figure 3.3

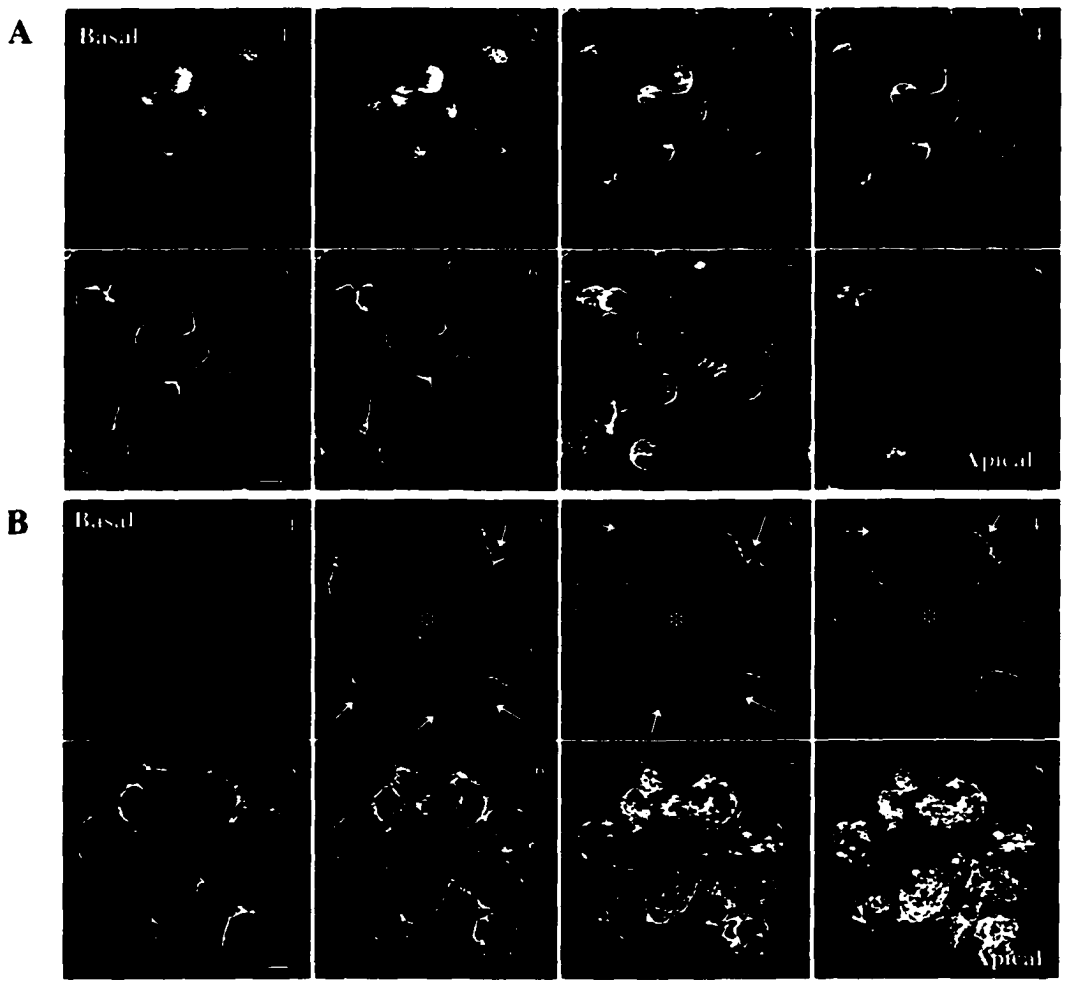
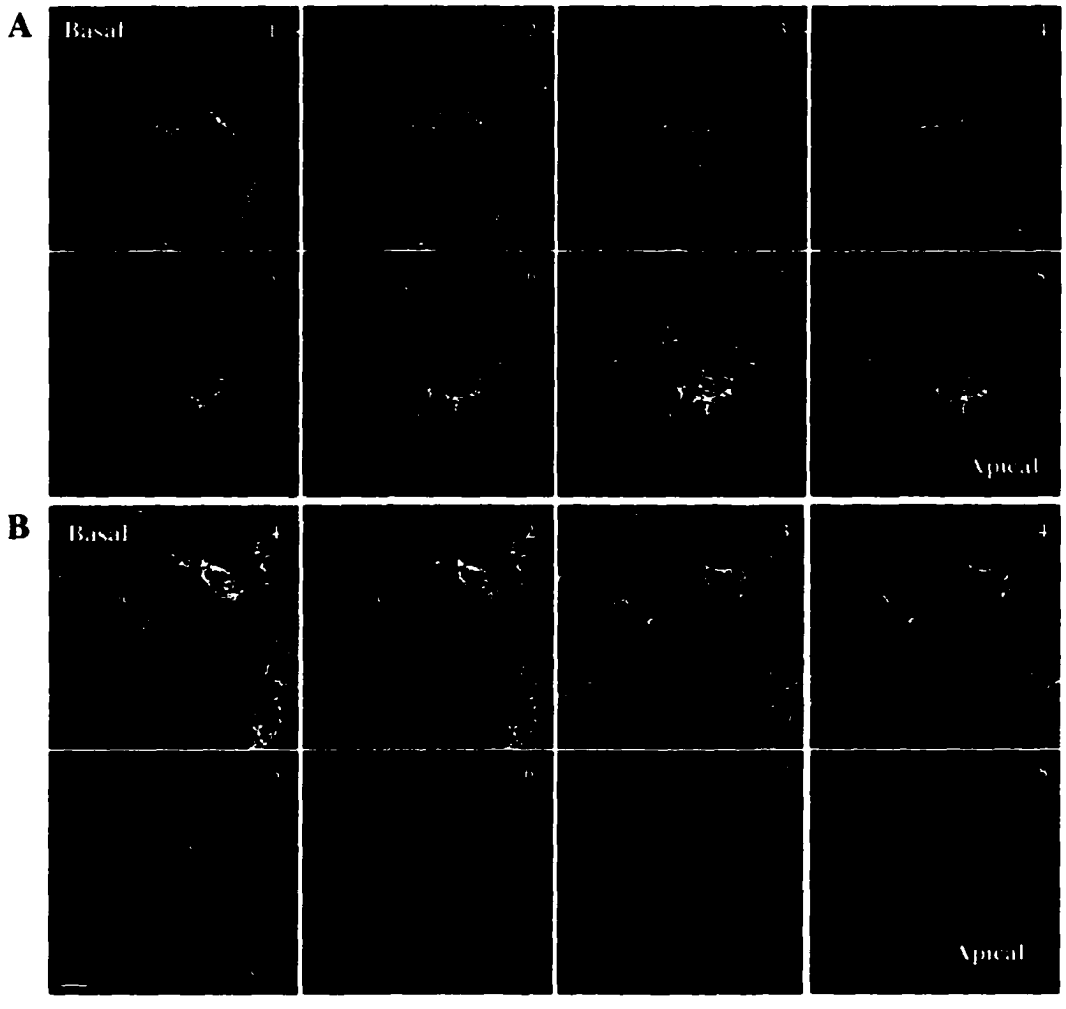


Figure 3.4 Distribution of transferrin receptor, a basolateral protein.

In control cells, transferrin receptor is found in all plasma membrane domains - apical, lateral and basal (A). Transferrin receptor is appropriately targeted and distributed in the basolateral membrane of P_0 expressors (B) indicating that P_0 cells regain functional cell polarity. (Bar 10 μm)

Figure 3.4



In P_0 expressing HeLa, PALP is found mostly at the apical surface. There is an absence of labeling in the areas of greatest cell contact (Figure 3.3 B:2-4; asterisk). Presumably, this is the region where the highest concentration of tight junctions exist, although due to incompatible fixation conditions we could not simultaneously localize PALP and tight junction proteins (Figure 3.3 B:2-4; asterisk). Conversely, in areas where there is less cell contact (Figure 3.3 B:2-4; arrows), PALP is found within all the plasma membrane subdomains. If there are no neighboring cells directly apposed, proteins can not be stabilized at the cell membrane, tight junctions do not assemble, and cell polarity is not maintained. Another interesting feature is that microvillar processes which are normally found at the apical surface of columnar epithelial cells can be seen radiating from the cell surface in the most apical sections (Figure 3.3 B:8; arrow).

P_0 expression changes anchorage-dependent and -independent growth parameters

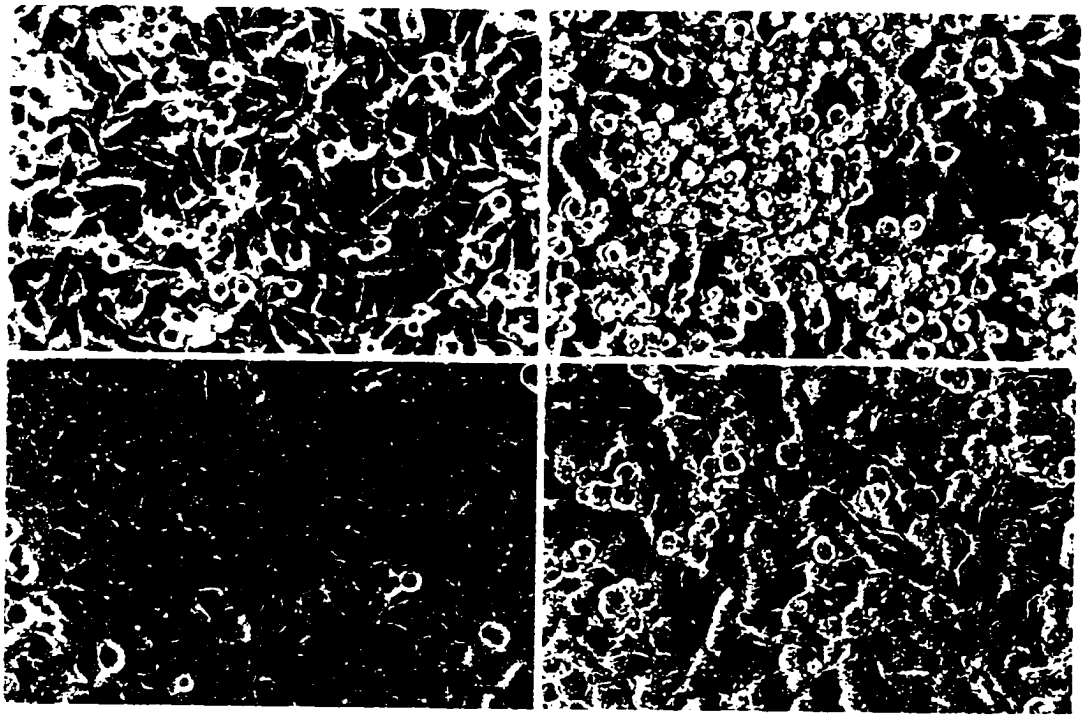
Normal epithelia grow in a controlled and contact inhibited manner. Carcinoma cells, including HeLa cells, have lost contact inhibition and, upon confluency, pile up and continue to divide due to the loss of density limited growth (Figure 3.5 B). Since P_0 expression restores cell to cell adhesion in HeLa cells, we considered the possibility that their growth properties might be altered.

Once P_0 expressors reach confluency, they become reversibly contact inhibited, stop dividing, and maintain their polygonal and flattened morphology (Figure 3.5 C, D). Cell to cell boundaries become indistinct due to close apposition of membranes (≈5 nm) mediated by P_0 adhesion (Doyle et al., 1995; Shapiro et al., 1996). In contrast, control cells continue to divide at confluency, forcing them to round up and pile on top of one another, and eventually to lose attachment to the plate surface (Figure 3.5 A, B). This data demonstrates that the anchorage-dependent growth pattern of P_0 expressors is similar to that of normal epithelial cells rather than to parental or control lines.

Figure 3.5 Anchorage-dependent growth parameters.

Phase contrast images show that pSV2-neo cells continue to divide and pile up on one another at confluency (A) and post-confluency (B). In contrast, P_0 expressors maintain their distinctive flattened and polygonal morphology even several days post-confluency (C, D); these cells never loose attachment to the plate surface. These results suggest that P_0 cells acquire contact inhibition and adhesion mediated growth control. (Bar 10 μm)

Figure 3.5



Additionally, epithelial cells require attachment to a basement membrane or matrix when undergoing cell division. Transformed cell lines, including HeLa, have a decreased requirement for cell-substrate attachment for continual proliferation which may lead to tumor formation. Anchorage-independent growth can be evaluated *in vitro* by determining if a particular line can form colonies when grown in suspension or in a semisolid medium such as soft agar. HeLa cells and the pSV2-neo transfected control, like most tumor cell lines, divide and form large colonies when grown in agar with a cloning efficiency of approximately 72% (Figure 3.6 A, C) (Celis et al., 1978). In contrast, virtually none of the P_0 expressors form colonies when grown under these conditions and have a much lower cloning efficiency (5%), similar to normal epithelial cell lines (Figure 3.6 B, C). The majority of the P_0 expressors remain as single cells.

P_0 expressors secrete low levels of matrixmetalloproteinases and are not able to invade an artificial basement membrane

Another property that contributes to the aggressiveness of a carcinoma is their ability to invade. One mechanism that enables carcinomas to invade basement membranes is the abnormally high synthesis and secretion of matrixmetalloproteinases (MMPs) which degrade the basement membrane and the surrounding matrix. Epithelial cells do not express and secrete these enzymes and are therefore less invasive.

An in vitro chemoinvasion assay (Albini et al., 1987) was used to compare and quantify the invasiveness of the P_0 expressors and the control cells. This assay measures the number of cells that are able to invade an artificial matrix (Matrigel) that is composed of laminin, type IV collagen, heparin sulfate and other substances found in the basement membrane. We find that after twelve hours, 293 control cells on average invade the matrix whereas only about 4.5 P_0 cells can invade (Figure 3.7 A-E).

We compared the enzyme activity and expression of two type IV collagenases (MMP-2 and MMP-9) in the P_0 expressors and other carcinoma cell lines by gelatin

Figure 3.6 Assessment of anchorage-independent growth.

Control HeLa and P_0-HeLa were grown in soft agar to assess anchorage-independent growth. PSV2-neo formed large colonies with a cloning efficiency of 72% (A, C), whereas P_0 cells were unable to proliferate in the absence of a matrix and had a cloning efficiency of only 5% (B, C). This indicates that P_0-HeLa could not grow in an anchorage-independent manner. (Bar 10 μm)

