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Disruption in Mammary Gland Development and Increased
Tumor Incidence

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INTRODUCTION:

We have been studying a gene, netrin-1, that was identified in the developing nervous system as a guidance cue for neurons and their axons. We hypothesized that netrin-1 also functions outside the nervous system and may regulate cell interactions during mammary gland development. We also hypothesized that loss of this activity may play a role in mammary tumor formation. We have been testing this hypothesis by analyzing the netrin-1 null phenotype in the murine mammary gland. In the last funding period, we identified the netrin-1 receptor neogenin as an attractant receptor for netrin-1 in the mammary gland. Furthermore we elucidated the mechanism that underlies the activity of netrin-1 and neogenin. We discovered that neogenin, expressed in cap cells, acts as a short-range adhesive receptor for netrin-1, expressed in preluminal epithelial cells.

BODY:

In the first year of my Career Award, I proposed to master techniques of mammary gland manipulation as outlined in the approved Statement of Work below. I have accomplished all these goals.

Task 1. Master Techniques of Mammary Gland Manipulation (months: 1-12)

- a) Basic gland histology: recognizing the mammary epithelium embedded in the opaque fatty stroma of the living animal; identifying mammary rudiments in female embryos; removing and spreading glands on capsules for flat preparations; whole gland staining and defatting.
- b) General surgery: administering and monitoring anesthesia; incision and wound clip closing of skin; suturing; post-operative care.
- c) Transplant techniques: clearing fat pads of epithelial elements at 3 weeks of age; visualizing tissue outgrowths in the living animal; transplanting ductal fragments (placement, size); freezing mammary fragments for long term storage.

I have also accomplished Task 2 as outlined which would allow me to proceed with Task 3 in the next funding period.

Task 2. Generate Tissue for Experiments (periodic task: 2-7 months, depends on the developmental stage)

- a. Generate knock-out (KO) and wildtype (WT) mammary gland outgrowths from embryos.
- b. Expand gland outgrowths to increase source of tissue.

In addition, we published a paper detailing our characterization of the netrin-1 phenotype which served as the foundation for my grant application to the Department of Defense. In this manuscript we show the expression patterns of netrin-1 (*ntn1*) and its receptor neogenin (*neol*) in the terminal end bud of the virgin mammary gland (Srinivasan et al. 2003, Fig. 1). Netrin-1 is expressed in preluminal cells and neogenin is expressed in a complementary pattern in the cap cells. We analyzed the phenotypes of mammary glands carrying homozygous mutations in *ntn1* and *neol* by harvesting mammary anlage from embryos and transplanting them into cleared fat pads of immunocompromised mice. We discovered that loss of either gene results in disorganized terminal end buds characterized by exaggerated subcapsular spaces, breaks in basal lamina, dissociated cap cells and increased influx of cap cells into the preluminal compartment (Srinivasan et al. 2003, Fig. 2). We rescued the phenotype by creating mosaic glands confirming that loss of netrin-1 is responsible for the observed changes (Srinivasan et al. 2003, Fig. 3). We showed that the phenotype is present in heterozygous animals providing evidence that a strict requirement for adequate levels of netrin-1 in the terminal end bud is not met by a single functional allele (Srinivasan et al. 2003, Fig. 3). Immunohistochemical analysis of *ntn1*^{-/-} and *neol*^{-/-} terminal end buds revealed breaks in basal lamina and identified the dissociated cells as cap cells (Srinivasan et al. 2003, Fig. 4). Cadherin staining was normal in the glands, suggesting that this cell-cell adhesion system remains intact (Srinivasan et al. 2003, Fig. 5). We found that the dissociated cap cells either die by apoptosis or migrate inappropriately into the preluminal compartment (Srinivasan et al. 2003, Fig. 6). We performed adhesion assays to demonstrate that neogenin mediates netrin-1 dependent cell-cell adhesion (Srinivasan et al. 2003, Fig. 7).

In the next funding period, the task in the Approved Statement of Work is Task 3: cDNA microarray analysis comparing netrin-1 null and wildtype tissue, (months: 12-24). This task generated some criticism during the review process. For example, Reviewer A commented, "The microarray experiments are the least impressive; sorting out its meaning may yield little useful information. Time might be better spent looking for netrin-1 receptors in these tissues, which would be important for the interpretation of the action of this molecule in the mammary gland". Reviewer B wrote, "Although identification of differentially expressed genes may lead to a better understanding of pathways involved in breast cancer progression or the maintenance of normal mammary development, the microarray analysis will likely generate large amounts of data that may not prove insightful but instead be overwhelming and confusing."

Recommended Changes:

In light of the reviewers' criticisms and our positive results looking for netrin receptors in the mammary gland (Srinivasan et al. 2003, Fig. 1), we propose to switch the focus of Task 3 to examining netrin-1 receptor expression and function in the developing mammary gland. Neogenin is expressed not only in the cap cells but also in fibroblasts (stromal compartment) that encircle the duct.

UNC5H1 (*u5h1*), a repellent receptor for netrin-1, is also expressed in these cells. I propose to look at the consequences of loss of *neo1* and *u5h1* in the stroma. Again this will require transplantation experiments. In these experiments, *neo1* $-/-$ and *u5h1* $-/-$ fat pads (the stromal compartment containing fibroblasts) are transplanted into cleared $+/+$ hosts, followed by transplantation of *neo1* $-/-$ and $+/+$ epithelium into *neo1* $-/-$ fat pads and similarly *u5h1* $-/-$ and $+/+$ epithelium into *u5h1* $-/-$ fat pads. Interactions between the stromal and epithelial compartments of breast tissue is known to be important during malignant transformation (Chrenek et al. 2001; Silberstein 2001; Shekhar et al. 2003). These experiments will give us a comprehensive understanding of netrin-1 function during mammary gland development and insight into how deregulation of this function contributes to the disease process.

KEY RESEARCH ACCOMPLISHMENTS:

- I learned key techniques necessary to perform surgical manipulations of the mouse mammary gland.
- Together with my laboratory, we:
 - Determined the expression pattern of netrin-1 and its receptor neogenin in the virgin mammary gland.
 - Analyzed the phenotype of the netrin-1 $-/-$ virgin mammary gland.
 - Analyzed the phenotype of the neogenin $-/-$ virgin mammary gland.
 - Demonstrated that neogenin functions as a netrin-1 dependent cell adhesion protein.

REPORTABLE OUTCOMES:

Manuscript:

Srinivasan K., Strickland P., Valdes A., Shin, G.C., Hinck, L. 2003. Netrin-1/neogenin interaction stabilizes multipotent progenitor cap cells during mammary gland development. *Developmental Cell* 4: 1-20.

Abstract: 2003 Mammary Gland Biology and Breast Cancer Gordon Conference

Strickland P., Hinck, L. Netrin and Slit axon guidance molecules play a role during mammary gland development.

CONCLUSIONS:

Changes in the function of molecules that regulate cell-cell and cell-extracellular matrix (ECM) interactions have been implicated in the initiation and progression of malignancy. During this funding period, we discovered that netrin-1 is secreted by preluminal cells of the developing mammary gland and its receptor neogenin is expressed in a complementary manner in the adjacent cap cells. We demonstrated that the netrin-1/neogenin adhesion system maintains normal topology of cap cells during a period of invasive growth. These results suggest that netrin-1 functions as a short-range adhesive protein that maintains normal tissue morphology during development. Loss of these adhesive contacts during malignancy potentially contribute to an invasive phenotype.

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APPENDICES: see attached 6-17, double sided reprint

Netrin-1/Neogenin Interaction Stabilizes Multipotent Progenitor Cap Cells during Mammary Gland Morphogenesis

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Summary

Netrin-1 and its receptors play an essential role patterning the nervous system by guiding neurons and axons to their targets. To explore whether netrin-1 organizes nonneural tissues, we examined its role in mammary gland morphogenesis. Netrin-1 is expressed in preluminal cells, and its receptor neogenin is expressed in a complementary pattern in adjacent cap cells of terminal end buds (TEBs). We discovered that loss of either gene results in disorganized TEBs characterized by exaggerated subcapsular spaces, breaks in basal lamina, dissociated cap cells, and an increased influx of cap cells into the preluminal compartment. Cell aggregation assays demonstrate that neogenin mediates netrin-1-dependent cell clustering. Thus, netrin-1 appears to act locally through neogenin to stabilize the multipotent progenitor (cap) cell layer during mammary gland development. Our results suggest that netrin-1 and its receptor neogenin provide an adhesive, rather than a guidance, function during nonneural organogenesis.

