Title: *Neuregulin-3* regulates epithelial progenitor cell positioning and specifies mammary phenotype

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Short title

Nrg3 function in embryonic mammary development

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Abstract:

Mutation of *Neuregulin-3* (*Nrg3*) results in defective embryonic mammary gland development. Here, we investigate functions of Nrg3 signalling in embryonic mammary morphogenesis. *Nrg3* regulates the distribution of epithelial progenitor cells within the presumptive mammary-forming region during early mammary morphogenesis. Basal and suprabasal epithelial cells are significantly smaller within the hypoplastic mammary primordium that forms in *Nrg3* mutants, indicative of failure to acquire mammary epithelial cell morphological phenotype. Activation of Erbb4 JM-a CYT-1, an Erbb4 isoform expressed in the developing mammary primordium, leads to mammary epithelial cell spreading and migration. Nrg3 promotes the accumulation of epithelial progenitor cells at the mammary primordium site in embryo explant cultures. Our results implicate Nrg3 signalling in mediating key events of mammary mesenchyme specification, including mesenchymal condensation, mitosis, and induction of mammary marker expression. Taken together, our results show Nrg3 has a major role in conferring specification of the mammary phenotype to both epithelial and mesenchymal progenitor cells.

INTRODUCTION

The mammary primordium (MP) consists of the primitive mammary epithelium and the adjacent mesenchyme [1]. Together these two tissues will direct the formation of the mammary gland during embryogenesis through a series of reciprocal interactions in which juxtacrine and paracrine signals are transmitted to mediate morphogenetic processes [2]. The mammary mesenchyme has inductive properties such that it can confer mammary fate to undifferentiated simple epithelium in tissue recombination experiments [3]. Based on these experiments, it is thought that during initial stages of embryonic mammary development, signals from the underlying mesenchyme provide an instructive signal to the overlying epithelium. The epithelium responds to the mesenchymal signal(s) by forming elliptically-shaped aggregates of epithelial cells which first appear on the ventral flanks of mouse embryos during embryonic day (E) E11 and five pairs of MP are morphologically distinct by E12 [4]. Several genetic pathways have been identified that are required during early embryonic MP development including *Eda*, *Fgfs*, *Gli3*, *Nrg3*, *Pthrp*, and *Wnt* signalling components [4,5]. Mechanistic insights into the precise cellular processes regulated by these genetic pathways are fairly limited and the genetic hierarchies among these key regulators remain to be completely clarified.

The initial stages of mammary primordium 3 (MP3) formation fail to occur in *Nrg3*^{ska}, hypomorphic mutants that express reduced *Nrg3* levels. Our previous studies of *Nrg3*^{ska} mutants identified *Nrg3* as a key mediator of mammary primordial specification, linking Nrg3 to the establishment of the mammary lineage during embryogenesis [6,7]. *Nrg3* is expressed in the dermal mesenchyme underling the site where MP will subsequently form between E10.5-E11.25 [8]. When MP3 becomes morphologically distinct at ~E11.5, *Nrg3* expression is found within the epithelium and is no longer expressed in the underlying mesenchyme [8]. Expression of *Nrg3* throughout the epidermal progenitor cell compartment in K14-*Nrg3* mice led to formation of supernumerary mammary gland formation, confirming a role for Nrg3 as a promoter of mammary cell fate during embryogenesis [7,9]. Dramatic alterations were also observed in postnatal K14-*Nrg3* epidermis skin including changes in expression pattern of Tenascin C, an extracellular matrix protein of stem cell niches [9]. The receptor for Tenascin C, integrin β1, as well as integrin α6, a marker for epithelial progenitor cells, also showed perturbed expression in K14-*Nrg3* epidermis, further suggesting a link

between Nrg3 signalling and progenitor cell regulation [9]. The cognate receptor of Nrg3, Erbb4 is expressed in dermal mesenchymal tissues of the mammary-forming region between E10.5-E11.0, before becoming strongly expressed in the surface epithelium of the site where the MP forms and remains expressed in the epithelium once MP3 has formed at E11.5 [8]. Erbb4 is alternatively spliced and exists as four major isoforms: JM-a CYT-1, JM-a CYT-2, JM-b CYT-1 and JM-b CYT-2 [10]. The Erbb4 isoforms regulate distinct aspects of postnatal mammary epithelial cell (MEC) and breast cancer behaviour including mammary cell differentiation, migration, proliferation and apoptosis [11-13]. In fact, chemoattractant properties of Nrg3 and another Neuregulin member, Neuregulin-1 (Nrg1) have been demonstrated: both chemoattract interneuronal cells, a type of neural progenitor cell that expresses Erbb4 in the developing cerebral cortex/forebrain [14]. Ligands for the Erbb receptor tyrosine kinases, the Neuregulins, are compelling candidates to mediate early mammary morphogenesis due to their expression patterns in relevant MP tissues [8].

Until recently, a lack of suitable reporter models existed for monitoring the early events occurring during MP formation. As a result, much remains to be understood regarding the cellular and molecular mechanisms by which the initial MP is developed from the simple epithelium. It has been clearly established that a very low level of cell proliferation is detected within the mammary primordial epithelium during the initial stages of organ formation from E11 through E14 stages [15,16]. Previous studies have concluded that early mammary morphogenesis is mediated primarily via localised cell movements [15,17]. Cell hypertrophy is also thought to contribute to increase in MP size [16]. It is not yet clear which signals mobilise the epithelial progenitors that are thought to locally migrate from along and near the mammary line to form the MP [1].