Figure 3.6

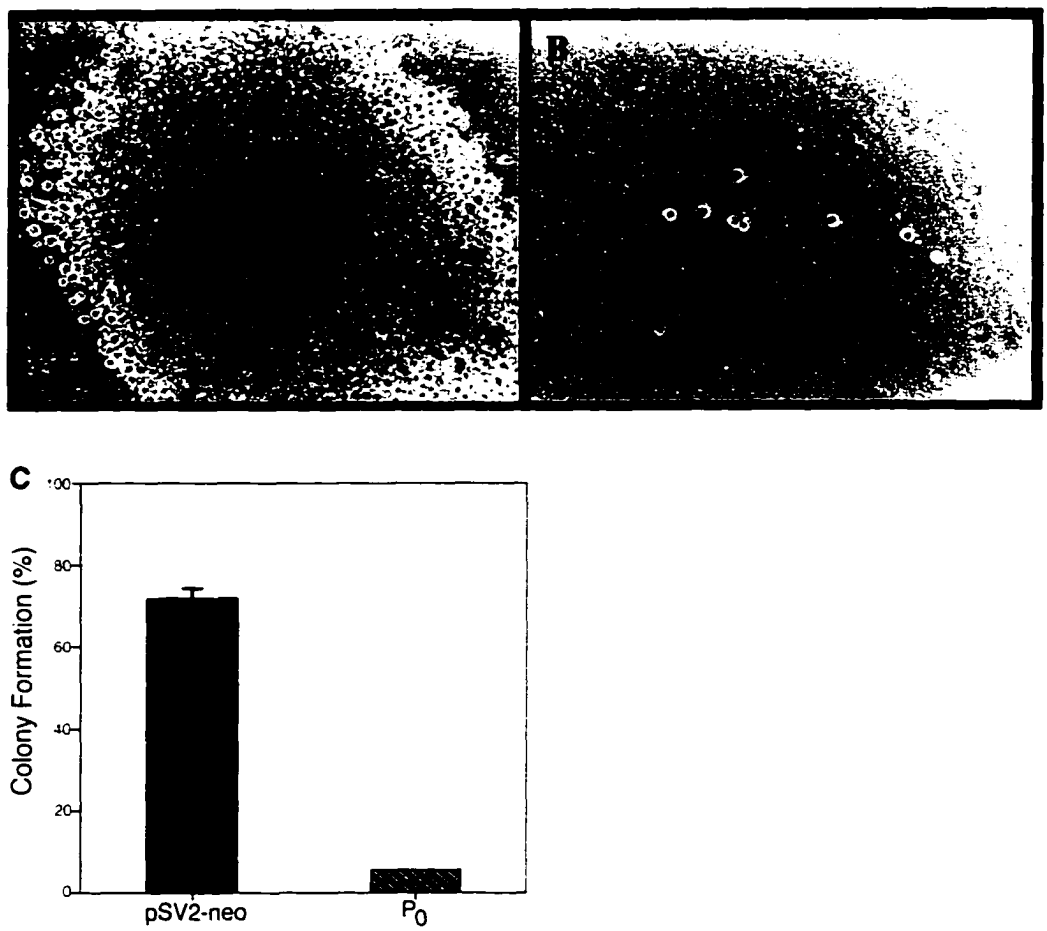
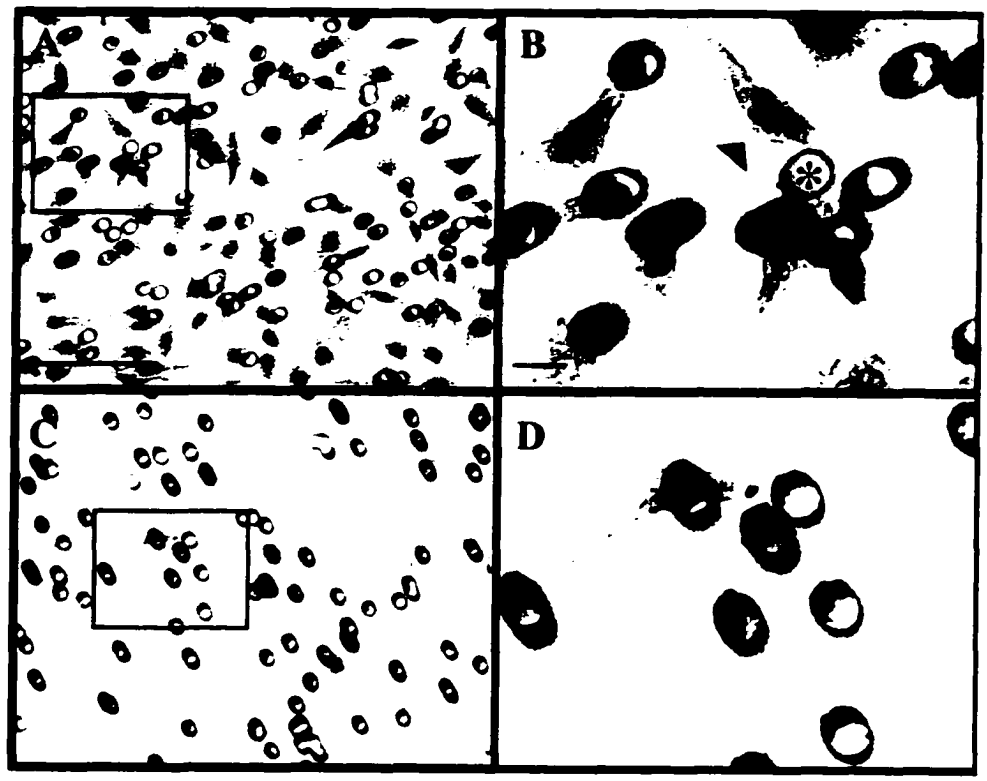
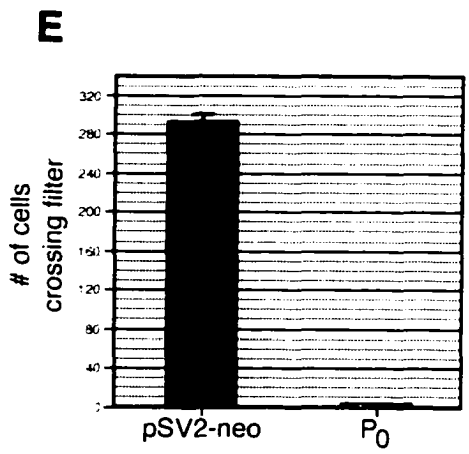


Figure 3.7 Comparison of invasion through a basement membrane.

The invasiveness of the P_0 and control cells was determined by comparing their ability to cross an artificial basement membrane (Matrigel coated filters). On average, 296 of the control cells (A, B, E) were able to invade through the matrix whereas only 4.5 of the P_0 cells could (C, D, E). Qualitatively (A-D) and quantitatively (E), it appears that the P_0 expressors are less invasive that control cells. B and D are high magnification of the boxed in areas in A and C. (Asterik: pore: Arrowhead: cells; Bar 10 μm)

Figure 3.7





zymography. Aliquots of conditioned media are run on non-denaturing gels containing gelatin, which serves as a substrate for the MMPs. The resolved gel is placed in buffer to allow development of enzyme activity, and then stained. The clear bands reveal the extent to which the gelatin has been proteolyzed (Heussen and Dowdle, 1980). Two highly invasive breast carcinoma cell lines MDA-MB-231 and 453 secrete high levels of MMP-9 or MMP-2 (Figure 3.8, lanes A, B). Control pSV2-neo HeLa cells (+ or - butyrate, Figure 3.8, lanes C, D) secrete both MMP-9 and MMP-2. P_0 expressing cells have lost the ability to elaborate these enzymes (Figure 3.8, lanes A, B).

These results show that the P_0 expressors cannot invade the artificial basement membrane to the same degree as the controls due to their decreased secretion of matrix degrading collagenases. This raised the possibility that these cells may be in fact less tumorigenic and invasive *in vivo* than parental or control cell lines.

P_0 expressors do not form tumors when injected into athymic nude mice

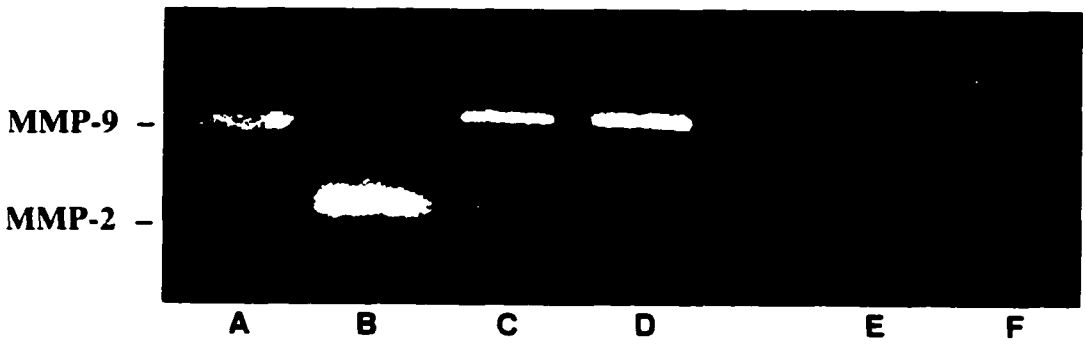
One well-documented test of tumorigenicity of a cell line in vivo is to inject cells into athymic nude mice and observe tumor formation over a period of weeks (Freedman and Shin, 1974; Celis et al., 1978). Mice injected with cells harvested from highly aggressive cancer cell lines form more tumors in a shorter period of time. In order to assess how the P_0 expressors behaved in situ, we injected athymic nude mice with either P_0 or pSV2-neo expressing cells and evaluated gross and microscopic tumor growth over a period of two months. HeLa cells, including P_0 expressors, express high levels of cytokeratin 18 (Doyle et al., 1995). Thus, we could specifically identify even small micrometastases within the liver using antibodies to this protein.

Six mice from each group were sacrificed at three and four weeks . All of the mice injected with control HeLa cells exhibited pronounced ascites which was found to contain viable pSV2-neo HeLa cells that were then cultured. Small tumors were found at the injection site and within the peritoneal cavity. Two mice had small tumor lesions attached

Figure 3.8 Secretion of matrixmetalloproteinases -- MMP-2 and MMP-9.

The secretion of matrixmetalloproteinases was assessed by gelatin zymography in several cell lines. The P_0-HeLa do not secrete MMP-2 or MMP-9 at the same levels as the control cells or the breast carcinoma cell lines (MDA-MB-231 and 453). Lane A. MDA-MB-231 cells; B. MDA-MB-453 cells; C. pSV2-neo cells + Butyrate; D. pSV2-neo cells; E. P_0-HeLa + Butyrate ; F. P_0-HeLa.

Figure 3.8



to the outer surface of the liver (Figure 3.9 A). High power examination of the livers in three other mice revealed that single cells or small groups of cell within the liver parenchyma that were cytokeratin positive (Figure 3.9 B) and thus pSV2-neo cells that had metastasized to this organ. By seven and eight weeks, four mice injected with the pSV2-neo cells died, presumably due to tumor overload and surviving mice in this group were extremely sick with abdomens distended by ascitic fluid. These mice had multiple subcutaneous and peritoneal tumors, and had gross metastases on the outer surface and within liver. Micrometastases were found disrupting the normal architecture of the liver parenchyma (Figure 3.9 C, D).

The mice injected with the P_0 expressors showed no physical or morphological signs of tumor formation. Mice did not have ascites and their organs were healthy and well-perfused. There were no gross tumors within the peritoneal cavity or on any organs, including the liver. In order to verify that there were no micrometastases, the organs were analyzed immunohistochemically with cytokeratin antibody and P_0 antibody. The liver parenchyma had no disruption in its normal architecture at either 4 or 8 weeks (Figure 3.10 A, C). The cytokeratin antibody reveals that the liver parenchyma was free of any micro-metastases at both 4 and 8 weeks (Figure 3.10 B, D); results are identical with the P_0 antibody (data not shown). The spleens of the P_0 mice were 1.5 - 2 times larger that the pSV2-neo injected mice. The spleens from both animals had no abnormalities in the architecture in the spleens from either group (data not shown) and were tumor free. All other organs are tumor-free as well (data not shown). In summary, 0/14 mice injected with P_0 expressors developed tumors and all of the mice with pSV2-neo cells formed tumors and metastasis. P_0 is not a promiscuous adhesion molecule and can only act homophilically to produce cell adhesion. We also considered that P_0 cells might be able to "home" to myelinated peripheral nerves where in the normal animal P_0 is found exclusively. Thorough histologic examination of the sciatic nerves revealed no tumors.

Figure 3.9 Nude mice injected with pSV2-neo cells had small tumors attached to the outer surface of the liver capsule by 3 weeks (A) and tumor had metastasized within the liver parenchyma by 8 weeks (C). Cytokeratin immunofluorescence showed that isolated cells can be identified within the liver parenchyma at 3 weeks indicating that tumors cells have begun to metastasize at this early time point (B). By 8 weeks the tumor cells have formed nodules that disrupt the architecture of the liver parenchyma (D). (Bar 125 μm A; 5 μm B; 15 μm C; 30 μm D)

Figure 3.9

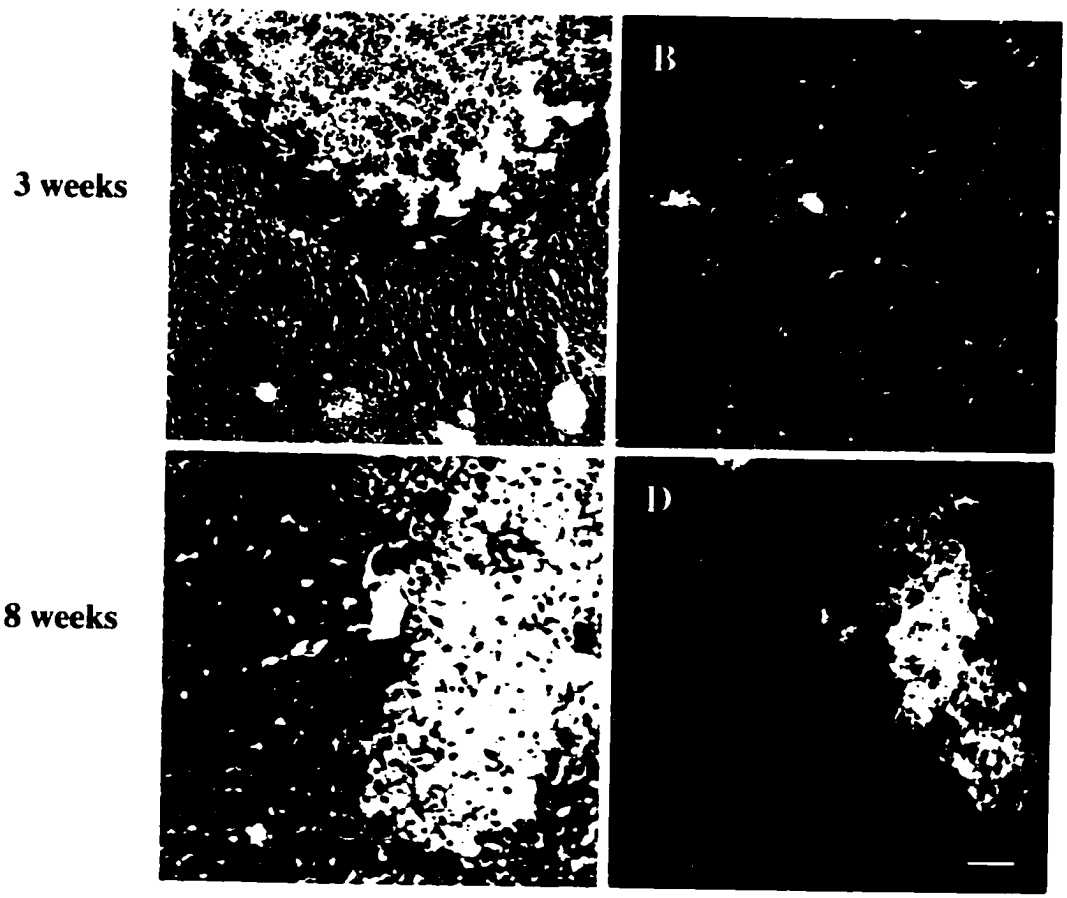
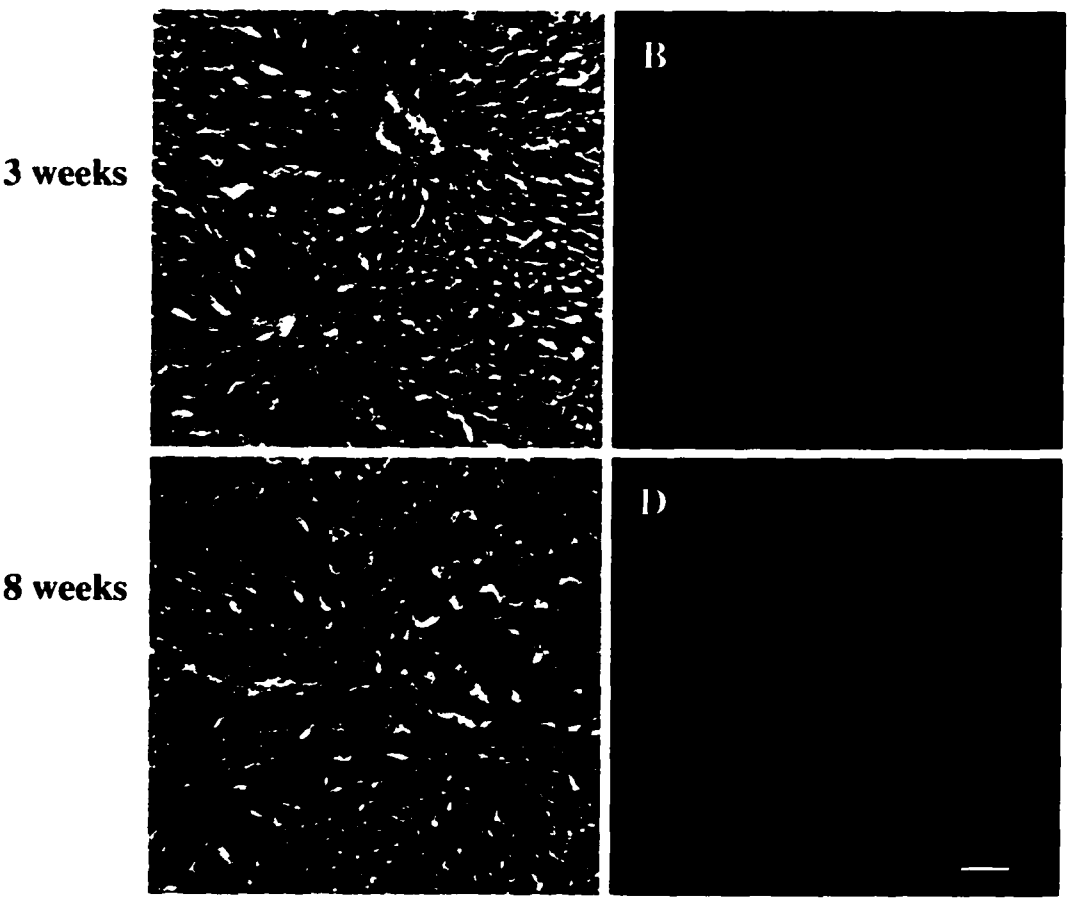


Figure 3.10 Nude mice injected with P_0 expressors do not exhibit tumor formation within the peritoneum or metastatic lesions in any organ. At 3 weeks (A) and 8 weeks (C) the liver parenchyma showed no evidence of tumor cells. Cytokeratin immunofluorescence confirmed that there were no micro-metastases in these livers at 3 (B) or 8 weeks (D). (Bar 30 μm A-C; 5 μm D).