Introduction

Netrin-1 (*ntn1*) plays a well-established role both as a short- and long-range guidance cue, directing neurons and their axons to targets during development of the nervous system (Tessier-Lavigne and Goodman, 1996). It is bifunctional, acting either as an attractant or a repellent depending on the receptors expressed on neurons and the levels of intracellular cAMP (Hong et al., 1999; Ming et al., 1997). Two vertebrate families of netrin-1 receptors have been identified, the Deleted in Colorectal Cancer (DCC) family, comprising *DCC* and *neo1* (Keino-Masu et al., 1996), and the UNC5H family, comprising *unc5h1-h4* (Engelkamp, 2002; Leonardo et al., 1997; Przyborski et al., 1998). The DCC family mediates attraction to netrin-1, while the UNC5H family forms a netrin-1-dependent complex with DCC to mediate repulsion (Hong et al., 1999). The function of DCC as a neuronal attractant receptor for netrin-1 has been well established (Fazeli et al., 1997; Keino-Masu et al., 1996), but relatively little is known about the function of neogenin. Although widely expressed during development (Gad et al., 1997), no phenotype has been reported for the *neo1* $-/-$ mouse (Leighton et al., 2001). Indeed, no functional role for neogenin has been demonstrated in either an in vitro or in vivo context.

While netrin-1 is primarily thought of as an axon guidance cue, guidance is unlikely to be its only function, since expression studies have shown that many netrins are widely expressed outside the nervous system (Koch et al., 2000; Meyerhardt et al., 1999; Salminen et al., 2000; Wang et al., 1999). Nevertheless, the developmental role of netrin-1 and its receptors in the morphogenesis of nonneural organs remains largely unexplored. Since netrin-1 is an early cue that patterns the nervous system by guiding millions of growth cones on their journeys, one way to think about its function in morphogenesis is as a molecule that provides spatial and temporal information to shape tissue and organ development.

The mammary gland is an organ that undergoes an elaborate and regulated morphogenesis (Silberstein, 2001). During development, mammary epithelium grows from the nipple subdermally, to invade the fat pad, establishing a mammary tree through a process of ductal elongation and branching. The ducts of the mammary gland comprise an outer tube of myoepithelial cells juxtaposing an inner tube of luminal epithelial cells surrounding a central lumen. Terminal end buds (TEBs) are the enlarged termini of ducts responsible for prodigious pubertal growth controlled by hormones and growth factors (Figure 1H). During development, the motile TEB penetrates the stromal fat pad at a rapid pace (0.5 mm/day), establishing the complex ductal architecture of the virgin mammary gland (Williams and Daniel, 1983). This growth is driven by the proliferation of a single layer of cells, termed cap cells, at the tip of the TEB, and by the underlying preluminal epithelium. Cap cells are multipotent progenitor cells of the virgin mammary gland (Williams and Daniel, 1983). As the TEB grows rapidly toward the edge of the fat pad, cap cells translocate laterally to differentiate into myoepithelial cells (Williams and Daniel, 1983). In addition, a fraction of the cap cells detach and move into the subjacent preluminal cell population, where they are thought to give rise to a fraction of luminal epithelial cells (Williams and Daniel, 1983). Cap and preluminal cell layers are maintained as distinct populations through different adhesion systems. Cap cells adhere to each other through P-cadherin, and preluminal cells adhere to each other through E-cadherin (Daniel et al., 1995). However, the mechanism that maintains the close apposition of the cap and preluminal layers during the dynamic growth of the TEB has not been identified.

Here we analyze the functional role of netrin-1 and neogenin in mammary gland morphogenesis. We examine in vivo localization of both proteins in the TEB and defects resulting from loss of either gene product. We also explore the mechanism underlying the netrin-1/neogenin interaction using in vitro assays. The results show that, in the developing mammary gland, netrin-1 acts locally through neogenin to maintain close apposition of cap cells and preluminal cells at the leading edge of the TEB. Furthermore, our results suggest that, in this context, netrin-1 acts as a short-range attractant and has an adhesive, rather than a guidance, function.

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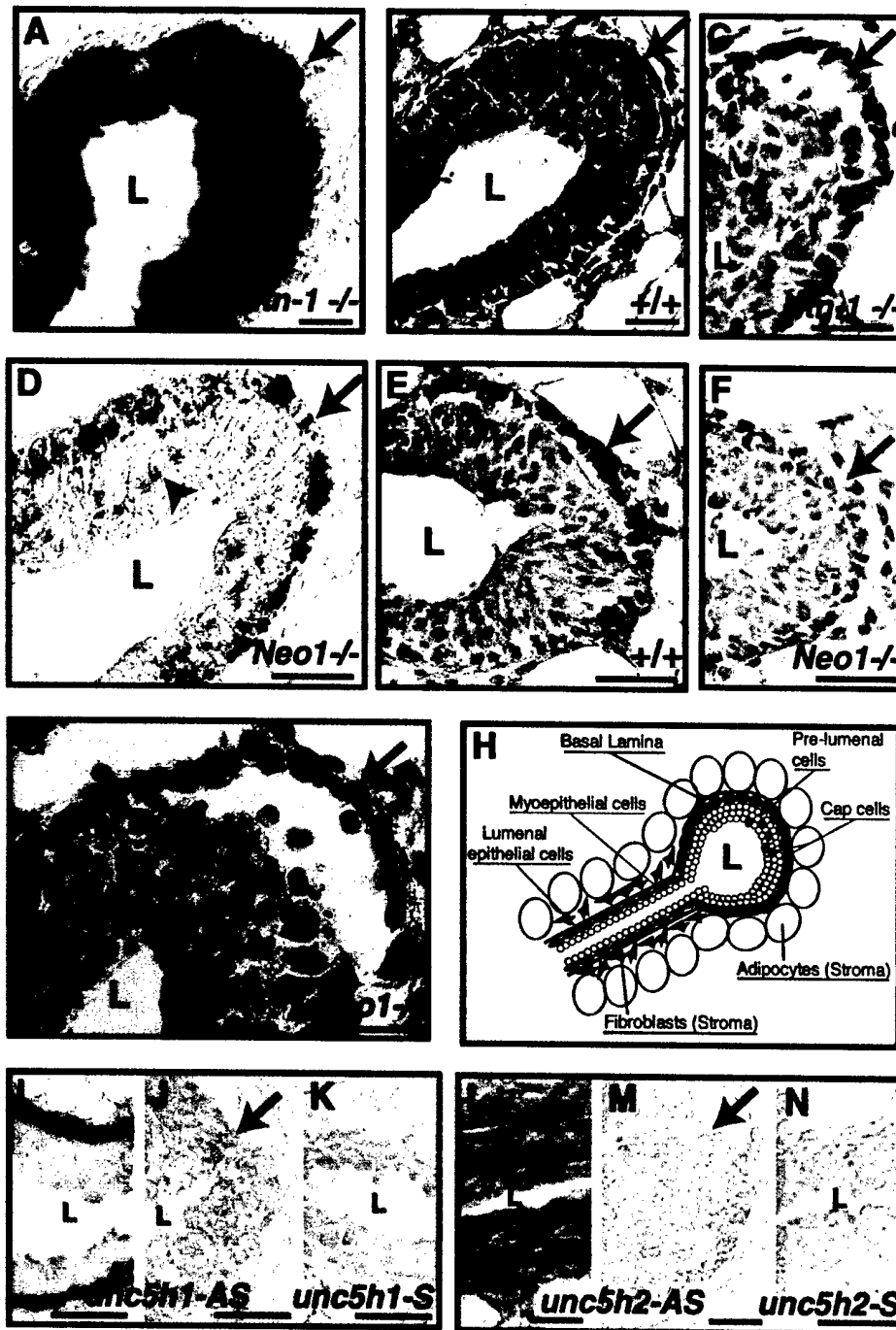


Figure 1. Expression Patterns of *ntn1* and Its Receptors *neo1*, *unc5h1*, and *unc5h2* during Early Mammary Development

(A) *ntn1* $-/-$ outgrowth stained for β-galactosidase activity.

(B, C, and G) Netrin-1 immunostaining on TEBs (B) $+/+$, (C) *ntn1* $-/-$, and (G) *neo1* $-/-$.

(D) *neo1* $-/-$ outgrowth stained for β-galactosidase activity.

(E and F) Neogenin immunostaining on TEBs (E) $+/+$ and (F) *neo1* $-/-$.

(H) Schematic of a TEB.

(I–N) *unc5h1* and *unc5h2* in situ hybridization on $+/+$ tissue; (I and J) with an anti-sense (AS) probe to *unc5h1*, (K) with a sense probe to *unc5h1*, (L and M) with an anti-sense (AS) probe to *unc5h2*, and (N) with a sense probe to *unc5h2*.

(A–F, G, J, M) Arrows point to cap cell layer.

(D and E) Arrowhead points to cap cells in the preluminal compartment that stain positive for β-galactosidase and neogenin, respectively.

All outgrowths were generated by transplantation technique and taken at 3 weeks for analysis. L, lumen; scale bar, 5 μm.