MP formation and initial organ growth appear to be elicited primarily through epithelial cell migration. We hypothesised that Nrg3 might facilitate epithelial progenitor movements in the presumptive mammary-forming region as the mammary primordium forms. We aimed to study the effects of Nrg3 and Erbb4 signalling on morphogenesis of embryonic mammary epithelium and mesenchyme, as well as cell migration and spreading of MECs. Here we show that Nrg3 has a major role in progenitor cell positioning and conferring specification of the mammary phenotype to both epithelial and mesenchymal progenitor cells.

MATERIALS AND METHODS

Sample collection

All animal work was carried out under UK Home Office project and personal licenses following local ethical approval and in accordance with local and national guidelines. *TOPGAL* mice (stock no. 4623) were purchased from the Jackson Laboratories. *s-SHIP-GFP* mice were kindly provided by the late Professor Larry Rohrschneider (Fred Hutchinson Cancer Research Center, Seattle, WA, USA).

Expression analyses

Immunohistochemistry (IHC) and whole-mount immunofluorescence (WM-IF) of MP were performed as described [9,18]. Antibodies used for immunostaining were: GFP (clone FM264G) from BioLegend UK (London, UK); phospho-Ser10-Histone H3 (clone 3H10) from Millipore (Hertfordshire, UK); ERα from Santa Cruz Biotechnology (Dallas, TX, USA); Lef1 (clone C12A5) and Cleaved Caspase-3 (Asp175) from Cell Signaling Technologies (Danvers, MA, USA). Antibody-labelled tissues were three-dimensionally analysed using Leica TCS SP II confocal scanning microscope as described [18]. Mitotic cells within control and $Nrg3^{ska}$ MP3 were scored by manually counting phospho-Histone positive cells through confocal images of MP3 made every 1.5 µm. X-gal staining was performed as described [6]. Quantitative real-time RT-PCR (qRT-PCR) was performed on freshly extracted total RNA from total MP at E12.5, the microdissected components of the MP (ME, MM) and separated epidermis and mesenchyme. qRT-PCR was performed using the following Taqman Gene Expression Assay probes from Life Technologies (Paisley, UK): *Actb* (Mm00607939_s1), *Esr1* (Mm00433149_m1), *Ar* (Mm00442688_m1), *Pth1r* (Mm00441046_m1) and *Wnt10b* (Mm00442104_m1). Results were analysed with the comparative Δ-ΔCt method normalised to Actb value and compared to comparator in triplicate. RT-PCR detecting Erbb4 isoforms *Erbb4 JM-a/b* and *CYT-1/2* were performed as described [9].

Morphological analysis of MP

GFP pictures of *s-SHIP-GFP*-expressing embryos at E11.5 and E12.5 were obtained on EVOS FL Color Imaging System from Life Technologies (Paisley, UK) using either x2 or x4 objectives. GFP

pictures were represented as black-and-white images to highlight where ME progenitor cells expressing *s-SHIP-GFP* accumulated on the embryo's surface. Briefly, brightness/contrast and HDR toning in the raw images were adjusted using Adobe Photoshop CS5, and threshold was adjusted to display GFP-positive region of interest as black dots on a white background. Arrangement of MP along the ML was further assessed by measuring the angle of the line connecting MP1 to MP3 and MP3 to MP5 using ImageJ. To examine mammary cell morphology, cell borders were visualised using CellMask Orange Plasma Membrane Stain from Life Technologies (Paisley, UK). Briefly, WM-IF was performed on microdissected MP3 with a modified permeabilisation step using 1 mg/ml digitonin in PBS since detergents and methanol in general method are not compatible for the dye. The stained tissue was photographed three-dimensionally every 1.5 μm slice, using a confocal microscope equipped with x40 objective. To measure three-dimensional sphericity and volume of basal and suprabasal MEC, individual cell periphery was manually traced using plasma membrane stained images through each slice using the Contour Surface tool in Imaris software from Bitplane (Zurich, Switzerland).

Recombinant proteins

Mutagenised Nrg3, referred to as Nrg3mut is described in Rakic *et al*. [14]. Recombinant protein rNrg3-EGF wt and rNrg3-EGF mut were prepared in S2 Schneider cells as previously described [6]. Nrg1β-EGF (396-HB-050) and Wnt3a (1324-WN-010) were purchased from R&D systems (Minneapolis, MN, USA).

***Ex vivo* culture of embryonic flanks**

Embryonic flanks from forelimb to hindlimb were carefully bisected from freshly isolated E11.0 *Nrg3^{ska} s-SHIP-GFP* embryo in D-PBS (Life Technologies) taking care not to damage the epidermis around the presumptive mammary region. By monitoring GFP under fluorescent stereomicroscope, only intact *Nrg3^{ska}* flanks at a stage in which the MP had not formed yet were used for experiments. Flanks were placed in humidified centre-well organ culture dishes, Becton Dickinson, (Oxford, UK) equipped with polycarbonate filter membrane (P9699-100EA, Sigma) and culture medium at 37ºC

with 5% CO_2. Freshly reconstituted EmbryoMax KSOM Embryo Culture media, Merck Millipore (Billerica, MA, USA) containing 75 µg/ml ascorbic acid (Sigma) was used. 2 µg/ml rNrg3-EGF or PBS was added to the media. After 24 hr incubation, the explants were evaluated for MP3 formation by GFP appearance and were fixed in 4% PFA in PBS and then subject to phospho-Histone staining which was used to score the number of mitotic cells in the mammary epithelial and mesenchymal tissues.