Figure 3.10



Components of the cadherin/catenin signaling pathway are upregulated in P_0-HeLa

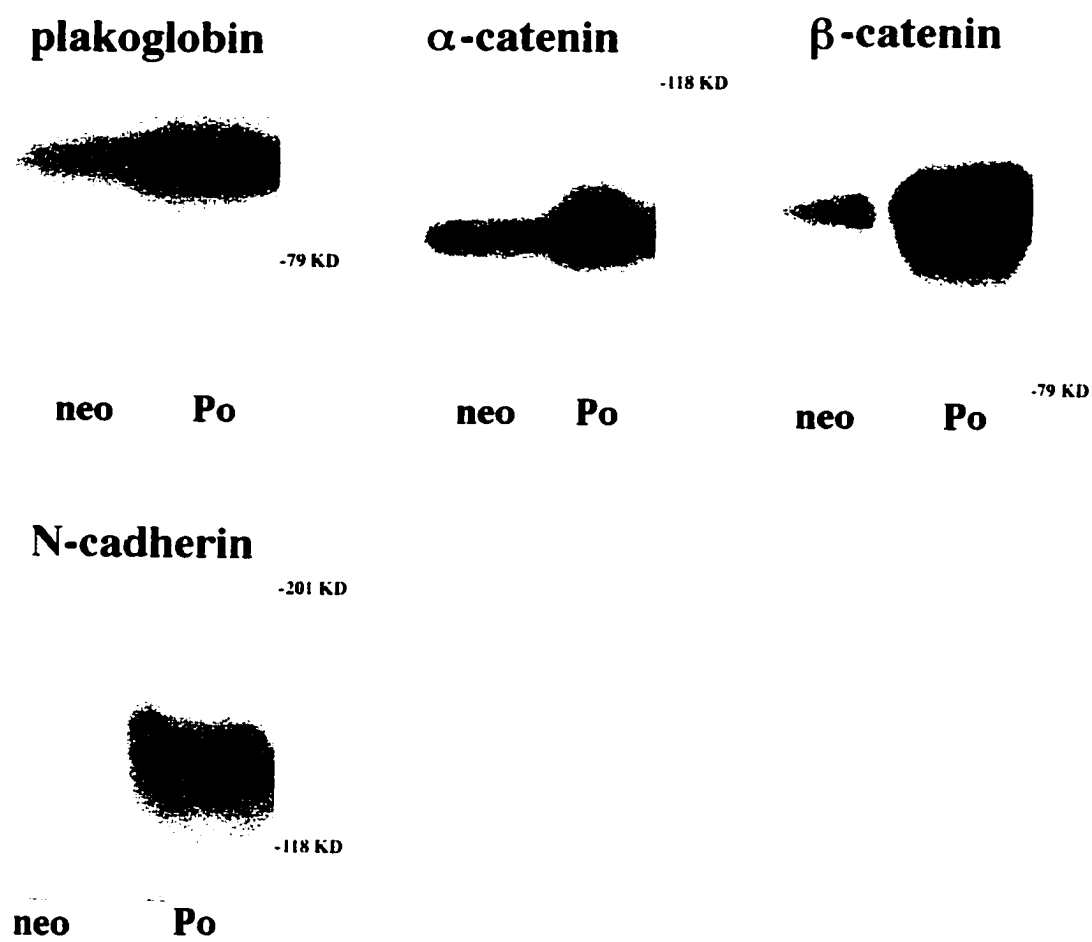
We have shown previously that P_0 expression in HeLa induces increases in the synthesis of N-cadherin as well as its associated proteins α-catenin and β-catenin (Figure 3.11; (Doyle et al., 1995). The catenins are downstream mediators of cadherin adhesion and are absolutely necessary for classic cadherins to function appropriately in epithelia. Another member of this family essential for cadherin mediated signaling is plakoglobin, and its expression is severely reduced in HeLa cells as well as in other carcinoma cell lines (Figure 3.11; (Simcha et al., 1996; Lewis et al., 1997)). P_0 expression in HeLa increases the synthesis of plakoglobin (Figure 3.11). The upregulation of N-cadherin and the associated catenins, α-catenin, β-catenin, and plakoglobin in P_0-HeLa suggests that P_0 adhesion may elicit epithelialization of this carcinoma through the activation of cadherin/catenin signaling pathways.

Taken together, our data reveal that the P_0 expressing HeLa cells have made the reverse transition from a carcinoma to a functioning epithelial-like cell line. It appears that P_0 expression re-engages a dormant, but undamaged program in this cell line that effectively suppresses the carcinoma phenotype.

Figure 3.11 Components of the cadherin/catenin signaling pathway are upregulated in P_0-HeLa.

Western Blot analysis of cytoskeletal enriched cell lysates demonstrate that control pSV2-neo cells express low amounts of N-cadherin and the catenins. P_0-HeLa upregulate the synthesis of N-cadherin, plakoglobin, α-catenin, and β-catenin.

Figure 3.11



Discussion

P_0 is solely the natural product of Schwann cells where it adheres a single plasma membrane surface to itself to generate compact myelin. Atomic structural modeling predicts that individual P_0 molecules emanate from the plasma membrane as tetramers that interdigitate to yield a virtually infinite, highly adhesive lattice network (Shapiro et al., 1996). Membrane surfaces are brought within 5 nm of each other, thus allowing very close apposition. In order to maintain compact myelin, P_0 lattice interactions are strongly adhesive, and once formed are probably not subject to modulation (Colman et al., 1996). The molecular mechanisms that mediate P_0 adhesion are general and "obligatory", since P_0 when expressed in a variety of cell lines, can mediate intracellular adhesion (D'Urso et al., 1990; Filbin et al., 1990; Schneider Schaulies et al., 1990; Doyle et al., 1995)

When the plasma membranes of HeLa are brought into close apposition by P_0, the eventual consequence is "epithelialization". These cells assemble functional tight junctions, restore cell polarity, and acquire adhesion mediated growth patterns including contact inhibition and the loss of anchorage-independent growth (Figures 3.5, 3.6). Additionally, P_0:HeLa are unable to invade an artificial matrix and have decreased secretion of matrix-degrading enzymes (Figures 3.7, 3.8). It appears that changes induced by initial P_0 expression are sustained; once this program is initiated, it is not dependent upon the maintenance of P_0-based adhesion. We have shown that individual P_0 cells injected into nude mice do not form tumors or metastatic lesions as control cells do (Figure 3.9, 3.10). P_0 adhesion triggers complex, dormant but intact pathways that lead to these observed changes in HeLa morphology and physiology sustained within each individual cell.

Multiple complex and interacting signaling pathways involving specific gene transcription and translation lead to the regression from carcinoma to the epithelial phenotype. In epithelia, members of the classic cadherins are thought to initiate certain molecular pathways involving the catenins, which lead to the organization of epithelial junctions and the physiological properties of epithelia -- adhesion mediated growth control,

lack of MMP- secretion and absence of tumorigenicity and invasion (Frixen et al., 1991; Vleminckx et al., 1991; Watabe et al., 1994; Miyaki et al., 1995).

How might P_0 act to induce these complex sets of events that ultimately leads to "epithelialization" of this aggressive carcinoma? One hypothesis is that P_0 directly signals these physiologic changes through its cytoplasmic domain via the catenins. In myelin, P_0 has no demonstrable outside to inside signaling properties because once P_0 is synthesized, it is completely sequestered in PNS myelin and does not appear on Schwann cell surfaces. As myelin is compacted, cytoplasm is completely excluded from the wraps of Schwann cell membranes, allowing no signaling proteins or cytoskeletal elements to associate with the P_0 cytoplasmic domain. However, mutational analysis and truncation studies have demonstrated that the intracellular domain is needed to activate the adhesive properties of P_0 (Wong and Filbin, 1994; Wong and Filbin, 1996) and so this domain may affect the conformation of the ectodomain, thereby modulating strength of adhesion during maturation of compact myelin; in effect, mediating *inside to outside* signaling.

It is perhaps most likely that, since P_0 lattices bring membranes very close together (Doyle et al., 1995; Shapiro et al., 1996) the small amount of endogenous N-cadherin molecules on opposing cell surfaces are able to engage. Cadherins only require a distance of about 20-30 nm interact adhesively with one another to form adherens junctions and desmosomes (Farquhar and Palade, 1963; Schmidt et al., 1994; Shapiro et al., 1995a). According to this scenario, N-cadherin interactions would trigger upregulation of N-cadherin synthesis as well as the catenins (Figure 3.11). The catenins are a distinct set of cytoplasmic proteins that interact with the cytoplasmic tail of cadherins (Gumbiner, 1993; Gumbiner, 1996). These proteins coordinate extracellular adhesive signals of the cadherin with intracellular signaling pathways that ultimately induces or inhibits specific gene transcription and translation (i.e., genes responsible for transformation) and leads to the assembly of adherens junctions, desmosomes and functional tight junctions (Gumbiner and McCrea, 1993; Ranscht, 1994; Watabe et al., 1994; Aberle et al., 1996; Bracke et al.,

1996; Jiang, 1996). In particular, it has been speculated that plakoglobin relays signals from classic cadherins to the desmosomal proteins to trigger desmosome formation (Lewis et al., 1994).

If the expression of any one of the catenins is disrupted, regardless of the levels of cadherin expression, this may lead to carcinomatous transformation (Shiozaki et al., 1994; Andrews et al., 1997; Hao et al., 1997; Hiscox and Jiang, 1997; Krishnadath et al., 1997; Richmond et al., 1997; Umbas et al., 1997; vanderWurff et al., 1997). In certain renal carcinomas that lack cadherin expression, overexpression of plakoglobin triggers epithelial characteristics -- cells gain adhesion-mediated growth control and are less invasive and tumorigenic, but there are no changes in cell morphology probably due to the lack of cadherin expression (Simcha et al., 1996). One highly invasive lung carcinoma cell line (PC9 cells) and some ovarian carcinomas, express normal levels of E-cadherin and β-catenin, but either lack α-catenin or have a mutant form (Nagafuchi and Takeichi, 1988; Watabe et al., 1994; Bullions et al., 1997). Once full length α-catenin is transfected into these cells, cell to cell adhesion is restored, junctional complex proteins are redistributed to the appropriate position in the membranes, and cells are no longer invasive (Nagafuchi and Takeichi, 1988; Watabe et al., 1994; Bullions et al., 1997). P_0 expression in HeLa significantly upregulates components of the cadherin/catenin pathway including N-cadherin, α-catenin, β-catenin, and plakoglobin suggesting that it works upstream of the cadherins. P_0 adhesion triggers these dormant pathways leading to the reverse transition from a carcinoma to an epithelium.

These data and the finding that obligatory P_0 mediated adhesion in HeLa is followed by sustained increases in cadherin and catenin levels suggest that this IgCAM can elicit an inherent but dormant or "sluggish" intracellular pathway which, when activated, triggers epithelialization and the suppression of the tumorigenic and transformed properties of this cervical carcinoma cell line.

CHAPTER 4

CADHERIN EXPRESSION AND THE INDUCTION OF EPITHELIALIZATION IN A CERVICAL CARCINOMA

In the previous chapter I showed that P_0 induced epithelialization and suppression of tumorigenicity in the cervical carcinoma cell line, HeLa. Additionally, N-cadherin and several catenins which are known to be involved with pathways leading to epithelial differentiation, were upregulated. I propose that P_0 may be mediating epithelialization directly upstream of the cadherins and activates the inherent cadherin-catenin signaling pathways.

I generated HeLa cells constitutively expressing N-cadherin or E-cadherin. I found that both N-cadherin and E-cadherin expressing HeLa are similar morphologically to intact epithelium. Junction-associated proteins are upregulated and targeted to lateral cell membranes consistent, with the observed formation of tight junctions, adherens junctions, and desmosomes. Additionally, the physiology of the cadherin expressing HeLa was similar to epithelia. Tight junctions were able to generate and maintain cell polarity as well as a permeability barrier. Cadherin expressors grew in an anchorage-dependent, contact inhibited manner. Lastly, the cadherin expressing HeLa were non-invasive and secrete less matrix degrading enzymes. These studies support the idea that adhesion brought about by cadherins or other obligatory adhesion proteins like P_0 can activate cadherin/catenin signaling pathways that lead to suppression of tumorigenicity.

Results

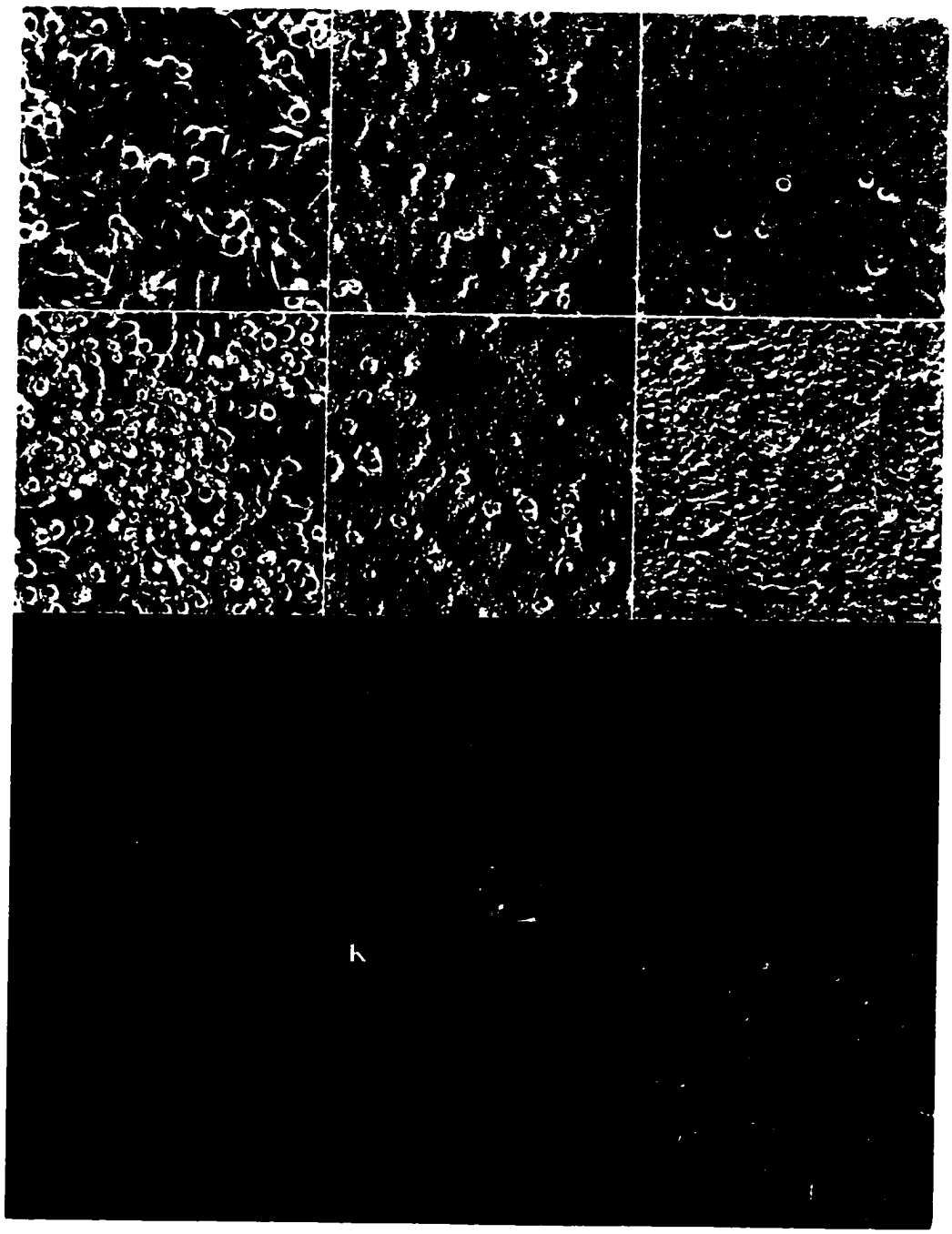
Cadherin expressing HeLa resemble epithelia morphologically

HeLa cells and control cell lines exhibit morphological features common to many carcinomas. They are heterogeneous, ranging in shape from round to spindle-like with clearly defined cell boundaries devoid of N-cadherin or any other known adhesion proteins (Figure 4.1 A; (Doyle et al., 1995)). In contrast, HeLa cells constitutively expressing N-cadherin or E-cadherin (Figure 4.1 B, C) are flatter and have a more uniform and polygonal shape. Additionally, cell contacts between the N- and E- cadherin expressors are difficult to discern and appear almost fused by phase contrast light microscopy. This may be due to

Figure 4.1 The morphology of N-cadherin and E-cadherin expressing HeLa cells is similar to normal epithelia

Phase contrast images show that neo control HeLa cells continue to divide and pile up on one another at confluency (A) and post-confluency (D). In contrast, the N-cadherin (B) and the E-cadherin (C) expressors maintain their distinctive morphology even several days post-confluency (E, F) suggesting that the cadherin expressing cells acquire contact inhibition and adhesion mediated growth control. Immunofluorescent analysis shows that control HeLa does not express detectable amounts of either N-cadherin or E-cadherin (G, J). N-cadherin cells express N-cadherin (H), but no E-cadherin (K) at their lateral cell borders. Likewise, the E-cadherin expressors had high levels of E-cadherin at its lateral cell borders (L) and did not express detectable amounts of N-cadherin (I).(Bar 20 μm A-F; 10 μm G-L)

Figure 4.1



the precise targeting and accumulation of the respective cadherins to lateral cell borders as shown in the corresponding immunofluorescent images (Figure 4.1 H, L). It had been shown through electron microscopy of cadherin-based junctions that cadherins bring plasma membranes into close apposition (30 - 50 nm). Additionally, HeLa cells membranes devoid of cadherins are not in close proximity (Farquhar and Palade, 1963; Doyle et al., 1995).