Results

ntn1 and *neo1* Display Nonoverlapping, Complementary Expression Patterns in the TEB

For *ntn1* to play a role in regulating early mammary gland development, it must normally be expressed during this stage. Since the construct used to generate *ntn1* $-/-$ mice inserted *lacZ* under the control of the *ntn1* promoter (Serafini et al., 1996), we assayed $+/-$ glands and $-/-$ outgrowths at 3 weeks for β -galactosidase activity. Accumulation of β -galactosidase reaction product revealed that *ntn1* is present exclusively in the preluminal epithelial cell compartment; cap cells do not stain positive (Figure 1A). We confirmed this pattern by in situ hybridization (data not shown). Since netrin-1 is a secreted glycoprotein, we determined the distribution of netrin-1 protein by performing immunohistochemistry (Deiner et al., 1997). This reveals strong staining of netrin-1 around all cells of the TEB, including cap cells (Figure 1B), showing that, even though cap cells do not express *ntn1* mRNA, they are surrounded by secreted netrin-1 protein. Light staining is also seen in the stroma immediately adjacent to the TEB, but not at a distance. With the same antibody, little or no background staining is observed in *ntn1* $-/-$ glands (Figure 1C). Unlike netrin-4, which is detected in the basement membrane surrounding various organs (Koch et al., 2000), we do not detect netrin-1 in basal lamina surrounding the TEB.

We next examined the expression of known netrin-1 receptors and discovered the presence of neogenin. Using *neo1* $-/-$ mice that also have *LacZ* inserted downstream of the *neo1* promoter (Leighton et al., 2001), we assayed $+/-$ glands and $-/-$ outgrowths at 3 weeks for β -galactosidase activity and observed *neo1* expression in the cap cell layer (Figure 1D). A fraction of cells in the preluminal compartment underlying the cap cells also stain positive for the β -galactosidase reaction product. It is likely that these are cap cells that have detached and migrated down into this region (Williams and Daniel, 1983). We also detected neogenin in cap cells using immunohistochemistry, including the occasional cap cell in the preluminal compartment (Figures 1E and 1F). Analysis of netrin-1 protein distribution in *neo1* $-/-$ outgrowths reveals netrin-1 staining around cap cells of the TEB similar to the staining in $+/+$ TEBs (Figures 1G and 1B), suggesting that neogenin does not simply bind and distribute netrin-1. Thus, the expression studies show that netrin-1 and neogenin are expressed in complementary expression patterns in the TEB. Together with the fact that netrin-1 binds neogenin (Keino-Masu et al., 1996; Wang et al., 1999), the data suggest that they function as ligand and receptor to signal between preluminal and cap cell layers during mammary gland development. The *neo1* homolog, *DCC*, is not expressed in the mammary gland at this stage of development, as determined by in situ hybridization and immunohistochemistry (data not shown). *Unc5h* receptors are detected by in situ hybridization, but only in fibroblasts that encircle the mammary ducts and not in epithelial cells (Figures 1I–1N).

Loss of Either *ntn1* or *neo1* Results in Abnormal TEBs

The perinatal lethality of both *ntn1* $-/-$ and *neo1* $-/-$ mutations prevented the study of mammary glands in

mice carrying the homozygous mutation. To circumvent this problem, we followed standard protocols (Robinson et al., 2000; Young, 2000) and harvested mammary anlage from *ntn1* $-/-$ embryos and transplanted them into cleared fat pads of immunocompromised mice. The contralateral fat pad received control anlage from $+/+$ embryos from the same litter. To ensure that only the epithelial compartment of the anlage was transplanted, we performed serial transplants of tissue fragments to generate outgrowths. At least three independently derived lines were isolated and serially transplanted for up to three generations to provide outgrowths for experiments. In all studies, littermate control $+/+$ outgrowths were generated on the contralateral side for comparison, so that both $+/+$ and $-/-$ outgrowths were subject to the same systemic environment.

We sectioned *ntn1* $-/-$ outgrowths 2–3 weeks post-transplantation to analyze TEBs. The sections were stained with Fast green and Sirius red to visualize the epithelial compartment and basement membrane, respectively. Compared with TEBs from control $+/+$ transplants (Figure 2A), *ntn1* $-/-$ TEBs displayed severe abnormalities. In *ntn1* $-/-$ TEBs, cap cells are significantly pulled away from preluminal cells (Figures 2B–2D), creating an exaggerated subcapsular space ranging from 5–20 μ m, compared with a subcapsular space of 0.1–1 μ m, typically observed in $+/+$ TEBs (Figure 2A). There are often detached cells in this subcapsular space (Figure 2C), and, in some morphologically abnormal TEBs, the basal lamina in front of the cap cell layer is disrupted (Figure 2D). The structure of the preluminal compartment of many $-/-$ TEBs is also disturbed, with epithelial cells appearing disorganized. Analysis of outgrowths at different time points posttransplantation reveal such defects throughout the ductal growth phase. The phenotype is 100% penetrant, and, within any given outgrowth, approximately 60% of TEBs are affected ($60\% \pm 5.5\%$, $n = 60$ TEBs, five animals). TEBs at the leading edge of the array display the most severe phenotype. These TEBs have the largest subcapsular space, the greatest number of detached cells, and, also, breaks in basal lamina. The severity of the phenotype in these TEBs is most likely due to the fact that they are the largest and most proliferative in the array. Although netrin-1 is expressed in ductal epithelial cells (data not shown), examination of ducts did not reveal any obvious defects in either lumen formation or stromal investment.

A similar analysis was performed on *neo1* $-/-$ outgrowths (Figures 2E and 2F). These TEBs are also disorganized, and we observe all the characteristics of *ntn1* $-/-$ TEBs (Figures 2E and 2F). The phenotype is 100% penetrant, and approximately 58% of TEBs are affected ($58.25\% \pm 8.8\%$, $n = 61$ TEBs, four animals). The observation that *neo1* $-/-$ TEBs phenocopy *ntn1* $-/-$ TEBs strongly supports the notion that *neo1* is the receptor for *ntn1*.

Netrin-1-Expressing $+/+$ Cells Restore Normal TEB Topology

The data document the functional involvement of netrin-1 in organizing the leading edge of the TEB. A stringent confirmation that the observed phenotype is solely due to lack of netrin-1 is to rescue disorganized TEBs by adding it back. Since mammary glands can be reconstituted with suspensions of mammary epithelial cells

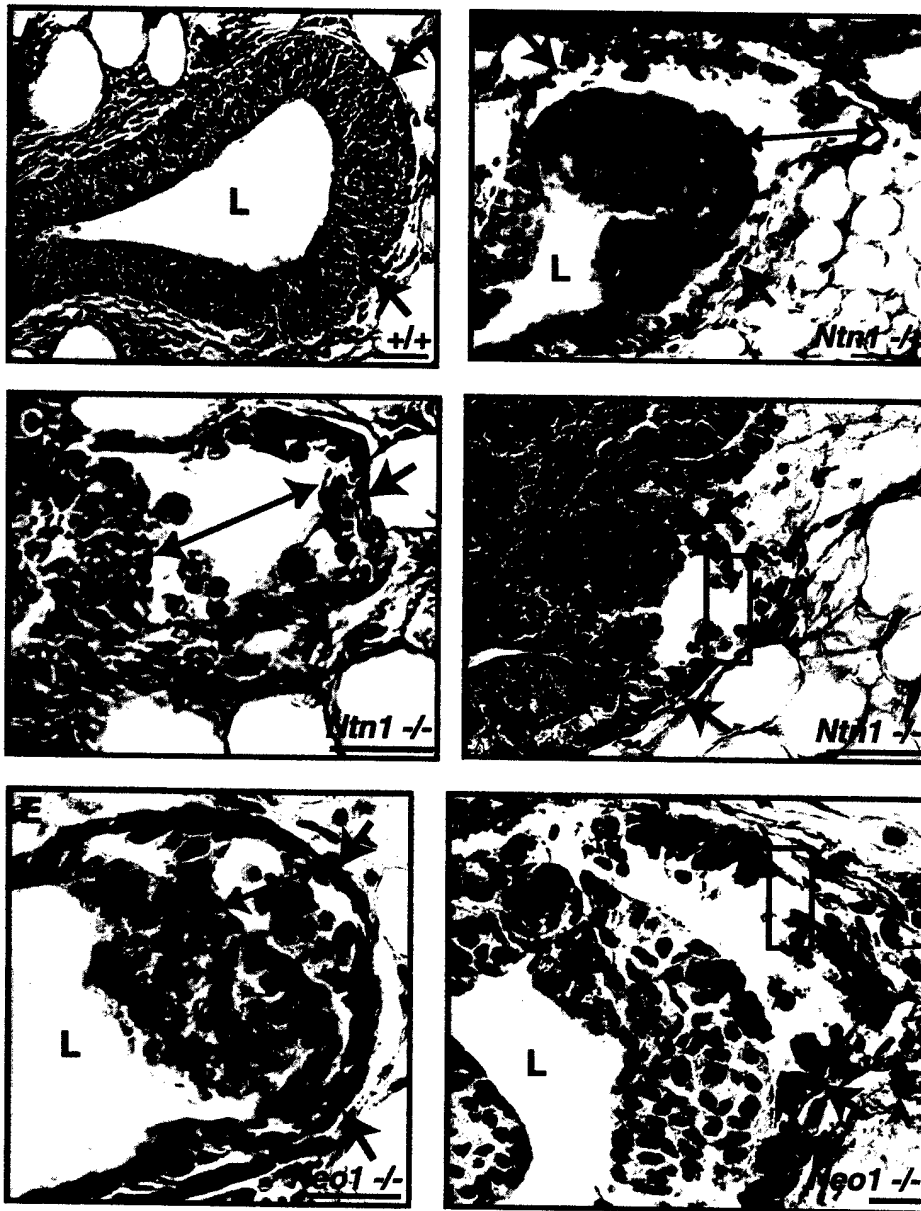


Figure 2. Loss of Either *ntn1* or *neo1* Leads to Abnormal TEBs

All the outgrowths shown in this panel were generated by serial transplantation technique. Every animal had a +/+ outgrowth in one #4 fat pad and either an *ntn1* -/- or *neo1* -/- outgrowth in the contralateral fat pad.