Cell culture and transfection

HC11 cells were maintained with RPMI1640 (Life Technologies) supplemented with 10% serum, 1% Penicillin/Streptomycin, 5 µg/ml Insulin (19278, Sigma), and 10 ng/ml human recombinant EGF (236-EG-200, R&D systems). To develop stable cell lines expressing isoform-specific Erbb4, 10 µg of human Erbb4 JM-a CYT-1 and JM-a CYT-2 cDNAs from expression constructs cloned in pcDNA3.1 were introduced using Amaxa Nucleofector II device and Cell Line Nucleofector kit V with a program T-024 (Lonza, Basel, Switzerland). After nucleofection, cells were maintained with media containing 1 mg/ml G418 (Life Technologies). After 2 weeks, a number of surviving colonies were tested for Erbb4 overexpression. Two independent clones were used for each isoform in this study: JM-a CYT-1 isoform, clone 5 and 6; JM-a CYT-2 isoform, clone 3 and 43.

***In vitro* chemotactic assay**

Polycarbonate Membrane Transwell inserts (8 µm pore size, 24-well format) were coated with 50 µg/ml rat tail Collagen type I (Becton Dickinson). HC11 cells expressing Erbb4 JM-a CYT-1 and JM-a CYT-2 were treated with serum-free medium for 72 hr prior to the transwell migration assay. The starved cells were harvested using HyClone HyQtase cell detachment reagent from Thermo Scientific (Waltham, MA, USA) and resuspended to 4 x 10^5 cells/ml with RPMI1640 supplemented with 1% serum, 1% BSA and 20 mM HEPES. 200 µl of cell suspension was added onto the Transwell inserts. The inserts were then transferred to 24-well plates filled with medium containing PBS, 2.0 µg/ml rNrg3-EGF wt, 1.0 µg/ml Nrg1β-EGF, or 0.01 µg/ml EGF. After 6 hr incubation, cells on the membrane insert were fixed with 4% PFA in PBS followed by DAPI staining. The transwell

migration was scored by counting the number of DAPI-labelled cells on the membrane at the bottom side.

Cell adhesion staining and spreading assays

0.5 x 10^5 HC11 cells expressing Erbb4 JM-a CYT-1 or JM-a CYT-2 per well were placed on fibronectin-coated 12-well plate in culture medium overnight. Cells were incubated with serum-free medium prior to stimulation with growth factor. After 72 hr starvation, cells were treated for 15 min with serum-free medium containing PBS, 0.5 µg/ml rNrg3-EGF wt or mut, 0.2 µg/ml Nrg1β-EGF, and 0.05 µg/ml Wnt3a. Cells were immediately fixed with 4% PFA in PBS followed by staining for phospho-Tyr397-Focal adhesion kinase (p-FAK) from Cell Signaling Technologies, Alexa555-Phalloidin and DAPI from Life Technologies. p-FAK-labelled cell adhesions were photographed using EVOS FL microscope equipped with x40 objective. Actin-based cell cytoskeleton and DAPI-labelled nuclei were photographed using x10 objective. Segmented shape of cell and nucleus was abstracted and automatically measured using CellProfiler cell image analysis software (http://www.cellprofiler.org, Broad Institute, Cambridge, MA, USA). 5-95% percentile box and whiskers plots were generated using median, quartiles, highest and lowest values.

Statistical analysis

The data in the graphs are presented as mean and the standard error of the mean (SEM). The data were analyzed by two-tailed ANOVA or Student's t-test using GraphPad Prism 6 software. P-value<0.0001 is considered as extremely significant (****), p-value=0.0001 to 0.001 as highly significant (***), p-value=0.001 to 0.01 as significant (**), and ≥0.05 as not significant (ns), respectively.

RESULTS

***Nrg3^{ska}* mutants display aberrant distribution of epithelial progenitor cells along the presumptive mammary-forming region during early mammary gland morphogenesis**

We previously found that 55% of *Nrg3^{ska}* hypomorphic mutants fail to form mammary gland 3 when mice were assessed for the presence or absence of nipples 7-10 days after birth [19]. We

backcrossed *Nrg3*^{ska} hypomorphic mutants and controls (C57Bl6/J) onto the *s-SHIP-GFP* reporter background, which expresses GFP in a subset of epithelial-lineage progenitor cells during embryonic development, including those present in the mammary-forming region [20]. We were then able to easily examine the formation of the MP between E10.5 and E12.5 since small aggregates of *s-SHIP-GFP*+ cells are readily apparent at, and in the vicinity of, the sites where the MP form in controls (Fig. 1a,b). *s-SHIP*+ epithelial progenitor cells appear less focally distributed along the mammary-forming region of *Nrg3*^{ska} hypomorphic mutants; more GFP+ cells remain on the dorsal-most aspect of the ventral flank when compared to control embryos in which a much denser ventral accumulation of GFP+ cells is observed at E11.5 (Fig. 1a). GFP+ cells observed along the mammary line fragment that corresponds to the site of MP3 formation appear to be shifted dorsally in *Nrg3*^{ska} compared to controls (Fig. 1a,b). These observations are further supported when changes in MP3 location were assessed by measuring the angles of the lines that connect MP1 to MP3, and MP3 to MP5 and compared them to those from control embryos and found this is significantly reduced in *Nrg3*^{ska} embryos (Fig. 1c).

Fewer *s-SHIP-GFP*+ cells are observed within the nascent *Nrg3*^{ska} MP3 when compared to control embryos at E12.5 (Fig. 1b). We restricted phenotypic analysis to MP3 since MP3 exhibits the most striking morphological defects in *Nrg3*^{ska} embryos. We found when E12.5-stage embryos are assessed for *s-SHIP-GFP*+ cell accumulation at the presumptive site of MP3, that 9% of *Nrg3*^{ska} mutants fail to show any *s-SHIP-GFP*+ cell accumulation and were scored as aplastic; 37% form hypoplastic MP3 with substantial reduction in accumulation of *s-SHIP-GFP*+ cells and are greatly reduced in size, and the remaining 57% form a relatively-normal sized organ and were scored as MP3+ (Table S1). This variable phenotype permits analysis of the consequence of reduced *Nrg3* signalling in the epithelial progenitor cells of MP3 scored as either hypoplastic or MP3+ and the mesenchymal cells associated with them.