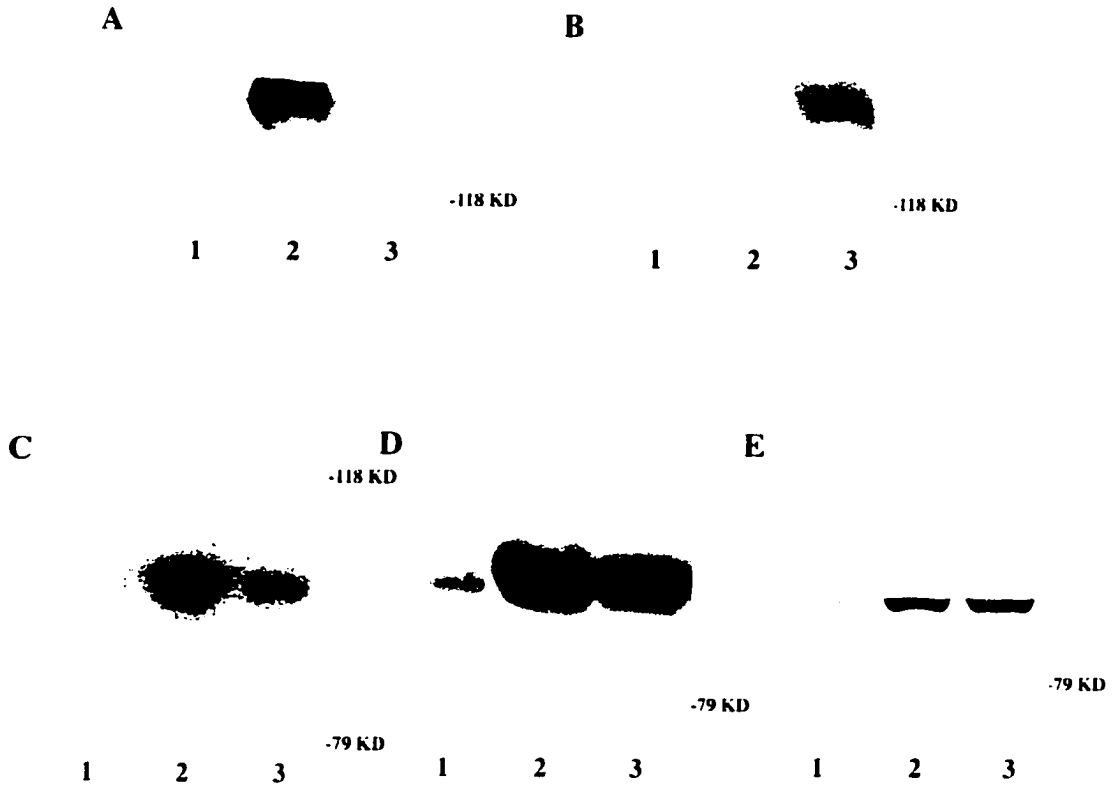
Another property of epithelial cells is they exhibit contact inhibition and grow in a density dependent manner. Transformed cell lines, including HeLa and control neo cells (Figure 4.1 D) lose this growth characteristic and at confluency continue to divide and pile up on one another eventually losing attachment to the plate surface. In contrast, both N-cadherin and E-cadherin expressors stop proliferating once they reach confluency (Figure 4.1 E, F). These cells maintain their flattened morphology and never lose attachments to plate surfaces. These observations indicate that expression of either N- or E-cadherin into HeLa restores the morphologic features as well as density limited growth control characteristic of intact epithelia.

Adherens junction associated proteins: α- and β- catenin and plakoglobin are upregulated and organized at cell to cell contacts of cadherin expressors

Epithelial cell morphology and communication are maintained by junctions referred to as the junctional complex, which includes tight junctions, adherens junctions, and desmosomes. The adherens junctions are composed of classic cadherins (e.g., E-cadherin, N-cadherin) that intracellularly associate with α-catenin, β- catenin and plakoglobin. These cytoplasmic proteins link to the actin microfilament network and coordinate cadherin adhesive activities with intracellular signaling pathways. Control HeLa cells, as their parental cell line, do not express significant levels of the components of the adherens junction (Figure 4.2). The synthesis of α-catenin, β- catenin and plakoglobin were all upregulated in the N-cadherin and E-cadherin expressors (Figure 4.2 C, D, E).

Figure 4.2 Expression levels of adherens junction proteins in N- and E-cadherin cells
Western blot analysis of total cell lysates indicate that N-cadherin cells (A; lane 2) and not control (lane 1) or E-cadherin (lane 3) expressing HeLa express N-cadherin. Likewise, only E-cadherin HeLa express E-cadherin (B; lane 3); this protein is not found in control neo or N-cadherin HeLa (B; lane 1, 2). Cytoskeletal enriched fractions show that both N-cadherin (lane 2) and E-cadherin (lane 3) cells upregulate the synthesis of a-catenin (C), b-catenin (D), and plakoglobin (E) over control levels (lane 1). (Lane 1: neo HeLa; lane 2: N-cadherin HeLa; lane 3: E-cadherin HeLa.

Figure 4.2



Additionally, these proteins were enriched in cytoskeletal extracts indicative of associations with actin cytoskeletal network. Indirect immunofluorescence reveals that the α- and β-catenins and plakoglobin co-localized at cell perimeters in a similar distribution pattern as N-cadherin in N-cadherin expressors and E-cadherin in E-cadherin expressors (Figure 4.1 G-L; 4.3).

N-cadherin expressors do not increase the synthesis of E-cadherin (Figure 4.2 B). Although E-cadherin expressors do not upregulate the expression levels of N-cadherin protein levels (Figure 4.2 A), these cells do increase levels of N-cadherin mRNA synthesis (data not shown). This implies that the N-cadherin mRNA is never translated (or alternatively, any endogenous N-cadherin protein produced is rapidly degraded). One explanation for this is that quite possibly, overexpression of E-cadherin is able to substitute for N-cadherin in cadherin-based junctions in HeLa. This is in contrast to HeLa cells expressing another adhesion protein. P_0 (See Chapter 3). P_0 could not substitute for N-cadherin at these junctions so N-cadherin was required for adherens junction formation and its expression was sustained (Doyle et al., 1995; Spiryda and Colman, 1998).

In summary, this data demonstrates that adherens junction-associated proteins are found to localize at cell perimeters of the cadherin expressing cells and are associated with the cytoskeleton.

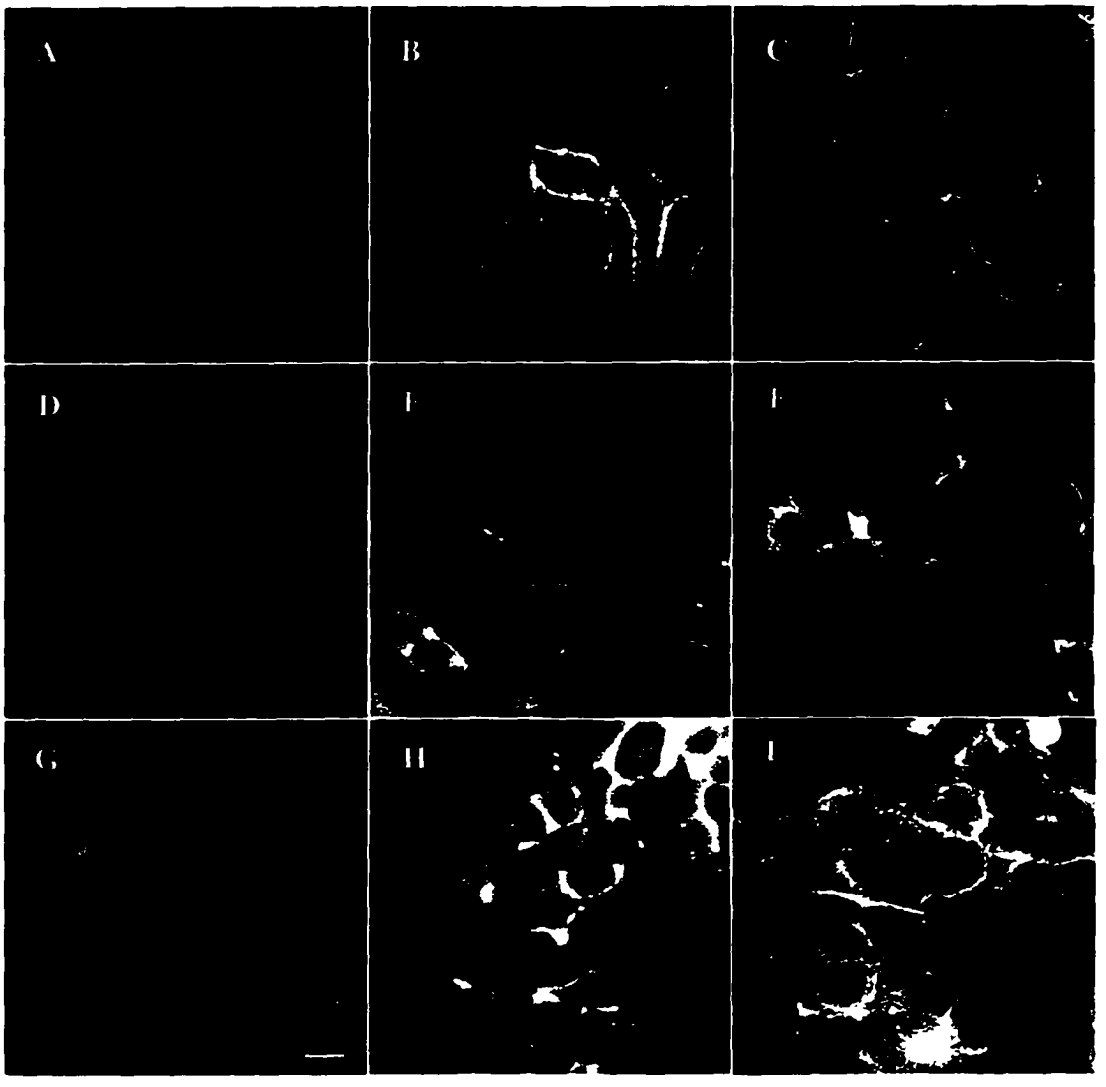
Cadherin expressors have increased expression of desmosomal-associated proteins at cell perimeters

The expression of classic cadherins and their assembly into adherens junctions are absolutely required for the assembly of other epithelial junctions, including desmosomes. The formation of desmosomes has been shown to be dependent on E-cadherin expression in maturing keratinocytes and corneal fibroblasts (Gumbiner et al., 1988; Lewis et al., 1994; Amagai et al., 1995; Vanderburg and Hay, 1996). The transmembrane components of desmosomes are desmosomal cadherins, desmocollin and desmoglein. Intracellularly,

Figure 4.3 Adherens junctions proteins are targeted to the lateral cell surfaces of cadherin expressing HeLa

The adherens junction associated proteins, a-catenin, b-catenin and plakoglobin co-localize at the lateral cell borders of the N- and E-cadherin expressing cells but are undetectable in the neo control cells. Panels A-C: a-catenin; D-F: b-catenin; G-I: plakoglobin. Panels A, D, G: neo HeLa; B, E, H: N-cadherin HeLa; C, F, I: E-cadherin HeLa.(Bar 10 μm)

Figure 4.3



this junctions contain desmoplakin and plakoglobin that link to the intermediate filaments (Koch and Franke, 1994; Green and Jones, 1996).

HeLa cells express low levels of desmoglein, desmocollin, and desmoplakin which are found randomly at the cell surface as well as in vesicles within the cytoplasm (Figure 4.4; (Doyle et al., 1995)). Both N-cadherin HeLa and E-cadherin HeLa express and enrich desmosomal proteins at areas of cell contact in a pattern is indicative of desmosome organization. Comparison of the amount of desmoglein produced in the controls and cadherin expressors are essentially identical (Figure 4.4 J). These results suggest that in cadherin expressing HeLa, endogenous pools of desmosomal proteins are redistributed to the cell surfaces and maintained at the lateral cell to cell contacts in an expression pattern characteristic of desmosome assembly.

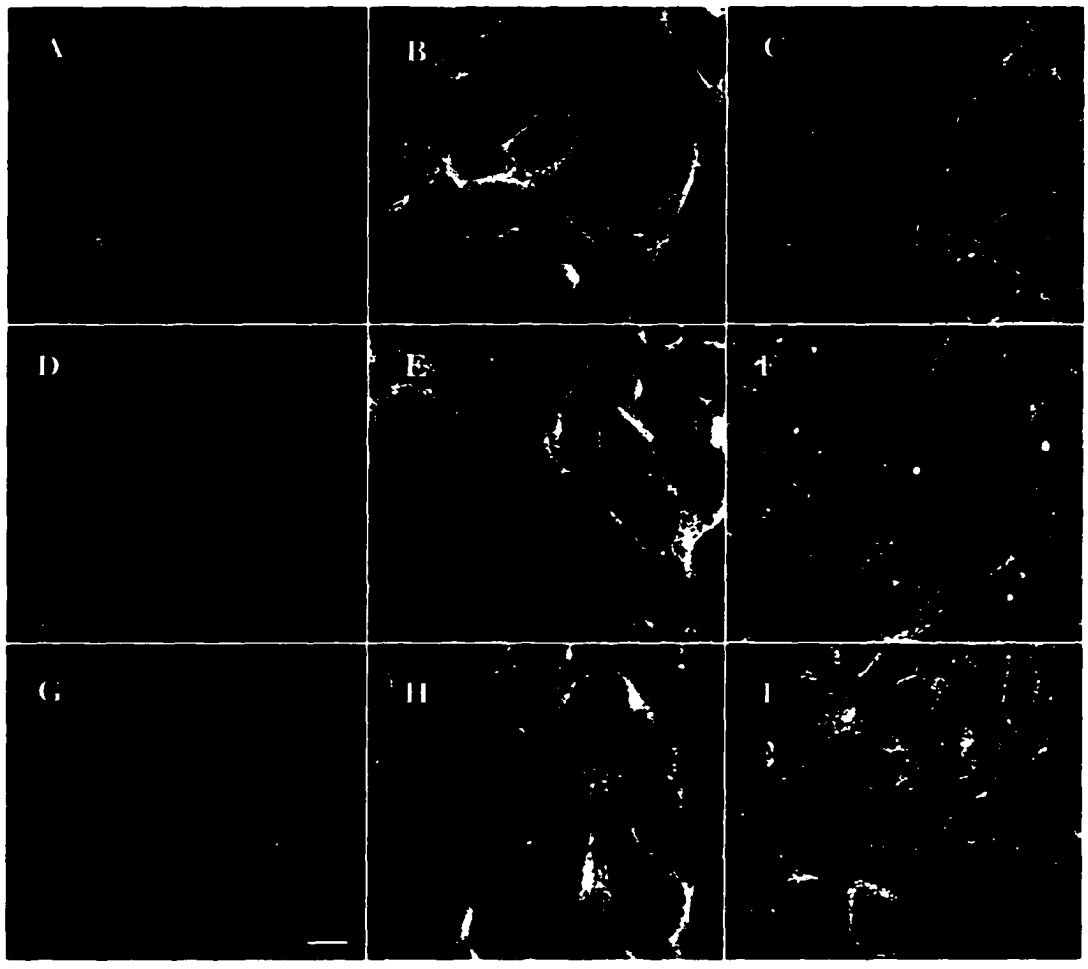
Tight junctional proteins, occludin and ZO-1, are at lateral cell contacts of cadherin expressing HeLa cells

The most apical junctions of the junctional complex are tight junctions. These junctions serve as paracellular permeability barriers and maintain cell polarity. They are composed of occludin, a tetraspan protein and several intracellular proteins, including ZO-1 (Furuse et al., 1993) and cingulin (Citi et al., 1988). Electron microscopic analysis revealed that HeLa cells as well as control cells assemble tight junctions only occasionally (Doyle et al., 1995); These cells express high amounts ZO-1, but very low levels of occludin (Figure 4.5 G, H). These proteins are found intermittently at the cell surface as isolated puncta (Figure 4.5 A, D). N-cadherin and E-cadherin HeLa express levels of ZO-1 that are similar to control cells, but occludin expression is vastly upregulated in these cadherin expressing cell lines (Figure 4.5). Immunofluorescent microscopy, showed that occludin was arranged in long arrays at cell perimeters with shorter bands of ZO-1 at the cell interfaces of both N- and E-cadherin HeLa as is expected for tight junction assembly (Figure 4.5).