(A) Longitudinal section through +/+ TEB demonstrates the tight juxtaposition of the cap cell layer with preluminal cells.

(B-D) Longitudinal sections through *ntn1* -/- TEBs.

(B) Exaggerated space between the cap cell layer and preluminal cells.

(C) Loose cells detected in the subcapsular space.

(D) Break in basal lamina at the leading edge of the cap cell layer (box) and loose cells outside the TEB (arrowheads).

(E and F) Longitudinal sections through *neo1* -/- TEBs.

(E) Exaggerated space between the cap cell layer and preluminal cells and loose cells detected in the subcapsular space.

(F) Break in basal lamina at the leading edge of the cap cell layer (box).

(A-F) Arrows point to the cap cell layer. Double-headed arrow indicates exaggerated subcapsular space between the cap and preluminal cell layers. Boxed region indicates a break in the basal lamina. L, lumen of the TEB; scale bar, 5 μ m.

(Young, 2000), netrin-1 can be added back by reconstituting outgrowths with mixtures of +/+ (netrin-1 secreting) and -/- cells. We injected *ntn1* -/- cell suspensions and determined that these outgrowths have a similar phenotype to those generated from ductal fragments (disorganized TEBs/outgrowth = 88.6% \pm 1.5%, n = 114 TEBs, four outgrowths).

To rescue the phenotype, we injected mixtures of cell suspensions containing different ratios of +/+ and *ntn1* -/- cells into cleared fat pads. Analysis of these mosaic outgrowths reveal TEBs that are composed entirely of +/+ cells, -/- cells, and mixtures of +/+ and -/- cells (Figures 3A and 3B). As a result, every outgrowth displays both normal and morphologically ab-

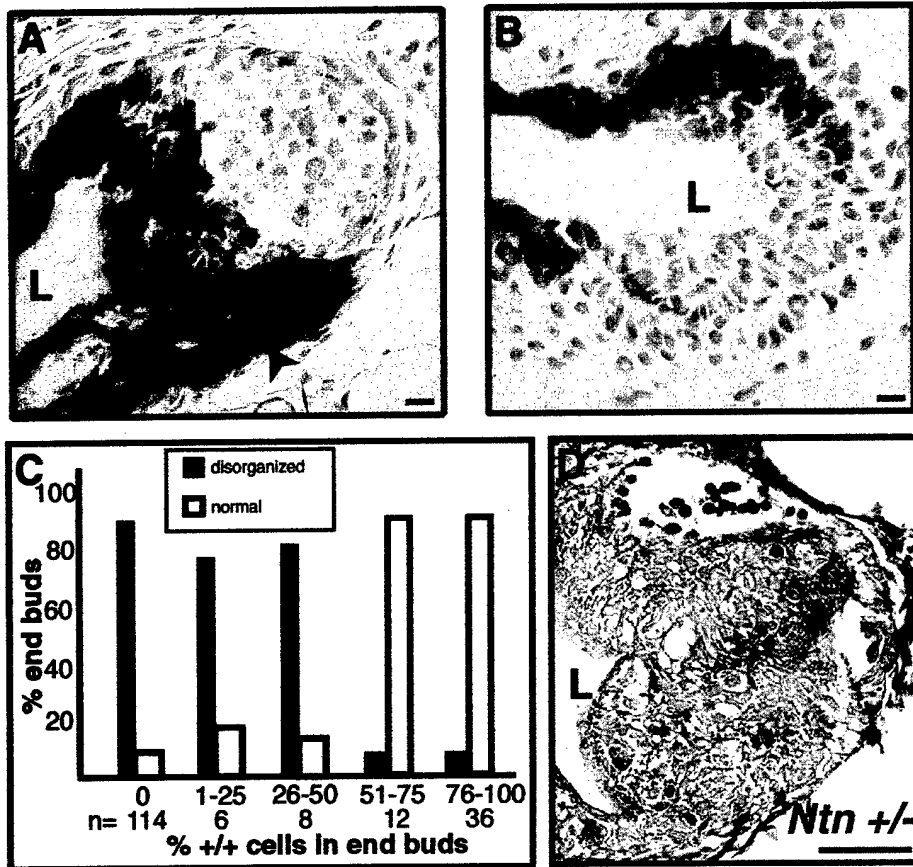


Figure 3. Mosaic Analysis Reveals that over 50% Netrin-1 Is Required for Normal TEB Morphology

(A and B) Mosaic TEBs from reconstituted outgrowths generated with mixtures of $-/-$ and $+/+$ cells stained for β -galactosidase and counterstained with hematoxylin (nuclei). Arrowheads point to $ntn1^{-/-}$ cells staining positive for β -galactosidase activity.

(A) TEB comprising a majority of $ntn1^{-/-}$ cells displays the phenotype.

(B) TEB comprising a majority of $+/+$ cells displays normal TEB structure.

(C) Distribution of TEBs with normal and abnormal morphology in reconstituted outgrowths reveals that the majority of TEBs composed of greater than 50% netrin-1-expressing cells are normal. n, the number of TEBs analyzed for each category.

(D) Longitudinal section of a representative TEB from an $ntn1^{+/-}$ animal displays the phenotype, as indicated by the exaggerated subcapsular space and dissociated cells. L, lumen of the TEB; scale bar, 5 μ m.

normal TEBs, but the ratio depends on the starting ratio of $+/+$ and $-/-$ cells used to reconstitute the outgrowth. For example, outgrowths generated with 90% $-/-$ cells have more disorganized TEBs compared with outgrowths generated with 50% $-/-$ cells. Within each TEB, the location of $+/+$ cells does not correlate with rescue, but the number of $+/+$ cells does. To determine the minimum number of $+/+$ cells required for rescue, we counted $+/+$ versus $-/-$ cells in each TEB and discovered a striking trend. With fewer than 50% netrin-1-expressing cells, the majority of TEBs display the $ntn1^{-/-}$ phenotype (Figures 3A and 3C). In contrast, with greater than 50% netrin-1-expressing cells, the majority of TEBs are normal (Figures 3B and 3C). The ability to restore normal TEB structure demonstrates that netrin-1 is responsible for the observed phenotype.

The observation that some TEBs containing 50% netrin-1 still display the phenotype suggests that $ntn1$ is haploinsufficient. To confirm $ntn1$ haploinsufficiency, we analyzed heterozygous mammary glands and discovered the phenotype (86% penetrant) (Figure 3D). However, compared with $ntn1^{-/-}$ outgrowths, which display approximately 60% expressivity, the expressivity

of the heterozygous phenotype is reduced to only 17% TEBs displaying morphological abnormalities ($17\% \pm 12.6\%$, $n = 128$ TEBs, seven animals). This analysis provides strong evidence that a strict requirement for adequate levels of netrin-1 in the TEB is not met by a single functional allele.

Detached Cells Are Multipotent Progenitor Cells from the Cap Cell Layer

Having confirmed that the observed phenotype is due to absence of $ntn1$, we next characterized the nature of the phenotype. First, we determined the origin of detached cells in disorganized TEBs. These cells could either be cap cells that move downward, or preluminal cells that move upward, into the subcapsular space. To distinguish between these two, we used an immunohistochemical marker.