The *s-SHIP-GFP* reporter shows a similar expression pattern when compared to the *TOPGAL-F* reporter mouse [21] in both control and *Nrg3*^{ska} mutants between E10.5 and E12.5 (Fig. S1a). In *Nrg3*^{ska} mutants, fewer *s-SHIP-GFP*+ and TOPGAL+ cells accumulate along the mammary line fragment that spans the site of mammary MP3 formation. When profiled by qRT-PCR, *Wnt10b*, a

mammary line marker [22], is reduced in the epithelium isolated from hypoplastic *Nrg3*^{ska} MP3 compared to control MP3 (Fig. S1b). *Wnt10b* expression is higher in the surface epithelium of the epidermis from the region dorsal to MP3 of *Nrg3*^{ska} embryos compared to control embryos, which is consistent with *Wnt10b*-positive epithelial progenitor cells remaining dispersed in the mammary-forming region of *Nrg3*^{ska} embryos near MP3. These results suggest Nrg3 regulates *s-SHIP*+ and TOPGAL+ progenitor cells recruitment to form MP3.

Maturation to achieve mammary epithelial cell phenotype fails in hypoplastic MP3 that form in *Nrg3*^{ska} mutants

We used plasma membrane staining to assess epithelial cell morphology (Fig. 2a). *Nrg3*^{ska} hypoplastic MP3 populations are composed of smaller epithelial cells when compared to control cells, which occupy larger volumes, but no significant change in cell sphericity is detected (Fig. 2b). Basally located cells show striking reductions in volume and suprabasal cells also show a significant reduction in cell volume in *Nrg3*^{ska} MP3 when compared to controls. Changes in sphericity are detected between basal and suprabasal cells, suggesting that morphological differentiation of the MECs is initiated, but does not mature to a full mammary phenotype, in *Nrg3*^{ska} MP3. We observed no changes in the number of apoptotic cells in hypoplastic *Nrg3*^{ska} and control MP3 epithelium when assessed by cleaved caspase-3 labelling (Fig. S2). Nuclear staining shows that epithelial multilayering is observed in MP3 of *Nrg3*^{ska} hypomorphic mutants (Fig. 2). We found no significant change in the number of mitotic cells observed in hypoplastic *Nrg3*^{ska} and control MP3 epithelium as assessed by phospho-Histone H3 staining (Fig. 3). It is notable that the surface epithelium has similar mitotic rate in *Nrg3*^{ska} hypomorphic mutants and control embryos (data not shown). Results from these morphometric analyses are consistent with our confocal and histological observations of the morphology of the cells present at the site of hypoplastic *Nrg3*^{ska} MP3 (Fig. 2, Figs S1, S2) and indicate Nrg3 mediates transitioning to the mammary epithelial phenotype.

***Nrg3*^{ska} mutant MP3 display defective specification of mammary mesenchymal cells**

Morphological analyses indicated that defects are also present in mesenchymal cells associated with *Nrg3*^{ska} MP3. The concentric rings of mesenchymal cells aligned around control MP3 epithelial cells start to appear at E12.5 but are not observed in mesenchymal cells adjacent to *Nrg3*^{ska} MP3 and these cells do not appear to condense (Figs 2-4). Lef1 stains both epithelial and mesenchymal MP3 control cells at E12.5 (Fig. 3c). We found that although *Nrg3*^{ska} MP3 are comprised of fewer epithelial cells compared to control MP3, Lef1 was expressed in *Nrg3*^{ska} MP3 epithelial cells. Lef1 expression was observed in mesenchymal cells adjacent to control MP3, but not in the mesenchymal cells associated with *Nrg3*^{ska} MP3. We also stained MP3 for expression of other mammary mesenchymal markers, including ERα (Fig. 4a). We detected a reduced number of ERα+ mesenchymal cells surrounding *Nrg3*^{ska} MP3 and lack of their condensation, even in those that are a similar size as control MP3 (denoted MP3+); both are indicative of failed specification of mammary mesenchymal cells (Fig. 4a). Fewer mitotic cells are detected in *Nrg3*^{ska} mesenchymal cells associated with MP3 at E12.5 when compared to control (Fig. 3a,b). To ascertain if *Nrg3* is required for the Pthrp/Pth1r-mediated specification of mesenchymal cells to a mammary phenotype to occur, we assayed levels of *Pthrp* and *Pth1r,* as wells as mammary mesenchymal markers *ERα* and *Ar*, in RNA isolated from E12.5 mammary primordial tissues. Using qRT-PCR, we found similar levels of *Pthrp* in epithelial cells from *Nrg3*^{ska} and control MP3 (data not shown). Mesenchymal cells surrounding the epithelium from aplastic and hypoplastic *Nrg3*^{ska} MP3, expressed the transcripts of *ERα, Ar,* and *Pth1r* at significantly reduced levels compared to those from control MP3 and at levels more similar to those found in dermal mesenchymal cells and indicate failed mammary mesenchymal specification **(**Fig. 4b).