Figure 4.4 Desmosomal proteins are enriched at lateral cell contact of N- and E-cadherin expressing cells

The desmosomal proteins, desmoglein (A-C), desmocollin (D-F), and desmoplakin (G-I), are highly enriched at lateral cell contacts of the N-cadherin (B, E, H) and E-cadherin cells (C, F, I), but are barely detectable by immunofluorescence in the control HeLa cells (A, D, G). Western blot analysis of cytoskeletal enriched extracts (J) indicate that virtually identical levels of desmoglein are expressed at similar levels in control HeLa (lane 1), N-cadherin (lane 2), and E-cadherin (lane 3) (Bar 10 μm)

Figure 4.4



J

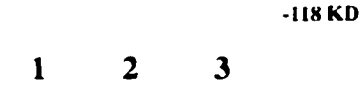
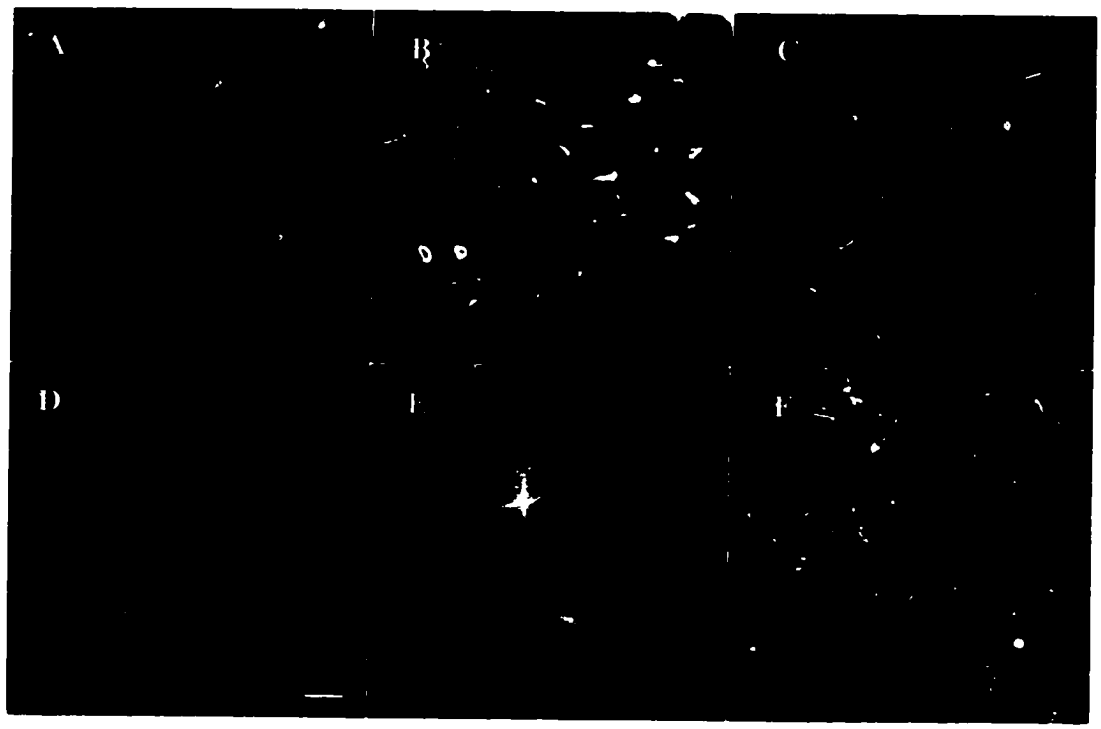


Figure 4.5 Expression and distribution of tight junction associated proteins, ZO-1 and occludin.

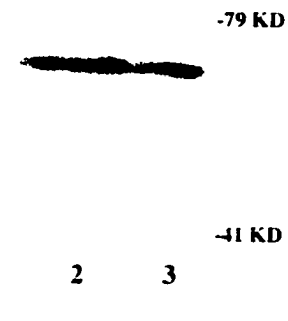
ZO-1 and Occludin immunofluorescence in control cells (A, D) is intermittent and punctate. These proteins are organized in long arrays about the cell perimeter in N-cadherin HeLa (B, E) and E-cadherin HeLa (C, F). Western blot analysis of cytoskeletal-enriched fractions indicate that the levels of ZO-1 (G) are similar in control cells (lane 1), N-cadherin (lane 2) and E-cadherin (lane 3) cells. There is a substantial upregulation of occludin synthesis (H) in the N- and E-cadherin expressors (lanes 2, 3) as compared to control cells (lane 1) (Bar 10 μm)

Figure 4.5



G

H



One role of tight junctions is to maintain a transcellular and paracellular permeability barrier that regulates the flux of electrolytes and macromolecules, thereby assisting in the maintenance of the particular environment characteristic of each tissue type. One way to assess this function in situ is to measure the transepithelial electrical resistance (TER) across cell monolayers. Cells that have a high TER form a tighter barrier (e.g., endothelial cells of the blood-brain barrier). Conversely, cells that lack tight junctions (e. g., carcinoma cells, like HeLa) have low resistance values. Occludin is one of the functional elements of tight junctions that enable cells to restrict the flux of ions and macromolecules (Balda et al., 1996; McCarthy et al., 1996; Wong and Gumbiner, 1997).

We measured the TER of control HeLa, N-cadherin and E-cadherin expressors to assess the functionality of assembled tight junctions. The TER of control cells was 5.3 Ω cm^2 as cells devoid of tight junctions would behave whereas N-cadherin and E-cadherin expressing monolayers had TER readings of 45 Ω cm^2 and 58.5 Ω cm^2 respectively (Figure 4.6).

We can conclude from these experiments that cadherin expressors form a significant permeability barrier to electrolytes and macromolecules, in contrast to the control carcinoma. This increase in TER correlates with the incorporation of occludin into the tight junctions and is compatible with formation of functional tight junctions in both the N-cadherin and E-cadherin expressing cells.

Cadherin expressors establish functional cell polarity

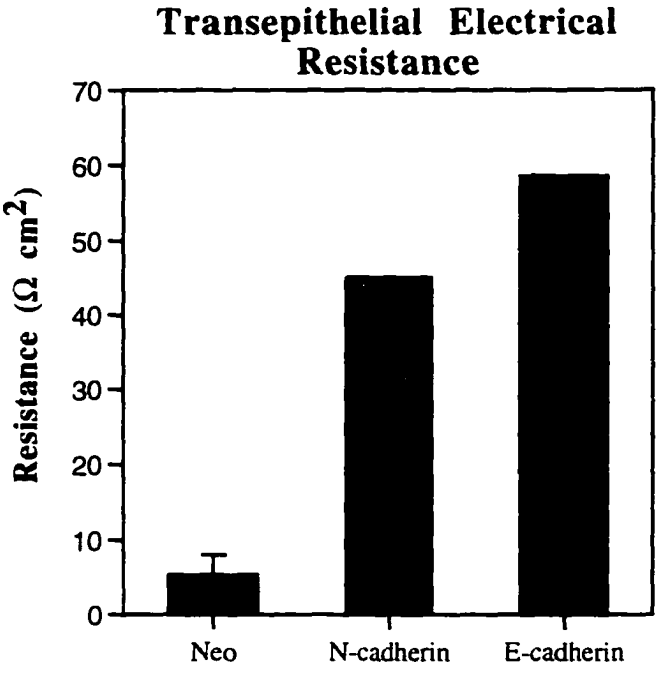
Another role of tight junctions is to act as the defining border between the apical and lateral plasma membranes, enabling cells to maintain specific proteins and lipids within a particular plasma membrane subdomain. In general, cell lacking functional tight junctions distribute proteins at random in the cell membrane.

We examined optical sections through N-cadherin, E-cadherin expressors and control cells to assess the distribution of placental alkaline phosphatase (PALP; Figure 4.7), as

Figure 4.6 Comparison of Transepithelial Resistance.

Both N-cadherin and E-cadherin expressing cell lines have a higher TER that control cells indicating that the assembled tight junctions are functional and can maintain a paracellular permeability barrier. The TER of control HeLa was 5.2 Ωcm^2 and the TER of the N-cadherin cells was 45 Ωcm^2 and E-cadherin cells had a TER of 58.2 Ωcm^2.

Figure 4.6



described in Chapter 3, an apically-directed protein, and the transferrin receptor (TR; Figure 4.8), a basolateral protein. In Figures 4.7 and 4.8, representative images are arranged from basal to apical (numbered 1-8; 1 is the most basal and 8 is most apical). Placental alkaline phosphatase (Figure 4.7 A) and transferrin receptor (Figure 4.8 A) are randomly distributed in all subdomains of the plasma membrane in control $pCXN_2$ cells. In both the N-cadherin and E-cadherin expressors, placental alkaline phosphatase and transferrin receptor were appropriately sorted and targeted to the apical and basolateral domains, respectively (Figure 4.7 B, C; 4.8 B, C).

The data presented thus far demonstrates that the expression of N-cadherin or E-cadherin in this cervical carcinoma can induce morphologic features similar to that of normal epithelia. The expression of many junctional proteins was upregulated and these proteins were precisely targeted to the lateral surfaces of neighboring cells consistent with the formation of adherens junctions, desmosomes and tight junctions. It has been previously reported that morphologic changes induced by cadherin do not always correlate with changes in cell physiology (Simcha et al., 1996). Several additional physiologic properties of N-cadherin and E-cadherin HeLa were assessed to ascertain their functional properties were characteristic of normal epithelia or if they still behaved like tumor cells.

N- and E-cadherin cells require a matrix for cell proliferation

Attachment to a basement membrane or matrix is absolutely necessary for cell division of epithelial cells; these cell cannot grow and divide if this relationship is disrupted. Many carcinomas cells lose this requirement for cell to substrate attachment for continual cell proliferation and can grow in an anchorage-independent manner. Anchorage-independent growth can be evaluated *in vitro* by determining if a particular line can form colonies when grown in suspension or in a semisolid medium such as soft agar.

Figure 4.7 Distribution pattern of placental alkaline phosphatase, an apically directed protein.

Control HeLa cells distribute placental alkaline phosphatase at random in all subdomains of the plasma membrane (A). N-cadherin (B) and E-cadherin (C) expressing HeLa appropriately target and maintain this protein exclusively in the apical domain which is the native domain for this protein. In all sections 1 is the most basal section and 8 is the most apical section. (Bar 10 μm)

Figure 4.7

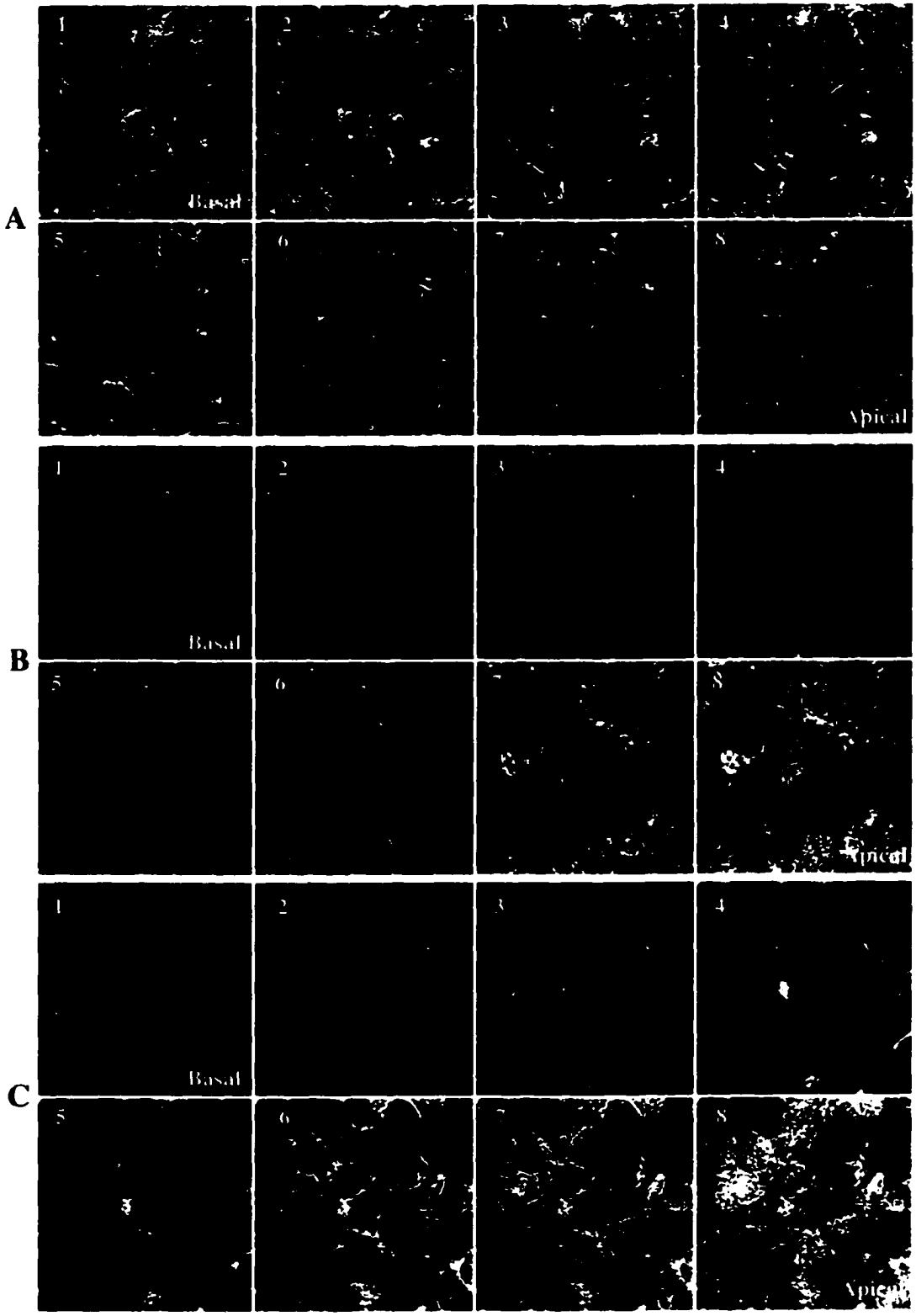
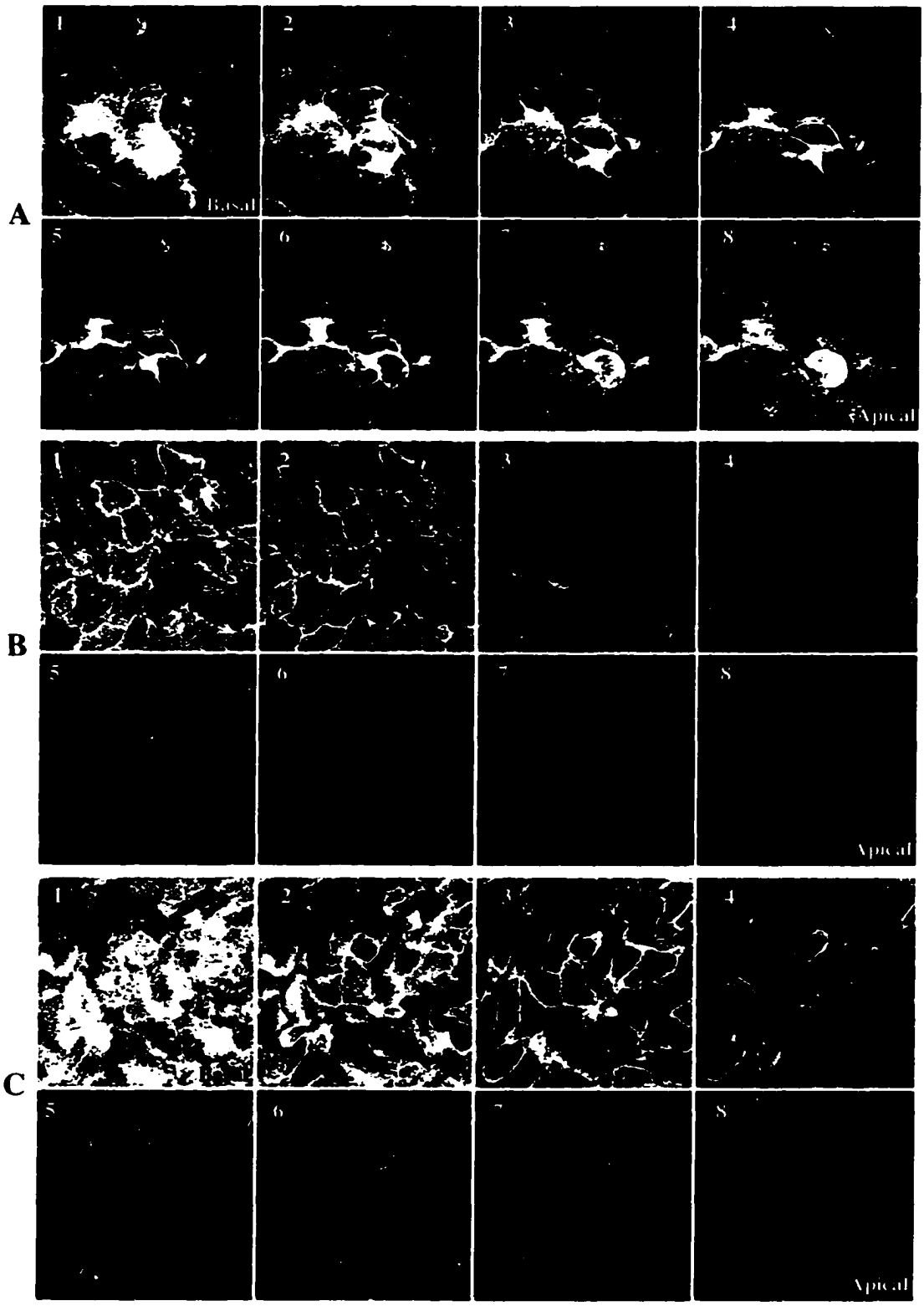


Figure 4.8 Distribution of a basolateral protein, Transferrin Receptor

In control cells, transferrin receptor is found in all plasma membrane domains - apical, lateral and basal (A). Transferrin receptor is appropriately targeted and distributed in the basolateral membrane of the N-cadherin (B) and E-cadherin (C) expressors indicating that cadherin expressing cells regain functional cell polarity. In all sections, 1 is the most basal section and 8 is the most apical. (Bar 10 μm)

Figure 4.8



HeLa cells and the $pCXN_2$ transfected control, like most tumor cell lines, divide and form large colonies when grown in agar with a cloning efficiency of approximately 73% (Figure 4.9 A, D) (Celis et al., 1978). In contrast, virtually none of the N-cadherin expressors or E-cadherin expressors form colonies when grown under these conditions (Figure 4.9 B, C). These cell lines have a much lower cloning efficiencies (14% for N-cadherin cells and 12.3% for E-cadherin expressors) which are similar to normal epithelial cell lines (Figure 4.9 D). This indicates that the expression of either N-cadherin or E-cadherin can restore signaling pathways involved with adhesion-mediated growth control in this cervical carcinoma.