Smooth muscle actin (*sma*) is a marker for both cap and myoepithelial cells (Deugnier et al., 1995), but cap cells can be distinguished on the basis of their location and morphology. Using an antibody to *sma*, we performed immunohistochemistry on $+/+$, $ntn1^{-/-}$, and

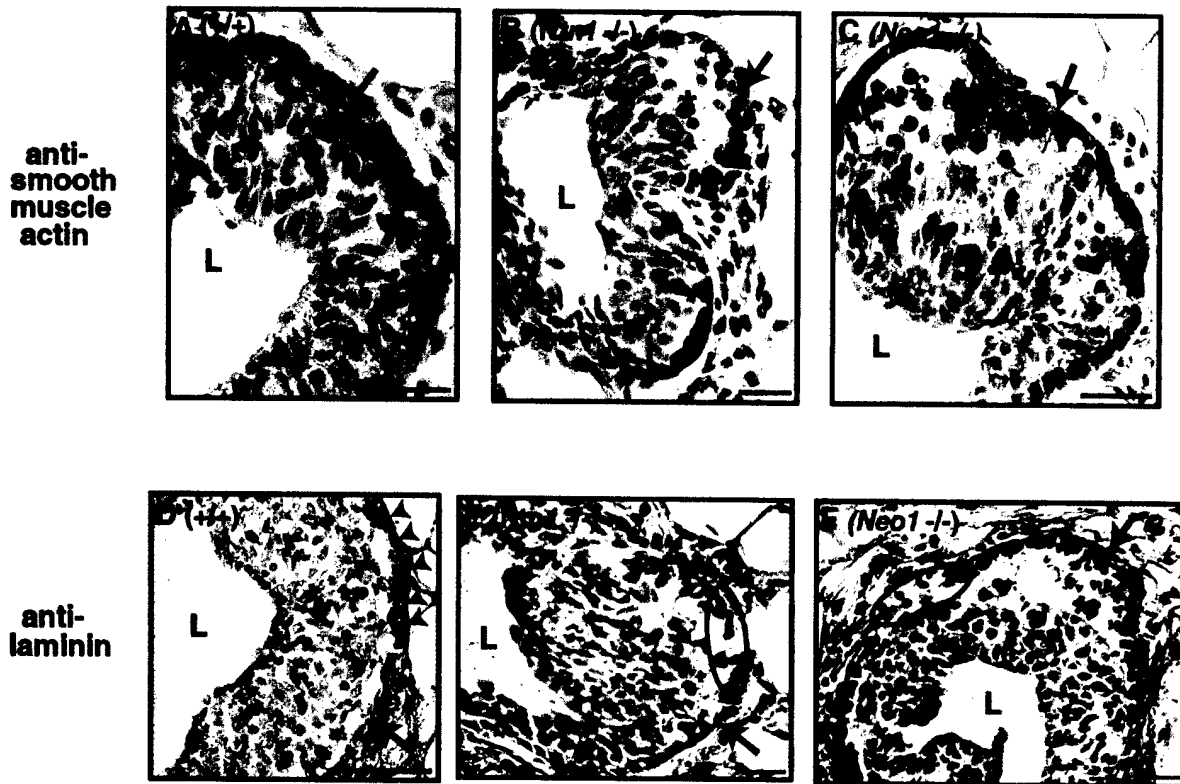


Figure 4. Immunohistochemical Characterization of Disorganized TEBs Reveals the Identity of Dissociated Cells and Breaks in Basal Lamina at the Leading Edge of the TEB

(A–C) Immunostaining with a smooth muscle actin antibody on TEBs from 3 week outgrowths generated by transplantation.

(A) Cap cells stain positive for smooth muscle actin in $+/+$ TEB.

(B and C) Cap cells and dissociated cells stain positive in TEBs (B) $ntn1^{-/-}$ and (C) $neo1^{-/-}$.

(D–F) Immunostaining with a laminin-1 antibody on TEBs from 3 week outgrowths generated by transplantation.

(D) Cap cells stain intracellularly for laminin-1 in $+/+$ TEB. The basal lamina stains extracellularly for laminin-1.

(E and F) Cap cells in the cap cell layer and a fraction of dissociated cap cells stain intracellularly for laminin-1 in TEBs (E) $ntn1^{-/-}$ and (F) $neo1^{-/-}$. Arrows point to the cap cell layer. Arrowheads point to the basal lamina. Asterisks point to dissociated cap cells. Circled region shows lack of basal lamina at the leading edge of the TEB. L, lumen of the TEB; scale bar, 5 μm .

$neo1^{-/-}$ outgrowths. As expected, cap and myoepithelial cells in the $+/+$ TEBs stain positive for *sma* (Figure 4A). A fraction of cells present within the prelumenal cell population also stain positive for *sma* and very likely represent cap cells that have migrated into this region (Williams and Daniel, 1983). Importantly, a majority of nonadherent cells present within the subcapsular space of disorganized $ntn1^{-/-}$ and $neo1^{-/-}$ TEBs stain positive for *sma*, confirming their identity as cap cells and further demonstrating the similarity of $ntn1$ and $neo1$ phenotypes (Figures 4B and 4C). A few detached cells do not stain positive, but their nuclei appear shrunken, and it is likely that these cells are dying by detachment-induced apoptosis.

Lack of Either $ntn1$ or $neo1$ Leads to Breaks in the Basal Lamina

While performing the analysis of $ntn1^{-/-}$ and $neo1^{-/-}$ outgrowths, we frequently observe breaks in basal lamina (Figures 2D and 2F) and occasionally observe cells in the stroma that stain positive for *sma* (data not shown). Cap cells make and secrete basal lamina, depositing it at the stromal interface. Therefore, their disorganization

may destabilize the laminin network in front of the TEB, allowing cap cells to escape their boundaries in morphologically abnormal TEBs. We examined this possibility by staining both $ntn1^{-/-}$ and $neo1^{-/-}$ outgrowths with an antibody to laminin-1, which is the major laminin in the basal lamina at the leading edge (Klinowska et al., 1999). This antibody can also be used to identify cap cells, since it intracellularly stains these cells, but not luminal or myoepithelial cells (Daniel and Silberstein, 2000).

Immunohistochemistry on $+/+$ control outgrowths with laminin antibody reveals staining in the basal lamina, extracellularly, and in the cap cells, intracellularly (Figure 4D). We also observe a fraction of cells staining positive intracellularly within the prelumenal compartment, providing additional evidence that, during normal development, cap cells migrate into this region (Williams and Daniel, 1983). A majority of detached cells in $ntn1^{-/-}$ and $neo1^{-/-}$ TEBs stain positive for intracellular laminin, further confirming their identity as cap cells (Figures 4E and 4F). In many disorganized TEBs we see striking breaks in laminin staining, indicating that basal lamina has been compromised (Figures 4E and 4F). Detached cells that have escaped their boundaries, pre-

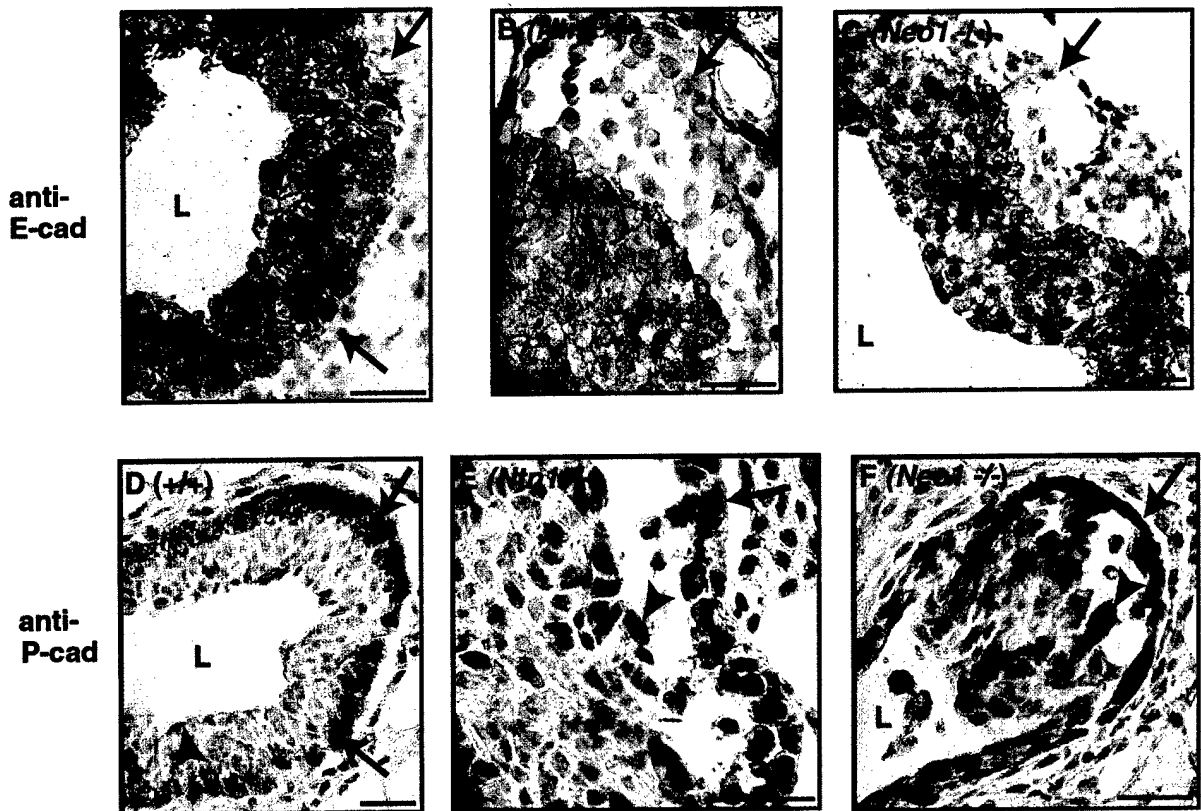


Figure 5. E- and P-Cadherin Adhesion Systems Are Intact in the Cap Cell Layer of the Disorganized TEBs