Rescue of *Nrg3*^{ska} MP3 cultured with Nrg3 is not achieved through increasing epithelial mitotic activity

When using conventional protocols that include serum-containing media for *ex vivo* culture of embryonic flanks, we observed a significant amount of epithelial proliferation within MP forming in culture (data not shown). We therefore optimised an *ex vivo* culture system using serum-free media so that it would mimic *in vivo* development where very little epithelial proliferation contributes to early

stages of MP development (Fig. 5a). *Nrg3*^{ska};*s-SHIP-GFP* ventral flanks were explanted at E11.0, prior to MP3 formation. Following 24 hours in culture *ex vivo* using these serum-free conditions, we assessed each *Nrg3*^{ska};*s-SHIP-GFP* ventral flank for the accumulation of *s-SHIP-GFP*+ cells at the site of MP3. We found that, 50% of the *Nrg3*^{ska};*s-SHIP-GFP* flanks formed MP3, whilst the remaining 50% were scored as having aplastic MP3. We found that when rNrg3-EGF was added to the culture media, after 24 hours in culture *ex vivo*, 87% of the explanted *Nrg3*^{ska};*s-SHIP-GFP* flanks formed MP3 and only 13% were scored as having aplastic MP3**.** No difference in the number of mitotic cells was observed in *Nrg3*^{ska};*s-SHIP-GFP* MP3 epithelial cells whether culture media contained rNrg3-EGF or not (Fig. 5b). In contrast, a significant increase in the number of mitotic cells was observed in the mesenchymal cells adjacent to MP3 epithelium when explants were cultured in media containing rNrg3-EGF compared to control media (Fig. 5b).

MP selectively express cleavable Erbb4 JM-a Isoforms

To further investigate the role of Nrg3 in facilitating the accumulation of epithelial progenitor cells at the site of MP3, we profiled the *Erbb4* isoforms expressed during early embryonic mammary gland development by RT-PCR. We found that only the cleavable *JM-a* isoform is expressed and no *JM-b* isoform is detected in mid-gestation MP (Fig. 6a). Both *CYT-1* and *CYT-2* isoforms are expressed (Fig. 6a).

MECs expressing Erbb4 JM-a CYT-1 isoform are more motile than cells expressing JM-a CYT-2 isoform

We transfected normal mouse mammary epithelial (HC11) cells with either the *JM-a CYT-1* or *JM-a CYT-2* isoform of ERBB4 to create stable cell lines expressing the isoforms relevant to embryonic mammary development. Using transwell assays, we found that cells expressing JM-a CYT-1 were significantly more migratory than either HC11 cells expressing JM-a CYT-2 isoform, which exhibited minimal migratory ability that is comparable to levels detected with untransfected HC11 cells (Fig. 6b). Unstimulated HC11 cells expressing JM-a CYT-1 express activated full-length (180 kD) and cleaved (80 kD) forms of Erbb4 and activated Erbb2 (Fig. S3); these cells were slightly

more migratory when treated with either rNrg1β-EGF or rNrg3-EGF, ligands for Erbb4 (Fig. 6b). The effects of stimulating cells with rNrg3-EGF wt and rNrg3-EGF mut were evaluated by the levels of Erbb4 as well as Erbb2 phosphorylation as shown in Fig. S4. No increase in canonical Wnt signals was observed when HC11 cells expressing either JM-a CYT-1 or JM-a CYT-2 isoform were stimulated with rNrg3-EGF in TOPflash assays (Fig. S5). These results indicate that Nrg3 does not increase canonical Wnt signals in MECs expressing either JM-a CYT-1 or JM-a CYT-2 isoform of Erbb4.

Nrg3 promotes spreading of MECs expressing Erbb4 JM-a CYT-1 isoform

To investigate the ability of Nrg3 to modulate MEC morphology, HC11 cells expressing either the JM-a CYT-1 or JM-a CYT-2 Erbb4 isoform were stimulated with rNrg3-EGF. Mammary cells expressing JM-a CYT-2 isoform showed no cell spreading in response to Nrg3 stimulation (data not shown). Mammary cells expressing JM-a CYT-1 isoform responded to both rNrg3-EGF and rNrg1β-EGF stimulation with a modest and significant amount of cell spreading (Fig. 7a-b). Cells expressing JM-a CYT-1 isoform treated with Wnt3A showed no cell spreading response, suggesting that growth factor-mediated cell spreading is specific for Erbb4-ligands in this cell type.

MECs expressing Erbb4 JM-a CYT-1 isoform form focal adhesions after stimulation with rNrg3-EGF

Under non-stimulating conditions with rNrg3-EGF mut, JM-a CYT-1 expressing HC11 cells have sparse distribution of p-FAK rather than the large focal adhesions at actin bundles that are observed in HC11 cells and HC11 cells expressing JM-a CYT-2 (Fig. 7c). HC11 cells and HC11 cells expressing JM-a CYT-2 show no obvious response to stimulation with rNrg3-EGF wt, whereas large focal adhesions are formed in JM-a CYT-1 expressing HC11 cells after stimulation with rNrg3-EGF wt (Fig. 7c). HC11 cells expressing JM-a CYT-1 isoform show a discernible change in cell morphology after stimulation with rNrg3-EGF (Fig. 7c.)

DISCUSSION

Alterations in epithelial progenitor cell distributions in *Nrg3* hypomorphic mutants suggest that Nrg3 signals influence the behaviour of epithelial progenitor cell populations relevant to embryonic mammary development from E10.5 through E12.5. The failure of a signal that normally leads to *s-SHIP-GFP* progenitor cell aggregation along the mammary-forming region would explain the striking phenotypes observed uniquely with MP3 in $Nrg3^{ska}$ mice [1]. The results presented here demonstrate the power of using the *s-SHIP-GFP* reporter model which permits analyses of epithelial progenitor cells through sequential developmental stages *in situ* using *ex vivo* embryonic cultures, which had not been previously possible for studies of embryonic mammary morphogenesis. Results from our embryo explant cultures in the *s-SHIP-GFP* reporter background strongly suggest Nrg3 promotes epithelial progenitor aggregation as MP3 initially forms. We also demonstrate that Nrg3 regulates epithelial progenitor accretion without increasing epithelial mitosis. The limited epithelial proliferation detected cannot account for the observed increases in mammary primordial size that occur between E10.5-E13.5 and that influx of cells from the adjacent surface epithelium accounts for the increases in epithelial cell numbers that accrue within the nascent organ [15-17]. Our results indicate Nrg3 signals mediate the distribution of epithelial progenitor cells along the ventral thoracic flank at the time that they are thought to be locally migrating to form the MP. A number of transcription factors including *Gata3* and *Tbx3* are required for the formation of all five pairs of MP, whilst others, such as *Gli3* and *Lef1*, are only required for formation of certain MP, including MP3 [23-27]. It is not yet clear precisely how the factors that regulate development of MP3 control epithelial progenitor cell distributions in the mammary-forming region, how these factors interact or whether any directly induce *Nrg3* expression.