N-cadherin and E-cadherin expressors cannot invade an artificial matrix

The hallmark feature of aggressive carcinomas is their ability to invade basement membranes and their subsequent metastasis to other organs. The ability of carcinoma cells to grow detached from a basement membranes correlates with their ability to form invasive and metastatic tumors.

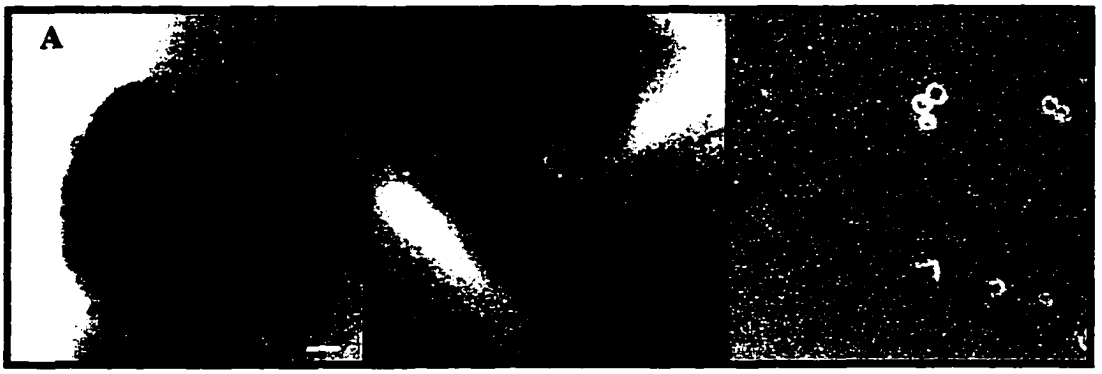
The invasiveness of a carcinoma was compared and quantified with an *in vitro* chemoinvasion assay as described in Chapter 3 (Albini et al., 1987). Cells were seeded on filters coated with Matrigel matrix and after 12 hours the number of cells that were able to invade through were counted with a light microscope. We observed that on average 290 of control cells are able to invade through the matrix whereas only an average of 15 N-cadherin cells and 37 E-cadherin cell were able (Figure 4.10 A-G).

One mechanism that enables carcinoma cells to invade basement membranes is the abnormally high synthesis and secretion of matrixmetalloproteinases (MMPs) which degrade the basement membrane and the surrounding matrix. We compared the enzyme activity and expression of two type IV collagenases (MMP-2 and MMP-9) in the N-cadherin, E-cadherin expressors and control HeLa by gelatin zymography. Aliquots of conditioned media were run on non-denaturing gels containing gelatin which serves as a

Figure 4.9 Assessment of anchorage-independent growth .

Control, N-cadherin, and E-cadherin expressing HeLa cell lines were grown in soft agar to assess anchorage-independent growth. PSV2-neo formed large colonies with a cloning efficiency of 72% (A, D), whereas N-cadherin (B) and the E-cadherin (C) cells were unable to proliferate in the absence of a matrix and had a cloning efficiency of 14% and 12% respectively (D). This indicates that N-cadherin HeLa and E-cadherin HeLa could not grow in an anchorage-independent manner. (Bar 100 μm)

Figure 4.9



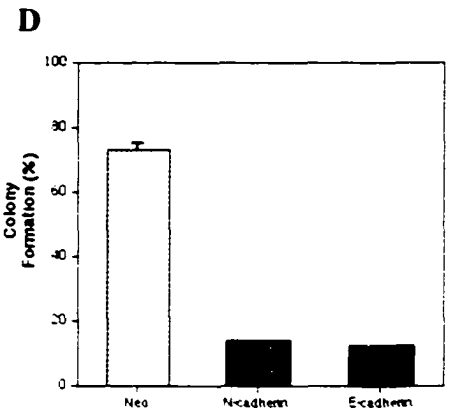
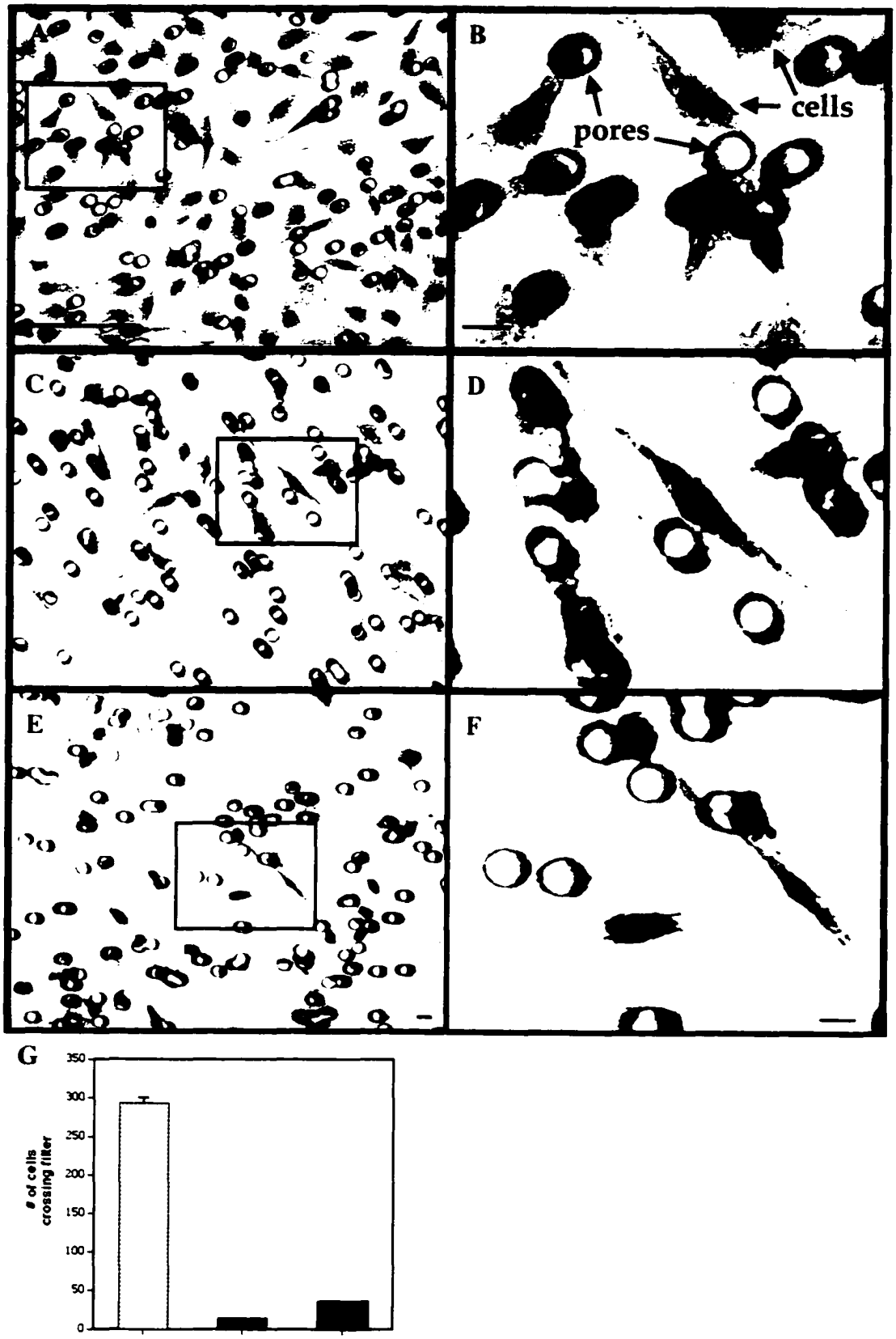


Figure 4.10 Comparison of invasion through a basement membrane.

The invasiveness of the cadherin expressing HeLa and control cells was determined by comparing their ability to cross an artificial basement membrane (Matrigel coated filters). On average, 296 of the control cells (A, D) were able to invade through the matrix whereas only 15 of the N-cadherin expressing HeLa (B, D) and 37 of the E-cadherin expressing HeLa (C, D) were able to invade. Qualitatively (A-C) and quantitatively (D), it appears that the cadherin expressors are less invasive that control cells. The boxed in areas in A, C, E are blown up in B, D, F, respectively. (Bar 10 μm)

Figure 4.10



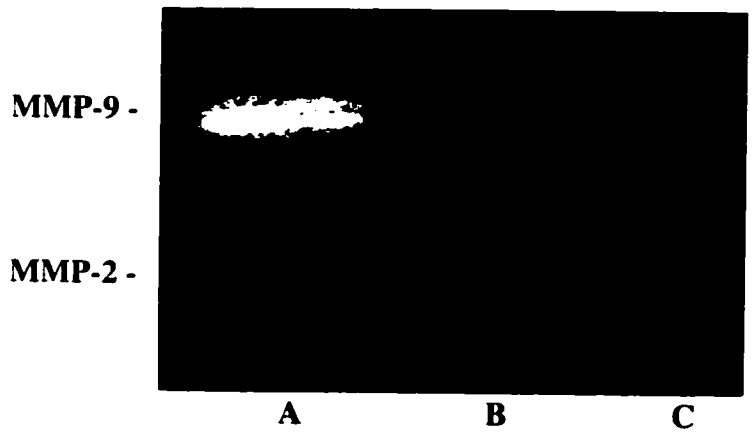
substrate for the MMPs. The resolved gel was placed in a buffer to allow development of enzyme activity. The clear bands reveal the extent to which the gelatin has been proteolyzed (Heussen and Dowdle, 1980).

Control cells secrete high levels of both MMP-9 and MMP-2 whereas the N-cadherin and E-cadherin cells have lost the ability to elaborate these enzymes (Figure 4.11). These results show that the cadherin expressors are not as invasive as the control HeLa cells possibly due to their decreased secretion of matrix degrading collagenases.

Figure 4.11 Secretion of matrixmetalloproteinases -- MMP-2 and MMP-9.

The secretion of matrixmetalloproteinases was assessed by gelatin zymography in control HeLa, N-cadherin HeLa and E-cadherin HeLa. The control HeLa cells secrete higher levels of MMP-2 or MMP-9 than either of the cadherin expressing cell lines. Lanes A neo HeLa cells; B. N-cadherin HeLa; C. E-cadherin HeLa.

Figure 4.11



Discussion

Cadherins are ubiquitously expressed proteins involved with many aspects of development and organization of tissue and organs. In epithelia, cadherins are believed to be initiators and mediators of cell aggregation that ultimately leads to the organization of cellular junctions that define the polarized and adhesive phenotype of epithelial cells. E-cadherin has been shown to initiate the organization of epithelial junctions and mediate subsequent molecular events that leads to the development of normal physiological properties of epithelia -- contact inhibition, decreased anchorage-independent growth, decreased MMP secretion and tumorigenicity in many different carcinomas including cervical carcinomas (HeLa) as we show here (Frixen et al., 1991; Vleminckx et al., 1991; Watabe et al., 1994; Miyaki et al., 1995). It has been difficult to ascertain in which order these properties occur following E-cadherin expression or adhesion. It is generally accepted that E-cadherin-mediated adhesion may be one starting point for the molecular cascade that ultimately leads to the development of a differentiated phenotype characteristic of epithelial cells (Gumbiner et al., 1988; Nelson, 1989; Rodriguez-Boulan and Nelson, 1989; Takeichi, 1991; Geiger and Ayalon, 1992; McNeill et al., 1993).

The role of N-cadherin in epithelial junction formation and tumor suppression has not been fully elucidated. In my thesis I show that the expression of N-cadherin can induce the reverse transition of a transformed cervical carcinoma (HeLa) to a differentiated epithelial phenotype.

N-cadherin expressing HeLa display morphologic features characteristic of epithelia; these cells upregulate the synthesis of and organize junctional associated proteins at cell interfaces and gain adhesion mediated growth control. Additionally, these cells are less invasive and secrete less matrixmetalloproteinases. The expression of E-cadherin in this carcinoma can elicit identical repertoire of events without inducing the expression of N-cadherin. E-cadherin can substitute for N-cadherin in these cadherin/catenin based signaling pathways that lead to epithelialization. As discussed in the previous chapter, P_0

mediated adhesion can also induce epithelialization through the activation of N-cadherin/catenin based signaling pathways. It appears that specifically N-cadherin expression is not required these phenotypic and physiologic changes. The activation of the cadherin/catenin signaling pathways may be sufficient to induce epithelial differentiation and suppression of tumorigenicity and invasion in this cervical carcinoma.

This is in contrast to other types of carcinomas where N-cadherin expression is associated with increased tumorigenic and metastatic behavior and has been proposed to work antagonistically to other classic cadherins. Some invasive and metastatic breast carcinomas express low or undetectable levels of E-cadherin, but high levels of N-cadherin (Hazan et al., 1996). It has been postulated that the N-cadherin expressing breast cells can interact homotypically with the surrounding N-cadherin expressing stromal cells, thus, hastening metastasis (Hazan et al., 1996). Likewise, several invasive fibroblastic squamous carcinomas express high levels of N-cadherin, but low levels of E- and P-cadherin (Islam et al., 1996; Li et al., 1998). When these cells were treated with antibodies to the N-cadherin ectodomain, both E- and P-cadherin synthesis increased with a concomitant loss of N-cadherin at the cell perimeter leading to increased cell adhesion (Islam et al., 1996). When engineered to express E-cadherin, endogenous N-cadherin levels were decreased through rapid degradation, whereas the stability and synthesis of the catenins increased. These molecular changes conferred density-dependent growth inhibition and increased aggregative properties (Li et al., 1998). Additionally, it has been shown that when two cadherins are co-expressed in the same cell type, they may compete with one another for junctional localization. Human vein umbilical cells, express VE-cadherin and N-cadherin, but only VE-cadherin is associated with junctions; N-cadherin is distributed diffusely at the cell surface suggesting that N-cadherin may interact with surrounding pericytes and vascular smooth muscle cells which also express N-cadherin (Salomon et al., 1992; Navarro et al., 1998). CHO cells expressing both VE-cadherin and N-cadherin have an identical expression pattern for these proteins, VE-cadherin is localized

at cellular junctions and N-cadherin is expressed diffusely over the cell surface. When these cells were co-cultured with N-cadherin CHO cells, N-cadherin was now localized at cellular junctions and VE-cadherin had a diffuse expression pattern (Navarro et al., 1998).

These studies suggest that cadherins, in general, may be able to elicit multiple signaling pathways that have very different consequences for epithelial cell biology. Several groups have begun to experimentally determine the structure of the cadherin ectodomain to help discern why cadherins appear to have multiple antagonistic functions. Although the NMR structures and x-ray crystallographic data do not support a unified view of cadherin ectodomain structure, there are certain central themes that can be interpreted from these experiments (Overduin et al., 1995; Shapiro et al., 1995a; Nagar et al., 1996; Tamura et al., 1998). Taken together, x-ray crystallographic analyses and NMR spectroscopy data of sole EC1 domains or combined EC1-EC2 ectodomains of either N-cadherin and E-cadherin have suggested that cadherins may have multiple conformation states that exist at a dynamic equilibrium at cell surfaces. One state may be active in engaging adhesion and exist as strand dimers emanating from cell membranes. Another conformation state may exist where individual monomers are at cell surfaces and are considered to be inactive and unable to confer tight adhesion (Colman, 1997). It can be proposed that these different conformational states of the cadherins may also allow the cytoplasmic domain to engage or trigger different intracellular pathways. It can be postulated that monomeric forms of cadherins may be expressed when pathways involved with cell migration and cell sorting need to be activated such as during embryonic development. However, cadherins that cluster and dimerize will be more apt to form adhesive junctions and reinforce the multiple signaling pathways that are responsible for cell differentiation. It can also be inferred from this model that the conformational state of the cadherin may also be dependent upon internal events within the cell which is then relayed by the cytoplasmic tail of the cadherin. This suggests that cadherins may be

involved with both outside to inside signaling and also inside to outside signaling as has been described for integrin heterodimers.

This model may help explain why certain carcinomas may express high levels of N-cadherin (squamous carcinomas;(Islam et al., 1996; Li et al., 1998)) or E-cadherin (infiltrating lobular breast carcinomas and adenosquamous carcinomas; (Moll et al., 1993; Bohm et al., 1994) but still display the carcinomatous phenotype. It can be postulated that the expressed cadherins in these particular carcinomas are unable to form stand dimers and therefore cannot confer tight adhesion that is necessary to engage cadherin signaling pathways involving the catenins that push the cell toward differentiation. Instead, abundance of the monomeric form of cadherins triggers other pathways that lead to the synthesis and expression required for tumor development, abnormal growth properties and proteins that promote invasion and metastasis. Overexpression of N-cadherin in a transformed fibroblast cell line partially changed cell morphology to be more "epithelial-like" but could not reduce tumorigenicity. Complete reversion to a more epithelial-like phenotype did not occur unless plakoglobin was co-expressed (Simcha et al., 1996). Additionally, in certain carcinomas, E-cadherin is expressed at normal levels, but these cells lack α-catenin. Epithelialization and decreased tumorigenicity only occurs when α-catenin is added back to these cells (Nagafuchi and Takeichi, 1988; Watabe et al., 1994; Bullions et al., 1997). These studies reinforce the importance of internal signaling events in epithelial cell biology.