(A-C) Immunostaining with an E-cadherin antibody on TEBs from 3 week outgrowths generated by transplantation. Preluminal cells display normal E-cadherin staining in TEBs (A) +/+, (B) *ntn1* -/-, and (C) *neo1* -/-. (D-F) Immunostaining with a P-cadherin antibody on TEBs from 3 week outgrowths. Cap cells display normal P-cadherin staining in TEBs (D) +/+, (E) *ntn1* -/-, and (F) *neo1* -/-. Arrows point to cap cell layer, and arrowheads point to cap cells. L, lumen of the TEB; scale bar, 5 μ m.

sumably by migrating through breaks in basal lamina, are occasionally observed in the stroma and also stain positive for intracellular laminin.

Cadherin Systems Are Intact in the TEBs

In TEBs, cells of the cap and myoepithelial layers adhere to each other through P-cadherin, while preluminal and luminal epithelial cells adhere through E-cadherin (Daniel et al., 1995). Since we observe loose cap cells in disorganized TEBs, we examined whether the cadherin adhesion systems are intact in these TEBs. Immunohistochemistry on +/+ control outgrowths with E-cadherin antibody reveals, as expected, membrane staining only on preluminal cells (Figure 5A). Similarly, in disorganized *ntn1* -/- and *neo1* -/- TEBs, preluminal cells stain positive (Figures 5B and 5C). Cap cells in the cap cell layer, in the exaggerated subcapsular space, and in the preluminal compartment do not stain positive for E-cadherin (Figures 5B and 5C). Since 2-fold-more cap cells migrate into the preluminal compartment in both *ntn1* -/- and *neo1* -/- TEBs (Figure 6C), patches of E-cadherin-negative (cap) cells are observed (Figures 5B and 5C), but contacts between preluminal cells do not appear dramatically altered.

We performed a similar analysis using a P-cadherin antibody. This reveals normal P-cadherin staining in

cells of the intact cap cell layer in both +/+ and -/- TEBs, providing evidence that *ntn1* -/- and *neo1* -/- TEBs maintain their P-cadherin adhesion systems (Figures 5D-5F). The majority of detached cap cells also stain positive for P-cadherin, suggesting that they have not downregulated it (Figures 5E and 5F). We also observe an increased fraction of cells staining positive for P-cadherin in the preluminal cell population of *ntn1* -/- and *neo1* -/- TEBs (Figures 5E and 5F), reflecting the increased number of cap cells that migrate into this region (Figure 6C). Together, the E- and P-cadherin staining demonstrate that preluminal and cap cell layers maintain their distinct adhesion systems, even though the interaction between these two layers is compromised in *ntn1* -/- and *neo1* -/- TEBs.

A Fraction of Detached Cap Cells Die by Detachment-Induced Apoptosis

Having established that detached cells are cap cells, we next wanted to determine their destiny. Normally, cap cells exhibit a high rate of proliferation and low rate of apoptosis (Humphreys et al., 1996). However, since some cap cells in the *ntn1* -/- and *neo1* -/- TEBs are detached, they might die by detachment-induced apoptosis (anoikis), as do many nonadherent cells (Boudreau et al., 1995), or continue their downward migra-

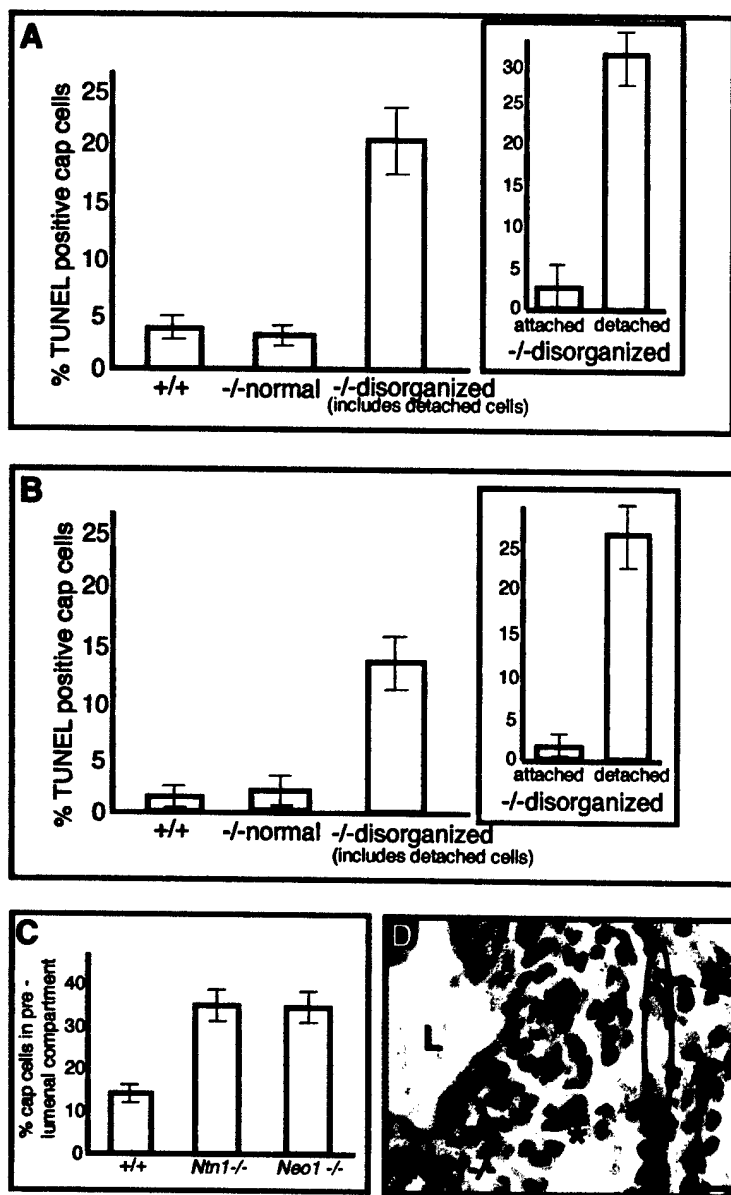


Figure 6. Cap Cells that Are Detached from the Cap Cell Layer Show an Increased Apoptotic Index Relative to Cap Cells that Are Attached to the Cap Cell Layer

(A) TUNEL on cap cell fraction of TEBs from +/+ and contralateral *ntn1* -/- outgrowths (both normal and abnormal TEBs) reveals a 5-fold increase in apoptosis in disorganized TEBs compared with normal TEBs (+/+, 3.9% ± 0.6%, n = 47; -/- normal TEBs, 2.6% ± 0.6%, n = 22; -/- abnormal TEBs (attached and detached cap cells), 20% ± 2.3%, n = 51). Inset shows TUNEL on abnormal TEBs comparing the fraction of TUNEL-positive attached versus detached cap cells (attached, 1.9% ± 3.3%, n = 31; detached, 27% ± 8.5%, n = 12).

(B) TUNEL on cap cell fraction from +/+ and contralateral *neo1* -/- outgrowths (both normal and abnormal TEBs) reveals a 7-fold increase in apoptosis in disorganized TEBs compared with normal TEBs (+/+, 1.6% ± 1.6%, n = 14; -/- normal TEBs, 2.0% ± 2.3%, n = 14; -/- abnormal TEBs, 14.5% ± 4.5%, n = 12). Inset shows TUNEL on abnormal TEBs comparing the fraction of TUNEL-positive attached versus detached cap cells (attached, 1.4% ± 2.5%, n = 10; detached, 27% ± 8.5%, n = 12).

(C) A 2.5-fold increase in number of cap cells in preluminal compartment of disorganized TEBs (+/+, 15.8% ± 0.8%, n = 19; *ntn1* -/-, 37.5 ± 1.9%, n = 25; *neo1* -/-, 37.2% ± 1.5%, n = 13).