Using 3-D morphometric analyses of MP3, we have quantified hypertophy that occurs in the epithelial progenitor cells within MP3 during maturation of the embryonic mammary epithelium. Our results indicate that $Nrg3^{ska}$ MP3 cells achieve transition from cuboidal to columnar morphology, which normally occurs as the epithelium stratifies and during embryonic mammary development [25]. Further enlargement in size to acquire a mammary cell phenotype fails in $Nrg3^{ska}$ MP3 cells. Defective epithelial hypertrophy is observed in $Nrg3^{ska}$ MP3 and indicates Nrg3 mediates

transitioning to the mammary epithelial phenotype. Insufficient *Nrg3* levels leads to formation of abnormal MP3 comprised of small aggregates of epithelial progenitor cells that have not fully differentiated to the mammary phenotype. Cell-based studies indicate Nrg3 promotes cell spreading and an ability to modulate cell morphology. Our results are consistent with a potential role for Nrg3 in promoting cell hypertrophy and contributing to initial organ growth via increase in cell area through cell spreading. However, it is not yet clear how Nrg3 elicits the columnar hypertrophy observed *in vivo*.

Subsequent mammary mesenchymal specification is incomplete in $Nrg3^{ska}$ MP3 as assessed by reduced mammary mesenchymal marker expression. This finding indicates that Nrg3-mediated mammary specification of epithelial progenitors cells also activates downstream effectors required for the transmission of signals from the mammary epithelial to adjacent mesenchymal cells. We also show that Nrg3 signalling increases proliferative potential of mammary mesenchymal precursor cells. Nrg3-mediated specification of the mammary epithelium is required for proliferation of the presumptive mammary mesenchymal precursor cells and their specification to a mammary mesenchymal phenotype to occur.

Threshold levels of Nrg3 are likely to be required to form MP3 with fully specified mammary epithelial tissue capable of inducing *Pth1r* expression in the adjacent mesenchyme by some unknown mechanism. Sufficient *Pth1r* levels are required so stimulation of Pth1r by Pthrp leads to induction of mammary mesenchyme marker expression, including AR and ERα [28], which show reduced expression in $Nrg3^{ska}$ MP3. The Pthrp signalling axis is a master regulator of mammary mesenchymal specification which it mediates by activating canonical Wnt signaling in mesenchymal cells [28,29]. Similarities exist between $Nrg3^{ska}$ and *Pthrp*-deficient MP with respect to their reduction in mammary mesenchymal marker expression and reduced proliferation of mammary mesenchymal cells. The mammary mesenchymal condensation defect observed in $Nrg3^{ska}$ MP3 distinguishes its mesenchymal phenotype from that of *Pthrp* and *Pth1r*-deficient MP, which appear to undergo mesenchymal condensation [30]. We did not observe induction of *Pth1r* expression when mesenchymal tissues from

Nrg3^{ska} were cultured and treated with rNrg3 (data not shown) so Nrg3 does not appear to directly induce expression of *Pth1r*.

Cell-based assays show that Nrg3 does not increase canonical Wnt signals in MECs expressing either JM-a CYT-1 or JM-a CYT-2 Erbb4 isoforms. Changes in expression of TOPGAL+ cells observed within the mammary-forming region of *Nrg3*^{ska} mice are unlikely to be due to Nrg3 inducing canonical Wnt expression, but are consistent with Nrg3 stimulating migration and local aggregation of TOPGAL+ precursor populations at the site of MP3 but this remains to be formally demonstrated.

MECs expressing the Erbb4 receptor of JM-a CYT-1 and JM-a CYT-2 show that these isoforms elicit distinct effects. CYT-1 and CYT-2, the cytoplasmic variants differ by the presence (CYT-1) or absence (CYT-2) of a short sequence containing a putative binding site for phosphatidylinositol (PI) 3-kinase p85 subunit [31]. In our studies, cells stably transfected with full-length receptor for the JM-a CYT-1 isoform were significantly more migratory than those expressing JM-a CYT-2 isoform. Directional cell migration is regulated by assembly and turnover of integrin-mediated adhesions [32]. Mammalian cells attach to surfaces through focal adhesions, cellular structures characterised by complexes of the transmembrane protein integrin and intracellular proteins including paxillin and focal adhesion kinase [33]. Since Erbb4 JM-a CYT-1 expression enhances migration ability in HC11 cells, we examined the effect of JM-a CYT-1 expression on cell adhesion formation, which was assessed with immunostaining with phospho-focal adhesion kinase (pFAK), a regulator of focal adhesion turnover. Under non-stimulating conditions, JM-a CYT-1 expressing HC11 cells display sparse distribution of p-FAK, rather than the large focal adhesions at actin bundles observed in HC11 cells and HC11 cells expressing JM-a CYT-2 (Fig. 7a). This data is in agreement with the observation that JM-a CYT-1 expressing cells are motile since migrating cells develop small adhesions rather than stabilised focal adhesions. MECs expressing JM-a CYT-1 isoform exhibit distinct responses to Nrg3 stimulation by forming focal adhesions and spreading, when compared to MECs expressing JM-a CYT-2 isoform, which show negligible changes in focal contacts and cell morphology after Nrg3 stimulation. These results suggest that Nrg3 stimulation affects cell adhesion dynamics and morphology, specifically for MEC cells expressing the JM-a CYT-1 isoform.