This leads to the hypothesis that cadherins expressed in certain carcinomas are mainly at the cell surface in the "inactive" monomeric forms. Once its intracellular partners are appropriately expressed, they help cluster the existing cadherins though their interactions with the cytoskeleton, pushing the equilibrium at the cell surfaces to the adhesive strand dimer conformation. This enables the cadherins to elicit tight adhesion which in turn engages or activates multiple molecular pathways required for the biochemical, morphological and physiologic properties of epithelia.

In conclusion, I have shown that cadherin expression in this cervical carcinoma cell line restores normal morphological features and, most interestingly, leads to the recovery of the normal physiology associated with epithelia. These studies provide evidence supporting the hypothesis that in P_0:HeLa, P_0 adhesion appears to be working directly upstream of the cadherins.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Several major conclusion can be drawn from my studies. Constitutive P_0 expression in HeLa cells restores several important aspects of normal epithelial cell physiology, including formation of functional tight junctions, establishment of cell polarity, contact inhibited and adhesion-mediated growth control, and loss of elaboration of MMPs. Of greatest interest, P_0:HeLa do not form any primary tumors or metastatic lesions in athymic nude mice. These data and the finding that obligatory P_0 mediated adhesion in HeLa is followed by sustained increases in cadherin and catenin levels suggest that this IgCAM can elicit an inherent but dormant or "sluggish" intracellular pathway which, when activated, triggers epithelialization and the suppression of the tumorigenic and transformed properties of this cervical carcinoma cell line. The overexpression of N-cadherin in HeLa also restores the morphological and physiologic characteristic of epithelia. Additionally, in this cervical carcinoma it appears that E-cadherin can substitute for N-cadherin and elicits an identical sequence of events leading to complete reversion of tumorigenicity. These studies taken together suggest that adhesion, no matter how it is initially brought about can mediated the reversion of this carcinoma to a differentiated phenotype.

Another interesting feature of cervical carcinomas is that more than 90% of these carcinomas, including HeLa, are associated with infection with human papillomavirus (HPV) types 16 (≈50%), 18 (≈12%), 45 (≈8%) and 31 (≈5%). It has been found that two viral proteins, E6 and E7, are considered to be transforming factors which contribute to the cellular growth characteristics and metastatic potential. It has been previously shown that keratinocytes immortalized with HPV E6 and E7, expressed decreased amounts of E-cadherin and α-catenin and were highly invasive (Wilding et al., 1996). Once E-cadherin was overexpressed in these cells, however, catenin expression and distribution was restored, and the invasive properties were reversed, suggesting that E-cadherin expression and activity of the E6 and E7 may be inversely correlated (Wilding et al., 1996). The inactivation of E6 and E7 adds another layer of complexity to the epithelialization pathway

in cervical carcinomas. It may be that these viral proteins are directly or indirectly inactivated by either P_0 or cadherins in P_0 HeLa and N-cadherin and E-cadherin HeLa.

Model of Epithelialization

A model of how epithelialization may be brought about by P_0 can be proposed based on the presented experimental evidence (Figure 5.1). It is important to note that multiple pathways need to be activated/inactivated to elicit the morphologic and functional properties of an intact epithelium.

The data support a model in which P_0 adhesion operates upstream of the cadherins. It is still not known how P_0 adhesion activates cadherin expression and its organization at lateral cell contacts. Although unlikely, perhaps P_0 binds to N-cadherin directly, but with low affinity, creating a heterophilic interaction. P_0 would help cluster the low endogenous levels of cadherin, presumably in the "inactive" monomeric conformation at cell surfaces. This may drive the formation of strand dimers leading to strong cadherin-based adhesion and junctions. Alternatively, P_0 adhesion brings membranes very close together (3 - 5 nm) and this enables low amounts of endogenous cadherins to cluster and dimerize to an "active" adhesive conformation. Once cadherins are in the adhesive dimer form, catenins are recruited and multiple intricate pathways are activated, leading to changes in gene transcription and translation that allow these cells to revert to a morphologically, biochemically, and functionally intact epithelium.

We conclude that, in general, obligatory adhesion molecules, such as E-cadherin, N-cadherin, or P_0, may serve as inducers of dormant intracellular events in carcinoma cells that ultimately triggers them to regain epithelial characteristics. It may be that there is a single "master" signaling event triggered by intercellular adhesion no matter how it is brought about, which in turn induces all subsequent events, and if so, this would have significant implications for cancer therapeutics.

Figure 5.1 Model of epithelialization by P_0.

P_0 engages a dormant but undamaged epithelialization and differentiation program in this cervical carcinoma. The data presented here suggests that P_0 mediated adhesion allows cadherins to be clustered and dimerized at the cell surface which in turn induces the synthesis and sequesters catenins to these areas of cell contacts, reinforcing the adhesive strand dimer formation. The formation of adherens junctions triggers the synthesis and assembly of desmosomal and tight junctional proteins. Concurrently, other pathways are activated leading to density limited growth, loss of anchorage independent growth, development of cell polarity and a paracellular permeability barrier, inhibition of matrixmetalloproteinases and the expression of other invasion genes. Additionally the transforming genes of HPV are inhibited. Collectively these multiple pathways all lead to the suppression of the tumorigenic and metastatic phenotype associated with these cells. Although the exact signaling events have not been fully determined as of yet, it is clear that their activation is dependent on adhesion as one of the upstream events. (X's indicate where pathways are blocked or inhibited from progressing due to upstream events)

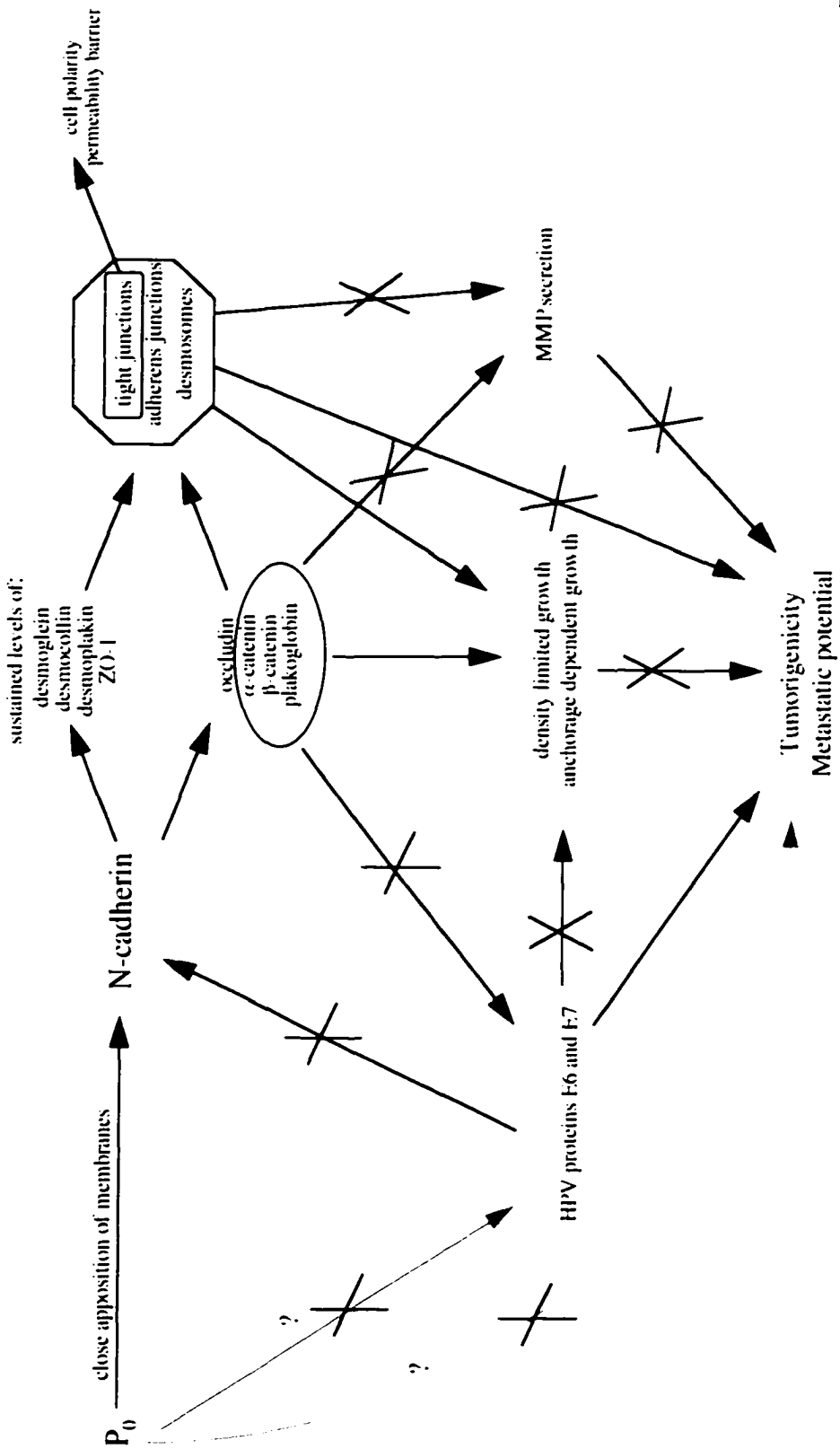


Figure 5.1

Preliminary Future Work

Several questions arise from these studies and should be addressed in the future.

1) Is P_0 a universal mediator of epithelialization and tumor suppression ?

It would be of interest if P_0 can universally induce epithelialization and behave as a tumor suppresser in other carcinoma types. More specifically, it should be determined if P_0-mediated adhesion can upregulate the synthesis of other members of the cadherin family. Several aggressive breast carcinomas have been shown to express normal, low, or null levels of E-cadherin and I have confirmed by Western blot analysis (Figure 6.1 A, B).

I have begun preliminary studies investigating whether expression of P_0 may be able to induce E-cadherin expression in certain breast carcinomas, MCF7, MDA-MB-453 and 231 cells. Transient transfection studies have helped determine the distribution of P_0 through indirect immunofluorescence. Concurrently, through double labeling immunofluorescence, I was able to determine if P_0 transfected cells upregulated their synthesis of E-cadherin.

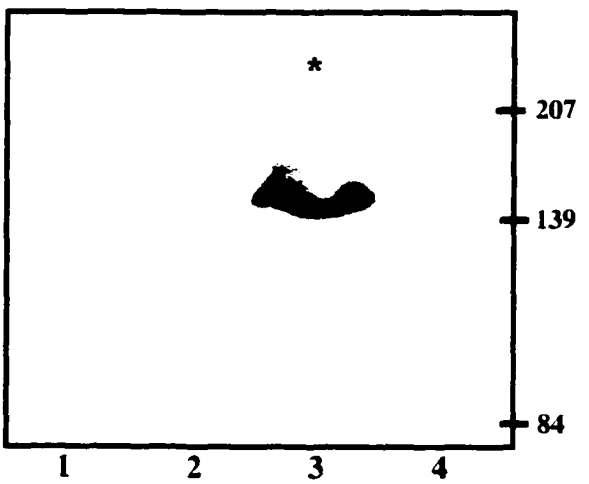
48 - 60 hours post-transfection in the MCF7 and MDA-MB-453 breast carcinoma cells, most of the expressed P_0 was transported to the cell surfaces and was concentrated at interfaces between two adjacent cell expressing P_0 as has been observed for other cell types (D'Urso et al., 1990; Filbin et al., 1990; Schneider Schaulies et al., 1990). The third cell line, MDA-MB-231, was unable to transport all of P_0 to cell surfaces, even by 60 hours post transfection; it appeared that the majority remained within vesicles throughout the cytoplasm (Figures 6.2 - 6.4). Interestingly, all three transfected cell lines had an increase in E-cadherin expression in juxtaposed transfected cells. The upregulated E-cadherin was specifically concentrated at the areas of cell to cell contacts in a similar distribution pattern as P_0 (Figures 6.2 - 6.4). I have generated cell lines constitutively expression P_0. In these cells, including P_0:231 cells, P_0 is primarily found at lateral cell borders, concentrated at areas of cell contacts (Figure 6.5). These cells lines will enable me to determine if P_0 expression and adhesion induce morphological changes or changes in

Figure 5.2 Cadherin expression in several breast carcinoma cell lines

Western blot analysis of total cell lysates (A) indicate that MDA-MB-231 (lane 1) and 453 (lane 2) cells like HeLa (lane 4), do not express E-cadherin. MCF7 cells were positive for E-cadherin expression (lane 3; *). The table in B summarizes characterization of these breast cell lines in regards to cadherin expression levels and junction formation (Soule et al., 1973; Cailleau et al., 1974; Brinkley et al., 1980; Sommers et al., 1991)

Figure 5.2

A



B

Cell Line	Cell Shape	Cadherin Expression	Junctions
MCF7	polygonal	E-cadherin	Adherens junctions Tight junctions Desmosomes
MDA-MB-231	spindle to round	None detectable	None
MDA-MB-453	round	None detectable	None

Characterization of Breast Carcinoma Cell Lines

synthesis or expression of E-cadherin or any of the other junctional associated proteins leading to assembly of epithelial junctions. Additionally I can examine functional properties such as growth parameters, secretion of MMPs, invasiveness as well as tumorigenicity and metastatic potential in nude mice. This studies will help determine if P_0 can behave as a universal tumor suppresser in a variety of carcinomas and may provide clues in designing therapeutic agents.

Figure 5.3 Transient P_0 expression in MCF7 breast carcinoma cells

Prolonged transient expression of P_0 in MCF7 leads to an accumulation of P_0 (A, C; arrows) and increased concentration or synthesis of E-cadherin (B, D; *) at lateral cell borders of expressing cells.

Figure 5.3

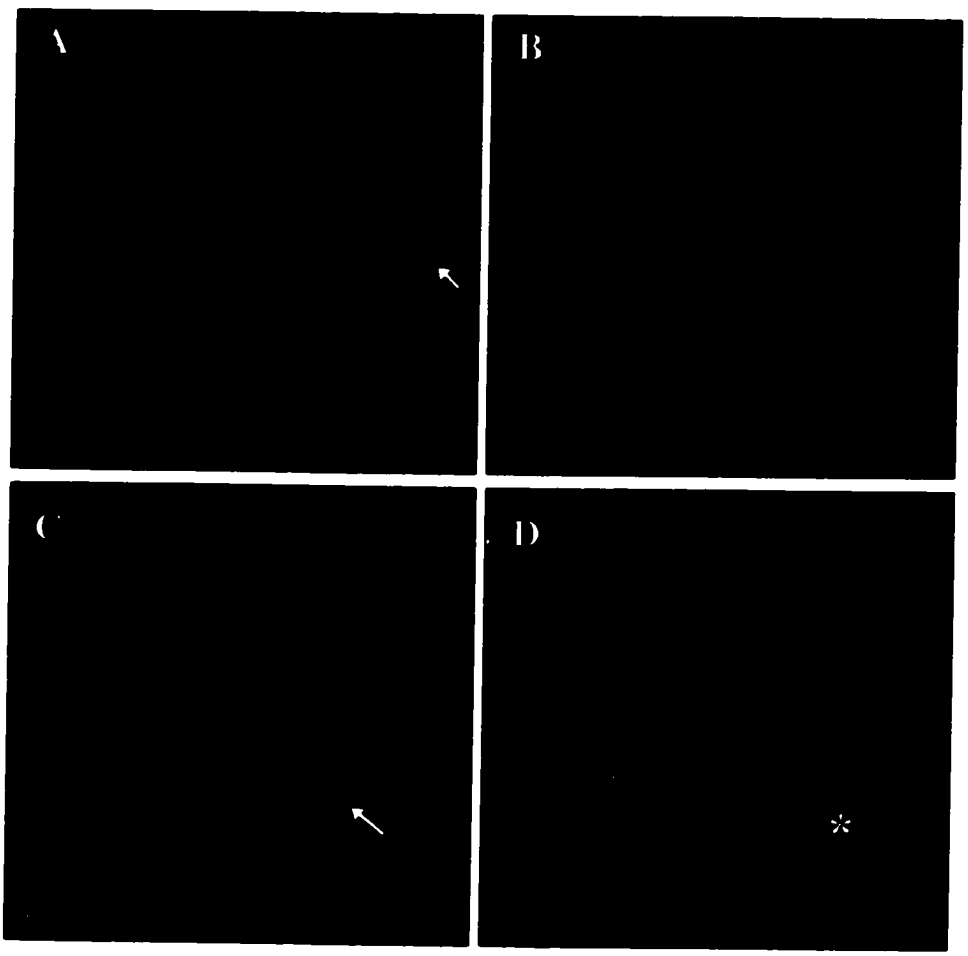


Figure 5.4 Transient expression of P_0 in MDA-MB-453 breast carcinoma cells

Transient expression of P_0 in MDA-MB-453 cells leads to the accumulation of P_0 at lateral contacts expressing cells (A, C, E; arrows). It also appears that E-cadherin synthesis is induced and is transported to the interfaces of expressing cells (B, D, F; *).