(D) Anti-laminin staining on *neo1* -/- TEB shows break in basal lamina (circled region) and loose cells in stroma (arrows). Asterisks indicate cap cells that migrated into the preluminal compartment. L, lumen; scale bar, 5 μm.

tion. The appearance of shrunken nuclei in a fraction of these detached cells suggested that at least some detached cap cells are apoptotic. Cap cells from +/+ TEBs and morphologically normal *ntn1* -/- TEBs display a low apoptotic index (Figure 6A). In contrast, in disorganized TEBs, cap cells, both attached to the cap cell layer and detached, display an approximately 5-fold-higher apoptotic index (Figure 6A). If this increased apoptosis is due to anoikis, then the apoptotic index of detached cap cells should be significantly greater than the apoptotic index of attached cap cells. Quantitation of these indices reveals a 17-fold-greater apoptotic index in the detached cap cell fraction of disorganized -/- TEBs than in the attached cap cell fraction of the same TEBs or +/+ TEBs (Figure 6A, inset). These data strongly support the idea that detached cap cells die by anoikis, resulting in the increased overall apoptotic index observed in disorganized TEBs. Examination of the apoptotic index of cells in the preluminal

fraction of *ntn1* -/- compared to +/+ TEBs shows no statistically significant difference (data not shown). Furthermore, examination of the proliferation rate of *ntn1* -/- compared to +/+ also shows no statistically significant difference (-/-, 36.5% ± 6.7%, n = 24 TEBs, three animals; +/+, 40.8% ± 5.0%, n = 21 TEBs, three animals).

We performed a similar TUNEL analysis on the *neo1* -/- TEBs, and we find a similar low rate of apoptosis in +/+ and normal -/- TEBs (Figure 6B). Examination of disorganized -/- TEBs (attached and detached cap cells) reveals an approximately 8-fold increase in apoptosis (Figure 6B). Again, the increase in death may be due to anoikis of detached cap cells, since we observe an approximately 18-fold-greater apoptotic index in detached versus attached cap cells in these TEBs (Figure 6B, inset).

The increased cell death observed in detached cap cells of -/- outgrowths likely explains the slightly

slower growth observed in $-/-$ compared with $+/+$ outgrowths. For example, at 3 weeks posttransplantation, the average percentage of fat pad filled (%fpf) by $ntn1$ $-/-$ outgrowths is 18% less than the %fpf by contralateral $+/+$ outgrowths ($n = 5$ contralaterally transplanted animals, $p < 0.0005$). Despite the slower growth rate, $ntn1$ $-/-$ outgrowths eventually fill the fat pad and establish normal looking ductal architecture (data not shown).

Increased Influx of Cap Cells into the Preluminal Compartment

Since only 30% of detached cells die by apoptosis, we investigated whether the remaining cells migrate into the stroma when the basal lamina is compromised or into the preluminal compartment. We occasionally observe a few cap cells in the stroma ahead of the TEB (Figure 6D), but we always observe more cap cells in the preluminal compartment, whether the basal lamina is intact or compromised. We counted these cells in both $ntn1$ $-/-$ and $neo1$ $-/-$ TEBs (Figure 6C) and discovered a 2-fold increase compared with $+/+$ TEBs (Figure 6C). As a result of this influx, the preluminal compartment contains a mixed population, making it appear disorganized. The propensity of cap cells to migrate into the preluminal compartment in the absence of $ntn1$ or $neo1$ suggests that netrin-1 is not responsible for guiding individual neogenin-expressing cap cells into this compartment. Lack of a point source of netrin-1 in the TEB likely precludes a traditional long-range guidance role for netrin-1. Instead, the close apposition of receptor-expressing cap cells with ligand-expressing preluminal cells suggests a short-range role for $ntn1$ in maintaining and stabilizing the layer of $neo1$ -expressing cap cells at the leading edge.

Neogenin Mediates Netrin-1-Dependent Cell Aggregation

To determine the nature of interaction between netrin-1 and neogenin, we shifted to an in vitro cell culture system using L1 mouse fibroblast cells. L1 cells do not express netrin-1 or DCC (data not shown) but endogenously express neogenin (Figure 7A). This indicates that neogenin does not function as a homotypic cell adhesion protein in the absence of ligand, since L1 cells are well known to be nonadherent. We established that neogenin-expressing L1 cells bind netrin-1 (Figures 7B and 7C) and then performed cell aggregation assays to determine whether these cells cluster in the presence of netrin-1 protein (Albelda et al., 1991; DeLisser et al., 1993). In the absence of netrin-1, approximately 10% of L1 cells are in aggregates of greater than three cells, similar to previous observations (Albelda et al., 1991; DeLisser et al., 1993). Addition of netrin-1 leads to a 2-fold increase in aggregation (Figures 7E-7G). This aggregation is blocked in the presence of an antibody against the extracellular domain of neogenin, but not a control antibody (Figures 7E, 7H, and 7I). Thus, neogenin mediates netrin-1-dependent aggregation of L1 cells, suggesting that the short-range attractive interaction between netrin-1 and neogenin may be adhesive, rather than instructive, in nature.

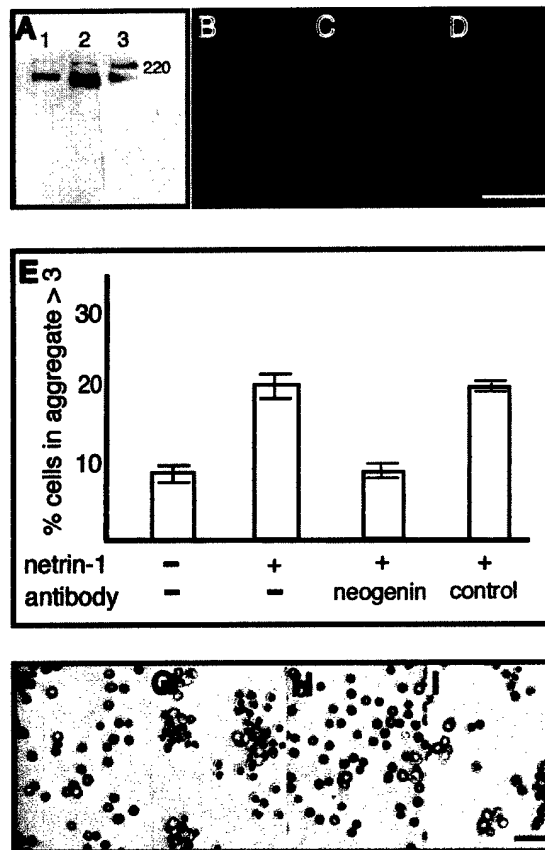


Figure 7. Neogenin Mediates Netrin-1-Dependent Cell Aggregation (A) Western blot showing neogenin expression in (1) COS cells transfected with control expression vector, (2) COS cells transfected with neogenin expression vector, and (3) L1 cells. (B) Immunostaining of neogenin expressed on L1 cells. (C) Netrin-1 binding on the same cells shown in (A) was detected with an antibody specific to netrin-1. (D) Background binding to L1 cells was detected with an antibody specific to netrin-1. (E) Quantitation showing 2-fold increase over control in the aggregation of L1 cells in the presence of netrin-1. Application of an antibody to the extracellular domain of neogenin blocks aggregation mediated by neogenin. Application of a nonspecific antibody (anti-PLAP) does not block cell aggregation mediated by neogenin. (F-I) Representative L1 cells in (F) absence of netrin-1, (G) presence of netrin-1, (H) presence of netrin-1 plus anti-neogenin, or (I) presence of netrin-1 plus anti-PLAP. Scale bar, 5 μ m.

Discussion

In the developing mammary gland, we discovered that netrin-1 is expressed by preluminal cells and that its receptor neogenin is expressed by the adjacent cap cell layer, raising the possibility that their interaction mediates local, short-range attraction between these two epithelial layers. Several lines of evidence support this hypothesis. First, netrin-1 and neogenin are expressed in close apposition, and netrin-1 appears to diffuse a short distance surrounding cap cells that do not express it (Figure 1G). Second, absence of either $ntn1$ or $neo1$ results in detached and inappropriately migrating cap cells, as may be expected when a stabilizing interaction is lost. Third, expression of *unc5h* family

members is not detected in cap cells, suggesting that neogenin functions alone as a short-range attractant receptor for netrin-1, rather than as a component of a repellent complex. Fourth, E- and P-cadherin-mediated contacts in the preluminal and cap cell layers, respectively, are maintained, even though the two layers lose their interaction. This suggests that a local mechanism, distinct from cadherin-mediated adhesion, maintains the apposition between these two layers. Finally, neogenin acts as a ligand-dependent cell adhesion protein in cell aggregation assays. Together the data support a model in which netrin-1, secreted by preluminal cells, stabilizes and maintains the close proximity of neogenin-expressing cap cells.

Netrin-1 Acts as a Short-Range Attractant through Neogenin

The TEB is the highly invasive growth structure of the developing mammary gland, and cap cells present at the leading edge direct this rapid growth. Cap cells are very motile, and the mechanism that restrains their motility is unknown. Here, we present a model where cap cell motility is kept in check by neogenin-mediated attraction of the cap cell layer to the underlying preluminal compartment. In the absence of this attractive force, there is an overall destabilization of the cap cells at the leading edge. One result of this destabilization is that cap cells pull away from the preluminal compartment, creating exaggerated subcapsular spaces. Another result is the inappropriate influx of cap cells into the preluminal compartment. Binding of netrin-1 to neogenin presumably triggers downstream signaling molecules, which ultimately lead to cytoskeletal reorganization of cap cells and their stabilization at the leading edge. Thus, in the TEB, neogenin-expressing cap cells are not guided to a netrin-1 source; rather, they are kept in place at the stromal interface and prevented from moving inappropriately.