Divergent cellular responses by Erbb4 isoforms in MECs have previously been reported. Another study using inducible mouse models to express CYT-1 and CYT-2 isoforms in postnatal MECs showed that the ICD of these two isoforms exert opposing effects on the postnatal mammary epithelium when transgenically-expressed *in vivo* [12]. MECs from transgenic mice induced to express CYT-1 isoform were found to have decreased cell growth, increased lumen formation in 3-D culture, increased Stat5a activation, and precocious lactogenic differentiation when compared to non-induced transgenic control postnatal mammary epithelium. Conversely, MECs from mice expressing transgenic CYT-2 isoform showed increased proliferation and no lumen formation in 3-D cultures compared to controls. The effect of expressing these isoforms on MEC migration was not explored in this study so it is not clear whether CYT-1 isoform has the ability to mediate migration of both embryonic and postnatal MECs.

To conclude, we present evidence that Nrg3 promotes changes in cell adhesion dynamics and morphology of MECs and have identified a role for *Nrg3* in promoting the maturation of the primitive mammary epithelium until it is fully specified to a mammary cell fate. Nrg3 is likely to promote mammary epithelial aggregation through stimulation of the JM-a CYT-1 isoform of Erbb4. We propose a working model (Fig. 8) for the formation of MP3 in which Nrg3/Erbb4 signalling facilitates progressively maturation of epithelial progenitor cells until they achieve a fully specified mammary epithelial phenotype. This is achieved by Nrg3 promoting cell spreading using autocrine signals, which leads to cell hypertrophy, an event that coincides with maturation to acquire the mammary cell phenotype. Nrg3-mediated signals are needed to regulate condensation of mammary mesenchymal precursor cells, presumably by stimulating signal(s), which remain to be identified. Mesenchymal condensation may induce Pth1r expression in mammary mesenchyme precursors, which are then activated and undergo mammary mesenchymal specification. Mammary specification coincides with expression of mammary mesenchymal markers and cell population expansion through increased cell mitosis. In summary, we show Nrg3 signalling is required for both epithelial and mesenchymal mammary specification of embryonic progenitor cells.

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FIGURE LEGENDS

Fig. 1. *Nrg3*^{ska} mutants display aberrant distribution of mammary epithelial progenitor cells during early mammary primordial morphogenesis.

s-SHIP-GFP-expressing cells are shown visualised in whole-embryos. GFP-positive cells relevant to embryonic mammary gland development are indicated in black in the digitised images shown below the GFP images. The mammary line fragments and dorsal line are also visible with *s-SHIP-GFP*+ cells and indicated by red and green dotted lines, respectively..

a. At E11.5, *s-SHIP-GFP*+ cells in the vicinity of the level of ~somite 16/17 indicate the site where MP3 has formed in control (*s-SHIP-GFP*+; C57BL/6J) embryos. Compared to the control, alterations in distributions of *s-SHIP-GFP*+ epithelial progenitor cells at the site MP3 forms and along the thoracic mammary line fragment in *Nrg3*^{ska} embryos with hypoplastic MP3 and aplastic MP3 are displayed.

b. At E12.5, *s-SHIP-GFP*+ cells indicate the sites where the 5 MP have formed in control embryos. The distribution of *s-SHIP-GFP*+ cells in *Nrg3*^{ska} embryos typically observed with hypoplastic MP3 and aplastic MP3 are shown (Note that MP3 position in *Nrg3*^{ska} is dorsalised compared to the control).

c. Angle between MP1-3 and MP3-5 were measured in control and *Nrg3*^{ska} embryos at E12.5 and E13.5. The horizontal lines in the graph indicate the mean value; n = between 11 to 25. **** indicates p-value<0.0001; ns indicates not significant (p=0.9268).

FL, forelimb; HL, hindlimb; k, kidney; ml, mammary line (red dotted line); dl, dorsal line (green dotted line); 1, MP1; 2, MP2; 3, MP3; 4, MP4; 5, MP5.

Fig. 2. Morphological maturation and transition of surface epithelium to a mammary epithelial cell phenotype does not occur in MP3 of *Nrg3*^{ska} mutants.

a. Confocal images of MP3 at E11.5 and E12.5 from control and *Nrg3*^{ska} embryos visualised with DAPI (nuclei) and plasma membrane stain (cell border). MP cells marked as red indicate suprabasal cells, blue indicate basal cells. Epithelial-mesenchymal boundary is indicated by yellow dotted line. A heterogeneous population of cells is obvious in mammary epithelium from E11.5 onward, which includes columnar cells marked with blue ovals. Early epithelial multilayering is observed in MP3 of *Nrg3*^{ska} hypomorphic mutants at E11.5-stage but these cells do not progressively mature to a mammary cell phenotype observed in control embryos at E12.5. Scale bar is 50 μm.

b. Three-dimensional images were generated using Imaris software, which was used to quantify cell sphericity and volume of mammary epithelial cells in *Nrg3*^{ska} and control MP3 at E12.5. Values calculated with this software for individual cells are shown in graphs. The horizontal lines in the graph indicate the mean value, n = 10. Scale bar is 50 μm.