Figure 5.4

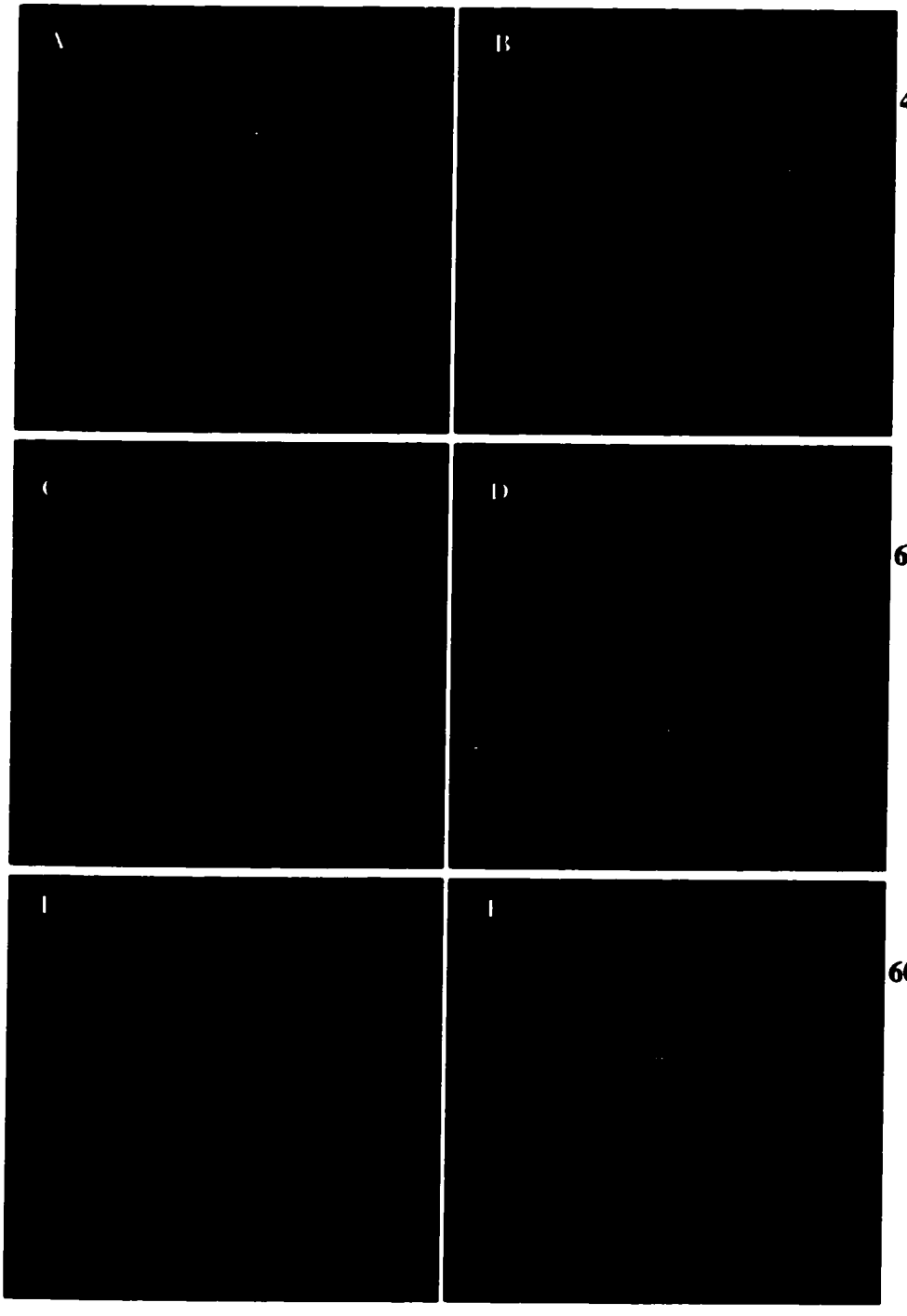


Figure 5.5 Distribution pattern of transient expression of P_0 in MDA-MB-231 breast carcinoma cells

At early time points following transfection, 36 hours, P_0 accumulates within vesicles within the cytoplasm (A) and E-cadherin expression is not induced (B). By 72 hours, P_0 vesicles fill the cytoplasm and some reaches the cell surface of expressing cells (C). Additionally, E-cadherin expression is induced in some of the P_0 expressing cells (D).

Figure 5.5

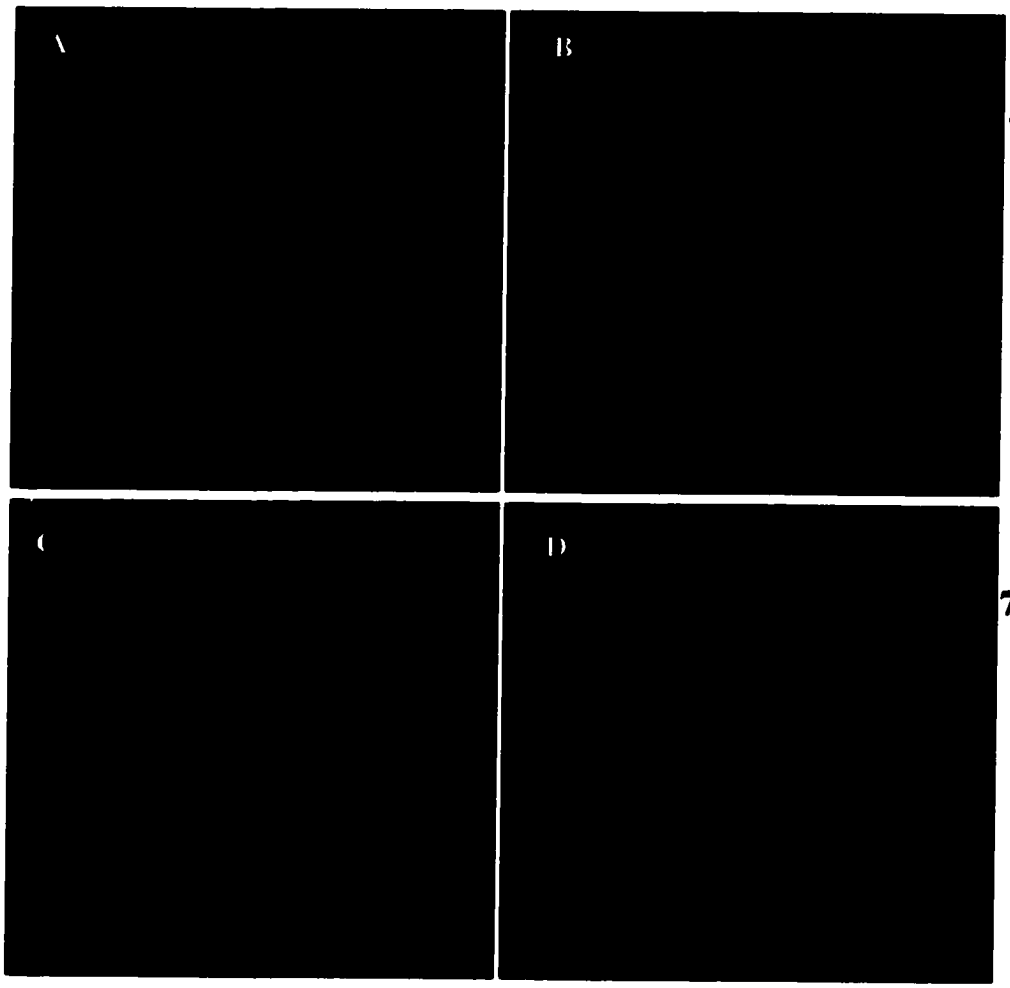
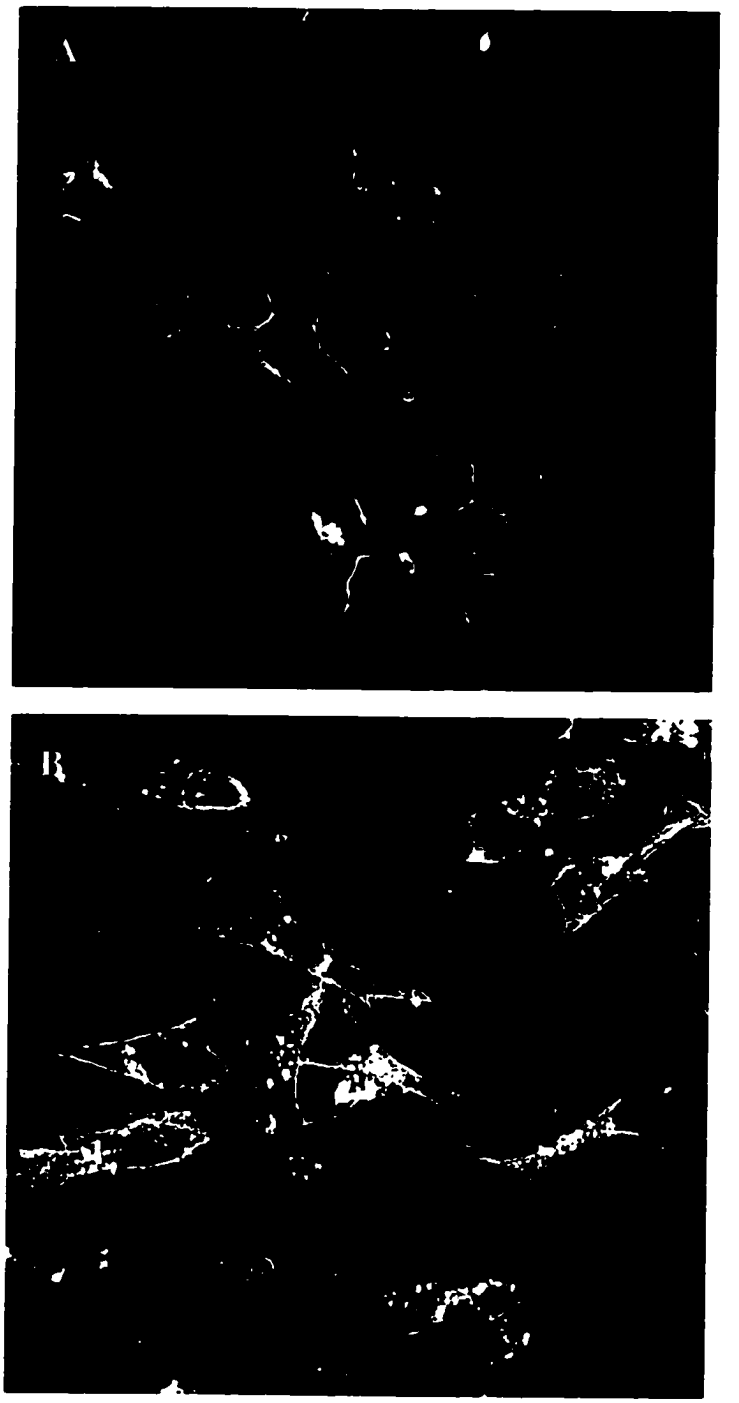


Figure 5.6 Distribution pattern of P_0 in MDA-MB-231 and 453 breast carcinomas constitutively expressing P_0

P_0 accumulates at lateral cell borders in monolayers of MDA-MB-453 and 231 cells constitutively expressing P_0.

Figure 5.6



2) What is the mechanism of P_0 induced epithelialization and suppression of the transformed phenotype?

It can be postulated from the studies presented that P_0 probably mediates morphological and physiological changes in HeLa though the cadherin-catenin signaling pathways. P_0 expression in HeLa causes the upregulation of several proteins in these pathways. Additionally, expression of N- or E-cadherin in HeLa was able to trigger an identical repertoire of events leading to epithelialization. The role of this pathway should be investigated further.

a) I have proposed that the close membrane apposition elicited by P_0 promotes cadherins to their active conformation leading to the activation of epithelial pathways. One way to directly test this hypothesis is to transfect HeLa with P_0 cDNA that has been mutagenized at amino acids suggested to be important for cis tetramer formation as well as lattice formation of P_0 and thus are believed to be essential for tight adhesion. If I find that these mutant P_0's are unable to elicit adhesion and morphologic and physiologic epithelialization, then it will confirm the proposed hypothesis. If these mutated P_0's can elicit these changes, it may be that P_0 is directly interacting with an intracellular signaling partner. Alternatively, these domains may not be the most important adhesive domains as has been predicted by the crystallographic model and may need to be re-evaluated

b) Additionally I have suggested that the active strand dimer form of the cadherins is necessary for the pathway to initiate following P_0 adhesion. This can be tested by expressing cadherin cDNA (N- or E-cadherin), that has mutations in amino acids predicted to be intrinsic to strand dimer formation and thus strong adhesion associated for adherens junctions. If these mutagenized protein can not elicit adhesion or epithelialization, this would confirm the proposed crystallographic models as well as confirm a causal role for cadherin function in normal epithelial cell physiology.

c) HeLa cells might also be transfected with constructs containing the P_0 ectodomain ligated to the highly conserved intracellular domain of a classic cadherin. These

experiments would answer two questions. First of all, is the cytoplasmic domain of P_0 required to initiate these pathways? Additionally, is cadherin-based adhesion required or is just the catenin binding domain essential to convey and integrate the signaling pathways.

d) Lastly, to test if the cadherin-catenin pathway is involved in the epithelial program HeLa cells could also be engineered to express a chimeric molecules containing the ectodomain of P_0 or a cadherin linked to α-catenin, β-catenin, or plakoglobin. Alternatively, full length catenins could be expressed in HeLa to see if this is sufficient to activate the epithelial program.

CHAPTER 6

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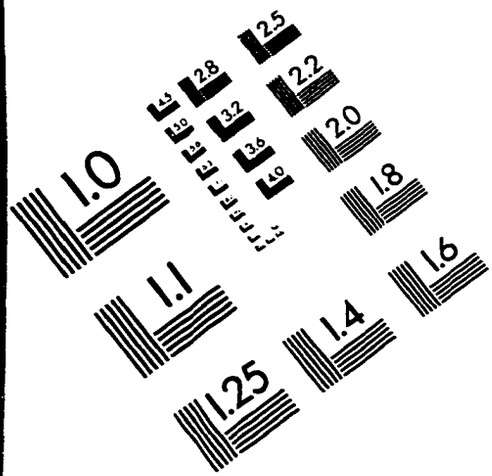
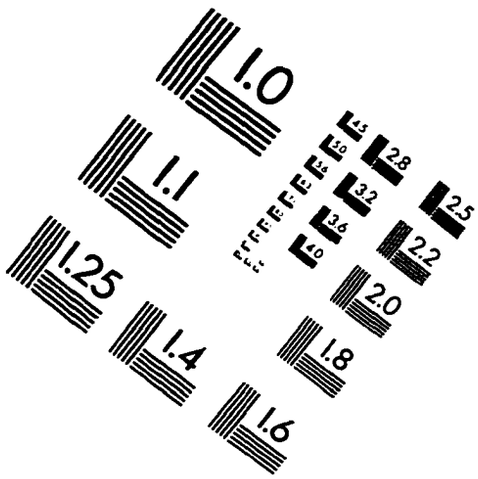
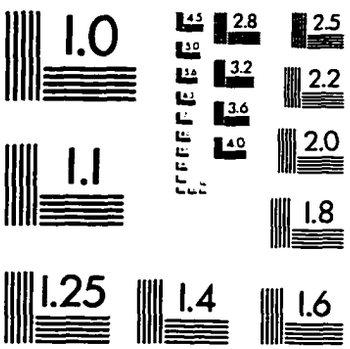
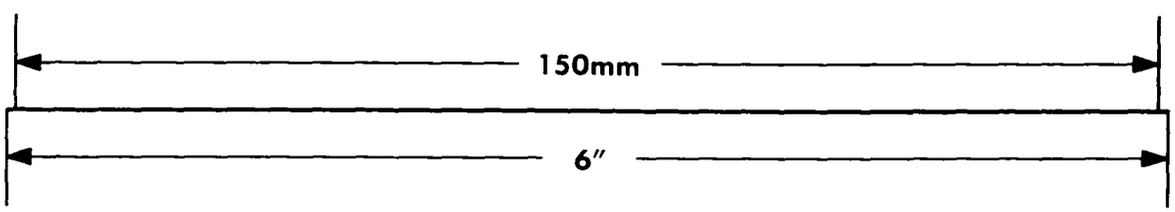
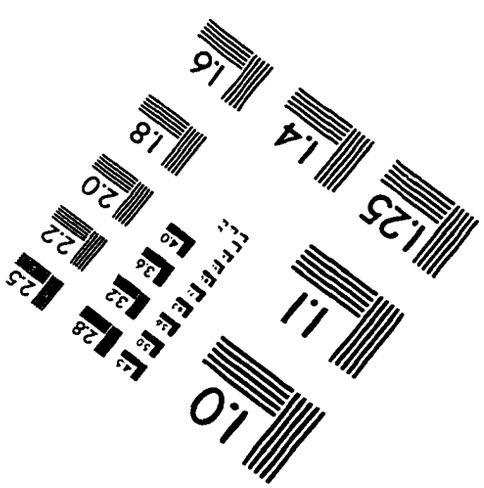
IMAGE EVALUATION TEST TARGET (QA-3)

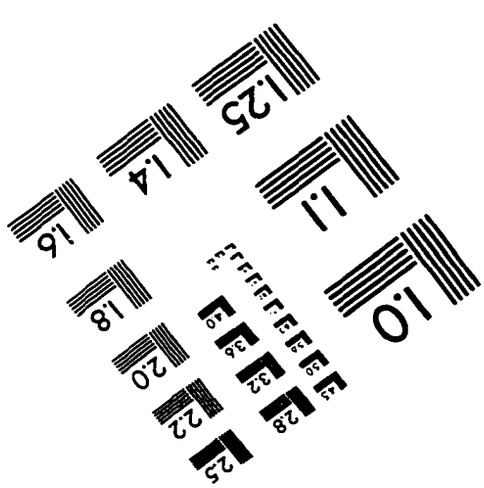












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