We describe the interaction between netrin-1 and neogenin as short-range attraction because netrin-1 is a secreted ligand that binds to receptors with relatively low affinity (Keino-Masu et al., 1996; Wang et al., 1999). This description is also consistent with the short-range activity of netrin-1 in the nervous system. For example, in the optic disc, netrin-1 acts locally as a short-range attractant to ensure the proper exit of DCC-expressing retinal ganglion cell axons from the eye (Deiner et al., 1997). In the mammary gland, it seems likely that short-range attraction between netrin-1 and neogenin subserves a low-affinity adhesive, rather than a guidance, function. In this context, we propose that the interaction between them preserves the structure of the end bud and the compartmentalization of cap cells without compromising the dynamic nature of these highly proliferative, multipotent cells.

Neogenin Is Not a Netrin-1 Dependence Receptor in the TEB

DCC has been reported to induce cell death in the absence, but not the presence, of netrin-1 (Mehlen et al., 1998). This dependence on ligand to prevent constitutive cell death raises the possibility that the phenotype of the *ntn1*^{-/-} outgrowths is simply due to neogenin

acting as a dependence receptor and inducing apoptosis in cap cells in the absence of netrin-1. However, it is unlikely for the following reasons. First, all cap cells express *neo1*, but we show that the increase in apoptosis in *ntn1*^{-/-} outgrowths is due to anoikis of detached cap cells. The apoptotic index of the attached cap cell fraction is unaffected by the loss of netrin-1 (Figures 6A and 6B). Second, myoepithelial cells also express *neo1* (K.S., P.S., and L.H., unpublished data), but we do not observe any apoptosis in these cells in *ntn1*^{-/-} outgrowths. Third, our TUNEL data on *neo1*^{-/-} TEBs show a similar amount of apoptosis in *neo1*^{-/-} TEBs to that in *ntn1*^{-/-} TEBs, which is inconsistent with a dependence role for neogenin (Figures 6A and 6B). In fact, all of our analyses of the phenotype demonstrate that *ntn1*^{-/-} and *neo1*^{-/-} TEBs have very similar defects, strongly indicating that neogenin is a netrin-1 signaling receptor in the TEB.

Signaling pathways that are activated by DCC-mediated attraction to netrin-1 have been identified, and it seems likely that similar molecules would be activated by neogenin. In the nervous system, netrin-1 signaling through DCC has been shown to activate two different second messenger pathways: small GTPases (Li et al., 2002; Shekarabi and Kennedy, 2002) and MAPK (Forcet et al., 2002). Furthermore, levels of intracellular cAMP play a key role in determining the response of a growth cone to netrin-1 (Ming et al., 1997). In mammary epithelium, cAMP induces proliferation and new TEB formation (Ethier et al., 1989; Silberstein et al., 1984), and MAPK signaling is associated with cytoskeletal reorganization (Subbaramaiah et al., 2000). Furthermore, p190B, a Rho-GTPase that promotes motility by cytoskeletal reorganization, is preferentially expressed by cap cells (Chakravarty et al., 2000). Thus, molecules that are potential transducers of a neogenin signal are present in the mammary gland. Future studies will focus on whether these molecules mediate neogenin signaling in the TEB.

Physiological Relevance of Netrin-1 and Neogenin Loss in the Mammary Gland

We show that, during early mammary development, absence of *ntn1* and *neo1* removes the constraints on cap cells and increases the number of these motile cells migrating into the underlying preluminal compartment (Figure 6C). This change in population dynamics of cap cells may have long-term consequences for the mammary gland. Cap cells are multipotent progenitor cells of the TEB, and retaining this type of cell in the gland during maturation and aging may increase the risk for tumorigenesis (Russo et al., 1982). These cells may retain their mitotic potential and accumulate genetic alterations over time. Since *ntn1*^{-/-} and *neo1*^{-/-} outgrowths also display breaks in basal lamina (Figure 6D), these alterations would occur in a "primed" background, as breaks in basal lamina have been linked to cancer progression in the uterus and breast (Gudjonsson et al., 2002; Nair et al., 1997). Experiments are in progress to study whether there is an increased incidence of tumorigenesis due to loss of *ntn1* and *neo1*.

While we focused on the stabilizing function of *ntn1* and *neo1* in the TEB, both genes are expressed during pregnancy, lactation, and involution of the mammary

gland (K.S., P.S., and L.H., unpublished data). Detailed analyses of these stages may provide further insights into the biology of netrin-1 and its receptors during epithelial remodeling. Furthermore, *ntn1* and its receptors are expressed in other developing organs. For example, *neo1* is expressed in simple epithelia of the developing lung, gut, and stomach and in stem/progenitor cells of the nervous system (Gad et al., 1997). Future studies will elucidate whether netrin-1 and neogenin stabilize interepithelial interactions in other tissues during remodeling and organogenesis.

Experimental Procedures

Animals

The study conformed to guidelines set by the UCSC animal care committee (CARC). *Ntn1* severe hypomorphs and +/- and +/+ controls were generated and genotyped as described (Serafini et al., 1996). Mice carrying gene trap insertions in *neo1* were genotyped and maintained as described (Leighton et al., 2001).

Transplant Techniques

Female athymic mice (nu/nu) were obtained at 21 days of age, and both #4 fat pads were cleared for use as transplant hosts (Young, 2000). Embryonic anlage rescue and fragment transplants were done as described (Robinson et al., 2000). Reconstitution experiments were performed as described (Briskin et al., 1999; Emerman et al., 1977), with the following modifications. Donor mice were primed with a single injection of both 5 μ g Depo-Estradiol and 5 mg Depo-Provera (Upjohn) 8 days prior to use. Donor glands were mixed on the basis of wet weight prior to mincing. A total of 2.5×10^4 cells were injected into each cleared fat pad and analyzed after 18 days. Glands were stained for β -galactosidase activity prior to sectioning (Briskin et al., 1999). Numbers shown were pooled data from four different outgrowths generated in four individual mice. As a control, the contralateral sides were injected with 100% -/- cells.

Tissue Analysis

Whole-gland preparations were analyzed with hematoxylin or β -galactosidase staining as described (Briskin et al., 1999). Phenotype was characterized with 6 μ m serial sections. Fat pad filling was calculated as a relative percentage compared with +/+ control outgrowth as described (Wiesen et al., 1999). Standard deviation was reported when animals from one transplant generation were analyzed. Standard error was reported when animals from more than one transplant generation were analyzed.

Expression Studies

The following protocols were performed as described: in situ hybridization (Friedmann and Daniel, 1996); β -galactosidase staining (Briskin et al., 1999); and immunohistochemistry with anti-netrin-1, 11760 (Salminen et al., 2000), anti-smooth muscle actin (Deugnier et al., 1995), and anti-E-cadherin (ECCD-2; a gift from Dr. Takeichi) (Daniel et al., 1995). The following antibody staining used standard protocols: anti-neogenin (SCBT, #sc-15337), anti-P-cadherin (SCBT, #sc-1501), and anti-laminin (Sigma L9393). For all quantitation purposes, tissue was sectioned (6–10 μ m) and mounted serially. To ensure that cells were counted only once, we followed TEBs through serial sections and counted the number of positively stained cells in every third section.

BrdU and TUNEL

BrdU and TUNEL analyses and quantitation were performed as described (Gavrieli et al., 1992; Humphreys et al., 1996), with the following modifications. A Zymed kit was used to detect incorporation of BrdU. TUNEL-positive cells were detected with a Vectastain ABC elite HRP kit.

Netrin-1 Binding, Cell Aggregation

Netrin-1 binding assays were performed as described (Keino-Masu et al., 1996), and neogenin was visualized with a specific antibody

(SCBT, #sc-15337). Cell aggregation assays were performed as described (Albelda et al., 1991; DeLisser et al., 1993) with $7-10 \times 10^5$ cells/ml in HBSS with calcium. Three micrograms of netrin-1 protein was added, and, for antibody blocking, cells were preincubated with 3 μ g of antibody for 20 min, washed with $1 \times$ HBSS, and resuspended prior to netrin-1 addition (neogenin antibody, SCBT, #sc-15337; PLAP antibody, SCBT, #sc-15337). Aggregation was quantified in a blind manner in triplicate by examining representative aliquots (at least 10) from each sample on a hemocytometer grid. The number of particles containing cells in aggregates >3 versus the total number of particles were counted (at least 1200 cells) from four 1 millimeter squares.

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