Fig. 3. *Nrg3*^{ska} mutants display impaired mammary mesenchymal proliferation.

a. Expression of phospho-Histone H3 (red) in E12.5-stage control and *Nrg3*^{ska} MP3. Scale bar 50 μm.

b. Mitotic cell numbers three-dimensionally detected in epithelial and mesenchymal cells in control and *Nrg3*^{ska} MP3. ME, mammary epithelium; MM, mammary mesenchyme. n = between 7 to 8.

c. Expression of Lef1 (white) in E12.5-stage control and *Nrg3*^{ska} MP3. Enlarged image (bottom) highlights reduced Lef1 expression observed in *Nrg3*^{ska} mesenchymal cells adjacent to MP3 epithelium compared to those of control mesenchymal cells, whilst *Nrg3*^{ska} MP3 epithelium has Lef1 expression level comparable to control epithelium. Epithelial-mesenchymal boundary is indicated by yellow dotted line. Scale bar 50 μm.

Fig. 4. *Nrg3*^{ska} mutants display defective specification of mammary mesenchyme.

a. Expression of ERα (red in top and white in bottom panel), *s-SHIP-GFP* (green) in E12.5-stage control and *Nrg3*^{ska} MP3. Mammary mesenchymal marker ERα-expressing cells are reduced in *Nrg3*^{ska} MP3. Scale bar 50 μm. Epithelial-mesenchymal boundary is indicated by yellow dotted line.

b. Results showing relative fold expression levels of *Esr1, Ar,* and *Pth1r* as assayed by qRT-PCR of mesenchymal tissues surrounding *Nrg3*^{ska} and control MP3 and adjacent dermal mesenchyme when normalised to mammary mesenchyme in control MP3. MM, mammary mesenchyme.

Fig. 5. Formation of MP3 in *Nrg3^{ska}* mutants can be restored by rNrg3-EGF treatment *ex vivo* without increasing epithelial mitosis and is associated with stimulation of mammary mesenchymal mitosis.

a. *s-SHIP-GFP* expression of E11.0-stage *Nrg3^{ska}* flanks before starting culture (Day 0). The same flanks photographed 24 hours after *ex vivo* culture in serum-free media with PBS or with rNrg3-EGF added to media (Day 1). The % of flanks that do not form MP3 (Aplastic MP3) or do form MP3 (MP3 formed) after 24 hours of *ex vivo* culture is indicated below an image depicting a representative result for the two outcomes in both treatment groups. n = 46 to 52.

b. Mitotic cell numbers detected in epithelial and mesenchymal cells of MP3 that form 24 hours after *ex vivo* culture of *Nrg3^{ska}* flanks in serum-free media with PBS or with rNrg3-EGF. n = 20 to 22.

Fig. 6. Expression of Erbb4 JM-a CYT-1 isoform enhances migration of HC11 cells.

a. E12.5-stage whole-embryos were used as a positive control (p.c.) that express four isoforms of Erbb4. Only cleavable JM-a Erbb4 isoforms are expressed in the embryonic MP. RT-PCR data indicates that *JM-a*, *CYT-1* and *CYT-2* isoforms are expressed during initial stages of mammary morphogenesis.

b. Results from transwell migration assays show that HC11 cells expressing the JM-a CYT-1 isoform (clone 5 and 6) are more motile than cells expressing JM-a CYT-2 isoform (clone 3 and 43) and migrate very slightly in response to Erbb4 ligands, Nrg1β and Nrg3. Significance is shown in comparison with HC11 samples.

Fig. 7. Nrg3 promotes cell spreading and focal adhesion formation of HC11 cells expressing Erbb4 JM-a CYT-1 isoform.

a. Cell area of HC11/JM-a CYT-1 cells treated with PBS or rNRG3-EGF for 15 minutes as visualised with Phalloidin (red) and DAPI (blue) stain. Digitised images used for quantification are shown below with the cell border marked in white and nucleus circles in green. Scale bar is 100μm.

b. Cell area of HC11/JM-a CYT-1 cells measured after 15-minute stimulation with PBS, mutant rNRG3-EGF, rNRG3-EGF, rNRG1β-EGF or Wnt3A. 5-95% percentile box and whiskers plots were generated using median, quartiles, and highest and lowest values. Median values are indicated. Significance is shown in comparison with PBS samples.

c. Cell adhesions in control HC11 and HC11 cells expressing Erbb4 isoform are visualised by phospho-tyrosine 397-FAK (pFAK) labelling together with phalloidin staining. Under non-stimulating conditions with a mutated rNrg3-EGF (rNrg3-EGF mut), HC11 cells expressing Erbb4 JM-a CYT-1 appear to have a smaller number of large focal adhesions linked to actin bundles that appear prominently in both HC11 cells and HC11 cells expressing Erbb4 JM-a CYT-2 (Arrowheads). Focal adhesions appear in HC11cells expressing Erbb4 JM-a CYT-1 after cells are stimulated with rNrg3-EGF wt (Arrows). Scale bar is 100μm.

Fig. 8. Model for Nrg3/Erbb4 signalling in mammary epithelial and mesenchymal specification.

Nrg3 expression within the nascent mammary primordial epithelial cells from E11.5 onward leads to changes in morphology of Erbb4 JM-a CYT-1-expressing mammary progenitor cells, resulting in an increase in cell volume and mammary cell phenotype acquisition. At E12.0, Nrg3-mediated signals are transmitted from the mammary primordial epithelium to adjacent mesenchymal cells, leading to their condensation by mechanisms that remain to be identified. Either Nrg3-mediated condensation or other signals lead to *Pth1r* expression in adjacent mesenchymal cells. Mesenchymal cells can then respond to activation of Pth1r, which specifies mammary cell fate to mesenchymal progenitors and leads to their differentiation (MM marker expression) and increased mitosis.

Author Disclosure Statement

No competing financial interests exist.